SYSTEMS BIOLOGY FOR TRADITIONAL CHINESE MEDICINE

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FOREWORD

Professor Guoan Luo and I got to know each other in late 1990s because of the implementation of the Fundamental Research of Key Scientific Issues in Traditional Chinese Medicine Formula project in the National Basic Research Program of China, and we have been working together in a multidisciplinary team ever since then. During more than 10 years of collaboration, I was quite impressed by his sagaciousness, enthusiasm, and modesty. He has proposed a series of new approaches and methodologies for Traditional Chinese Medicine (TCM) formula research and explained these profound theories in a simple and understandable way in this treatise. Additionally, as the backbone of our team, he encourages innovation in research and has set himself as an excellent example to his followers, which truly fulfilled the connotation of "learn to be an excellent teacher and act as an exemplary person." As stated in his own preface, Prof. Luo is convinced that through the test of time and practice, TCM will be an advanced science, and the theories of TCM always have been, are, and always will be a guidance for its clinical practice, and vice versa. As a TCM scholar myself, not only will his remarkable contributions in theory, technology, and implements for TCM command my gratitude and admiration, but also his deep understanding and prospective assessment of TCM theories and practices as a medicinal chemist will help to promote its academic development.

Biomedical research development should be innovative as well as adaptive considering the dramatic changes in the scientific environment and the needs of public health in this new era. Therefore, medical scientists have integrated translational medicine, digital medicine, and regenerative medicine. Translational medicine requires establishment of a "basic research–clinical practice–industry–talent" system from laboratory to clinic, from

hospital to community village, and from result to benefit in order to benefit the people, which is a major livelihood project. Translational medicine also includes integrative medicine and pharmacy, so-called 3P medicine—including predictive, preventive, and personalized medicine, as well as participative medicine. According to the theory of combining natural sciences with humanities in translational medicine, some original theories and practical experiences in TCM, including the concept of holism, imagery thinking, and treatments based on syndrome differentiation, inseparability of the body and spirit, and prevention of disease, are interpreted with modern concept and then applied to research and practice so as to establish the leadership of TCM in the medical field. With the purpose of keeping the innovativeness and adaptability of TCM research on the basis of modern science, we first need the guidance of correct cosmology, for example, the "harmony between body and nature" theory that was originated from hieroglyphics and long-term agricultural civilization, and the "harmony but different" theory, which integrates macro and micro perspectives, synthesis and analysis methods, entity and relationship ontologies.

The essential processes to understand these theories and incorporate them with the Western theories include regression analysis based on systems biology, stratification research based on systematic method, and drawing of a preliminary conclusion. Second, we need to conduct interdisciplinary research. Since the era of Sir Newton and Madame Curie long ago, scientific study today requires the integration of all natural science fields, although the innovative studies following one's own interests are still respectable. For instance, biologists or chemists can be the leaders to manage research work. So besides personal skills, all-around consciousness, empathic ability, and humanistic literacy are more important at present. Third, we need to establish innovative teams and cultivate positive team spirit. Specifically, we call for good study and working styles, tolerance of failed attempts, and overcoming fickleness and eagerness for instant success and quick profits.

Nowadays, many intellectuals in the scientific and technological fields approve the scientific aspect of TCM; the public looks forward to its dedication to public health; and government actively supports the development of the TCM industry, so this is a perfect time for the modernization of TCM. However, there are still some problems and barriers. As a result, we should unite experts and scholars and learn as much from them as possible in all fields. For clinical medical research, the concepts and methods of scientific evidence-based medicine should be introduced to get high quality, high level, and consistent evidence in TCM and Western medicine to reflect the vitality of TCM. For basic medical research and industrial development, innovation of methodology should be treated as the first priority, and scientific hermeneutics that integrates understanding, interpretation, and application should be used to interpret the unique advantages of original theories in TCM to enrich modern medical science. Additionally, the method system should be updated by using advanced instruments and new techniques to promote the research and

development of innovative TCM in order to provide services for clinical medicine and bring benefits to society.

This book is a summary of years of research by Professor Luo's expert group. Prof. Luo elaborates on the formation, evolution, and research content of systems biology for TCM, as well as insightfully analyzing the methodology system based on holism and systematology of TCM. After reading this treatise, I noted with pleasure that there was guidance for both modern concepts and operable technical routes. This treatise is not only based on the TCM theory of prescription such as "seven conditions in making up prescription," "*Jun*, *Chen*, *Zuo*, *and Shi*," and intensive study of component compatibility and molecular biology, but also contains original thoughts on basic theories, information about product research and transformation, and updates on major prescriptions. Moreover, this treatise contains active explorations about the introduced complex intervention in refractoriness diseases, which can guide the transition from "Western learning spreading to the East" to the coexistence of both "Western learning spreading to the East" and "Eastern learning spreading to the West." As we remember, Prof. Mi Wu and Prof. Yinke Chen from the Chinese Culture College in Tsinghua University always called for independent spirit and free thought, arguing for respecting the Chinese cultural heritage and thinking back to the old virtuous people before seeking the development of Chinese culture. Furthermore, when it comes to education, Han Yu's "knowledge teaching, method introducing and problem solving" have been taken as the model, but only by breaking through the conservatism and advocating originality and research can academic advancements be improved.

Although Prof. Luo suggests some shortcomings about this book, his truth-seeking and difference-seeking spirit is very encouraging. In light of our profound friendship after many years of cooperation, it certainly is an honor to write this Foreword. I want to share these words with Professor Luo's group and hope they are encouraged by them. And last, I would like to express my gratitude and congratulations to Prof. Luo for this splendid treatise.

Prof. Yongyan Wang

Academician of Chinese Academy of Engineering
Former President of China Academy of Chinese Medical Sciences

PREFACE

Why did we write this book about systems biology for traditional Chinese medicine (TCM)? Because we have found that systems biology is one of the best tools among modern science and technologies to embody the essence of holism and systematology-based TCM (Eastern medicine) and to modernize TCM. At the same time, the philosophy and methodology of TCM theory can also benefit and promote the development of systems biology. In this book we applied systems biology to investigate TCM and attempted to combine the essential merits of Eastern and Western medicine. We believe that the research and development of TCM should be different from the "point to point" (P2P) methodology of Western medicine (which represents a single small molecule acting on a single target), and we propose a "system to system" (S2S) methodology (which represents a drug system interacting with the human body system) in order to develop modernized composite medicine and realize the modernization of TCM.

There are similarities as well as differences between TCM-based systems biology and systems biology in general that have been defined by Professor Lee Hood, Jeremy K. Nicolson, and others. The similarities lie not only in their philosophy, where holism and systematology both describe the study of a drug's action with a body system, but also in the platform, such as genomics, proteomics, metabonomics, and bioinformatics. Systems biology was defined to be the study of how a biological system responds to a given disturbance or stimulant of the environment. When considering Western medicine, the disturbance is usually just a single compound representing the action of a point to a biological system (P2S). On the other hand, the study of systems biology in TCM shows the interaction of a drug system to a biological system (S2S), since the formula-based medication commonly used in TCM constitutes a drug

system including a mixture of numerous compounds. Biological systems including the human body could be addressed by the network of genes, proteins, or metabolites. Additionally, the therapeutics of TCM is also quite different from Western medicine. Western medicine is focused on the treatment of diseases (with a specific target), while TCM is concerned with the ill body, and defines different states of the body as *Zheng* (syndrome) consisting of a set of symptoms of both pathological and physiological states. Therefore, the introduction of systems biology to the research of TCM should embody these intrinsic characteristics of TCM with a modern approach.

The discovery of Western medicine (synthetic drugs) that is based on the P2P mode has met with great success, especially for single-factor infectious diseases. However, in recent years drug research and development face serious challenges. For example, there are still no satisfying solutions for multi-factor complex chronic diseases. Furthermore, some drugs proven to be effective for a given target have been reported to have severe side-effects and are now forbidden to be marketed. Therefore, even for the study of a single-molecule drug, we should also consider its actions on the body in a systems approach (P2S mode) with the use of network pharmacology. In the Western pharmaceutical industry, some multi-component drugs have also emerged, which have been developed based on a combination of existing drugs or a combination of targets. However, the general principle and methodology for the development of multi-component drugs or composite drugs is still in demand.

TCM has a history of thousands of years. It has a systemic theory derived from long-term clinical experience, the philosophy of holism and systematology and the theory that man is an integral part of nature (emphasizing the harmony of the body and the environment). It also emphasizes the global balance of the body other than the simple inhibitory effect on a disease target, and on individualized therapy with medication of TCM formulae. But the system of theories of TCM expressed in words is in demand of support by evidence-based study. For example, the TCM theory of "*Zang* and *Fu* organs" (including heart, liver, kidney, and lung) is somewhat different from the understanding of the organs based on modern anatomy. One of the key issues of "TCM modernization" is how to interpret the rational principles of TCM using the tools and experimental data based on modern science and technology. If we simply use the viewpoint of conventional pharmacy, we must isolate all the constituents of a given formula (tens of compounds or even more) and interpret the interactions between each constituent and each target; then we have to find out how many constituents and targets are needed to interpret such a complex interaction. In fact it may be almost an impossible mission, and such a mode (multi-point to multi-point) is inefficient to embody the characteristics of TCM of treating the body system.

In the post-genome era, systems biology was developed, which recalls the ideas of holism, systematology, and network, and provides the possibility to build a connection between Eastern and Western medicine. We are now able to investigate the interaction of a drug system (including TCM and Western

medicine) and the whole body system at the level of genes, proteins, and metabolites. In order to adapt systems biology to TCM, the point-based disturbance should be developed into a drug system (composite medicine) that can be analyzed at several levels according to the constitute complexity. Based on the systems studies, the traditional formula consisting of medicinal herbs can be simplified into a multi-component drug that may consist of isolated partials or a compatible combination of several compounds. Similarly, synthetic drugs can also be combined into a composite drug that is considered as a drug system. The composite drugs refer to treatment drugs that are composed of multiple compounds or compound groups and are developed by combining a variety of treatment principles and under the guidance of multiple action mechanisms. These drugs can achieve the holistic optimal efficacy, rather than the best effect from the single-target.

In order to develop composite drugs, we must break through the limitation of target-based discovery and consider the whole response of the body system, which may be expressed at the basic levels such as genes, proteins, and metabolites. Therefore, the studies based on genomics (Chapter 5), proteomics (Chapter 6), and metabonomics (Chapter 7) are valuable for either the body of the diseased (viewed from TCM) or diseases of the body (viewed from Western medicine). Such omics studies provide the systematic understanding of the interaction of drug system with the body system, while the isolated studies of genes, proteins, and metabolites may lead to fragmentation of information. Therefore, we conducted an integrated study of genomics and metabonomics on the cases/controls of diabetic nephropathy (DN), in order to combine the knowledge of genes and metabolites and find the disease targets, which may provide a comprehensive and systematic understanding of the complex disease by integrating the global characterization (macroscopic) of the genetic network and the metabolic network with the specific characterization (microcosmic) of given pathways and targets. Moreover, we explored the possibility of unified expression of the body of the diseased (TCM's holistic view) or diseases of the body (Western reductive view). Taking the case of DN as an example, on one hand, the expression of *Zheng* was used to characterize the pathological and physiological state of the diseased body according to TCM's view; 34 symptoms of nine syndromes were scored and taken into correlative analysis with quantitative data. On the other hand, clinical biochemical and pathological indicators were combined to characterize DN according to the Western view. To connect TCM and Western medicine on the levels of genes, proteins, and metabolites, we tried to establish a comprehensive biomarker indicator system for the characterization of DN based on the platform of integrative metabonomics (Chapter 9). We reached the following conclusions: (1) The diagnostic systems of TCM and Western medicine gave consistent conclusions on most of the patients' conditions. For example, most of severe cases considered as stage V of DN were also discriminated to be the worst *Zheng* deficiency of both *Yin* and *Yang*. (2) An integrative biomarker indicator system is more suitable for new drug discovery based on the global

body condition. For Western medicine, the integration of pathological and biochemical indicators with the network of genes, proteins, and metabolites provides a better characterization of a complex disease. For TCM, the integration of pathological and biochemical indicators with a network of genes, proteins, and metabolites as well as *Zheng*-based indicators helps us to understand TCM theories and facilitate the research and development of composite drugs. (3) Some of the TCM's concepts such as *qi* that are difficult to understand may have some substantial basis. For example, our study related deficiency of *qi* with some genes related to transportation, thus suggesting the essence of TCM understanding of *qi*.

In this book, a keynote topic will be focused on the development of composite drugs (including TCM and Western medicine) with the integrated consideration of both pathological and physiological impact. Many of the existing formulae with a compatible combination of herbs are described as *Jun* (monarch), *Chen* (minister), *Zuo* (assistant), and *Shi* (guide) and were derived based on thousands of years of clinical experience. However, it is difficult to fully understand the therapeutic mechanisms of these drugs due to their complex composition. Therefore, we propose an approach of chemomics (Chapters 2 and 3) to characterize TCM and analyze TCM formula at three levels: compatibility of herbs, compatibility of fractions, and compatibility of constituents. The first two represent a drug system that should be characterized by a combination of fingerprinting (global characterization) and multicomponent quantitative determination (specific characterization) different from single-compound drugs (Western medicine). The compatibility of constituents is similar to the composite drug of Western medicine. In addition, the research and development routine of composite drugs of TCM is a process during which simplification and optimization of the compatible combination is conducted step by step based on the mode of a "system to system" interaction (Chapters 10 and 11). The research and development routine of composite drugs of Western medicine is a process during which complication and optimization occur. It represents a combination of drug compounds considering the interaction of drug systems with biological systems. In addition, development of composite drugs, including TCM and synthetic drugs, was explored.

This is a research monograph with an attempt to explore a new routine, and there are likely some limitations. Here we introduce some modern studies on TCM in China, and expect to raise more interest and collaboration in the research of TCM worldwide.

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ABBREVIATIONS

5-HT	5-hydroxyl tryptamine
AA	arachidonic acids
ACE	angiotensin-converting enzyme
AGEs	advanced glycation end-products
ANN	artificial neural networks
AR	aldose reductase
ATP	adenosine triphosphate
AUROC	area under curve of a receive operating characteristic curve
BMMSCs	bone marrow mesenchymal stem cells
CE	capillary electrophoresis
CE-MS	CE coupled with mass spectrometry
CK	creatine kinases
cMHC	cardiac myosin heavy chain
CSF	cerebrospinal fluid
cTnI	cardiac troponin I
CVDs	coronary diseases
DAG	diacylglycerol
DIP	Database of Interacting Proteins
DM	diabetes mellitus
DN	diabetic nephropathy
DPN	diabetic peripheral neuropathy
EBs	embryonic bodies
DQY-PQ	deficiency of both *qi* and *Yin*, particularly *qi* deficiency

DQY-PY	deficiency of both *qi* and *Yin*, particularly *yin* deficiency
DYY	deficiency of both *Yin* and *Yang*
ECG	electrocardiogram
ECM	extracellular matrix
ET-1	endothelin
FGF	fibroblast growth factor
GC	gas chromatography
GC-FTIR	gas chromatography coupled with Fourier-transform infrared spectroscopy
GC-MS	gas chromatography coupled with mass spectrometry
Glu	glutamic acid
GO	gene oncology
HCS	high content screening
HDG	high dose group
HDL	high-density lipoprotein
HE	hemotoxylin and eosin staining
HGP	Human Genome Project
HPLC	high performance liquid chromatography
HPLC–ESI-MS/MS	HPLC–electrospray tandem mass spectrometry
HPLC-MS	HPLC coupled with mass spectrometry
IBS	Integrated Biomarker System
IDF	International Diabetes Federation
IGF-1	insulin-like growth factor-1
IGT	impaired glucose tolerance
IL-1β	inerleukin-1β
IMPT	integrative metabonomics platform technology
iNOS	inducible nitric oxide synthase
ISO	isoproterenol hydrochloride
KEGG	Kyoto Encyclopedia of Genes and Genomes
KNN	K-nearest neighbor algorithm
LC-NMR	liquid chromatography tandem nuclear magnetic resonance
LC-DAD	liquid chromatography tandem photodiobe array detector
LC-ICP/MS	liquid chromatography tandem plasma mass spectrometry
LC-MS	liquid chromatography–mass spectrometry
LDA	linear discriminant analysis
LDG	low dose group
LDH	lactate dehydrogenase
LPC	lyso- phosphatidylcholine
LPO	lipoperoxide
LSP	*Liushen* pills
Lyso-PC or LPC	lysophosphatidylcholines

MCCM	multi-component Chinese medicine; multi-component drug; modernized composite medicine
MDA	malondialdehyde
MDG	medium dose group
MDLC	multi-dimensional LC
mESCs	mouse embryonic stem cells
MHC	myosin heavy chain
MI	myocardial infarction
MMDB	Molecular Modeling Database
MRM	multiple reaction monitoring
MSCs	mesenchymal stem cells
NCBI	National Center of Biotechnology Information
NEFA	nonesterified fatty acid
NIH	National Institutes of Health
NMR	nuclear magnetic resonance
NSLF6	new *Shuanglong* formula; *Shuanglong* derived formula
NTDs	neural tube defects
NTF	neurotrophic factors
OMIM	Online Mendelian Inheritance in Man
OSC	orthogonal signal correction
P2P	point to point
P2S	point to system
PC	phosphatidylcholine
PDGF	platelet derived growth factor
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PKC	protein kinase C
PK-PD	pharmacokinetics-pharmacodynamics
PLS-DA	partial least squares discriminant analysis
PMF	peptide mass fingerprinting
PMS	premenstrual syndrome
PN	Panax Notoginseng
PNS	Panax Notoginseng saponins
PS	phosphatidylserine
QMPT	quantitative metabonomics platform technology
RT-PCR	real time quantitative polymerase chain reaction
ROS	reactive oxygen species
RPG	remainder of ginseng
RSM	remainder of *Danshen*
S2S	system to system
SAB	salvianolic acid B
SAH	S-adenosyl-L-homocysteine
SAM	S-adenosyl-L-methionine

SIMCA	soft independent modeling of class analogy
SLF	*Shuanglong* formula
SM	sphingomyelin
SNP	single nucleotide polymorphism
SOD	superoxide dismutase
SPSS	Statistical Product and Service Solutions
STITCH	search tool for interactions of chemicals
SVC	support vector clustering
SVR	support vector regression
systems ADME/Tox	systems of absorption, distribution, metabolism, excretion and toxicity
TBA	total serum bile acid
TCA	tricarboxylic acid
TCM	Traditional Chinese Medicine
TGF-β	transforming growth factor-β
TGS	total ginsenosides
TLC	thin layer chromatography
TNF-α	tumor necrosis factor
TSA	total salvianolic acids
TTD	Therapeutic Target Database
TV	toads venom, *Chanshu*
UPLC-Q/TOF	ultra performance liquid chromatography tandem quadrupole/time of flight MS
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VIP	variable importance
WM	Western medicine
XCMS	various forms (X) of chromatography mass spectrometry

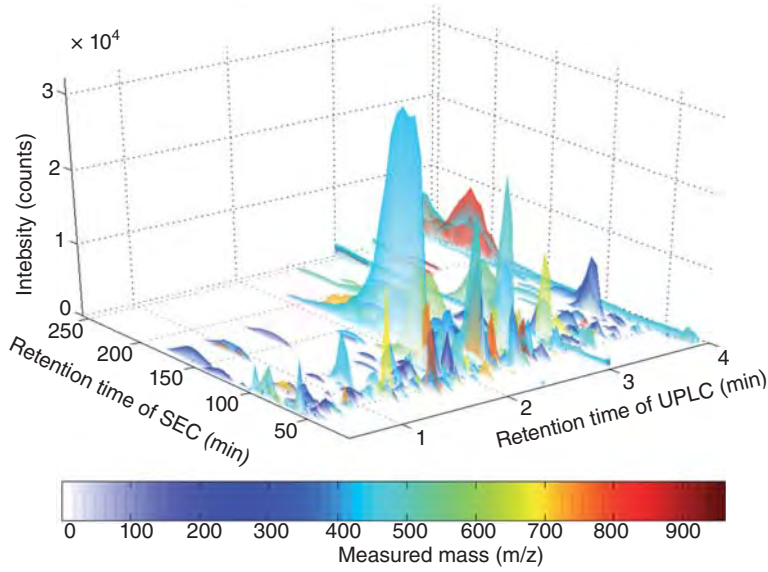

Fig. 3.7 Four-dimensional spectrum of HPLC × UPLC-TOF/ MS system.

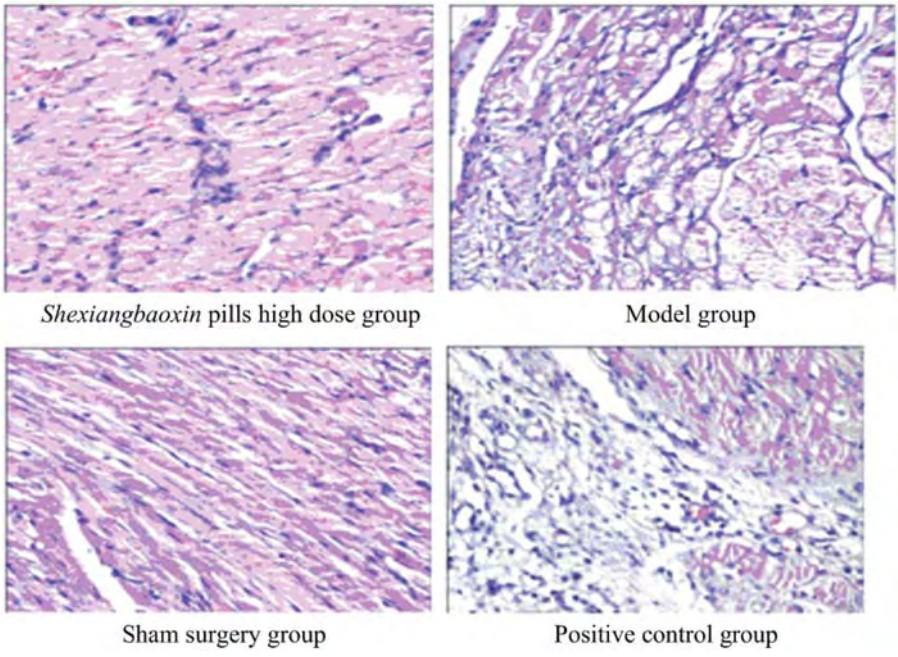

Fig. 5.1 Photomicrographs of myocardial tissues. The myocardial tissue biopsies were dyed by HE red.

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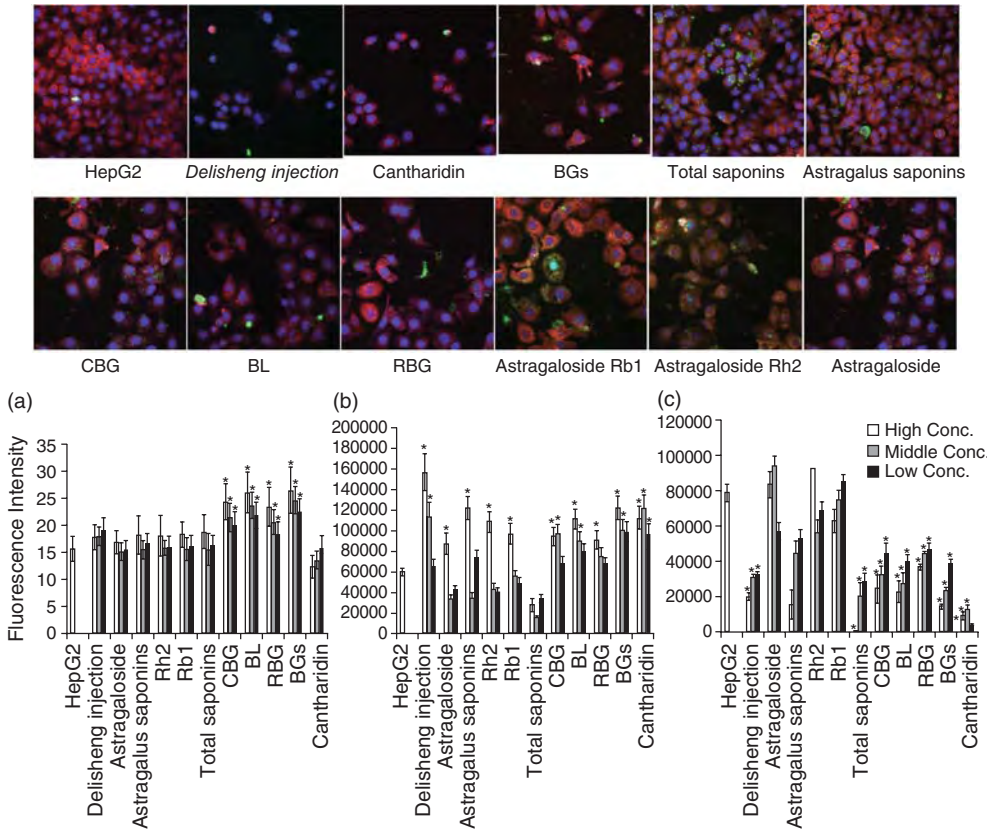

Fig. 6.4 High content screening results of *Delisheng* injection and its components on HepG2 apoptosis. A: the integrity of the nucleus; B: the cell membrane permeability; C: mitochondrial membrane potential. The blue represents the nucleus. If the cell was healthy, the shape of nucleus was a regular circle. The green color shows the cell membrane permeability, and more green represents greater membrane permeability and more cell apoptosis. The red represents the mitochondrial membrane potential. The higher mitochondrial membrane potential showed better activity of the cells. $P < 0.05$ versus the negative control group.

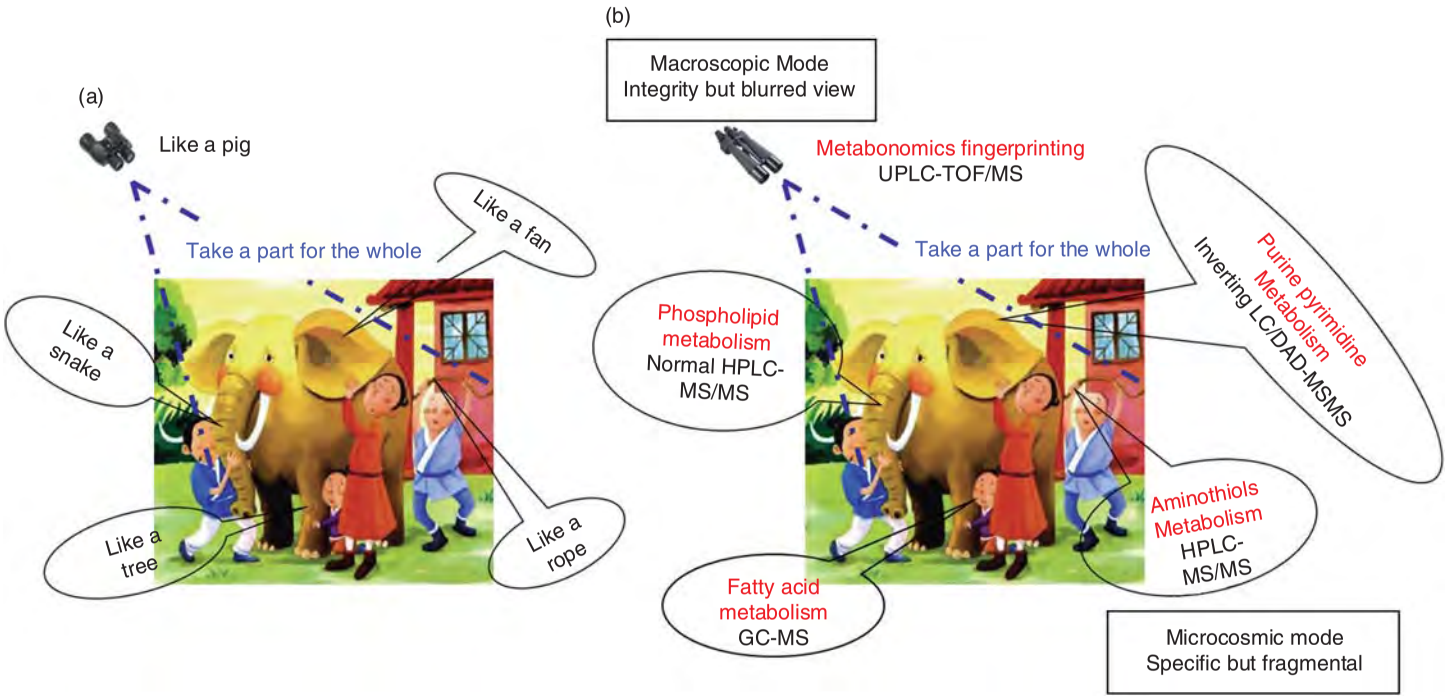

Fig. 7.1 Sketch map for blind men touching an elephant (a) and quantitative metabonomics platform technology for research on diabetic nephropathy (b).

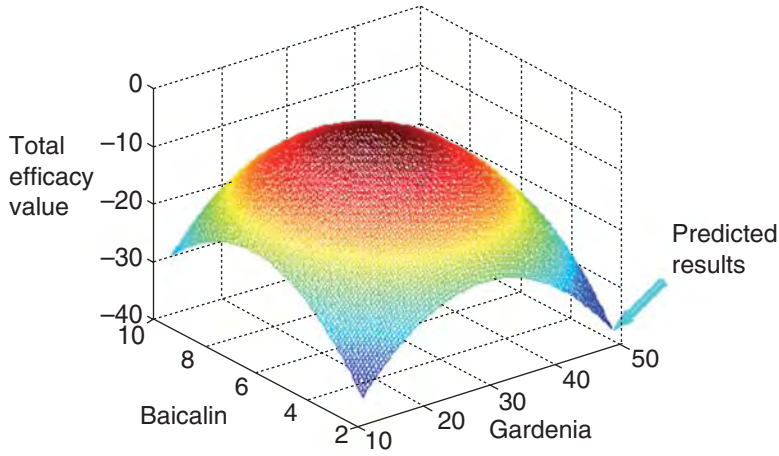

Fig. 10.19 Optimization of prediction map of the *Qingkailing*-derived formula.

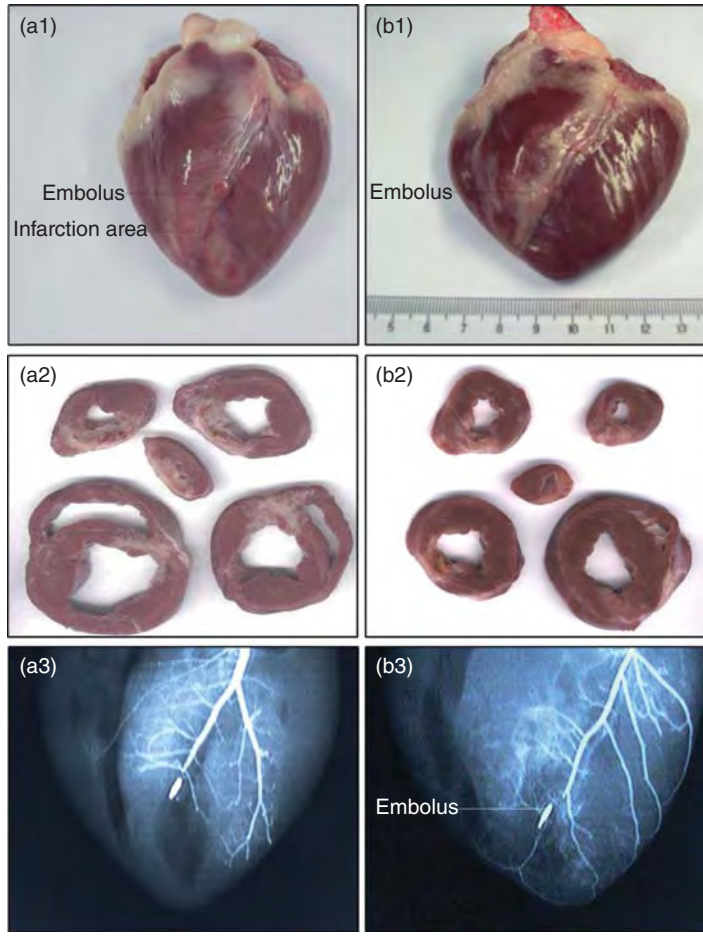

Fig. 11.1 Myocardial infarction of Chinese miniature pigs. A: model group; B: SLF + MSCs group.

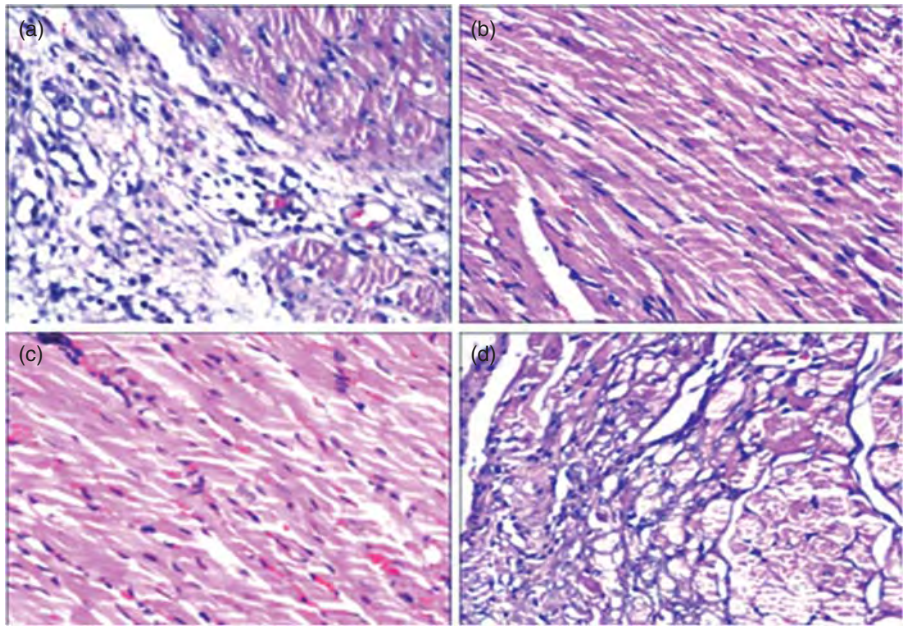

Fig. 11.8 Effect of NSLF6 on coronary artery ligation-induced myocardial injury. Hematoxylin and eosin staining was used to visualize the formalin-fixed sections of cardiac muscle tissue of rats (magnification 100 diameters). A: model group; B: sham surgery group; C: NSLF6 group; D: positive control drug group.

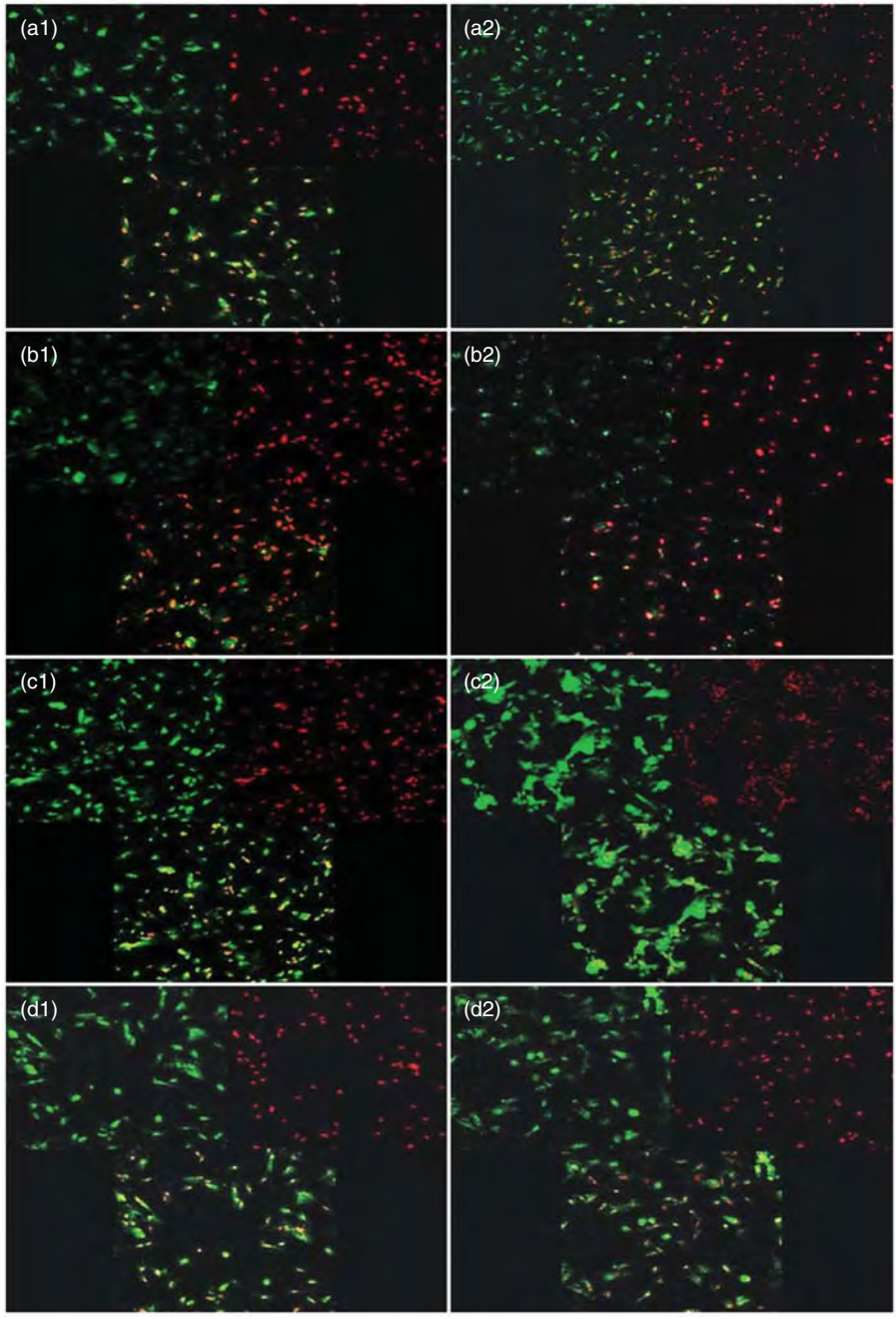

Fig. 11.11 Immunofluorescences of the MHC and cTnI of BMMSCs induced by different combinations of TCM on day 30. Each picture consists of three photos. The left superior one is a photo of fluorescent reaction antibody staining; the right superior one is a photo of PI nuclear staining; the lower one is a photo including both fluorescent antibody reaction staining and PI nuclear staining. The pictures marked 1 are results of MHC expression; the pictures marked 2 are results of cTnI expression. A, B, C, and D are, respectively, "ingredient" group, "ingredient + 5-aza" group, "component" group, and "component + 5-aza" group.

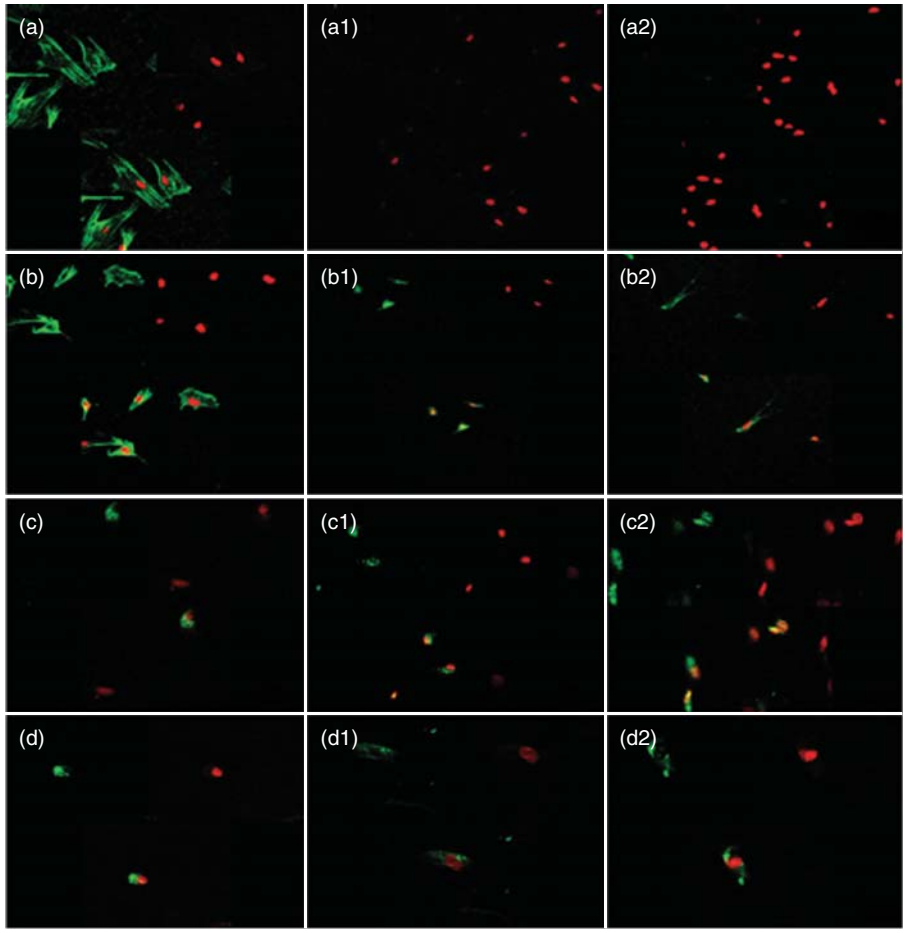

Fig. 11.14 Immunofluorescence results of MSCs induced by TCM. Undifferentiated MSCs were immunostained positively for actin (A), but negatively for cMHC (A1) and cTnl (A2), while rat cardiomyocytes were positive for all three examined antibodies (B: actin; B1: cMHC; B2: cTnl); differentiated MSCs induced by 5-aza (C1: cMHC; C2: cTnl), SLF (D1: cMHC; D2: cTnl), or SLF ingredients (E1: cMHC; E2: cTnl) were positive for both cardiac-specific protein markers, cMHC and cTnl.

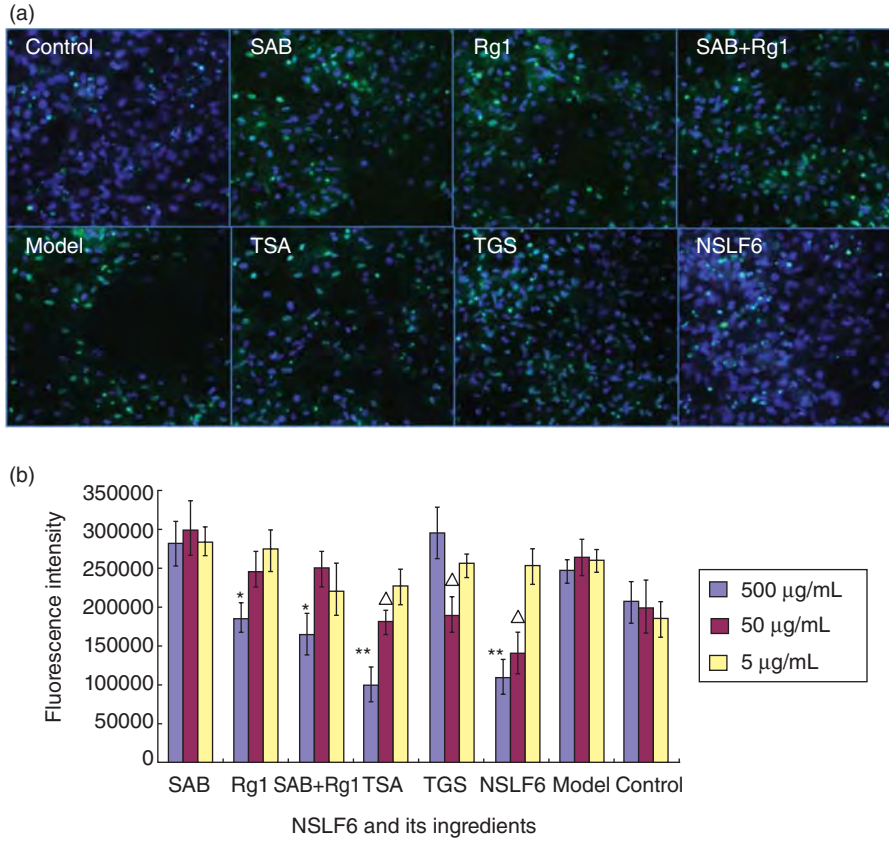

Fig. 11.24 Effect of NSLF6 and its effective ingredients on the membrane permeability of cardiomyocytes by H_2O_2. A: fluorescence expressions of membrane permeability of the damaged cardiomyocytes stimulated by NSLF6 and its effective ingredients. Different fluorescent shows the membrane permeability and nucleus stained by DAPI. B: Comparison of NSLF6 and its effective ingredients to the membrane permeability of cardiomyocytes by H_2O_2.

CHAPTER 1

INTRODUCTION OF SYSTEMS BIOLOGY IN TRADITIONAL CHINESE MEDICINE (TCM)

1.1 CHARACTERISTICS AND COMPATIBILITY PRINCIPLES OF TRADITIONAL CHINESE MEDICINE (TCM)

1.1.1 Special Features of Diagnosis and Treatment in TCM

Practitioners of TCM have accumulated valuable experiences in learning about life, improving health and fighting disease in its extensive history of production activities and real life practice. Chinese medicine has a unique theoretical system, rich clinical experience, and scientific ways of thinking. Based on natural sciences and the humanities and social sciences alike, it is a notable medical system in the multitudes of traditional medicines worldwide.

Eastern and Western medicines are obviously different in their treatment theories and drug forms (Fig. 1.1). These are the reflections of the differences and specialties between the East and the West. These differences cause great difficulties not only in medical communication, but also in the recognition of TCM by the Western medical system.

TCM has its unique treatment theory and long-term clinical experience, especially for the diagnosis and treatment of many complex chronic diseases. Meanwhile, it also faces the new challenge of inheritance and innovative development. However, the modernization and internationalization of TCM is the trend of the times. On the other hand, the Western medical system has

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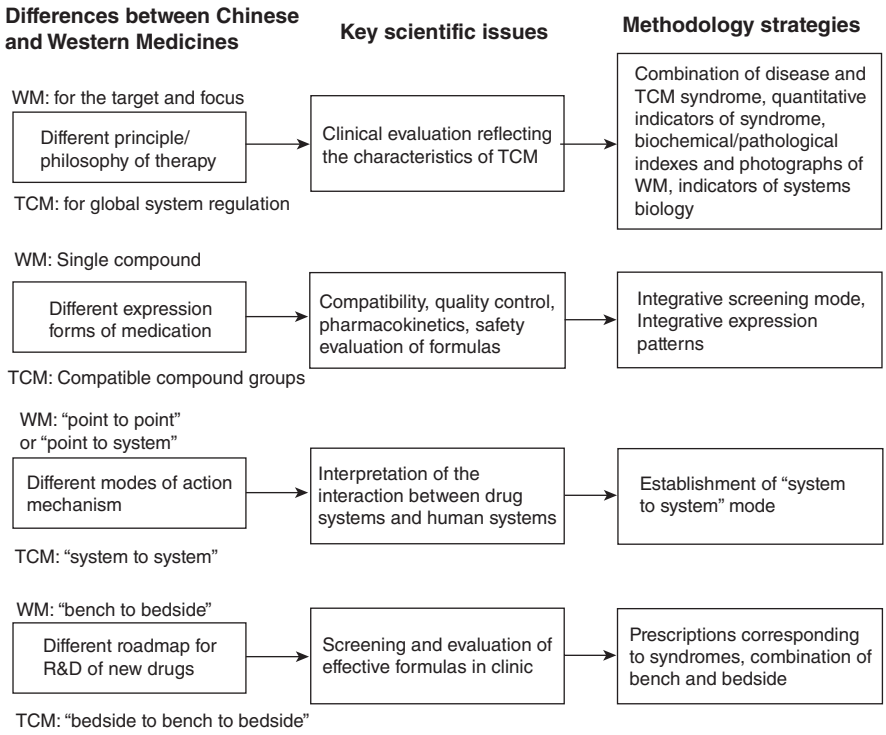

Fig. 1.1 Difference between TCM and Western medicine (WM) systems.

its own problems. Therefore, practitioners of Eastern and Western medicine should learn from each other and make use of others' experiences for reference. Through the conduction of modern research on TCM, for instance, the efficiency of TCM and its scientific value could be proved and further explained by modern scientific language through modern technology and scientific experiments, which will promote the development of TCM and modern medicine and life science as well.

1.1.1.1 Concept of Holism and System Theory The concept of holism is the core idea of TCM theory. TCM has a unique holistic, dynamic, and dialectical theory of the complex phenomena of life and diseases. For instance, it considers the human body to be an organic integrity; the various components of body composition on body structure are indispensable, coordinating in their functions and interacting pathologically with each other. In addition, it advocates "harmony between body and nature," and emphasizes that human activities should be adapted to geographical and seasonal changes, thus maintaining the body's health. Meanwhile, it makes appropriate diagnoses and recommends treatments based on the overall perspective of pathogenesis, location, and potential of diseases, combined with season, geographical aspects, and diet.

As a result, the holistic concept of TCM can be seen in the comprehensive and coordinative functions targeting regulation of many organs of the body. TCM formulas are based on the condition of patients; characteristics of the medication; the taste, functions, and indications of drugs; and relationships of the seven conditions of ingredients in prescription (single effect, mutual assistance, mutual reinforcement, mutual detoxication, mutual antagonism, mutual restraint, and mutual incompatibility). Prescriptions are made according to the principles of compatibility of "*Jun*, *Chen*, *Zuo*, and *Shi*" (roles of ingredients in the prescription with the functions of *Jun*, *Chen*, *Zuo*, and *Shi*, respectively). The mutual restraint, guidance, and synergies in the process of compatibility constitute the overall effect, which is more efficient than the simple combination of each part.[1] Professor Yongyan Wang, an expert of TCM, has stated that "TCM is not a simple allopath but an integrated regulation from the perspective of multichannels, multilinks, and multifaceted roles in the human body based on TCM formulas and the main procedure of the incidence of disease. Therefore, the human body at different levels, overall, organ, cell, subcell and molecule, can be adjusted effectively."[2] Moreover, the holistic concept is reflected in "the unity of medicine and treatment." TCM formulas originate from the clinical diagnosis and are applied to the clinical diagnosis. Diagnosis, methods, prescription, and drugs are combined through the three steps of "dialectical-legislation-prescription," and finally unified into a whole. Therefore, "systematic theory" and "holistic" ways of thinking are the essential in TCM theories, which coincide with the mainstream of modern life sciences.

The theory of "*Yin* and *Yang* balance" is the important manifestation of the concept of holism, which guides the development of TCM. In TCM, the human body is considered as a whole unit with the balance of *Yin* and *Yang* keeping a healthy condition. In TCM, we consider a disease to be caused by functional imbalance of *Yin* and *Yang*. There is a dialectical relationship between *Yin* and *Yang*. When *Yin* is too much it will lead to a deficiency of *Yang*, and vice versa (from "A Great Theory on *Yin* and *Yang* of *Su Wen*"). TCM treatment must follow the relationship between *Yin* and *Yang*, which will be regulated until the health recovers. Therefore, TCM medication is very different from the Western type. "Compatibility" is very important in TCM treatment. For example, medication should be adjusted according to the location, time, patient, and syndrome. Such a traditional theory of TCM plays an effective role in clinical diagnosis and treatment. However, its scientific mechanisms still need to be fully understood and validated.

With the changes in the human disease spectrum, the Western medical treatment, which aims at a single target, often fails to fully treat complex diseases. The defects of the reduction of humans to "organs–cells–genes" have become more and more evident. A combination of various interdisciplinary research ideas has also become a biological topic in the forefront of life science research. Therefore, based on the overall concept and system theory ideas, the TCM treatment system has unique advantages in complex chronic disease

diagnosis and treatment. Its theory, technology, and medicine have important practical significance. The development of modern medicine and the influence of life science research will further promote scientific contents and better explanations of TCM.

1.1.1.2 Syndrome Differentiation Treatment and Individualized Treatment

Treatment based on syndrome differentiation is characterized in diagnosis and therapy with TCM, in which relevant information about a disease, especially symptoms and signs, at a particular stage, is collected in order to discern the nature of the disease.

In a TCM clinic, doctors collect as many symptoms and signs as possible from the patients by inspection, auscultation and olfaction, interrogation, and palpation of the pulses. However, the body anatomy and biochemical metabolic processes are poorly understood (like a black box in TCM). With thousands of years of clinical practice, functional linkages from the body have been summarized as the Meridian, although it still lacks accurate measurement. After the cause and syndrome of the disease are confirmed, appropriate prescriptions according to previous experience can be used with close observation. The physicians can make some revision on the prescription for the patients according to variation of syndromes and signs.

For individualized treatment, TCM holds that each body is unique due to the physical differences, when confronted by different virus and diseases, various syndromes will naturally form. For the elderly, children, and certain individuals, the dosage will be different from that for others. For instance, the amount of bitter cold gypsum, honeysuckle, and mild *Mahuang* (Ephedrae Herba), which have strong effects, should be reduced. Here, the syndrome differentiation and the principle of individual treatment are fully reflected in the course taken.

Therefore, there might be a case for different treatments for the same disease, and the same treatment for different diseases. In recent years, systems biology has discovered that many diseases (especially the complex polygenic diseases) are often attributed to combined results of genetic and environmental factors. There may be a variety of pathogenic factors (mechanisms). As a result, it is possible that the inner basis of individualized treatment principles can be explained in the perspective of systems biology.

1.1.1.3 Theories of Compatibility of Complex Formulas and Prescriptions Corresponding to Syndromes

The preparations and usage of TCM are completely different from those of Western medicine. TCM emphasizes "compatibility," and the prescriptions are adjusted according to the changes of location, time, patients and syndrome, so the body reaches a new balance of *Yin* and *Yang* after the coordination of the overall system. The earliest record available in prescription literature is the *Prescriptions of Fifty-two Diseases* and 13 records of *Huangdi's Inner Classic of Medicine* collected in the spring and autumn period, which summarized the theory of syndrome

differentiation treatment principle and rules, prescriptions, and compatibility, and provided solid theoretical foundation for further development. Zhang Zhongjing of the East Han dynasty wrote the two famous books, *Treatise on Exogenous Febrile Disease* and *Synopsis of Golden Chamber*. Zhang created the syndrome diagnosis theory, combining "theory, principle, formula, and medicine," and established a close relationship between medical theory and clinical practice, which was regarded as the "ancestor of prescriptions." *Shen Nong's Herbal* is the earliest pharmaceutical monograph in China with a total of 365 types of *materia medica*, which proposed medicinal theory of "four properties and five flavors." It clearly stated that "cold disease should be treated with hot drugs, and vice versa" and the compatibility theory of "seven functional relations for drugs," providing an important theoretical basis for making prescriptions.

"Prescriptions corresponding to the syndromes" is the principle for TCM formula. For example, *Liuwei Dihuang* pills mainly cure fevers, sweating, weakness at the waist and knees, vertigo, and tinnitus; *Wuling* powders are mainly used in the treatment of urination disorder, vomiting inverse, edema, diarrhea, pale tongue with white and thin fur, and smooth or deep pulse.

"Prescriptions corresponding to the syndromes" not only refers to the interaction between one prescription and one syndrome, but also uses one prescription for many other syndromes and many prescriptions for one individual syndrome. This correspondence may change with time. For instance, when the syndrome changes after the prescription is taken for some time, there would be a gap period between this prescription and the syndrome. At this time, the prescription has to be adjusted based on the syndrome.

1.1.2 Compatibility of Prescriptions

1.1.2.1 Compatibility of "Jun, Chen, Zuo, and Shi" (Monarch, Minister, Assistant, and Guide) in the Prescription The formula of TCM is neither a simple piling up of drugs of the same concoction, nor a simple efficacy pile of various drugs. It is indeed guided by the essence of TCM theory of "concept of holism, syndrome differentiation treatment," and based on the compatibility principle of "*Jun*, *Chen*, *Zuo* and *Shi*," leading to the synergies of drugs, mutual restraint of overall function, and, finally, obtaining attenuated efficiency.

The basic principle of the "*Jun*, *Chen*, *Zuo*, and *Shi*" was first recorded in *Huangdi's Inner Classic of Medicine*. The "*Jun*" herbs are the major ingredients for treatment of the main disease and play a key role in the drug treatment. Their efficacy dominates in the first place. "*Chen*" herbs are the secondary ingredients, which help *Jun* herbs in producing therapeutic effect against the main disease and syndrome or for treatment of other diseases. *Chen* herbs have weaker efficacy compared with the *Jun* herbs. *Zuo* herbs are of adjuvant effects, which are divided into three categories according to their functions. These adjuvant drugs can enhance the therapeutic effect of *Jun* and *Chen* herbs. Junior drugs are used to eliminate the drug toxicity of *Jun* and *Chen*

herbs; anti-adjuvant drugs are contrary to the *Jun* herbs and have enhanced effects in the medication. In the prescription, the efficacy of *Zuo* herbs is weaker than that of *Chen* herbs. *Shi* herbs, the adjusting drugs, play a guiding role in the medication. Moreover, they provide the regulating effect with a minor dosage and mild efficacy.

The *Mahuang* decoction, made from the *Mahuang* (Ephedrae Herba), *Guizhi* (Cinnamoli Ramulus), *Xingren* (Armeniacae amarum Semen) and *Zhigancao* (processed Glycyrrhizae Radix), is the main prescription in the treatment of exogenous cold. When the human body has been bothered by wind chill, sweat excretion is inhibited and the syndrome of no sweat and severe chills can be observed. The function of skin is controlled by the lung, the skin pores are closed, and the lung *qi* cannot be diffused; then cough symptoms appear. In the formula, *Mahuang* can encourage sweat and relieve the exterior to disperse the wind chill, diffuse lung *qi* to relieve cough and panting, and is therefore the *Jun* herb. *Guizhi* can induce sweating and relieve muscle pain, and is the *Chen* herb. *Xingren* can alleviate cough, and is recognized as the *Zuo* herb. *Zhigancao* can adjust the whole features of the herbs in the formula and is the *Shi* herb. With the mutual coordination and restraint of these herbs, the *Mahuang* decoction is used in the treatment of cough and asthma.

1.1.2.2 Unity in Prescription Compatibility The principle of the unity of opposites is the fundamental law of materialist dialectics, which not only runs through the basic theory in TCM, but also plays a guiding role in clinical practice. Ancient physicians attached great importance to the compatibility of heat and chills, *Yin* and *Yang*, reinforcement and reduction for complex syndromes. They emphasized the adjustment of chills and heat, complementary of *Yin* and *Yang*, the combination of nutrition and purgation, and the compatibility of promoting and control.

(1) Combination of cold and heat medication: This kind of treatment can be used for a variety of syndromes. For example, in the formula of *Banxiaxiexin* decoction, *Banxia* (Pinelliae Rhizoma) is pungent and warm and can be used to downbear counterflow to suppress vomiting, as a *Jun* herb. Dried ginger is very hot and can cure cold syndromes. *Huangqin* (Scutellariae Radix) and *Huanglian* (Picrorhizae Rhizoma) are bitter and cold and used against fevers as *Chen* herbs together with dried ginger. These four herbs can keep a balance between cold and heat.

(2) Compatibility complementary of *Yin* and *Yang*: Drugs are used for syndromes in deficiency of *Yin* or *Yang*, or both *Yin* and *Yang*. For example, in the formula of *Shibu* pills, *Lurong* (Cervi Pantotrichum Cornu) and *Fuzi* (Aconiti Lateralis Preparata Radix) are *Jun* herbs and have warming effects for *Yin* and *Yang*. *Wuweizi* (Schisandrae Fructus) is acidic and mild, that helps *Jun* herbs to nourish the kidney. *Shudi* (Rehmanniae Preparata Radix), *Shanzhuyu* (Corni Fructus) and *Shanyao* (Dioscorea opposita) can nourish kidney and essence, increase *Yang* from *Yin*, and as *Chen* herbs together with *Rougui*

(Cinnamomi Cortex) can tonify fire, assist *Yang* and conduct fire back to its origin.

(3) Compatibility of nutrition and purgation medication: It is used for syndromes with intermingled deficiency and excess. For instance, in *Liuwei Dihuang* pills, *Shudi* (Rehmanniae Preparata Radix) can nourish *Yin*, the kidneys and essence as a *Jun* herb. *Shanzhuyu* (Corni Fructus) nourishes the liver and kidneys. *Shanyao* (Dioscorea opposita) tonify spleen and *Yin*, and tonify kidney to control nocturnal emissions as *Chen* herbs with *Shanzhuyu*. The three *Jun* and *Chen* herbs are normally called "Three reinforcing herbs." *Zexie* (Alisma Rhizoma) can remove dampness and turbidity, and control the overnourishing of *Shudi*. *Danpi* (Moutan Cortex) can clear ministerial fire and restrain the warmness and astringent effects of *Shanzhuyu*. *Fuling* (Poria) can eliminate dampness and it can assist not only *Zexie* to remove turbidity of the kidneys but also *Shanyao* in transporting the essence of food and water. These three herbs are called "Three inducing herbs."

(4) Compatibility of promoting and controlling medication: It can be used normally with drugs for invigorating blood circulation and eliminating stasis as well as with astringents. For the formula of *Wenjing* decoction, *Wuzhuyu* (Evodiae Fructus) and *Guizhi* (Cinnamomi Ramulus), as *Jun* herbs, can warm the meridian to dissipate cold and smooth the circulation of blood. *Danggui* (Angelicae Sinensis Radix) and *Baishao* (Paeoniae Alba Radix) can nourish blood and astringe *Yin. Chuanxiong* (Ligustici Wallachii Rhizoma), *Danpi* (Moutan Cortex) and *Ejiao* (Corii Asini Colla) can promote the circulation of blood and nourish blood and *Yin*. The formula can be used for treatment of menstrual disorders, large amounts of bleeding and even infertility.

The complementary principles of the prescriptions were based on the *Yin* and *Yang* balance of human body, physiology and pathology, therapy and the characteristics of drugs, which have crucial clinical significance.

1.1.2.3 Systematic Theory of TCM Formulas The principles of holism, relevance, time-order and dynamics for TCM formulas are based on the idea of diagnosis and treatment, as well as systematic theory. With the aid of modern systematic theory, the TCM formula combines qualitative with quantitative, macro with micro, and global with local investigation. A new theory of the modern system of TCM formula compatibility will be developed.

(1) Holism principle of TCM formulas: The core idea of systems theory is the concept of holism. In TCM, the concept of holism lies in the fact that drugs with opposite and complementary functions can be used based on compatibility to achieve the best therapeutic effect. For example, in the formula of the *Qingkailing* injection, bile acid and deoxycholic acid, as *Jun* herbs, can clear heat, expel miasma, calm the heart and induce resuscitation. Using a Buffalo horn as a *Chen* herb has an effect of protecting the heart and eliminating fever. *Huangqin* (Scutellariae Radix), *Zhizi* (Garbeniae Fructus), *Jinyinhua*

(Lonicerae Flos), and *Banlangen* (Isatidis Radix) as *Zuo* herbs can clear heat and detoxify. *Zhenzhumu* (Margaritifera Usta Concha) used as a *Shi* herb can calm the nerves. As a result, the combination of these eight ingredients, focusing on the core issue of detoxication, can be used for clearing heat, expelling miasma, calming the heart and inducing resuscitation. In the clinic, this formula has been widely used against fever, coma, stroke, paralysis, unconsciousness, acute hepatitis, upper respiratory tract infection, pneumonia, cerebral thrombosis, and cerebral hemorrhage. This formula follows the principle of holism for TCM formula compatibility.

(2) Relevance principle of TCM formulas: Systematic approaches focus on the linkages between the elements and roles. Drug prescription fully reflects the composition and drug interactions. It is not simply a combination of drugs; rather, it is an optimized composition for the specific disease according to the interrelated functions of different causes, locations, the nature of the sickness, and its developing trend. Its diagnosis and treatment are based on the drug's nature, smell, category, effectiveness, and function to finally achieve the desired therapeutic purposes. For example, in the composition of *Liushen* pills, six ingredients—bezoar, realgar, musk, borneol, pearl myogenic, and *Chansu* (Venenum Bufonis)—have different functions individually; however, they interact with each other to achieve the overall function of clearing heat and detoxifying, reducing swelling, stopping pain, and promoting tissue regeneration.

(3) Time-order principle of TCM formulas: The time-order principles of TCM formulas can be found in the use of drugs following the roles of "*Jun*, *Chen*, *Zuo*, and *Shi*." Without the order of these roles in prescriptions, physicians easily lose their logical thinking processes and the prescription cannot achieve an optimized structure. In addition, the cooking of the drugs in prescription follows an order; otherwise, the best efficacy of the formula cannot be achieved. Principles such as "relieving the exterior syndrome and then catharizing," "supplying deficiency and then expelling excess," and "ascending the clear and descending the turbid" are typical time-order principles in TCM formulas.

(4) Dynamic principles of TCM formulas: In TCM theory, the balance (or imbalance) of *Yin* and *Yang* are regarded as an orderly (or disorderly) state. Also, the dynamic nature of the prescription is shown by regulating the imbalance between *Yin* and *Yang* with a medication. Furthermore, the prescription has its orderly structure. Change of composition or drugs leads to different functions and therapeutic effects, which might be suitable for other diseases. For instance, the formula of the *Mahuang* decoction for dispersing lung-*qi* and relieving asthma can also be used against exogenous cold. If plaster, ginger, and jujube are added, the formula is called a *Daqinglong* decoction, which can be used against heat, chills, fever, headache, pain, and irritability as well as cold. Without *Guizhi*, it is a formula called *Sanniu* decoction, which can be used against heavy nasal sound, weak voice, cough, and chest tightness.

1.2 KEY SCIENTIFIC ISSUES IN TCM MODERNIZATION

1.2.1 A Comprehensive Evaluation System under the Guidance of the Concept of Holism

For the diagnosis and treatment of disease, Western medicine mainly pays attention to "patient's disease," using subtypes to express the characteristics of the patients at different stages. However, TCM emphasizes "patients with disease," using different types of symptoms to differentiate the individual characteristics and adjusted prescriptions in clinic. Western medicine diagnosis and treatment tend to have a clear target lesion or organ, and use clinical biochemical laboratory or imaging test results to establish clear diagnostic criteria. In TCM, the diagnosis of symptoms lays comparatively far behind that of Western medicine in objectivity, standardization, and quantity. By reviewing the current TCM clinical approaches, we find it is necessary to combine ancient medical research with the current systematic research to achieve the unity of TCM treatment, dialectical characteristics. The evaluation of human health should be more comprehensive and systematic. A feasible way to integrate Eastern and Western treatment is to coordinate systematic concepts and methods based on quantitative evaluation, TCM (syndrome, behavior, and life quality assessment), Western biochemical pathology (clinical biochemistry, imaging, pathologic anatomy, etc.) and systems biology (genomics, proteomics, metabonomics, etc.) evaluation to establish a comprehensive evaluation indicator system.

1.2.2 Key Scientific Issues That Need to Be Addressed in Modern TCM Research

The traditional theories of characteristics, meridian, and compatibility only show some intuitive and analogism instructions on the efficacy of the prescriptions, which cannot reveal the substance, process of the activity, or the exact mechanisms. The traditional TCM theories cannot indicate the material basis for the mechanism and pathology. It has a great limitation in clinical use although it can guide the clinical application of formulas.

TCM formulas can reflect the concepts of holism and syndrome differentiation and treatment. There are complicated interactions between the active components in the formulas and human body. Therefore, the research on the mechanisms and compatibility of herbs must reflect the interaction between two complex systems of TCM and human body, and create a higher system, based on the holistic features of complex prescriptions.

1.2.2.1 Material Basis of TCM Formulas In China, the material basis of TCM formulas is studied through separating active chemical components from formulas with the model of Western medicine. For a single herb, active ingredients are still unclear. There are three main reasons for this. (1) The chemical

composition of TCM formula is very complicated, and a single herb may have a variety of chemicals. Take licorice as an example; to the best of our knowledge so far, it contains 18 types of flavonoids and terpenoids, 22 species and 14 amino acids, alkaloid, coumarin, cinnamic aldehyde, cellulose, starch, salt and 60 other chemicals. (2) Based on integrity of the prescription, clinical efficacy is evaluated with the overall effect on human body. The multiple and complex targets cannot be easily related to their exact active components. (3) Other issues, such as multiple use of one prescription, adjusted complex prescriptions and syndrome differentiation and treatment, make it difficult to reveal the active chemicals by traditional pharmacological methods.

To illustrate the complexity and the integrity of TCM formulas, it is necessary to add a new concept between raw herbs and effective compounds. That is "effective component groups of TCM formulas."[3]

The concept of "effective component groups of TCM formulas" refers to the fact that there is a large group of compounds with similar chemical properties. A complicated TCM formula can be divided into several effective component groups (there may be hundreds of chemical components) to illustrate the chemical substance basis of the compatibility of TCM formula with the aid of pharmacodynamics. As a result, we put forward a research strategy including using modern separation methods to extract effective component groups, fingerprint for qualification, indicate components for quantification, and identify the integrity of effective component groups and pharmacodynamics. Based on modern separation methods, herbs in a formula are divided into different effective component groups such as volatile oils, alkaloids, and flavones, or smaller classes. The qualification of effective chemicals can be achieved using the instruments of high performance liquid chromatography (HPLC), capillary electrophoresis (CE), HPLC coupled with mass spectrometry (HPLC-MS), CE coupled with MS (CE-MS) and chromatographic fingerprints. Several effective or indicator components are selected for quantification. Integrated with the pharmacologic activity, these chemicals are further investigated on mechanisms at four different levels (animal, organs, cells, and subcells, and molecular biology). With the information of the activity and compatibility of these component groups and compounds in *Jun*, *Chen*, *Zuo*, and *Shi* herbs, the material basis of TCM formulas can be explained clearly.

Therefore, the research on TCM formulas should start from the concepts of holism and systems biology. The suitable clinical cases are selected based on clinical efficacy. The quantitative pharmacologic models are established and one or more observational indicators are used to evaluate the activity. Botanical chemical separation methods are employed for extraction and separation. Closely integrated with pharmacologic tests, the chemicals and the relationship among activity, quality, quantity and size can be scientifically investigated. In research principles, the methods of integration of traditional TCM theories and modern science and technology, in vivo and in vitro evaluation, organic and inorganic chemicals, and static and dynamic analysis should be established.

1.2.2.2 Quality Evaluation System The quality of TCM formulas should be illustrated since they have complicated components and treatment. The modern quality evaluation system of TCM preparations should follow the material basis of herbs with effective component groups. The methods of fingerprints and quantification of multiple indicator components not only have the feature of integration but also show precise qualification and quantification, which is a suitable model for quality control and evaluation of TCM herbs.

(1) Qualification with fingerprints: For each effective component group, modern separation and analysis methods such as HPLC are used to get chromatogram containing dozens of chemical components. HPLC-MS (MS), CE-MS and other advanced identification methods are combined to identify the chemical constituents. The molecular structures of the peaks can possibly be studied by MS/MS. HPLC chromatogram can be considered as a fingerprint. Further investigation can be carried out with the comparison of fingerprints of some effective component groups and the original single herb. Novel compounds can be found and the structures and pharmacology of these compounds can be further investigated.

(2) Quantification with indicator components: The fingerprint of each effective component group is analyzed and those effective compounds or special compounds with high content are selected as indicator compounds. In some cases, effective and special compounds are the same. The quantification of indicator compounds must be considered the relationship of their representation and analytical technology for multicomponent quantification.

(3) Multiple types of information in fingerprints: The fingerprint of TCM herbs includes information on chemistry and efficacy. Based on literature describing the chemistry and pharmacology of each herb in TCM formulas and combined with the technological process, types of compounds can be elucidated. By modern separation methods (such as supercritical extraction, macroporous resin column, countercurrent chromatography, and a variety of preparative chromatography), the chemicals can be separated into individual chemical parts (compound groups). A variety of pharmacological experiments can be carried out to confirm the effective component groups and compounds as well as their ratios in content. With the relationship of the variation of compounds (type, number, and content) and efficacy (from experiment or clinic), the fingerprint contains information on chemistry and efficacy. For example, certain fingerprints can indicate pharmacology and clinical efficacy. Therefore, efficacy and quality assurance can be achieved.

1.2.2.3 Evaluation of the Efficiency and Safety of TCM Formulas As remedies used in clinical situations, TCM formulas must be compliant with the primary requirement of "safe, effective, stable, and controllable." Due to the lack of regulation and convincing scientific data to evaluate on the

effectiveness and safety for most of the TCM, new technology and methods are developed at a slow pace. TCM has had a difficult time to get approved and accepted by the international medical world; therefore, it is important to establish a complete evaluation system on its effectiveness and safety. Evaluation of efficacy on TCM formulas refers to the following two aspects: preclinical and clinical evaluation.

(1) Preclinical evaluation on TCM formulas: At present, animal models with similar clinical syndromes are often used for pharmacological investigation. It is, however, difficult to make an animal compliant with the clinical syndrome as TCM defines, especially for the more complex syndromes. Thus, it is necessary to develop some animal model corresponding to both symptom and disease. Furthermore, the final activity is caused by the compatibility of each herb in the prescription and the traditional investigation method based on single factor analysis cannot obtain entire and systematic information of mechanism. Therefore, the mechanism of TCM formulas is evaluated based on the overall effect.

(2) Clinical evaluation on TCM formulas: In ancient times, the classic TCM books recorded the procedure of the diagnosis and treatment in form of medicinal cases, paying attention to the standard of improvement of clinical syndrome. In recent years, TCM practitioners pay attention to both subjective and objective syndromes. The disease names used in Western medicine are also applied to TCM. Meanwhile, laboratory and physical examinations, especially the former, are also employed to indicate the effect and scientific nature, combined with the evaluation criteria of Western medicine or criteria self-developed by the researchers. However, the evaluation of TCM syndrome is ignored.

Therefore, the evaluation of TCM efficacy should be guided by the theory of syndrome differentiation and treatment based on the concept of holism, as well as by the recent achievement of modern medicine and life sciences. Based on clinical efficacy and animal experiments, comprehensive evaluation system should be established with quantification evaluation indices in TCM (such as syndromology, ethnology, and life quality), pathological and biochemical indices in Western medicine (clinical biochemistry, iconography, anatomy, etc.), as well as the evaluation indices in systems biology (genomics, proteomics, metabonomics, etc.).

At present, toxicity of TCM formulas is mainly evaluated by experiments of effective components, pharmacology and toxicology. For pharmacological and toxicological study, the routine methods for chemical drugs are often used. However, most chemical drugs contain only one single compound, different from TCM formulas. Therefore, different methods and analysis tools should be developed and utilized for evaluation of the safety of TCM formulas.

1.2.3 Main Ideas and Progress of Research on TCM Formulas in Recent Years

1.2.3.1 Compatibility of Effective Component Groups and the Relationship Between Effective Component Groups and Efficacy The compatibility of effective component groups has been widely studied by elucidating the material basis of TCM formulas using advanced techniques. Moreover, the relationships of compatibility, chemical components, and pharmacological effects can be investigated.

In the 1990s, a novel research system for TCM formulas was proposed which can be summarized as "One combination, two basic elucidations, three chemical layers and four pharmacological levels" ("1-2-3-4" system).[4, 5] "One combination" refers to the integration of chemical components and pharmacological investigation. "Two basic elucidations" means the effective components should be elucidated, as well as the efficacy and the mechanism of TCM formula. Based on "three chemical layers" (formula and raw herbs, effective component groups and effective compound mixtures), the research with "four pharmacological levers" (animal, tissues and organs, cells and subcells, molecular biology) can be carried out to illustrate the chemical basis of the compatibility theory of TCM formulas. This research system lays critically on the material basis of TCM formulas, emphasizes the interaction of ancient compatibility theory and modern pharmacological theory and the combination of substance analysis and observation on the pharmacological effect. It has been widely applied to TCM formulas.

New techniques and methods have to be developed to elucidate the effect and relationship of active substances in TCM formulas. The methodology of "effective component groups–efficacy" (analysis of the correlation between effective component groups and efficacy) is one of these effective models combining chemistry, pharmacodynamics, and informatics following the idea that: Under the guidance of TCM theory, the entire complex prescription is properly divided into n effective component groups, global chemome are presented with matrix $n \times k$ where n vectors represent n effective component groups and each vector element represents the number of compounds (k) in each component group (amended with 0 if insufficient). Thus there are $n \times k$ compounds in the global chemome. In the matrix of models, p pharmacological models are used including the animal, tissues and organs, cells and subcells, and systems biology (genomics, proteomics, etc.), each vector element represents the number of indexes (q) in each model (amended with 0 if insufficient). From the matrix ($n \times k$) of global chemome and the matrix ($p \times q$) of models, effective chemome ($m \times j$ matrix) can be obtained based on bioinformatics and experimental research, which is the objective of chemomics. Meanwhile, both the number of active component groups and effective compounds in the effective chemome are smaller than those in the global chemome. Therefore, the conditions of $m < n$ and $j < k$ are required, and irrelative

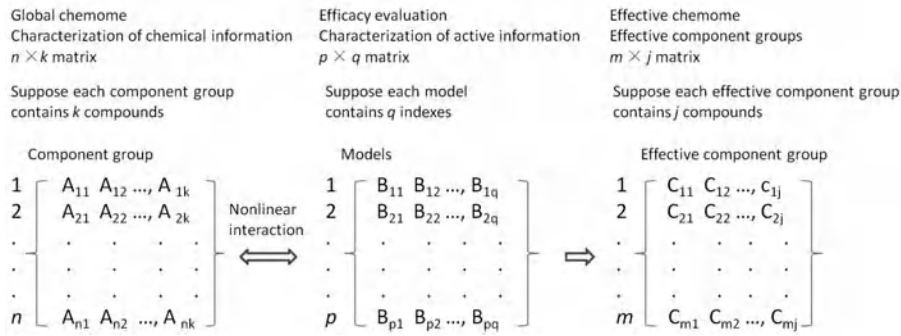

Fig. 1.2 Method to study the relationship between effective chemome and efficacy of TCM formula. A: compounds of component group; B: indicators of model; C: compounds of effective component group.

components are ejected from the global chemome. Chemomics provides a new "omics" approach to develop modernized composite medicine, where the phytochemical composition of a herbal formula with demonstrated clinical efficacy is regarded as a global chemome, which can be simplified successively through bioactivity-guided screening to achieve an optimized chemome with minimal phytochemical composition for further drug development, while maintaining its curative effect for a specific disease. This technique routine is shown as Fig. 1.2.

The chemical information of TCM formulas and effects can be characterized by fingerprints and quantification of multiple indicator compounds. The methodology of "effective component groups–efficacy" in TCM requires pharmaceutical informatics to identify the effective component groups related to efficacy and clarify the roles of fingerprint peaks. This methodology is different from relation investigation between quantification and structure of drugs in Western medicine system. A fingerprint chromatogram of TCM herbs focuses on the characteristics of active compound groups rather than one or more compounds. Moreover, it is different from the methodology of "chromatogram–efficacy," which confirms the relation between the entire fingerprint chromatogram and efficacy, rather than the relationship between the compounds or groups and the efficacy.

1.2.3.2 Serum Pharmacology and Serum Pharmacochemistry in TCM Drugs The method of serum pharmacology, developed by a Japanese expert, can be defined as an in vitro pharmacological test with serum containing medicinal components after oral administration of TCM preparations. In China, some researchers also investigated the methodology of serum pharmacology, such as the relationship between the activity and in vivo concentration, administration regime, dosage of serum containing medicinal components, time for blood collection, storage condition and the

impact of serum inactivation on the efficacy. For instance, the *Shuanghuanglian* formula showed an obvious effect on anti-RSV based on the method of serum pharmacology.[6]

For traditional in vitro analytical methods, it is difficult to identify and test the pharmacological effect of a number of compounds altogether, and the state analysis on these compounds cannot reveal the dynastic metabolism. However, serum pharmacochemistry focuses on the absorbed components including the metabolites. Through investigation of the components and their metabolites, active components are able to be identified. After the establishment of the fingerprint of serum chemical components and the determination of pharmacological effects, the analysis on active components can be carried out to find the relative active compounds. Several Chinese experts set up a HPLC fingerprint of rat serum after oral administration of *Liuwei Dihuang* pills. Eleven compounds were found in rat serum, four of which were new metabolites and the other seven of which were compounds from the original preparation.

1.2.3.3 Other Research Methods The main idea of the "*fenziyaoxing*" and "*yaoxiaotuanyaoxing*" hypotheses[46] is that the special structures of molecules are responsible for their bioactivity. Drug components have a variety of molecules and effective groups, leading to multiple targets.[44–49]

The theory of "TCM molecularomics" argues that the TCM formulas affect the integration of signal molecules with the integration of chemical components, leading to recovery of the signal molecular net and treatment of TCM syndromes.[47] It starts from a chart of essential syndromes with "syndrome–pathway of effect–signal molecules–molecular net" and investigates the influence of complex prescriptions, effective component groups, and effective compounds to the syndrome. The method is effective to some extent; however, there is no exact conclusion for recognition of signal molecules of TCM syndromes.

The theory of "histological pharmacology of TCM formulas" studies the relationship between the spectrum of main components and their concentrations in the target pathological tissues and the pathological morphology of target tissues as well as the efficacy.

The theory of "metabonomics of TCM formulas" investigates the therapeutic mechanism, targets, criteria of efficacy, toxic models, biological makers, and so on, based on the theory of metabonomics.

The theory of "differetial pharmacodynamic serum spectrum" establishes profiles of chemical compounds in serum based on the maximum and minimum efficacy by the chromatographic technique, and then separates the active components by the differentiation method. The components are tested in the animal models with identical TCM syndromes and are identified with further experiments until the active components are confirmed.

The limitations for the aforementioned strategies, however, underline the fact that there are no efficient experimental technical platforms under the

molecular level, and the concept of holism of TCM cannot be systematically expressed. Systems biology from the post-genomic epoch can be implemented into the research on TCM.

1.3 DEVELOPMENT OF SYSTEMS BIOLOGY

1.3.1 Proposal of Systems Biology

Systems biology is a new branch in the field of life sciences, which was first proposed by an American scientist, Leroy Hood, one of the pioneers for the Human Genome Project (HGP).[7] According to Hood's definition, systems biology investigates all the compositions (gene, mRNA, protein, etc.) in the biological system, and the interactions of these compositions in certain circumstances.[8] Therefore, different from the original experimental biology focusing on individual genes and proteins, systems biology focuses on global investigations of interactions among many genes, proteins and compositions. Hood pointed out that "systems biology would be the core driving force for medicine and biology in 21st century." Hood and two other scientists founded the first systems biology institute at the end of 1999. Thereafter, systems biology was gradually accepted by scientists, and aroused the attention by a flood of experts in the external field of biological research. In March 2002, a special issue for systems biology was published in the journal *Science*.

1.3.2 Technological Platforms of Systems Biology

With the development of the genomic sequencing, scientists have focused their studies on functional genes. A number of testing platforms, including genomics, transcriptomics, proteomics, metabonomics, interactomics, and phenomics, were developed to study the gene expression and regulation, the structure and function of genes and their metabolites, the interactions between molecules, and the correlation between genotype and phenotype at the genomics level.

(1) Genomics: Genomics includes technical platforms such as gene sequencing, genotyping based on single nucleotide polymorphism (SNP), and epigenomics. Gene sequencing reveals the entire genetic code in each organism, which is the basis of systems biology. Genotyping is now accurate at the level of single ribonucleotide. The haplotype omics research was initiated by the U.S. National Institutes of Health (NIH) in 2001 for determining the SNP of different ethnic groups, which illustrated the susceptivity for diseases, drug answering, and other individual differences for characteristics and behaviors, crucial for development of prospective medicine and individualized medicine.

(2) Transcriptomics: Transcriptomics investigates gene expression profiling at the mRNA level, providing a high-throughput and parallel tool on the

genomics level for determining mRNA abundance using DNA chips. DNA chips are used to determine the gene expression profiling for different cells and tissues, compare the expression profiling between diseased and normal tissues, and determine gene expression profiling for cells at different differentiation and development stages as well as in different environmental conditions. These results are not only adopted to investigate novel gene functions and signal transduction systems and the response to environmental factors in cells and organisms, but are also used to identify and validate the target genes for new medicines.

(3) Proteomics: Proteomics studies the structure, space-time distribution, and function for proteins in cells and organisms, including three branches: proteomics, functional proteomics, and structural proteomics. In recent years, development of two-dimensional LC-MS and protein chip-MS systems has further extended the scope of proteomics. Expression proteomics has been applied to discover and identify protein-labeled molecules at certain physiological and pathological stages, and the protein-labeled molecules can be used in drug discovery, clinical diagnosis, and disease monitoring.

(4) Metabonomics: Metabonomics is another important branch of systems biology besides genomics, transcriptomics, and proteomics. The concept of metabonomics was first derived from metabolic profiling.[9] The Nicholson group proposed the metabonomics method in 1999, to investigate metabolite changes in organisms under external stimulations, focusing on the changes of small metabolites with molecular weight of less than 1,000 in the metabolic cycle, by response to external stimulations and genetic modifications,[10] which has been successfully used in disease diagnosis and medicine screening. Fiehnfirst proposed the concept of metabolomics[11] and correlated the plant metabolites with gene functions. Since then, many studies on plant metabolomics have been conducted, and the two main branches of metabolomics were developed. Fiehn, Allen, Nielsen, Oliver, Villas-Boas, and others further developed some definitions of metabolomics, which are accepted worldwide.[12–16] The first level is target analysis, a quantitative study of the substrate and/or products for a target protein; the second level is metabolic profiling, quantitative analysis of relationships between preinstalled metabolites and structures and characteristics in certain metabolic processes, using specialized techniques; the third level is metabolic fingerprinting, qualitative or semi-quantitative analysis of all intracellular and extracellular metabolites; the fourth level is metabolomics/metabonomics, the quantitative study of all metabolites in organisms (which is difficult to achieve).

Metabonomics has been extensively used for the investigation of drug toxicities and mechanisms,[17–21] microorganisms and plants,[22] disease diagnosis and animal models,[23–26] and gene functions.[27] As a science branch, metabonomics has been developed quickly and applied to many other research aspects including safety evaluation for TCM compositions,[28] metabolic analysis for

medicines,[29, 30] toxicogenomics,[31, 32] nutrigenomics,[33] pharmacometabonomics,[34–36] systems of absorption, distribution, metabolism, excretion, and toxicity (systems- ADME/Tox),[37–39] and so on.

(5) Phenomics: Nowadays, phenomics studies are mainly at the cellular level, since cells, as the basic elements of vital activities, have the major characteristics of live organisms, such as signal transduction, space-time organization, breeding, homeostasis, and the response and adaption to the environmental changes. Cells can be used for studies at a high-throughput genome-wide level.

The major phenomics platforms are cell chips and tissue chips. Cell chips carry out various genetic manipulations for each gene at the genome-wide level, including gene knockout, gene transfer, gene suppression, gene activation, constructing corresponding cell lines, and high-throughput phenotype investigation. Tissue chips are mainly used in research on high-throughput pharmacology, toxicology, and pathology. Phenomics is the terminal of the omics platform for systems biology, implementing the whole process for genome sequence to basic vital activities by genomics, transcriptomics, proteomics, metabonomics, interactomics, and phenomics. Systematic investigations from genomics to phenomics have been conducted in the glycometabolism of colibacillus and yeast. More and more cell chips have been used for the discovery of new medicines and medicine targets and the evaluation of new medicines, which enables development of new drugs from a high-throughput to high-content base.

(6) Computational biology: Integration of experimental and computational results is necessary for explanation and quantitative forecasting in complex biological systems. Computational biology, including knowledge discovery and analysis based on simulation, provides a strong basis for proposing scientific questions, using modeling and theory exploration. Knowledge discovery is also called data exploitation, which extracts the implied law from the huge amount of data and information produced by each experimental platform of systems biology, and forms hypotheses. Analyses based on simulations predict the biologic experiments in vitro and in vivo, using a hypothesis from computational validation.

Knowledge discovery has been widely used in bioinformatics, as in the prediction of introns, exons, and the stereo chemical structures for encoded proteins based on the nucleotide sequence and gene regulation network by gene expression profiling. These studies are based on heuristic and statistics differential analytical methods. Analysis based on simulation goes in for predicting the system kinetics, and the production and accumulation for a large amount of quantitative data from high-throughput platform provide a solid basis for investigations based on simulation. Computational modeling and analysis provide useful biologic cues and prediction, such as bifurcate analysis for cell cycles, metabolic analysis, and comparison research on biologic tank loop homeostasis. Drug research and development is also an application

hotspot in computational biology. The computational modeling for virtual screening and molecule design, ADME, and studies of drug toxicity is an important tool for research and development of drugs.

1.4 CHEMOMICS INTEGRATED SYSTEMS BIOLOGY

In recent years, tools of systems biology have been applied in TCM investigations by many scholars.[42, 43] However, systems biology is just taken as a tool to illustrate some theories and regulations of TCM, and the disadvantages are the lack of globalization and systematicness. However, it still has limits explaining some of the theories and rules of TCM, and lacks integration and system. In other countries, significant results of TCM research using biology systems have been explored. However, because they are limited in their understanding of TCM theory, foreign scholars cannot get the essence to the full extent. Understanding the philosophy and scientific theories of TCM in the view of modern science is persuasive. Investigations in systems biology range from "compositions" to "functions."[7] Systems biology is unceasingly integrated and developed with the development of technologies. Leroy Hood first integrated genomics, proteomics, and numerology into integrative systems biology. Nicholson proposed the global systems biology by integrating genomics, proteomics, and metabonomics, based on establishment of metabonomics techniques.[40] More omics have been integrated into systems biology studies in recent years.

Systems biology uses integrative concepts and methods to elucidate the physiology, pathology, and dynamic rules of complex human systems. However, limited to the descriptions for biological internal system (response system) information, current systems biology cannot completely explain externally disturbing information (information such as the intervening chemical information of drugs).[41] When the external intervening system is relatively simple, for example, a single compound, it is summarized as disturbance point, which is the equivalent of action of "point to system." But when the external intervening system is a complicated system, for example, TCM formulas, the current systems biology is not yet compatible with the integrated research for the corresponding TCM formulas. Although it can provide methods for quantitative characterization of the syndromes and the biological effects of drugs (efficacy and safety evaluation), which may solve the problem of expressing a "syndrome," there is no integrated information of "formula" (drug intervening system). Thus it is difficult to reveal the internal relationship between the two systems (the biological response system and the complicated substance system of TCM formula).

Based on our studies on TCM and systems biology, we have proposed a number of new methods of systems biology, suitable for TCM research, called "chemomics integrated systems biology."[42]

1.4.1 Definition of Chemomics Integrated Systems Biology

Investigation of the mechanisms of TCM formulas and evaluation of compatibility should consider the integrity of the TCM effect, which in nature manifests the interaction between the two complex systems, TCM (external system) and human (internal system). Only under the guidance of TCM theories and in combination with modern scientific technologies can the interactions between these two systems illustrate TCM compatibility theories, mechanisms, and the substance basis of efficacy. To achieve this goal, two things should be considered: one is the global characteristics for human response system (internal system) during the interference of TCM, and the other is the compatible relationship of TCM formula (external system), which are the problems that should be solved by systems biology and chemomics, respectively. The interactions can be revealed by combining the two systems. The investigations of TCM require establishing a "system to system" (S2S) method corresponding to its characteristics. Therefore, we have proposed that chemomics integrated systems biology (global systems biology) should be developed based on current systems biology (Fig. 1.3). Global systems biology represents the compositions and interactions of drug interference systems using chemomics, delineates the response from biological systems, and further analyzes the interactions between the two systems. It reveals the correlations between the changes in chemomics and space-time response of the biological system (the correlation between formula and syndrome). Moreover, the biological system is studied not only with systems biology information at the molecular level (such as genomics, proteomics, metabonomics, etc.), but also with the information of pharmacology and safety evaluation at the level of animal experiments, organ tissues, cells, subcells, and molecular biology, respectively.

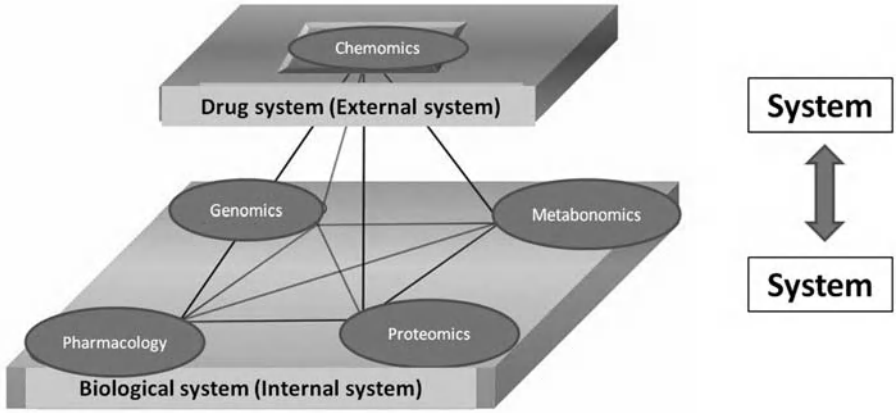

Fig. 1.3 Chemomics integrated systems biology.

1.4.2 Research Mode of Chemomics Integrated Systems Biology

The research system of chemomics integrated systems biology includes the investigation of changes in pharmacodynamic substances, transmigration laws during the process of the drug compatibility, and the component compatibility for prescription by chemomics. It explains rationality on the basis of substances and investigates the corresponding changes of pharmacodynamics and biological effects with the changes of pharmacodynamic substances during the compatibility process of TCM formulas, by chemomics integrated systems biology and the combination of chemomics integrated systems biology with global animals, organ tissues, cells, subcells, and pharmacodynamic studies at the molecular level. The advantages of TCM formulas in efficacy enhancement and toxicity reduction are also being studied. Moreover, the underlying pharmacodynamic mechanisms of TCM formula are investigated, and an evaluation system for TCM is established. The global technical route is shown in Fig. 1.4.

1.4.2.1 Definition of Chemomics Chemomics is an approach used to identify and quantify all of the chemical components in the chemome and to

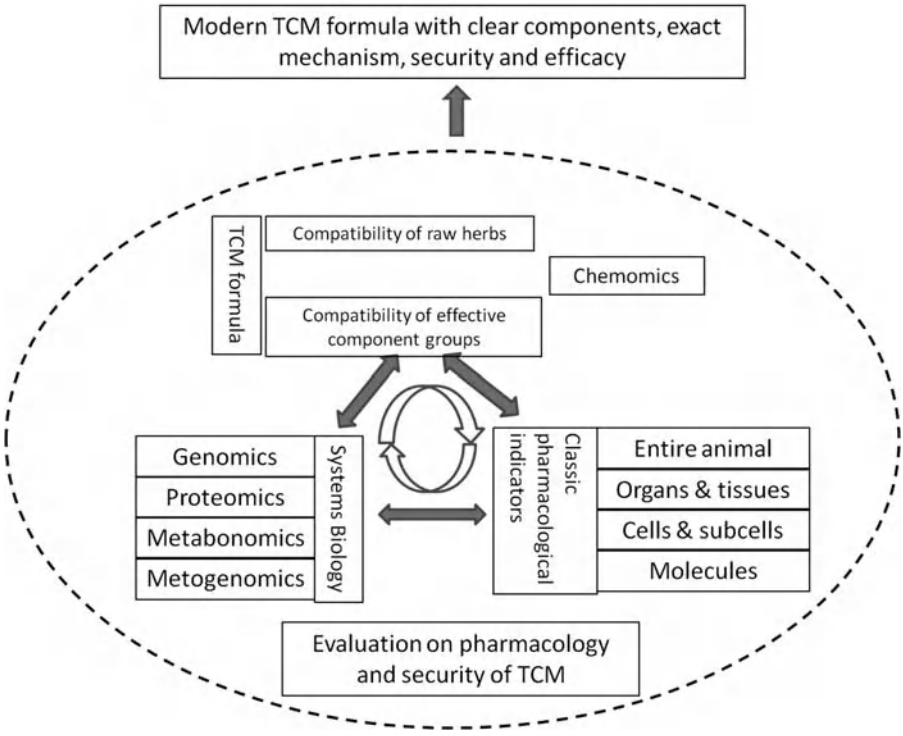

Fig. 1.4 Technical route for applying global systems biology to TCM formula investigation.

understand the relationships between the changes in the chemome and the time-related responses of biological systems, whereas the chemome is the aggregate of all the chemical components among a specified external intervention system interacting with the biological system.[43] For chemomics of TCM formulas, following the compatibility theories of TCM formulas, a strategy derives from system to part (top-down) and increases step by step, by correlation analysis of chemical information flow and multiparameter biological information flow for chemomics at different levels, to identify the composition of effective chemomes and to reveal the essential pharmacodynamic compositions from the global chemomics, and to illustrate the compatibility theories of TCM formulas based on raw materials, component groups, and compounds. Another strategy is the bottom-up mode. Raw herbs can be also considered as a global chemome, different compositions are screened, and the effective component groups are then recombined as a complexformula, which is the bottom-up mode of TCM chemomics. For a formula, the research mode of chemomics from top to down is recommended, which is derived from TCM formula with global effects and investigates the compatibility of a chemome under the condition of keeping the global compatibility of the formula, using the top-down mode and increasing step by step, by revealing the correlation between formula and effectiveness, to find an effective chemome and effective component groups from a global formula. We think that, on one hand, the effective and reasonable TCM formula compatibility should be retained and explained fully; on the other hand, during the development of compatibility of TCM component groups, the global idea from "top to down" should be emphasized to better manifest the characteristics of TCM formulas, to more easily optimize the compatibility under the guidance of TCM theories, and to reveal the mechanisms of TCM formulas. The two application modes of chemomics are shown in Fig. 1.5.

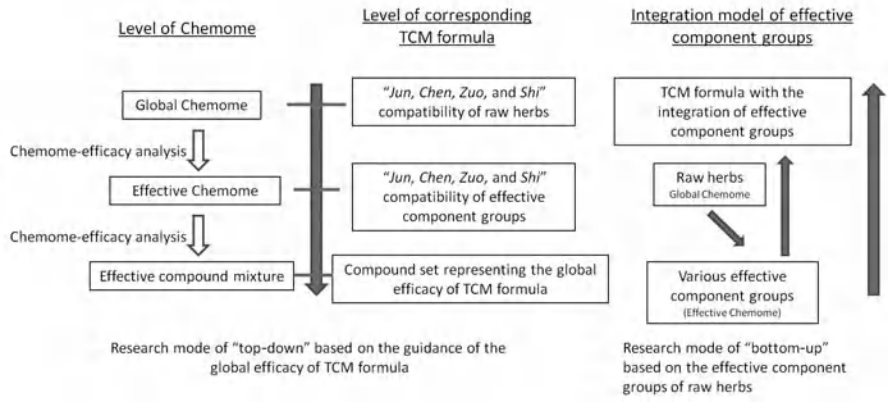

Fig. 1.5 Two application modes of chemomics.

1.4.2.2 Key Technologies of Chemomics Integrated Systems Biology

The key technologies of chemomics integrated systems biology include information access and processing technologies.

In systems biology, including all types of omics research, a great amount of information is obtained by high-throughput information access technologies that are suitable for development of chemomics, genomics, proteomics, and metabonomics, such as new methods like LC-MS/MS and ultra-performance liquid chromatography coupled with quadrupole-timeofflight MS (UPLC-Q/TOF), biological chips with the application of construction analysis for enterobacteria flora (metagenomics research), and the key technologies for TCM formula safety evaluation.

Information integration technologies include the information integrated in one system, as well as between two systems. It integrates bioinformatics and chemometrics technologies and develops correlation analysis, multisource fusion technology, the discovery technology for causality and the theory and method of multivariate statistics, including regression analysis methods of principal component regression (PCR), partial least squares (PLS), and support vector regression (SVR), as well as the classification methods of linear discriminant analysis (LDA), partial least squares discriminant analysis (PLS-DA) and support vector clustering (SVC), and the network theories that construct the interaction mode for two systems.

1.4.2.3 Application of Chemomics to the Compatibility of TCM Formulas

TCM formula compatibility is crucial for TCM's global view, syndrome differentiation, and treatment theories. Chemomics can reveal the compatibility rules of TCM *materia medica* and component groups based on the substances and mechanisms investigation at the molecular level, and TCM formula compatibility investigation can be carried out on formulas and syndrome correspondence.

(1) Combining the formula and syndrome correspondence with TCM formula compatibility is a key point in explaining the theories of TCM formula compatibility. Take the formula of the *Sijunzi* decoction for treatment of splenasthenic syndrome as an example. It is composed of four raw herbs (global chemomics), *Renshen* (Ginseng Radix), *Baizhu* (Atractylodis Macrocephalae Rhizoma), *Fuling* (Poria) and *Gancao* (Glycyrrhizae Radix). Based on the "top-down" mode and the strategy of global chemome, the component group compatibility research of the five classes of effective composition groups has been carried out, which are saponins (mainly derived from *Renshen* and *Gancao*), volatile oils (mainly derived from *Baizhu*), polysaccharides (mainly derived from *Fuling*, and partly from *Renshen*, *Baizhu*, and *Gancao*), triterpenes (mainly derived from *Fuling* and *Gancao*) and flavonoids (mainly derived from *Gancao*) are the most effective. With the pharmacodynamic model of splenasthenic syndrome, metabonomics, and metagenomics, the substance basis of splenasthenic syndrome was revealed; the changing law of the splenasthenic

syndrome models before and after interference by the material media and component group compatibility were investigated; and compatibility theories for the *Jun* (*Renshen*), *Chen* (*Baizhu*), *Zuo* (*Fuling*), and *Shi* (*Gancao*) herbs in the *Sijunzi* decoction were validated from the constructional relationship of effective substances and pharmacological correlation of integrated systems biology parameters. The essence of the TCM pharmacodynamic evaluation is how to establish the correlation between the syndrome and the drug's therapeutic effect. The global therapeutic effect of TCM formula is difficult to see manifested by a single target or combination of a few targets, whereas systems biology can provide a feasible method for evaluation of the treatment of syndrome fromTCM formulas at a global level.

(2) Understanding the *Jun*, *Chen*, *Zuo*, and *Shi* compatibility of raw herbs and component groups is crucial for development of the compatibility theories of TCM formulas. Traditional TCM formulas are composed of raw herbs according to some formula principles, and the *Jun*, *Chen*, *Zuo*, and *Shi* compatibility theories also refer to the raw herb compatibility. Moreover, the compatibility of effective components has been applied to research and development of compound formulas. Our chemomics study of *Qingkailing* injection suggested that, following the anti-cerebral ischemia cascade reaction and using group-effect analysis, four classes of effective component groups that play important roles in the compound are found to be composed of eight raw herbs, and a new TCM formula with compatibility of effective component groups has been successfully developed.[43] Component TCM formulas, as a new branch of TCM formulas, will definitely strengthen the theory and law investigation of component compatibility of TCM formulas. According to the opinion of chemomics experts, TCM formula is also a global chemome and can be regarded as the compatibility of several related subchemomes. Subchemome has two main styles: raw materials and component groups that represent some characteristics of formulas (also called effective component groups). Therefore, effective component group compatibility is consistent with the raw material compatibility. In the investigation of effective component compatibility for *Qingkailing* injection, the *Jun*, *Chen*, and *Zuo* correlation among the four effective component groups have been validated, which suggests that the compatibility also exists among the effective component groups.

1.4.2.4 Application of Global Systems Biology to the Action Mechanisms of TCM Formulas

1.4.2.4.1 Evaluation of the Pharmacological Model for TCM Formulas The premise of the study of the pharmacological mechanism is to elect the appropriate model of TCM formulas. However, it is difficult to evaluate the accordance between the pharmacological model of TCM theory and the characteristics of TCM formulas. The global systems biology and the theory of TCM share many features. Therefore, it is more reasonable to evaluate the pharmacological models of TCM formulas. The systematic relation and

rationality between selected models and the clinical syndromes can be evaluated under the guidance of TCM theory. The pharmacodynamic models and parameters of TCM formulas can be chosen combining the animal, organ tissues, cells, pharmacological evaluation in molecular level, and the evaluation parameters of systems biology together, to evaluate the correlation and rationality for the chosen model and clinical syndrome. Besides the animal model, high-throughput cell models can be explored, particularly stem cell models.

The past pharmacological studies in TCM utilized many evaluation methods and standards of Western drug pharmacology. Systems biology characterizes various pathologic and physiologic stages using the multilevel network style integrated by genomics, proteomics and metabonomics, to reveal the internal principle of the biological response system. Therefore, the relative parameters of systems biology may possibly be used for pharmacological evaluation. Finally, a comprehensive evaluation system can be established from the evaluation parameters for TCM quantification (semiotic symptomatics behavioristic, and life quality evaluation), pathological biochemistry parameters for Western medicine (clinical biochemistry, imaging, and pathologic dissection) and systems biology (genomics, proteomics, and metabonomics).

1.4.2.4.2 Safety Evaluation of TCM Formulas Based on Systems Biology

Due to the complexity of TCM formulas, the chemical composition is not clear, and neither are the mechanisms of toxicity and toxic targets. Thus it is difficult to evaluate the safety. The development of systems biology, in particular metabonomics and biological chip technologies, does not depend much on previous experience. By combining the evaluation of acute toxicity and long-term toxicity, the safety evaluation for *Liushen* pills using chemomics, metabonomics, and toxicokinetics was carried out, in order to reveal the major acute toxic injuries of Venenum Bufonis for the heart, as well as the long-term toxicity of realgar for liver. Our results have demonstrated that *Liushen* pills' compound compatibility has an effect to reduce toxicity and enhance the drug efficacy. The "dosage–effect–time–toxicity" relationship of *Liushen* pills was studied, and the safety and effective dosages were identified.

1.5 RESEARCH STRATEGY AND PROSPECTIVE OF SYSTEMS BIOLOGY IN TCM

1.5.1 Background of Systems Biology in TCM

In systems biology, systematic analysis is performed by integrating various omics information on genes, cells, tissues, and the body at different levels, which is a great tool for studying complex biological systems. TCM is also a complex system. For traditional TCM theories, the most idiomatical characteristics are "global," "dynamic," and "dialectical," which are all consistent with systems biology. New ideas and methods from systems biology are also utilized

in TCM, which facilitates modernization of TCM.[4] The development of TCM systems biology is important for life sciences. The famous scientist Xueseng Qian pointed out that "after we really understand and summarize the theories and practice of TCM, the whole modern scientific technologies will be influenced, and scientific revolution will be evoked."

1.5.2 Basic Strategy for TCM Systems Biology

Systems biology developed along with modern life sciences, which has been used in Western modern medicine. As forementioned previously, given the similarities between methods for systems biology and TCM, we can assimilate these two. However, due to the huge differences between Chinese and Western medicine systems, research of systems biology in TCM cannot be "borrow and used." We should follow the laws of TCM when using systems biology methods. Based on our previous study, the following two basic strategies or principles of systems biology can be applied in TCM.

1.5.2.1 "System to System" Research Strategy of Formula and Syndrome Correspondence "Formula and syndrome correspondence" is the basic principle of prescription research and clinic application of TCM and is crucial in establishing a pharmacological evaluation system of TCM. Thus, there is the saying in TCM that "drugs and medicines can't live apart." The interactions between the two complex systems, TCM and the human body, and the formation of a higher system entity are based on TCM globalization. The clinical TCM experts should participate in the research of TCM systems biology, which is based on the "system to system" (S2S) research strategy of formula and syndrome correspondence, and requires a combination of two aspects: one is the global delineation of system characteristics in the process of a biological body (response system) being interfered with by TCM; the other one is system disclosure of the internal relationship in the chemical substance system of TCM formula. The interactions can be disclosed in the global level with combination of the two systems. The current network pharmacology investigates the biological response system ("point to system," P2S), taking medicine as a disturbance factor. The P2S mode is different from the P2P mode ("point to point," single compound acts to single target). However, when a TCM formula as a complex substance system interferes with the body, being taken as "point," the interactions between the TCM formula's interfering system and the biological response system cannot be constructed. It is difficult to identify the effective component groups in a TCM formula and to further carry out the compatibility optimization of compounds. Thus, we developed chemomics, a method integrating chemomics of TCM formulas and systems biology (Fig. 1.6), to investigate the interactions between TCM complex system and human biological system, providing new insights into the mechanism of TCM formulas as well as evaluation of the compatibility of TCM formulas.[4, 5]

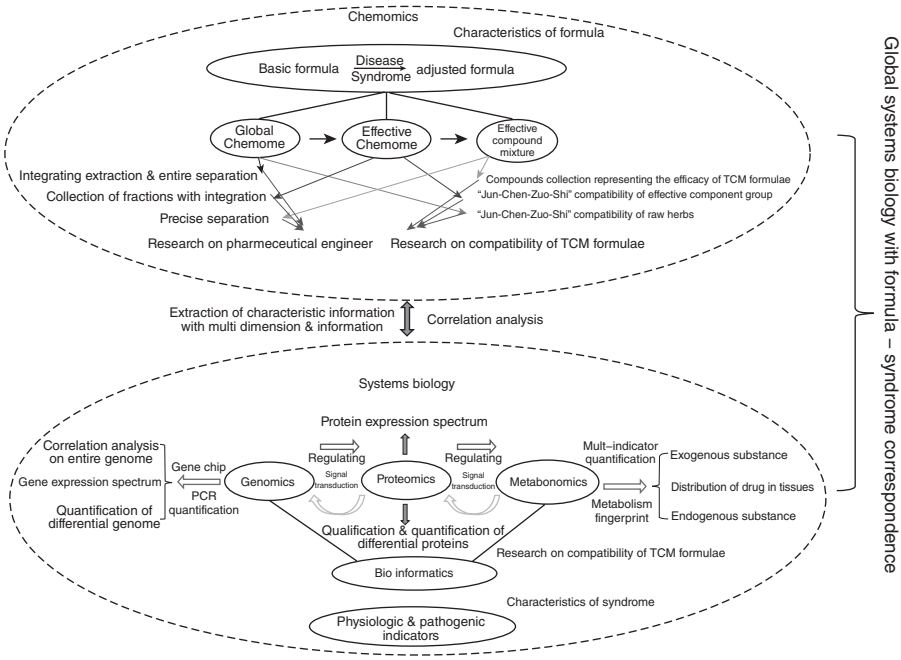

Fig.1.6 "System-to-system" research strategy of TCM formula–syndrome correspondence.

1.5.2.2 Research Strategy of Combination of Macroscopic and Microcosmic Representation TCM emphasizes globalization (macroscopic) but pays less attention to microcosmic characteristics. Western medicine is characterized by anatomy and molecular organism, focusing on the diseased organs, targets of the disease. Systems biology attempts to overcome the disadvantage of "missing the forest for the trees" of traditional biology, explaining the macroscopic characteristics of the system from a points of view that integrates various omics. The omics high-throughput screening models in current systems biology can be used for quantitative/semi-quantitative analysis of the macroscopic characteristics of genomics, proteomics and metabonomics. However, due to the technical limitation, the microcosmic characteristics cannot be quantitatively analyzed. Therefore, the macroscopic representation methods and accurate analysis of microcosmic characteristics should be combined in TCM systems biology, to delineate the characteristics of a complex system. For example, we have combined omics research and the confocal research on special signals to explore the levels of system biology, the gene expression profiling and special gene quantitative real time polymerase chain reaction (RT-PCR) in the level of genomics, and metabolic fingerprint profiling and multimetabolites qualification of special pathways in the level of metabonomics to develop metabonomics platform technologies.[6, 7]

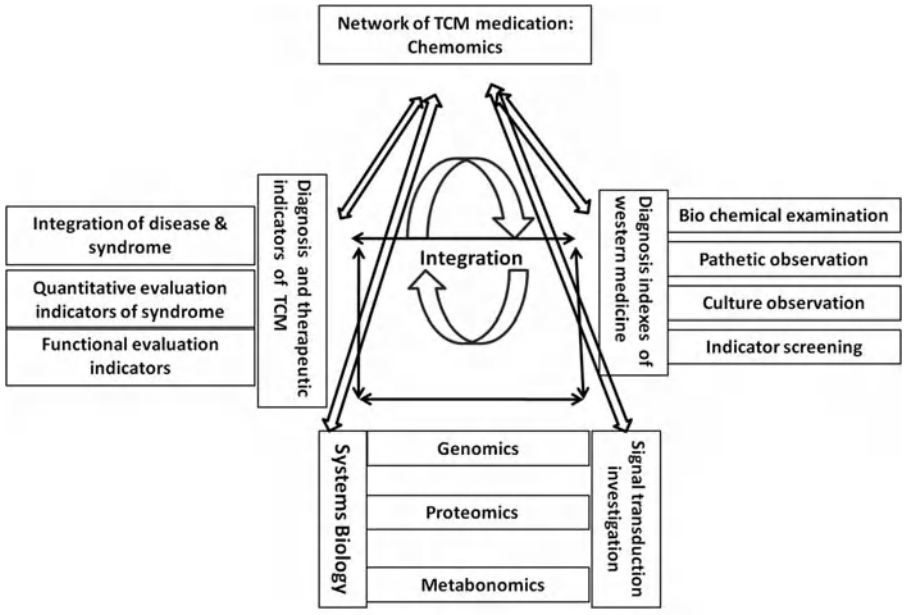

Fig. 1.7 Comprehensive systems for TCM disease–syndrome treatment and efficacy evaluation.

According to the strategy of combining macroscopic representation and microcosmic characteristics, integration of multitechnologies is used to establish the comprehensive systems for TCM disease-syndrome treatment and effect evaluation (Fig. 1.7).

1.5.3 Key Scientific Problems That Need to be Solved by the Systems Biology Approach of TCM

Based on the crucial scientific problems and its potential modernization of TCM, systems biology study of TCM should address the following important issues.

1.5.3.1 Basic Research on Systems Biology Regarding Individual Diagnosis and Treatment of TCM Individual diagnosis and treatment is the essence of differential treatment of TCM, which takes into account conditions in terms of locality, time and people involved. Individual treatment program can be used in accordance with the different objects (physique, cause, and pathogenesis) and different environment. Thus, the circumstance is that the same symptoms receive different treatment and different diseases with the same therapy during the cause of TCM diagnosis and treatment. In recent years, the development of systems biology indicates that many diseases (more complex genetic diseases) are caused by the interaction of hereditary factors

and environmental factors. Various pathogenic factors (mechanism) are likely to exist. The internal basis of individualized treatment principles of TCM may obtain support from studies of systems biology.

For example, to analyze the large amount of patients identified with TCM by genome-wide association studies (GWAs),[8] gene polymorphism and complexity can be used to understand why people with different physiques, genetic backgrounds, and pathogens (environment) suffer from the same diseases and have different responses to the same medication (Chinese herbal medicine), and why different drugs may cause similar treatment effects. It is also possible to find an answer for the material foundation of same disease with different treatment, and different diseases with the same therapy of TCM from genes–protein–metabolism regulatory and control level.

1.5.3.2 Basic Systems Biology Research of TCM Syndromes Syndrome differentiation is crucial for treatment. Characterization and evaluation of TCM syndrome is critical to TCM systems biology research. Modern research shows that TCM syndrome is complex. It is difficult to express with single factors as physiological and biochemical indicators. However, the overall regulatory and control network can be used to investigate the material base of TCM syndromes based on the net of "genes–protein–metabolism."[44–56]

Considering the lack of animal models with TCM syndromes, TCM syndrome systems biology should be combined with TCM clinical investigation. Clinical experts make clinical research programs, criteria of syndrome differentiation and suitable patients with enough cases, since mixed syndromes are widely prevalent.

Fig. 1.3 shows an evaluation system combined with quantitative diagnostic indicator (standardized scale) for different syndrome differentiation in TCM, pathological biochemical indicator of Western medicine, and system evaluation indicator. Based on the relationship among various indicators, the specific indicator can be found which is most helpful for illustration of the syndrome present. The difference found from functional genes, proteins, and metabolic control can be used to know the material basis and science in TCM syndrome differentiation and to improve the clinical diagnosis and treatment ability.

1.5.3.3 Overall Screening and Evaluation Research on TCM Formulas with Systems Biology According to the TCM principle (prescriptions corresponding to syndrome), the formula with exact clinical efficacy should be selected corresponding to syndrome.[4, 5] The efficacy evaluation models of disease and syndrome can be established (multiple combination of animals, organs and tissues, cells and subcells, molecular targets). Using chemomics to investigate the chemical basis of the formula and different compatibility, we can reveal the relationship between component groups and their efficacy, as well as compatibility. Modern TCM preparations have further been developed that not only have independent intellectual properties and keep the efficacy

of traditional TCM formula, but also have simpler and clearer components with basically clear mechanisms, great stability and safety.

The variation of both chemical component groups and biological efficacy caused by compatibility can be revealed by systems biology tools. The efficacy mechanism of TCM formulas can be explained from the relationship of "system to system." Through the relationship of chemical information of the change of efficacy and biological information of targets, processes, and controlling net, we can integrate the overall and special parameters to compare the effect of compounds with single or compatible use, to reveal the interaction among different compounds, targets, processes, and controlling net. Based on the net model controlled by multiple factors, the active material basis and the mechanism of activity can be further studied.

1.5.4 Main Development Direction and Key Technology of TCM Systems Biology

The major branches of TCM systems biology include TCM chemomics, TCM genomics, TCM proteomics, TCM metabonomics, and TCM bioinformatics. The combination of overall representation and individual characteristics is not only used in the study of TCM systems biology, but also in strategies and methods for future studies in each branch.

1.5.4.1 TCM Chemomics TCM chemomics is the study of the interrelationship between composition and changes of chemome and the active response of biological systems. Chemome is the assemblage of all chemical or/and chemical groups, and acts on the external disturbance systems (such as TCM formulas) of biological systems under certain circumstances. TCM chemomics usually adopt fingerprints as overall characterization means and multi-indicator components quantitative as precise expression mode of local features. For example, during the chemome study of the *Qingkailing* injection, we respectively set up three kinds of fingerprints as overall characterization, including organic components, inorganic ions, and components of biological macromolecules, and simultaneously separated more than 40 kinds of organic components and 10 kinds of inorganic ions. The content identification and separation were then conducted. By adopting the combination of multidimensional fingerprint chromatogram database and multi-indicator component quantification to the *Qingkailing* injection, we tracked the research on the whole formula, raw herbs, herb couples, herb teams and effective component groups. All these steps provided guidance to the research and development of *Qingkailing* injection.[42, 43]

1.5.4.2 TCM Genomics TCM genomics combines theory, means of genomics and TCM theory from the level of genes and gene–environment interaction to recognize the scientific connotation of TCM individual diagnosis and treatment systems. We attempted to establish a relation of TCM property,

functions, indications and influence to the regulation and control of related genes expression of specific diseases (TCM syndrome). At the molecular level, we gave an explanation to the scientific content of TCM syndrome differentiation and treatment, and the mechanism of TCM through genomics theory. TCM genomics includes research at the DNA level (such as the genome-wide association analysis and DNA methylation analysis) and RNA level (such as MicroRNA and mRNA), among which gene expression profile (mRNA) has been used in TCM research. Gene expression profile study of correlation between prescription and TCM syndrome finds the gene expression profile database of different TCM syndromes and discovers groups of distinctively expressed genes that have relationships with the specific TCM syndromes. This has significance for the objectivity of TCM syndrome differentiation and knowledge in the syndrome development discipline of TCM. We can further choose TCM with obvious curative effect and relative clear research on material basis and effect mechanism. Using various high-throughput technology (microarray, etc.) and quantitative analysis technique (real-time PCR, etc.), we can study the differences of gene expression during the process of TCM intervention, draw up gene expression profile by comparison and analysis of bioinformatics and statistics and establish gene expression profile database of TCM therapeutic processes. The link with the chemical characteristics database of TCM (chemomics study) and TCM theory offers the study of functional gene expression profile of the TCM role, and reveals signal pathway network of TCM. Based on the above theory and the influence of gene expression with different active component groups (compatibility) to TCM, we can further illustrate our traditional medical theory and its organic function. Meanwhile, we can interpret TCM theory and mechanism for functional gene networks. For example, using an integration screening chip containing 6,000 genes, we studied the overall level of animals and cells for pharmacodynamic evaluation of the *Shuanglong* formula to treat myocardial infarction. We established a gene expression profile database about normal group, model group, sham surgery control group, *Shuanglong* formula group (high, medium, and low dose), and positive medicine control group. Through the bioinformatics process, we found 180 genes differentially expressed and potentially related to myocardial infarction and effects of the *Shuanglong* formula. We further used the fluorescent quantitative PCR method to precisely analyze the ten functional genes of expression differences more than eight times. Through the complete feature of gene chips and by precisely analyzing important genes, we finally obtained the functional gene network of the *Shuanglong* formula established in the process of myocardial infarction treatment.

1.5.4.3 TCM Proteomics TCM proteomics combines theory, means of proteomics and TCM theory to determine the relation of its properties, functions, indications, and influence for the participating regulatory and control process of related protein expression of specific diseases (TCM syndromes). On the molecular level, an explanation of the scientific content of TCM

syndrome differentiation and treatment as well as the mechanism of TCM through the proteomics theory was given. The main content of the study is about the different protein mass spectra related with correlation between prescription and TCM syndrome. We can choose TCM formulas or herbal medicine materials with obvious curative effects and relatively clear research on material basis and effect mechanism, using high-throughput proteomics technical platforms, such as biological mass spectrometry protein chips (SELDI/TOF), two-dimensional electrophoresis (2D-Gel-MALDI/TOF) and multidimensional liquid chromatography/mass spectrometry (MDLC-ESI/MS/MS), and comparing the different protein mass spectra during the process of TCM intervention. By conducting bioinformatics and statistics analysis, we can establish a database for different protein mass spectra of TCM therapy linked with chemical characteristics database of TCM (TCM chemomics research) and TCM theory. The target protein of TCM can be further studied. At this point, combing with bioinformatics, we can reveal the influence of TCM effective substances on protein expression and regulation and interpret TCM theory and its role on protein regulation network. We adopted the comparative proteomics method to ensure the role mechanism of the *Shuanglong* formula promoting differentiation of stem cells into myocardial cells and interpreted the relevant protein signal pathway.

1.5.4.4 TCM Metabonomics TCM proteomics combines quantitative metabonomics methods and techniques as well as TCM theory to study the body's complete metabolism state, disease-related metabolic pathway, and changing features of metabolism during the process that TCM interposes specific diseases (TCM syndromes), clarifies pharmacokinetics, coordinates laws about the TCM effective component groups and their compatibility, and interprets TCM theory and its mechanisms on the metabonomics level. Quantitative metabonomics is a technical system that combines panorama pattern of the entire metabolic fingerprint with a focalization model of more specific metabolic pathways and multi-indicator components quantitatively.[7, 8] The main features are the combination of overall characterization and local characteristics, integration of qualitative and quantitative, and integration of multiple analysis methods as LC, gas chromatography (GC), MS, nuclear magnetic resonance (NMR), and so on. The primary research contents include the application of TCM quantitative metabonomics in TCM syndrome evaluation and disease diagnosis, application in effective components screening of TCM and overall efficacy evaluation of TCM, and correlation research between pharmacodynamics and pharmacokinetics of TCM. For example, in nephropathy disease research (see Chapter 7), we obtained the metabolic fingerprint of metabolites using UPLC-Q/TOF,[13] and adopted the PCA method to analyze more than 3,000 species of metabolites by cluster analysis. We obtained samples of diabetic nephropathy in different pathological stages and identified TCM syndromes and types. Combining this with the PLS-DA method, we further discovered more than ten species of new metabolism markers.

Simultaneously, according to prior knowledge, we conducted quantitative analysis accurately on several important substances in metabolic cycle, including fatty acids, phospholipids, amino acid, purine/pyrimidine, and nucleoside. By processing these analysis data, we achieved potential biomarkers of different angles and levels. Our results indicated that only a part of the biomarkers could be identified no matter what mode or circle was used. Even by obtaining overall fingerprints with the capacity of testing thousands of metabolites at the same time with UPLC-Q/TOF, we still lost information on some important metabolites. For example, nucleoside and amino acids-class metabolism markers' information did not reflect enough in the overall fingerprint, and it could be supplemented by using quantitative research of special pathway. Therefore, with the integration of different information, quantitative metabonomics platform technology can achieve more comprehensive, accurate, and specific explanation and characterization for whole-body metabolism states and specific metabolism pathways. The better prediction and judgment of the occurrence and development of disease can reveal the pathogenesis mechanism and provide guidance for clinical treatment.

1.5.4.5 TCM Bioinformatics TCM bioinformatics combines bioinformatics with TCM theory and develops bioinformatics methods of multiple omics information mining related to TCM syndrome, chemomics, genomics, proteomics and metabonomics, and so on. With the integration of gene–protein–metabolism, and multiple levels of network information, TCM theory and mechanisms could be explained comprehensively and systematically on level of overall systems biology from chemomics, genomics, proteomics, and metabonomics. Main research contents include information processing technology of TCM fingerprints, distinction and identification technology of key effective medicine components, distinction and identification theory, and method study of TCM composition effective relationships. It offers how to build a relationship between genes or protein network regulating and controlling model of multiple factors, multiple links, multiple levels, and the integrative adjustment mechanism of multiple targets, multiple links, and multiple levels of TCM.

1.5.5 Research Prospective of TCM Systems Biology

In recent years, much has been achieved by studying TCM formulas, using the pharmacological experiments and chemical analysis methods. However, the curative effect or mechanism of TCM formulas has mostly been explained through modern medical indicators (such as molecules, cells, organs, and overall levels). With the integration of TCM theory and the latest technology of systems biology, we have proposed "system to system" integrative systems biology methods of TCM, which is an innovative method system in accordance with the characteristics and patterns of TCM. With the development and improvement of technical platforms including chemomics, genomic, proteomics, metabonomics, and bioinformatics of TCM, the information obtaining

ability and processing capability of TCM integrative systems biology will be greatly enhanced. It is a combination of "TCM syndrome–design method–compound prescription–curative effect" and research of integration chemomics of the integrative systems biology. Therefore, it will reveal therapeutic material basis and mechanisms of TCM formulas, clarify the compatibility laws of TCM formulas, and guide new drug development. It also provides a communication platform for the integration of TCM with modern sciences, and further inheritance and development of TCM theory. The rapid development of TCM integrated systems biology will greatly expand the modernization research on TCM at the next stage.

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CHAPTER 2

CHEMOMICS OF TRADITIONAL CHINESE MEDICINE

Compound preparation is the major method used to prevent and cure diseases in TCM, composed sequentially according to illness needs, flavor and meridian tropism of medicinal materials, function, and use, together with the harmony of seven conditions of ingredients in preparation. Compound preparation's holistic effect can be greater than the sum of the parts through matching medicinal materials in order, and by determining which drugs restrict each other, guide each other, or coordinate with each other.[1–3] Therefore, we proposed, in the last chapter, to establish a "system to system" research mode that is suitable to understanding TCM formula characteristics. The research mode also clarifies the compatibility theory, efficacy mechanism, and material basis of composite preparation systematically and comprehensively by integrating it with systems biology and chemomics. In other words, it characterizes the composition of the medicine intervention system and elucidates the relationship of its components by chemomics. In addition, it describes the response process of biological systems through systems biology, analyzes interactive relations between the two systems by bioinformatics, and reveals the relationship between changes of chemomics and spatiotemporal response relevance to biological systems.

This chapter explains the characteristics of TCM, its research challenges, the background of TCM chemomics, definitions and research modes of chemomics, and its application in formula research.

Systems Biology for Traditional Chinese Medicine, First Edition. Guoan Luo, Yiming Wang, Qionglin Liang, and Qingfei Liu.

2.1 CHARACTERISTICS AND RESEARCH DIFFICULTIES IN TCM

Along with changes in the human disease spectrum, the problems become apparent that the single target antagonism treatment of Western medicine hardly solves complex diseases, and its "reductionism" mode requires research from organs to cells then to genes. TCM has gained international recognition and support by exploring life and disease, together with the complex medical and pharmaceutical phenomena, with its systematic, dynamic and customized (dialectical) mode of thinking, and its superior clinical efficacy.

As shown in Fig. 1.1, Eastern and Western medical systems differ greatly, but advantages in the integration of Eastern and Western have already appeared. Western medicine has gradually adopted Chinese medical theories such as "harmony between body and nature," "prevention of diseases," "treatments based on syndrome differentiation," and "TCM as a unified whole," and puts forward new goals and methods for TCM by combining it with modern life sciences and biotechnology. It certainly provides new opportunities for TCM.

However, how can Chinese medicine transform from its self-explanatory closed system to an accommodating open system? How to combine, integrate, and converge Chinese medicine with other modern sciences (such as systems biology)? How to embody the modern scientific mode, methods, and results with data, not words? These are the urgent scientific problems that are in the path of the development of TCM. The key to solving these problems is to establish an innovative system of TCM that consists of basic TCM characteristics and laws but is still able to fully integrate with the latest modern technological achievements. This system needs to integrate with modern scientific and technological achievements of theoretical innovation systems according to basic theory, characteristics and laws of TCM. It needs multidisciplinary theories of modern science, especially complex systems research, to study and explain the essence of Chinese medicine. Considering its globality, nonlinearity, fuzziness, and temporal sequence characteristics, TCM is rather complex. However, there are novel fields and methods to suit this compatibility, such as systematology, informatics, dissipative structure theory, coordination theory, nonlinear science, and fuzzy mathematics catastrophe theory. Utilizing these theories and methods to explore and reveal the connotation of TCM is the core of TCM modernization research.

2.1.1 The Globality and Systematicity of Chinese Medicine

The advantages and characteristics of TCM lie in that it takes the concepts of holism and systematology as guiding ideologies and studies the complex phenomenon of life and disease in a holistic, dynamic, and dialectical way. Additionally, it advocates "harmony between body and nature," balanced treatments, treatments based on syndrome differentiation, and personalized treatments. Different from the "single target confrontational therapy" of

Western medicine, TCM takes Chinese medicine preparations as its main component, a holistic comprehensive regulation as therapeutic form, aims at the main attack link of illness, and affects multiple levels and targets of body through multiple channels and links, to adjust the holistic level, organ level, subcellular level, and molecular level.[4]

2.1.1.1 Holism of Chinese Medicine There are fundamental differences between holistic efficacy and active ingredient efficacy. The holistic effectiveness of preparations and medicinal materials used in TCM differential treatment is a holistically oriented principle. That means either an herb or a preparation has differences between its holistic efficacy and its active ingredient's efficacy. There is a relationship that "the whole is not equal to the sum of its parts" between the holistic efficacy and the ingredients' efficacy; that is to say, the preparation efficacy is not equal to the sum of each compatible drug's efficacy, and Chinese medicine uses holistic efficacy. The question is whether the holistic efficacy can be broken down, restored, and summarized as the ingredient efficacies. Modern scientific research shows that it is essentially impossible, because the whole and parts (components) are two different levels in structure with differences and transitions in levels. Besides, the whole and parts (components) have principle differences on their properties, functions, and behaviors. For example, aqua regia has the effect of dissolving gold, but it does not do the same when it is decomposed down into concentrated hydrochloric acid or concentrated nitric acid or even separated and purified to the atomic level. Therefore, the effect of dissolving gold only exists on the holistic level of aqua regia.

The law of "the whole is greater than the sum of its parts" is universal. Systematic theory summarizes the integrity principle. Modern research in Chinese medicine has demonstrated that the holistic effectiveness is greater than the summed effects of various drugs. For example, the *Jiao tai* pill is composed of *Huanglian* (Coptidis Rhizoma) and *Rougui* (Cinnamomi Cortex). Experiments show that the two herbs have no excitatory or inhibitory effect on the cerebral cortex or central nervous system, but the combination of them can "communicate the kidney and heart in an instant," and cure insomnia caused by the disharmony between the heart and kidney. The *Yinchenhao* decoction is composed of *Yinchen* (Artemisia Scopariae Herba), *Zhizi* (Gardeniae Fructus) and *Dahuang* (Rhei Radix et Rhizoma), and experiments proved that only gardenia can slightly induce gallbladder contraction, while the preparation exhibits excellent inducing potency. A large amount of research on separating prescriptions has proved that the relationship elucidated as "the whole is greater than the sum of its parts" is universal. The holistic efficacy of a preparation cannot be decomposed in principle. The holistic efficacy is generated by a foundation that should be explained clearly. However, it is merely the material basis. It is not the holistic effect itself, and is not the essence of holistic effect; also, it cannot directly explain the holistic effect.

2.1.1.2. Relationism of Chinese Medicine Interaction is the decisive link to generate a new property on the material basis, which is also the carrier and foundation of the original holistic effect. And it is the same case for TCM preparations. The compatible drugs are a material basis for the corresponding formula.

The reason that "the whole is greater than the sum of its parts" lies in the composition of four parts including *Jun*, *Chen*, *Zuo*, and *Shi*, together with the harmony and integration of seven conditions of ingredients in preparation known as "mutual reinforcement (相须), mutual assistance (相使), mutual detoxification (相杀), mutual restraint (相畏), mutual antagonism (相恶), mutual incompatibility (相反)." This is the carrier of holistic effectiveness exhibited by the composite preparations, and it would be destroyed in splitting research. It is the same case as that of herbal medicines. The theory of Chinese herbs' properties is gradually formed by summarization of the functional properties of herbs from different aspects, which is the high recapitulation of its functional characteristic through the TCM theory. The content of this theory was consisted of four properties (cold, hot, warm, and cool); five flavors (pungent, sweet, bitter, sour, and salty); ascending, descending, floating, and sinking; together with meridian tropism. Undoubtedly, each of these properties has its own certain material basis, and will be fully understood in future. However, it is impossible to attribute the "cold, hot, warm, and cool" or "pungent, sweet, bitter, sour, and salty" characteristics as physical and chemical properties or pharmacological effects of certain ingredients. The carrier of TCM preparations' holistic performance depends on the interaction and combination way of the compositions. Therefore, the research into medicinal materials and preparations has to clarify not only the material basis of the holistic effect, but also the interaction and combination of the compositions and their mechanisms contributing to the holistic performance. Interaction and combination are the bridges between holistic and ingredient efficacy, and are the key link that the "not only composition" theory should grasp. It is important to study the carrier and basis after understanding the material base and ingredients' function.

2.1.2 Complexity of the Material Basis of TCM

Complex chemical systems have become the main analytical object in modern analytical chemistry. The complex material system of TCM has a characteristic of having various components. The main difference between complex material systems and simple physical systems is that complex material systems have numerous components, and generally simple system components have less. Besides, there are complex relationships among the components of a complex material system, and a simple system is opposite. Components of a complex system differ greatly from each other, while a simple system usually has clear, fixed content. The detecting response of components in complex systems is significantly different from one system to another and the nonlinear

relationship is significant in certain scope, while the responses of components in simple system are simple and linear.

Based on the above characteristics of the TCM complex system, it is necessary to adopt corresponding strategies to maximize the display of system components comprehensively.

2.1.2.1 Analysis of the Effective Parts of Chinese Medicine The so-called "effective part" refers to a large class of compounds that possess similar chemical characteristics in TCM. The concept of effective part differs from the active ingredients (often a single chemical composition) and effective sites (often plant parts containing effective sites, such as leaves, roots and fruits). A TCM formula may contain a number of effective parts according to its multi-target action characteristic. "Effective part" is the bioactive compound groups combined according to their chemical properties based on pharmacology and pharmacodynamics studies. Therefore, the analysis of effective parts is crucial for Chinese medicine.

Since the 1990 edition of "Chinese Pharmacopoeia," the identification of TCM and proprietary Chinese medicines has made marked progress. One of the most significant changes is the use of the reference medicinal materials in thin layer chromatography (TLC) to supplement or replace past methods that conduct identifications relying on a single chemical reference substance. The reference medicinal material provides a complete characteristic chromatogram whose image can provide accurate authentication of medicinal materials. Besides, quantitative sampling also provides quantitative information to estimate the delivery of raw materials and the overall composition stability during production and storage. It solved the problem of not being able to identify herbal medicine without reference substances or giving biased outputs even when chemical references are provided. It's an effective change for the progress of TCM, and has been certified in the practices of last three editions of "Chinese Pharmacopoeia" and the discovery of new drugs in the last 10 years. Actually, the prototype of fingerprint identification has already been established through reference medicinal material settings and chromatograph integral characteristic identification.

Observation of chromatographic fingerprints of holistic features allows one to identify the authenticity of medicinal raw materials, distinguish the different parts of medicinal plants, inspect the quality of commercial medicinal materials and formula, trace the changes of effective compositions in the preparation, and monitor the stability between raw materials and finished products, as well as differentiate between finished batches. Therefore, it is logical to analyze and conduct quality control on the effective parts of TCM with chromatographic fingerprints.

2.1.2.2 Global Analysis of Chinese Medicine In the research of TCM effective component groups, solvent partition and column chromatography

have been commonly used to purify components into single compounds, and their structures would be identified with various spectrums. Although this method in the analysis of chemical components has achieved fruitful results, it cannot provide TCM with comprehensive explanations because of the fuzziness and complexity of Chinese medicine. Many deficiencies have been found during the application of this strategy in separation and analysis, such as multiple operate steps, low degree of automation, and inefficiency; besides, trace analysis is very difficult. On the other hand, this method often represents the integrated TCM formula with several effective component groups of high contents, which obey the integrated principle of TCM artificially.

Modern high performance chromatographic techniques provide a good tool for the separation of multiple components in a single experiment. However, as stated before, a single chromatographic method is insufficient for the global representation of TCM, which is restricted by the limited detector response and sensitivity. Thus, the combination technology of chromatographic and spectroscopic means becomes an important part of modern TCM analysis. The chromatographic method is used to analyze components and spectroscopic means to identify structures, and the combination of them can enhance the chemical analytical ability of mixture compositions greatly.

Due to the increasing demand for the analytical technique and the rapid development of various microanalyses, preparative liquid chromatography, spectroscopy, and information technology, each coupling technique has developed substantially. One of the most prominent is the successful combination of chromatography and mass spectrometry, such as gas chromatography–mass spectrometry (GC-MS) and liquid chromatography–mass spectrometry (LC-MS). The multiwavelength ultraviolet (UV) detector works according to the ultraviolet spectrum characteristics of the analyte, so with the application of photodiode technology, liquid chromatography coupled with a photodiobe array detector (LC-PDA) has become the representative of commonly used traditional multiple wavelength UV detectors.

Since the development of fast superconducting nuclear resonance spectrometer with high frequency and high sensitivity, the combination of liquid chromatography and NMR (LC-NMR) has progressively increased in use in TCM research. In addition, gas chromatography coupled Fourier transform infrared spectroscopy (GC-FTIR), liquid chromatography coupled plasma mass spectrometry (LC-ICP/MS), as well as thin layer chromatography coupled mass spectrometry (TLC-MS) also have been applied recently. Meanwhile, the associations of several optical means have expanded the application range. Successful examples include liquid chromatography with photodiode array ultraviolet scanning-mass spectrometry (LC/PDA-MS), liquid chromatography and nuclear magnetic resonance spectrometry (LC-NMR-MS), as well as chromatographic and multistage mass spectrometry. Due to the diversity of

chromatography, spectroscopy and mass spectrometry, there are various combination modes for the chromatography coupling technique. On the basis of chromatography separations, it's possible to represent the holistic features of TCM by combining various qualitative and quantitative detection methods and displaying their advantage sufficiently.

2.1.2.3 Characteristic Analysis of TCM Recently, scholars have committed to researching TCM with molecular biological methods. Above all, the most encouraging progress is the application of genetic analytical technology in classification, identification and utilization of resources and other aspects of TCM. For example, gene cloning for important herbs is of significance for the protection and utilization of TCM resources. Transgenic technology has been used for the cultivation of efficient Chinese herbal medicines, which can affect the compositions of TCM purposefully, and to obtain efficient and cheap resistance components.

Traditional identification of crude drug material includes source, characteristics, microscopic and physiochemical techniques. The identification of Chinese medicinal herbs using this method was usually conducted according to the morphological classification and anatomic characteristics, which is comprehensive, clear, simple, and easy to operate. However, for some species, especially the multichannel-processed herb or animal medicines, as well as the formula, which are lacking in morphological structures, it is difficult to identify them with traditional techniques. A new, convenient and accurate molecular identification of TCM has been developed by genetic engineering analysis, in order to obtain herbal medicines' DNA characteristic spectrums. The principle of this method is that DNA of each individual organism is separated by electrophoresis after restriction enzyme cutting. The distribution of electrophoretic bands formed after hybridized with labeled DNA probes and autoradiograph should be different, that is, individual-specific, population-specific, and species-specific. To confirm the existence of some herbal medicines in the formula, one can use these molecular genetic marking methods to find the highly specific fragments, and prepare for the highly specific oligonucleotide probes, then identify the medicines after molecular hybridization fingerprint analysis.

As an identification method for the characteristics analysis of traditional Chinese herbs, the DNA analysis, which is based on the genetic specificity of herbs (plant or animal), was thought to be a more prominent one. This analysis technique exhibits many advantages such as accuracy and simplicity, and can avoid the interference of complex metabolites (including primary and secondary metabolites).

In addition, on the basis of the fingerprinting obtained by chromatography or coupling techniques, the analysis method utilizes chemometric methods to obtain fingerprint characteristics, thus the characteristic spectrum that is formed has a strong specificity for Chinese herbal medicine and its preparations.

2.1.3 Research Strategies

The decomposed recipes and phytochemistry research are popular in modern studies of herbal medicine and TCM preparations, which have made great progress in recent years. However, many difficulties have also been met. Specifically, it cannot illustrate the holistic effectiveness of TCM and composite drugs by physicochemical properties and pharmacological effects. In TCM preparation research, one must pay attention to the basic characteristics of the effects of Chinese medicine. To clarify the different contents, mechanisms and principles from Western medicine in terms of resistance, pharmacology, and efficacy, the following principles should be considered.

2.1.3.1 Based on the Guidance of TCM Theory The characteristics and the core contents of TCM theory are its holistic concept, the principles of syndrome differentiation treatments, and their comprehensive analysis. The trace elements and organic components coexist in a single herbal medicine or compound preparation, and one has to recognize and consider their actions in view of the holistic effect, because the interaction and mutual influence of these components may produce new effects and generate new complex compounds. The medication rule of TCM emphasizes the combination principle known as "*Jun*, *Chen*, *Zuo*, and *Shi*" particularly. It is important to remember those theories in the effective chemical component research of TCM.

2.1.3.2 Use Scientific System Theory and Principle Consciously The systematic method takes the research object as a whole to recognize and reform, which is significant for effective chemical component research. It should be noted that the systematic theory is not equal to the holism. "System" should be defined as a unity, which is composed of several parts with certain level and structure, in contact with the environment. Various chemical components in TCM are in such a system.

2.1.3.3 Integrity Principle The holistic function of a system is not simply equal to the sum of its compositions. The pioneer of "system theory," the biologist Ludwig von Bertalanffy from Austria, put forward the famous theory that "the holistic effect is greater than the sum of the isolated parts," which is an important guiding principle for the research of effective chemical components in TCM.

2.2 BACKGROUND OF TCM CHEMOMICS' PROPOSAL AND DEVELOPMENT

Two methods have been widely used in the research of TCM preparations for a long time. One is to discover and separate the single effective component, and then make structural modifications to obtain the herbal medicine. This method follows the thought of leading compound in Western medicine. Though

it can interpret the substance foundations and mechanisms of a formula to a certain degree, it is difficult to interpret correctly the content of TCM because of the multicomponent, multitarget and multilink features. The other method is to optimize the compatibility of the formula under the guidance of TCM theory. However, this method easily fragments the internal contacts among Chinese medicine active substances, and stays in the medicine compatibility level. From the early 1990s, in order to open the black box of TCM and grasp the efficacy material basis, scholars in China explored thoroughly the chemical systems of formulas. And the contention of a hundred schools of thoughts have been evoked by various strategies and methods.

2.2.1 Advancement of Chemical Research on Chinese Medicine

Yu Yagang et al. put forward the "triadic designing theory" for the systematic separation and identification of chemical components in certain formula.[5] Xue Yan et al. proposed the target shotgun theory for the chemical study of TCM preparations.[6] They used different methods to cure diseases. For example, several small functions in TCM combine to create a large function, and the interaction of several effective component groups produces new active substances. The research methods include holistic formula research, part of formula research, effective component research of single herb medicines and pharmaceutical analysis. The order of the research should be holistic formula research first, part of formula research second, effective component group research of single herb medicine third and pharmaceutical analysis last. Additionally, it also has the principle of setting the ingredients by disease and selecting essential active ingredients as the effective ingredients. This theory reflects the multicomponent characteristic of TMC, but does not reflect the compatibility theory of *Jun*, *Chen*, *Zuo*, and *Shi*. Academician Zhou Jun proposed the combinatorial chemistry library of formulas and many target mechanisms theory.[7] Several effective ingredients enter into the body at a lower dose than a single treatment, and act on several direct targets (temporary solution) and indirect targets (permanent solution) selectively and repeatedly, and then cure the disease. It is better to choose a simple effective formula for design, divide it into several parts first, and then select the divided parts by the multitargeting microscreening model. The active ingredients selected from the divided parts directed by the multitargeting microscreening model are accumulated and pharmacologically studied in depth; and finally the possibility of a new formula is discussed by considering the pharmacological research mechanism. Yang Kui et al. put forward the combinatorial chemistry research methods of formulas by citing modern combinatorial chemistry ideas.[8] It takes formulas into a natural combinatorial chemistry library and establishes an evaluation index that can reflect pathological and physiological characteristics of the formula. The most active compatibility would be found as the effective fraction of the formula by a multitarget integrated screen of the divided parts and

monomer compositions. In addition, they explored the methodology of compound combinatorial chemistry. *Chuanxiong* (Chuanxiong Rhizoma) and *Tianma* (Gastrodiae Rhizoma) extracts were studied by the binary indexed library screening method. After analysis and comparison, the two strongest activity groups were found. More specifically, the combinative formula of the ethanol extract of *Chuanxiong* together with *Tianma* is the material basis for preventing 5-HT release of blood platelet and the calcium channels in vascular endothelial cells. The above theory combined phytochemistry and pharmacology study, and proposed opinions and ideas of systematically studying TCM. However, these theories usually prove TCM's compatibility by the splitting research of formulas, and in these theories, they adopt the total and point extracts or determine roughly several chemical components. When it comes to drug efficacy and action mechanism, these theories refer little to chemical composition. Therefore, they cannot explain the integrity and complexity of TCM.[9]

Zhou Lidong et al. applied "quantitative composition activity" to the study of complex systems of TCM. It clarifies the relationship of multiple chemical components and the efficacy of TCM through multivariate analysis.[10] The method has the dialectical thinking mode of TCM theories, and has effective positive significance in explaining the mechanisms of formulas, effective part research and monomer drug screening. Cao Zhiquan[11] proposed the "coordination chemistry theory" for the effective ingredient study of TCM preparations. This proposes that the effective substances of a formula are combination compounds of organic matters and micrometallic elements, and the combination compounds can reflect the formula's substance foundation better than single organic principle. The theory considers chemical species form as the core and the interaction of organic components and trace elements as the basis. Wang Benxiang believes that the analysis of compound material basis should distinguish between the major components and minor constituents, and stated a theory of "major and minor components of effective component group on the integrated effect."[12] He also advocated that one should first grasp the major contradictions (the main ingredient), and then consider the minor contradictions (the minor constituents).

In summary, before the mid-1990s, Chinese medicine compound chemical component research mostly studied on two different chemical levels. Some combined these two levels, but the levels of TCM preparations (or medicinal materials) as a whole are too fuzzy and complex, thus making the research factors difficult to control. Owing to the lack of a suitable middle level or transition level, it is easy to fragment the inherent contacts of the effective substances in a formula or herbal medicine by directly jumping to "chemical components" levels. Therefore, it is difficult to unify the two research levels.

Therefore, in 1997 we proposed a middle level, or a concept of effective component group, that should be introduced into TCM study.[13] So-called effective component groups (components) refer to a class of compounds with similar chemical properties (compounds with similar properties group)

in herbal compound drugs. A complex TCM preparation (which could have hundreds of chemical components) often can be divided into several types of effective parts. When combining with pharmacodynamics, it is possible to illustrate the chemical material basis of compatibility and synergy in a formula. For this reason, we propose a global research strategy including modern separation methods of effective components, qualitative fingerprints and quantitative index components, in combination with pharmacodynamic study. The effective parts theory of TCM also promoted Chinese medical research to expand from two chemical levels to three chemical levels, and quickly become one of the important research directions in Chinese medical research. On the basis of effective part theory, we further propose a chemical research system of TCM preparations together with a research method searching for the correlation between the material bases and efficacy.[14] Through a combination of chemistry and pharmacodynamics research (one "combination"), the efficacy substance foundation and its mechanisms in a Chinese medicine compound should be illustrated definitely (two "basic elucidations"). One should then elaborate on the efficacy substances in TCM preparations from assistant and guide medicines (whole preparations), effective parts (components) and an effective constituents group (three "chemical levels"). Research on the mechanisms of compound formula was conducted in four "pharmacology levels," such as holistic animal, organ organization, cellular and subcellular, and molecular biology levels. This theory was summarized as the "one-two-three-four system." The fingerprint technique was used for the representation of the global characteristic of TCM. And with the guidance of the effective parts theory, according to the correlation analysis of the chemical and pharmacodynamics information of the different fractions, it is possible to discover and identify the effective component group (effective parts) for major therapeutic functions. Furthermore, we put forward the concept of multidimensional chemical fingerprinting, which identifies the chemical characteristics of the effective component group in TCM.[15] In 2000, under the lead of academicians Wang Yongyan and Zhang Boli, China started its first TCM "National Basic Research Program of China" (973 Project)—basic research on the key scientific issues of preparation of TCM. The concept of effective parts and the chemical research system of Chinese traditional medicine preparations ("one-two-three-four system") were absorbed as the main research method system for the chemical research of TCM in the global study thread of this project. More than 20 research institutions, universities, and companies were involved in this 973 Project, which greatly promoted the development of research on TCM preparation theory and study. One of the most prominent results was the development of the compatibility theory from medicinal herbs to effective component groups, which successfully explored the process of developing modern Chinese medicine from TCM.

The compatibility theory of effective parts and multicomponent Chinese medicine research is the further development of effective parts (effective

component group) theory, which proves that the abovementioned theory about effective component group is practicable. On the basis of assuring that the effective parts theory and the "one-two-three-four system" fully absorb the advancements in modern biological sciences and especially systems biology, we proposed a new methodology named chemomics.

2.2.2 Comparison of Related Concepts about TCM Material Groups

With the development of systems biology, post-omics studies based on holism and complex sciences, such as proteomics and metabolomics, are being widely used in TCM research, which means that TCM research has entered into the "omics" era. Recently, research based on the material foundation of TCM has formed the original state of "omics" fields, which are fitting to the holism of TCM. The emergence of the "material groups of TCM" and other, similar concepts and related omics methods has provided new approaches and methods for the modernization of TCM.

In 2000, Wang Shengqi put forward a strategy, "TCM genomics" and "TCM chemics," based on research development and trends of modern genomics, especially functional genomics, modern analytical chemistry, and some advanced biological techniques.[16] TCM chemics states the composition, structure, content, mutual role, and the nature of effective compounds that refer to the material basis related to TCM theories. It relates herbal properties, functions, and main indications through modern science technologies combining TCM theories and modern science theories. Furthermore, TCM genomics uses modern genomics, especially functional genomics and disease genomics, to explain the efficacies and mechanisms of TCM on the molecular level. The core of this research system is to interpret the mechanisms or functions of TCM by a set of relative genes; it represents TCM material as a group of chemical constituents to explain its function.

Du Guanhua et al. proposed the concepts of "effective compounds groups" and "bioactive compounds groups" of TCM compounds in 2002.[17] "Effective compounds group" refers to pharmacological ingredients closely related to the clinical applications of herbal medicine or TCM preparation; "bioactive compounds group" means the entire content of the effective compounds group and other biologically active substances, no matter what the active ingredient does, or the roles it plays in the clinical uses. After studying the anti-ischemic effective ingredient groups of the formula known as *Naodesheng*, the blood activating and stasis dissolving effective ingredient groups of the TCM formula *Xiaoshuan Tongluo* recipe and the anti-Alzheimer effective compounds group of TCM formula *Xiaoxuming* decoction, they established a new research mode for active ingredients and mechanisms of formulas to discuss the substance foundation of formulas.[18–20]

We proposed the concept of chemome and a new methodology of chemomics in 2005. It was established after the conclusion of effective part theory and the chemical research of formulas, fully absorbing the advanced

achievements of systems for modern life sciences, especially system biology. Chemome refers to complicated systems including all of the chemical substances in the biological system under a certain condition, such as drugs, food, and other chemical substances taken from the external environment. Chemomics is a method of studying the composition and change of chemome, and their mutual relationships with biological effects. Take TCM chemomics as an example: We explained research methods and levels of chemomics and discussed links between chemomics and systems biology. TCM chemomics utilizes drug effect-oriented systematical grouping and progressive optimization strategy, together with the integrative expression and holistic screening strategy based on the combination of global characteristic and local features. TCM chemomics has been successfully applied to the basic substance research for the efficacy of the *Qingkailing* injection.

In 2006, Professor Li Famei proposed to research the metabonomic strategy for studying the therapeutic basis matter of TCM by using various chromatographic techniques and new systems biology and omics research strategies based on the holistic understanding on TCM efficacy chemomes.[21] In another aspect, this is three-dimensional research of Chinese medicine's effective component groups by metabolomic strategies that can reflect the holistic features of organisms, or studying the relationship of chemical component spectrum, metabolism spectrum, and original metabolic spectrum in vivo, in order to examine the pharmacodynamic effective groups in Chinese herbal medicines. It also integrates studies of efficacy substance foundation and mechanisms of Chinese medicine.

Liang Xinmiao et al. proposed the herbalome project in 2007. This project should comprehensively disclose the composition, structure, and function of herbal medicine by modern efficient separation and characterization technologies, and construct the library of herb substance resources. The multiple components and multitarget comprehensive regulation mechanism of TCM is aimed to be elucidated sufficiently. The research of the herbalome project consists of herbal material structure groups, functional groups, and TCM theory. On the basis of inheriting the theory and practice of Chinese medicine, the herbal material structure group focuses on the material basis of Chinese herbal medicine, while the herbal substance functional groups focus on the biological functions. These two studies should be coordinated and both emphasize, coupling with systems biology technologies and clinical evaluations, the multicomponent multitarget regulatory mechanisms of TCM would be interpreted.[22]

Zhang Jiwen et al. proposed the concepts of "TCM chemome" and "TCM materiomics" in 2008. The former refers to the certain collection of all the compounds contained in TCM, which refers to all the compounds that could be released or dissolved from the formula and can be detected under certain conditions. TCM materiome is a material collection in the category of TCM, which refers to the components for treating different diseases under the guidance of TCM theory, but food, chemicals, drugs, and so on are excluded from

the materiomics. The research of TCM materiome was established on this basis, which takes TCM materiomics as the object of study, with supports from modern omics technologies and methods. It studies the features, stability, quality control standards and methods, pharmacodynamics, toxicity, pharmacokinetics and drug delivery system design method of TCM. Recently, related methodological and application for studies have appeared to illustrate the release kinetics of the *Yinqiao Jiedu* pill formula in TCM materiomics, which takes the whole material basis as object.[23–25]

Comprehensive comparisons about the connotations and research content of these TCMomics revealed that they are significantly different from each other in applicable scopes, definitions, characteristics and entry points.

The research objects of TCM chemics, effective compound groups, metabonomic strategy for studying therapeutic basis matter of TCM, TCM materiomics and herbalome are limited to TCM and its formulas. However, the chemomics we proposed not only includes Chinese herbal medicine, but could also be applied to other chemical matter systems, such as food and environment. Currently, we have already applied the system to tea leaf chemomics and tobacco chemomics.

Omics, such as genomics, proteomics, metabonomics, are usually defined as "a method by which to study the composition and mutual relationships of some group"—that is to say, methods to study the composition and mutual relationships of genomes, proteomes, or metabonomes. The objects of these omics methods can all be clearly defined, and have common features such as defined scopes or stages, all substances of the given system (human, model animal, etc.) in certain conditions, which are indicated by suffix -ome. The definitions we proposed for chemomics and chemome match perfectly with international standards. While "TCM chemics" and "metabonomic strategy for studying therapeutic basis matter of TCM" cannot meet the defined modes of general omic methods formally, because TCM chemics and the therapeutic basis matter of TCM cannot be studied as research objects, there is no official definition for the research object in the text when it was proposed. The effective compounds group of TCM has not been defined officially, so it can be thought of as the research object, but is not accurate enough to be defined as omics (which should represent a method). Moreover, the actually defined contents of these three group study methods are not consistent with the scope required by the definition of omics. According to general definition of "ome," when one takes TCM as a research object, all components in the herbs or preparations should be defined as research objects. While the defined scope of "TCM chemics," "effective compounds group of TCM," "metabonomic strategy for studying therapeutic basis matter of TCM," and "TCM materiomics" is clearly not all the constituents in TCM, it is different from chemomics. The four research approaches have certain theoretical values. They are similar to the level of effective chemome if chemomics is applied to TCM. More specifically, they are equivalent to the second level according to our chemomics strategy-effective chemome. The research objects of herbalome are Chinese medicinal materials.

We not only give the normal definition of chemomics, but also point out the three different study levels, and propose a hierarchical, systematic and gradually optimized research strategy. The main content of research systems including "TCM chemics," "effective compounds group of TCM," and "metabonomic strategy for studying therapeutic basis matter of TCM" demonstrated the necessity and significance of the research (corresponding to the levels named as" effective chemome of TCM formula") in the holistic view. "TCM materiomics" demonstrates the efficacy substance group foundation of Chinese medicine from the drug release dynamics perspective. Herbalome elaborates on the multicomponent and multitarget regulatory mechanism of Chinese herbal medicines through efficient separation and characterization technology and analysis of the compositions, structures, and functions of Chinese herbal medicines. It is equivalent to the "bottom up" research mode, which easily implements modern separation and analysis. However, this research mode has to fully embody the compatibility of Chinese formula, and preferably reflect the scientific connotation of TCM.

2.3 CHEMOMICS

Similar to other omics methods of life science, chemomics is a method system used to study interrelation of composition change and biological effect of chemomes. Although life science is a discipline to study the regulation of life activities in the process of interaction between biological systems and the external environment, the existing approaches, such as systems biology, pay close attention only to the biological system. It still lacks a viable approach that requires characterization of the external environment and study of the interaction between two complex systems. Therefore, chemomics is proposed mainly to research the effects of external chemistry complex system on biosystems. Chemome means, under certain conditions, a chemical convergence including complex chemicals in the external environment that affect biosystems, such as medicines, food, and other chemical substances. It considers TCM as a whole group of chemical substances, and researches changes of the chemome data corresponding to formula compatibilities (compatibility relations of the subchemome) and the correlation of changes and biological effects such as pharmacology, pharmacodynamics, and toxicity.

2.3.1 Definition and Hierarchy of Chemomics

Chemomics is directed by a complex scientific theory. It employs hierarchical, systematic, and progressive optimized strategies. It emphasizes the effectiveness on the whole (global chemome), which means that it can result in expected biological responses, including efficacy for diseases such as Western diseases and TCM syndromes. Then, as it turns from the whole to parts, the first step is to clarify the relationships among the various parts and confirm the effective

part (effective chemome). The second step is to study the mutual relations among the various ingredients of effective chemome, in order to find and identify the effective compound mixture. Since the research of chemomics is divided into different levels, it can use different characterization methods for chemical characteristics.

2.3.1.1 Global Chemome Global chemome is the most complex manifestation of an external chemical system, and it is a collection of all chemical substances that enter into a biological system. Global chemome can be divided into multiple subchemomes. The division of subchemomes is conducted generally according to the similarity and distinguishability of chemical properties, similarities, structures, or functions. For example, a TCM preparation is usually composed of several herbal medicines, and they constitute together the global chemome. With respect to the preparation, ingredients of each herb constitute a subchemome; or, rather, a preparation is divided into several fractions with different properties or functions by a certain method, and each fraction can be considered a subchemome of the global chemome. Global chemome is an extremely complex system. Just as proteomics cannot characterize all the proteomes, metablomics is not yet able to identify and quantify all the metabolites. At present, it cannot confirm all the ingredients of the complex chemomics. Just as in metabolic fingerprinting, the existing chemistry fingerprint technology is useful to characterize chemomics. A variety of methods and multiple fingerprints can be applied to fully express the rich chemical information of chemomics. Furthermore, it is possible to take examples from the differential proteomics to study the change of the differentially designed chemomics under certain conditions.

2.3.1.2 Effective Chemome In global chemome, the existence of some subchemomes has little effect on the efficacy of the global chemome. Or the existence of some subchemomes may offset the holistic effect. Sometimes the absence of some subchemomes will reduce the efficacy of the global chemome. So on the premise of taking global chemome as comparison, it is necessary to screen and select various subchemomes by designing systematically for certain functions. And the finally new subcollection composed by the essential subchemomes will not reduce the global chemome's function, which is called effective chemome or functional chemome. The chemical feature should be represented by the effective component group fingerprint together with multicomponent quantitative analysis.

2.3.1.3 Effective Compound Mixture Though there are some unnecessary chemical ingredients in the effective chemome, the majority of nonessential chemical components have been removed on the premise that this action will not reduce the holistic effect in comparison with the global chemome. It simplifies the chemical composition greatly and supplies the foundation and condition to conduct the research on effective ingredients thoroughly. It is

possible to identify the key effective ingredients or effective component group by further separation as well as perform relevance analysis on active ingredients. That means the minimum set of chemical components would be obtained by further screen according to certain efficacy. An effective compound mixture can be represented by multicomponent quantitative analysis. The simplified form of effective compound mixture is a single component, which is equal to a lead compound. The basic task of chemomics is to find the effective chemomes from the global chemome, then confirm the effective compound mixture and reveal their mutual relations.

2.3.2 Relationship Between Chemomics and Systems Biology

Systems biology refers to describing all the elements of a system under certain conditions, identifying the biological networks of various elements in a system, and characterizing the information flow of elements or networks that are associated with specific physiological stimulation. In short, systems biology is to study the relationships among the various elements in biological system. Recently, many existing technologies, such as genomics, proteomics, and metabonomics, can obtain a massive amount of various element data about the biological system, and these different types of data can integrate into a network model through bioinformatic technology to reveal certain operation processes of the biosystem. However, the existing systems biology is concerned only with the complex biological system by integrating multiple omics methods. It ignores the chemically complex system of the external environment and its impact on the biological system. In other words, the existing systems biology focuses on the biology information network of biologic internal systems in response to the specific stimulations (e.g., medicine treatment) in terms of the system by integrating multiple omics methods. There was no way to characterize the other system of that stimulation (external system) before we proposed the strategy of chemical chemomics, which has provided a solution for this problem. The study of chemomics is the study of the external system and how it reacts with biological systems in order to discover the effective chemomics (chemical information network), which are related to specific biology responses or functions. As a result, chemomics is a necessary complement to existing systems biology methods; and it makes the research of interaction of biological systems and external input system (environment) possible by supplying a good method. And then "chemomics-integrated global systems biology" was raised by integrating the existing systems of biology and chemical genomics. It can not only understand biological systems functions systematically in the view of genes, proteins, metabolism and other multilevel networks, but also represent the chemical substance information input externally, and discover the effective chemomics on the basis of correlation research between the chemical substance information and biological function information. Along with the development of chemomics and systems biology, when chemomics-integrated global systems biology researches the effect of complex biological systems and

external complex systems, it not only describes biological system responses, but also identifies the corresponding chemical materials that lead to response, and therefore further understands the process and the mechanism more profoundly.

2.3.3 Application Modes of TCM Chemomics

TCM chemomics has two application modes, "top-down" and "bottom-up." The "bottom-up" mode first separates active components or individual components from herbs, then composes an effective formula by the combination of effective component group or single ingredients. After we proposed the two chemomics application modes, the "bottom-up" mode was adapted to herbalome by Professor Xinmiao Liang of Dalian Institute of Chemical Physics Chinese Academy of Science. The "bottom-up" mode has also been developed internationally; for example, it has made significant progress in the recent multicomponent antimalaria drug discovery:[26] according to recognized multiple drug targets, this mode uses modern pharmacological methods to screen the best compounds from traditional antimalaria plant medicine, then screen for the optimal combination according to these lead compounds or the mutual functions of existing anti-malaria medicine. These multicomponent antimalarial drugs provide the best solution for the widespread drug resistance of strains of malaria, and it is possible to further improve the drugs' curative effect. This also makes us feel encouraged, since the international community can realize the advantages of component compatibility. However, the "top-down" mode (shown in Fig. 2.1) could gain a higher efficiency and success rate in the research of formulas for the research and discovery of TCM preparations that are clinically effective, especially for the big TCM preparations with complex combinations. On one hand, the compatibility of the existing approach is effective and reasonable if the traditional formula is clinically effective. Compatibility groups of active ingredients should exist and dependent on the compatibility of the original formulas, maintaining and utilizing the existing medicament structure is equivalent to having a relatively clear direction. On the other hand, the integration path of the "top-down" mode should be emphasized when developing component compatibility of Chinese medicine. It can reflect the characteristics of TCM better, optimize compatibility research under the guidance of TCM theory, and reveal the mechanisms of traditional formula.

The "top-down" mode has to be directed by the guidance of TCM theory and complexity theory when it is applied to the studies of formulas. In addition, it needs to set a fixed adaptation syndrome or disease (efficacies) and establish a viable model for evaluation and parameters. On the premise that the preparation is effective as whole, the formula is divided into several different components (subchemomes) according to particular herbs or chemical properties with the hierarchical, systematic and gradual optimizing research strategy proposed previously. Then after an appropriate mathematical design, the correlation between the chemical composition and efficacy information of

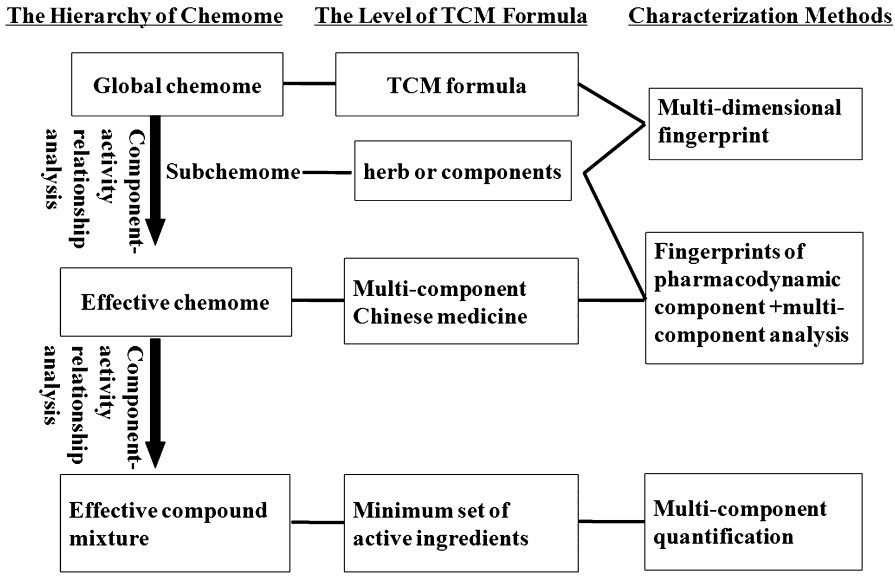

Fig. 2.1 "Top-down" mode of TCM chemomics diagram.

various subchemomes' combination will be studied to identify the relationships among the various components. After removal of unnecessary components under the principle of efficiency, eventually essential components are used to reconstitute an effective chemome. The effective compound mixture will be found and identified according to the relationships among various components of effective chemome (relationship between composition and effectiveness). The relationship between composition and effectiveness would be identified by the component-activity relationship analysis. The efficacy components or effective compound mixture can be identified in the nonlinear interaction functional relationship of two matrices with bioinformatic and chemical informatic technologies.

As the chemome research of a formula discovers the effective chemome gradually under the premise of effect guarantee, a more effective compound mixture could then be determined. As for the certain function and treatments, the determined effective compound mixture has to represent the effective chemome, and the effective chemome has to represent the global chemome. In other words, for a formula, multiple levels of expression of the chemical basis with a certain function and efficacy—global chemome, effective chemome, and effective compound mixture—have equivalent effects for a certain function and efficacy; and their research level gradually becomes more in-depth. Global chemome is the first level, so it is the most complex manifestation; effective compound mixture is the third level of the study, so it is the simplest form, expressing the material basis corresponding to the researched function

and efficacy. In other words, the absence of any ingredient will affect the efficiency greatly. Generally, an effective compound mixture is a group of compounds that have common functions. It is possible to find a single active ingredient (i.e., a lead compound) in the effective compound mixture whose potency is equivalent to the global chemome. So it is possible to find lead compounds in chemomics (if they exist in the original preparation), which can be considered to be the special case of the effective compound mixture. Compared with conventional screening, the application of chemomics research strategy with a higher success rate and efficiency decreases workload greatly. However, either effective chemome or effective compound mixture should correspond with particular function (or a disease). It may become "invalid" or "inefficient" for the other functions and efficacies of the global chemome. So component compatibility or active ingredient compatibility is exactly equivalent to the whole formula, and can only be referred to as its function and efficacy (or specific disease).

Depending on its complexity, a formula can be penetrated deeply by multilevel chemome research with the research needs and technique conditions. Global chemome research is equivalent to the holistic-level research of formula. For a long time, a commonly used method for the development of new TCM preparations was on the basis of optimization and combination of subchemomes (compatibility of medicines). As global chemome is complex, it is difficult to elucidate the effective ingredients and mechanism of action clearly. While just like metabolomics and proteomics, no matter whether the majority of contents are in the fuzzy state, it still does not impede their application as a research method. So the first level of chemomics research can be applied to the compatibility research of formulas. We believe that the research of global chemome should be maintained to reflect the characteristics of TCM preparations, which is beneficial for its genuine protection. Although some issues are not clear, the efficacies of TCM formulas are exact. Therefore, it is beneficial to keep the old flavors to further the succession and inheritance of TCM.

The screening and confirmation of effective chemome as the second level is the compatibility of effective component mixture of Chinese medicine, which is aimed at the discovery of multicomponent Chinese medicine, as well as clarifying the composition and mechanism, may be the key point of studying formulas at present and in the near future. It may become the research focused on TCM compounds for some time to come. With the heightened requirements for formulas and the progress of modern science and technology, research may also advance to the third level, and gradually enter the level of the effective compound mixture, including developing an herbal medicine with single components. Currently, TCM is evaluated by the general standards of Western medicine, which requires clarifications on all ingredients. This is unreasonable for TCM. Therefore, the chemomics we propose provides such a multilevel, sustainable development strategy and method of research; and it is likely to embody the characteristics of formulas as safe, effective, stable and controllable.

2.4 CHEMOMICS AND THE RESEARCH OF FORMULAS

A formula is a complex system whose efficacy material basis is the generalized chemical composition, including three categories which are inorganic substances, small molecular organic compounds (such as essential oils, alkaloids, flavonoids, and saponins), and biological molecules (such as peptides, proteins, and polysaccharides). Relying on these constituents, a formula with the nature of primary-secondary multitarget exhibits the holistic synergetic therapeutic effect. Therefore, it is necessary to understand the relevance between the material basis and the natural theory, compatibility theory, processing, efficacy, action mechanism, and pharmacokinetics, in order to elaborate on the connotations of TCM theory such as the essence, mechanism, and nature theory, and then clarify the efficacy chemical composition and the action mechanism for the purpose of research and development of multi-component Chinese medicine.[15] Multi-component Chinese medicine, also called modernized composite medicine (MCM), represents a category of innovative drugs with the credible assurance of safety, efficacy, and quality control while maintaining the advantages of compatibility of TCM, developed through such procedures where the ingredients or fractions of a TCM formula or herbal materials are isolated, purified, and then recombined to form a new optimal prescription under the guide of TCM theories and support of modern science and technologies..

2.4.1 Research on the Relevance of Material Basis, Efficacy, and Mechanism of TCM

Western medicine focuses on treatment and reversal of pathological changes of target organs, so most Western drugs only contain a single component with high specificity. However, Chinese medicine emphasizes syndrome differentiation, which summarizes the pathology and physiological changes at a certain stage of disease as "syndrome." According to evidence of the holistic level of awareness of the disease, from aspects such as the contact of organs and the relationship between humans and nature, Chinese medicine regulates the body's homeostasis on a multichannel and multitarget level. The medicine used in the treatment is formula. All these show the characteristics of TCM: the use of multiple active ingredients with multiple targets, multiple links, and dynamic treatment. The investigation on the efficacy and mechanism of TCM must be based on multiple indices and the research must not be only in multiple indices, but also on animal, organ, cell, and molecular biology levels. The "evidence" that a formula focuses on is how to design the four levels perfectly.

The holistic curative effect and the action mechanism of the formula with a good clinical efficacy have to be explained at four levels. Split preparation research is a means by which to study TCM. However, preparation study emphasizes the splitting side of research and the extraction of effective

component groups, but ignores the application characteristics of TCM preparations, leading to the results that many studies cannot explain on the holistic nature of preparation.

The research areas of formula mechanisms are not only their efficacy, but also their functional roles. The mechanism research should become the key point of research. It researches both the traditional efficacy and the new effect of formula. Discovering new pharmacological roles and extending the scope of treatment are the main purposes of formulas. For example, when the mechanism of *Danggui Shaoyao* powder (Angelica Sinensis Radix, Paeonia Lactiflora Radix, Chuanxiong Rhizome, Macrocephala Rhizoma, Poria, and Alismatis Rhizoma) was studied, the function of drug-induced memory impairment and the influence of neurotransmitters such as acetylcholine were found; and these functions are an effective clinical treatment for senile dementia. The combination of chemistry and pharmacology of formulas includes pharmacodynamics, pharmacokinetics, mechanisms and the preparation compatibility law of TCM and may solve the modernization problem of TCM fundamentally.

2.4.2 Research on the Relevance of Material Basis and Pharmacokinetics of TCM

Pharmacokinetics of TCM might include two aspects: one is TCM pharmacodynamics, using the biological half-life as an indicator to evaluate the biological effect; the other is TCM pharmacokinetics, using the half-life of the prototype drug or its metabolites in blood or tissues as indicators. Study of the pharmacokinetics of TCM focuses more and more on the mechanisms of TCM, clinical therapy, optimal treatment programs and new drug dosage design.

Pharmacokinetics of preparation and "*zhenghuo*" raised by Huang Xi and his research indicates that pharmacokinetics can be researched through changes of chemical composition of TCM formulas in the body.[27–28] For example, it was found that compatibility of *Chuanxiong* with *Danshen* could significantly change pharmacokinetics of TMPP by reducing its absorption and bioavailability after administered to rats with *Chuanxiong Danshen* decoction. Compatibility changes of *Jun*, *Chen*, *Zhuo*, and *Shi* can significantly affect their pharmacokinetic parameters, and are closely associated with efficacy and side effects. Glycyrrhetinic acid (GA) has anti-inflammation, anti-allergic and anti-cholesterol blood function. Enzyme immunoassay and HPLC determination of the concentration of GA were applied to compare the changes made by licorice decoction and GA after oral ingestion of glycyrrhizin licorice decoction, and found that GA content found in the blood improved greatly when combined with *Shaoyao* (Paeoniae Lactiflora Radix). In addition, GA in urinary and fecal excretion was determined, and the improvements in GA content might be due to some factors that make fecal excretion lower and prolong the time that GA stays in the body. There is not enough information to explain licorice

decoction on the levels of such a medicinal material nature. In order to explain exactly which components of *Shaoyao* play this role, perhaps we can divide *Shaoyao* into a number of effective component groups, or divide the decoction of peony into a number of effective component groups and redesign the experimental program.[29]

The fast development of pharmacokinetics of TCM benefited from the material basis of TCM—ideas of chemical constituents and high sensitivity, microvolume, and quick and efficient separation methods. At present, GC, HPLC, CE, and combined techniques such as GC-MS, HPLC-MS, and CE-MS, in particular the application of HPLC-MS/MS and HPLC-NMR, will provide a quicker, better result for pharmacokinetics.

2.4.3 Research on the Relevance Between Material Basis as Well as Efficacy of TCM Formula and Informatics

For many years, a small database of efficacy and material basis for TCM formulas has been established, and its regularities have been analyzed by computer. However, there are not enough samples. The design of the database system needs to be improved to explore the relationships between the chemical constituents of Chinese traditional compound medicines and their effects. All the above are the main problems of current Chinese traditional medicine database research. Research needs to develop the ability to clarify the relevance of chemical constituents and medicine effects from a large amount of data and to supply a basis for developing new formulas. Information distribution should be carried out comprehensively and systematically. It should begin with the establishment of databases, one of which is the TCM database, and it should include nature, taste, material distribution, administration site, chemical composition, pharmacological action, side effects, and adaptive diseases. The other one is formula database. It should include ancient preparations, preparations with confirmed efficiency, source of drugs, adaptive diseases, clinical curative effects, active components, effective chemical constituents, and pharmacological actions. In order to find the regularity of Chinese traditional medicinal compounds, the application of statistics, fuzzy mathematics, cluster analysis, artificial intelligence, and other information science, as well as the basic theory of TCM and expert experience, are needed. All the above will supply the basis for the idea of new drug development.

The goal of correlation research between material basis and efficacy is to describe the chemical compositions, effects, and mechanisms of formulas. To achieve this purpose, we must combine the research of chemical composition and pharmacology. In order to adapt the complexity and holism of formula, it is necessary to study the drugs' (compound, medicinal herbs) effective parts and active ingredients. This research also needs to be combined with efficacies, in order to confirm the effective part and active ingredients by the "monarch, minister, assistant, and guide" theory, and clarify the

material basis of compatibility; moreover, the research should be carried out for pharmacology on four levels, including whole animal experiments, organs and tissues, cells, and molecular biochemistry. We believe that modern Chinese preparations can be researched and made so that they are internationally accepted.

The important application of information science to chemomics and the modernization of TCM have also been reflected in the composition–activity relationship of Chinese medicine. The so-called composition–activity relationship is a method used to identify the efficacy related compounds group from the global chemomics (global fingerprint) of TCM by bioinformatics methods. It emphasizes the relevance of each compound in the fingerprints and its efficacy, but not the relationship of the entire fingerprint and efficacy. Composition–activity relationship research is not only applied on the relevance of each original compound and its efficacy but also on the process these compounds have in body. The Chinese medicine effective component pharmacokinetics-pharmacodynamics (PK-PD) correlation analysis has vital significance regarding the revelation and proof of the composition and effectiveness relationship of Chinese medicine. The PK-PD correlation analysis of effective component groups is vitally significant for the revelation and corroboration of TCM composition–activity relationship.

There will be a large amount of data in the pharmacokinetic research; and how to make full use of these data to identify useful knowledge (time–dose curve, time-effect, pharmacokinetics, pharmacodynamics parameters, and so on) is a key issue. Intelligent computing is an emerging branch of pattern recognition and data analysis. This data-driven branch aims at knowledge discovery and is widely used in data mining. In the last ten years, intelligent computing methods have been gradually applied to pharmacokinetic studies; pharmacokinetic models established by this method achieved better results than the traditional methods. This field has gained more and more attention and has become a fast-growing research branch. So far, intelligence computing methods applied to pharmacokinetics include mainly statistical learning theory (including artificial neural networks, support vector machines, etc.), fuzzy logic, linear change methods such as dynamic systems and fractal methods. Novel mathematical models have been widely used in pharmacokinetics, pharmacodynamics, and pharmacokinetics pharmacodynamics correlative research.

2.4.3.1 Application of Intelligent Computing Methods to Pharmacokinetic Studies Artificial neural networks can be used to predict plasma concentration and pharmacokinetic parameters. Compared with the traditional processing methods, artificial neural network has better accuracy. Yamanura et al.[30] predicted the plasma concentration of amino- glycosides on the basis of patients' physiological data, and compared the predicted results of artificial neural network and multiple linear regression analysis. The results showed that artificial neural networks can recognize complex relationships

between data, and the method used to predict the plasma concentration of antibiotics is better than standard statistics.

2.4.3.2 Application of Intelligent Computing Methods to Efficacy Studies Artificial neural networks have a strong nonlinear mapping ability, which has been successfully used for the prediction of drug effects.

2.4.3.3 Application of Intelligent Computing Methods to Pharmacokinetic-Pharmacodynamic (PK-PD) Correlation Research Artificial neural networks can determine the time-dose relationship of pharmacokinetics and time-effect relationship of pharmacodynamics through statistical analysis of the pharmacokinetics (PK) and pharmacodynamics (PD) correlation of biological active substances, and predict each other according to the correlation data. The artificial neural networks carry out information processing by simulating human neurons, and then a complex nonlinear mapping can be established. It has been successfully applied in variety of analytical fields such as pharmacokinetics and pharmacodynamics as well as in the relationship between pharmacokinetics and pharmacodynamics. The support vector machine developed recently, which is the second generation of artificial neural networks, further improved some shortcomings of artificial neural networking. It makes parameter adjustment more convenient, and the predictive ability was greatly enhanced. Therefore, it has become the hot topic and trend of researchers in fields such as intelligent information processing, artificial intelligence, pattern recognition, machine learning and data mining. Bayesian network is a graphic model that is used to represent the connection probability of the variables. It provides a natural way to indicate the causal information which can be used to find the potential relationships of data. In the Bayesian network, nodes represent variables, and directed edges represent the dependencies of variables. Bayesian networks have become one of the most dramatic focuses among various data mining methods with its characteristics such as unique uncertain knowledge expression form and abundant probability expression ability, together with increment learning characteristics by integrating prior knowledge. So the application of Bayesian networks to the analysis of PK-PD relationships in TCM pharmacokinetics has exhibited a unique advantage. However, there is no research report about the application of Bayesian networks.

Judging from international research status, the applications of intelligent computing methods on pharmacokinetics have obtained better results than traditional methods. However, since this technique has just started, it is not systematic and comprehensive enough yet. Some latest algorithms of intelligent computing methods have not been reported, such as support vector machines with strong predictive ability, nuclear methods based on positive definite nuclear methods and Bayesian networks.

According to the above analysis, it can be seen that intelligent computing methods are usually used to research pharmacokinetics of Western medicine,

which are usually single compounds. While TCM are always used as formulas, chemomics contains several hierarchies such as effective component group and effective ingredients. In addition, its pharmacological effect also has to be divided into multiple levels and multiple indicators. As a result, it is significant to apply intelligent computing methods, which are good at dealing with complex issues, to pharmacokinetic research on Chinese medicine and formulas and form a unique research method on pharmacokinetics of TCM.

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CHAPTER 3

TECHNOLOGICAL PLATFORM OF TCM CHEMOMICS

A gradational, systematic and stepping optimization strategy for the chemomics of TCM was proposed with the guidance of the scientific theory of complexity. First, the whole (global chemome) is effective (exhibiting the anticipated biological efficacy, such as the therapeutic effects). Then, the global chemome is divided into several sections (subchemomes) and the relationship between the parts should be clarified. After the effective fractions (the effective chemome or chemomes) are confirmed, the relationship between major components should be considered, and then the effective compound mixture should be found and determined. So far as the special function, the effective compound mixtures must be able to represent the effective chemome, while the effective chemome must be able to represent the global chemome. This chapter will specifically introduce the acquisition methods and techniques of TCM chemome based on the previous chapter.

3.1 ACQUISITION METHODS AND TECHNIQUES FOR TCM CHEMOMICS

Since the gradational strategy was adopted in the study of TCM chemomics, different methods should be used to acquire gradational chemomics.

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3.1.1 Acquisition Methods and Techniques for the Global Chemome

The global chemome is the most complex expression form of external chemical systems, which is the collection of all chemical substance and chemical compositions inputted to the biological system. It is considered to be composed of various chemical ingredients from the herbs of the TCM formula in question. In order to provide credible material basis for the continuous research, the aim of researching the global chemome should be to extract as many components as possible. Lately, a lot of new technologies have been used in the research and discovery of TCM, which greatly promoted the development of TCM industry, so that Chinese medicine research level rose to a new height.

3.1.1.1 Solvent Extraction According to the different solubilities of the chemical components in different mixtures, an appropriate solvent should be chosen to extract the compounds from tissues of herbs. The specific operation is to choose the exact solvent according to the characteristics of proper components, then add it to the grinded materials. The solvent will penetrate the cell wall and flow into the cell gradually, and the soluble material will be dissolved, which will result in the difference between intracellular and extracellular concentrations. Then the intracellular solution with high concentration will spread out continually, while the extracellular solvent will get into the herbal tissues. The saturated solution is filtered and concentrated until the concentration between intracellular and extracellular is balanced dynamically. The fresh solvent is added into the residue and the above procedure is repeated until most of the required compounds in the herbs are extracted. Then, all of the extracted solutions containing required active ingredients are mixed. Immersion, percolation, decoction, and reflux are commonly used as extraction methods.

3.1.1.2 Steam Distillation Steam distillation is applied for the components that are difficult to dissolve or insoluble in water, do not react with water, and are able to be distilled with water steam without being damaged. Usually, the boiling point of these ingredients is higher than 100°C. A certain vapor pressure may be achieved when the temperature is close to 100°C. The mixed liquid will be boiled when the sum of vapor pressure of the components and water arrives at 1 atm (1 atm = 101325 Pa), and the volatile substances should be taken away together with the steam.

3.1.1.3 Semi-Bionic and Bionic Extraction Technique Semi-bionic extraction (SBE method) is a new technique designed for gastrointestinal delivery of TCM preparations in terms of biopharmacy. Strategies such as the combination of global and molecular drug research methods, simulation of the gastrointestinal tract transportation absorption environment of oral drugs, and the adoption of activity-oriented separation have been applied in this method.

In order to obtain the "active mixture" with high-content marker components, acidic and alkaline water with suitable pH together with continual extraction were used.

The main characteristics of semi-bionic extraction are summarized as followed: first of all, the extraction process fits the features of compatibility with TCM, clinical medication, and transfer absorption of oral administration in the gastrointestinal tract as well. Second, since both the active mixed components and monomer ingredient are considered to be indicators during the selection of specific extraction process, the comprehensive roles of the active mixture are displayed. Meanwhile, the quality of the TCM preparations would be well controlled utilizing the index ingredients. Third, fewer active ingredients are lost. In many studies on single herb and formula preparation, semi-bionic extraction has been shown to have obvious advantages and broad application prospects. Zhaowang et al. took ferulic acid, matrine, total matrine alkaloids, and the weight of dry extract as indicators. They compared techniques of SBE and water extraction (WE) in the extraction of components of the *Danggui kushen* pill, and found that the SBE method was superior to WE on the basis of comprehensive evaluation of four indicators.[1] But the SBE method still utilizes a high temperature, which tends to influence a number of effective active ingredients and then decrease the potency. Therefore, scholars have suggested that the extraction temperature should be close to body temperature, and simulative physical digestive enzymes should be added to the extraction, in order to make the extraction process closer to drug's transit absorption in the human gastrointestinal tract, which is much more in line with syndrome differentiation and treatment theory of TCM.

The bionic extraction method[2] was proposed based on semi-bionic extraction, in which a biologically active enzyme is added and the principles of chemical bionics (artificial stomach, artificial intestine) and medical bionics (enzyme applications) are comprehensively utilized. Bionic extraction combines methods in global drug research (the compounds extracted from bionic extraction are closer to ingredient groups when they are balanced in the body) and molecular drug research (taking one of the ingredients as an indicator), so the Western medicine mode in phytochemistry is avoided, which makes it more suitable for the systematic treatment characteristics of TCM. The purpose is to overcome the shortcomings of semi-bionic extraction, which damages the active ingredient when boiling, and to obtain the maximum active ingredients in the original drug. Semi-bionic and bionic extraction methods have provided us with a new way to extract active ingredients together with a new idea for screening the active ingredients. Based on the perspective of biochemical engineering, combining with the thorough analysis of phenomena such as false-positive and false-negative, which resulted from TCM screening in cell culture, we proposed a biochemical engineering solution for TCM screening based on the idea of bionic science. It is easy to measure the processing parameters in the method,[3] and both the

fundamental role of drugs and the information about screening targets of drug mechanisms are provided. This method had been applied in a study taking gelatinase as a specific target.

3.1.1.4 Ultrasonic Extraction An ultrasonic wave is a kind of high-frequency mechanical wave, and the ultrasonic field provides energy to the system mainly through ultrasonic cavitations. The ultrasonic wave frequency ranging from 15 to 60 kHz is usually used in the process of hardening and chemical reaction. Ultrasonic extraction has been applied in the extraction of active ingredients in Chinese medicine. Its principle is to use the effect of ultrasonic cavitations to damage the cell membrane, which helps to release the active ingredient. And the extraction solvent can be vibrated by the ultrasound, which may make the solution diffuse easily. Simultaneously, since the thermal effects of ultrasonic waves keep the temperature of water at 57°C, it will increase the water-solubility of the raw material. Ultrasonic extraction has been widely applied in the extraction of various types of chemical compositions of TCM, such as polysaccharides,[4] saponins,[5] flavonoids,[6] and alkaloids,[7] and it has exhibited distinct advantages compared with the conventional methods. Currently, ultrasonic extraction is mostly used for the study of monomer ingredients in laboratory research, yet few studies pay attention to the processing parameters in extraction of TCM compounds, which limits its application.

3.1.1.5 Microwave-Assisted Extraction A microwave is a nonionizing electromagnetic radiation. Microwave-assisted extraction (MAE) is a new technology which uses microwave energy to improve the extraction efficiency. In a microwave electromagnetic field, the extracted polarized molecules undergo rapid spinning oriental alignment, in which the generation of tearing, friction, and heat ensures the rapid transfer and ample utilization of the energy, and in turn promotes dissolution and release.

As a new extraction technology, microwave extraction has exhibited many advantages compared with general extraction methods, such as having excellent selectivity, taking less time, and having high efficiency and energy conservation. So this technology has been widely used in research on natural products, especially in the extraction of effective components such as polysaccharides,[8] alkaloids,[9] flavonoids,[10] saponins,[11] and volatile oils.[12] The principle of MAE is that the rapid vibration with the ultrahigh frequency of 2.45 billion times per second produced by magnetrons makes the molecules collide and crush each other, which benefits the extrusion of effective components. During the process, herbal drugs will not aggregate and gelatinize, which may overcome such deficiencies in hot water extraction. Though the microwave-assisted extraction technology has showed many advantages, it is only used in the lab for now. Further, thorough study should be carried out to realize the industrialization of this technique.

3.1.1.6 Supercritical Fluid Extraction Supercritical fluid extraction (SFE) technology is a method using supercritical fluid as an agent to extract the effective ingredients from a liquid or solid. CO_2 is the most commonly used supercritical fluid. Supercritical fluid extraction has characteristics of both liquid phase extraction and distillation simultaneously, and the extraction solvents are easily to recycle. Compared with traditional extract methods of effective components, supercritical fluid extraction has many advantages such as high extraction efficiency, good product purity, simple operation flow, low consumption of energy, low operational temperature, and a closed system. SFE is especially suitable for the extraction of volatile ingredients with poor thermostability and which are susceptible to oxidization, as well as the fat-soluble and small molecular substances of TCM, such as volatile oil components.[13] In addition, this method has also been used in the extraction of other natural products, such as flavonoids,[14] alkaloids,[15] and glycosides.[16]

Although there are many reports about the application of the supercritical fluid extraction technique in the extraction of effective components in TCM, many engineering factors should be taken into account in practical application due to the special requirement for the high pressure device this technique uses.

3.1.1.7 Ultrahigh Pressure Extraction Ultrahigh pressure extraction is a novel technology for the extraction of effective components in TCM. For the purpose of extraction, hydrostatic pressure of more than 100 MPa was used to act on the extraction liquid of TCM compounds, and, after a period of time, the pressure is released rapidly. There is essential difference between the ultrahigh pressure method and thermal extraction. Owing to the smaller damaging effects on the active ingredient, ultrahigh pressure extraction exhibits unique advantages in the extraction of heat-sensitive substances, such as ginsenoside saponin[17] and tea polyphenol.[18]

The application of various new methods has greatly enhanced the extract efficiency, and significantly reduced the destructive effect of active components. However, because of strict requirements for the devices of these abovementioned new methods and techniques, some key parameters should be studied further.

3.1.2 Acquisition Methods and Techniques for the Effective Chemome

The effective chemome is the simplified chemome with the same effect as the global chemome, which was obtained by discarding the inefficient chemical groups on the basis of good knowledge of the TCM formula's function. In global chemomics, the existence of some subchemome may show little impact on the overall efficacy, and may even weaken the effects. Therefore, in order to display the efficacy of the remaining effective chemome, appropriate methods should be used to remove this kind of subchemome. As far as the

study of effective chemome is concerned, accurate acquisition should be considered as the guiding principle, and various appropriate separation means should be used to separate the effective chemome from the global chemome.

3.1.2.1 Water–Alcohol Method The water–alcohol method is the most common extraction method in current research on TCM. As for water-soluble components, water is usually used as the extraction solvent, then protein, starch, pigment, and resin are precipitated down from water extracts with a high concentration of ethanol because of the differences of solubility. The components with high solubility both in water and in ethanol will remain to the greatest extent.[19] As for alcohol-soluble components, certain concentrations of ethanol are used as the extraction solvent, and water is used to precipitate impurities from alcohol extracts.[20] The water–alcohol method is normally used in TCM extraction processes, and although this method has some drawbacks, such as being time-consuming, having low efficiency, and possibly causing the loss of heat-sensitive components, it is relatively simpler to transfer from the laboratory to the industry because the parameters of water–alcohol extraction process are easy to control.

3.1.2.2 Membrane Separation Technique The membrane separation technique (MST) is an emerging high-efficiency separation technique including ultrafiltration, microfiltration, nanofiltration, and reverse osmosis. The separation, classification, purification, or concentration for a multicomponent system is carried out using a natural or artificial selective permeable membrane,[21] which takes the external energy or chemistry potential difference as impetus. Compared with conventional separation methods, MST has garnered satisfactory technology descriptions such as high efficiency and low-energy consumption.

It is widely accepted that the effective components of TCM should be composed of inorganics, organic compounds with molecular weight about 1000 kDa (such as volatile oil, coumarins, anthraquinones, cardenolides, terpenes, saponins, flavonoids, alkaloids, organic acids, and polysaccharides), while the molecular weight of the ineffective ingredients (such as polysaccharides, protein, and starch) may be over 10,000 kDa. In theory, the active ingredients can be isolated from the extract with membrane microfiltration and ultrafiltration technology, so it has been applied in practice.[22]

3.1.2.3 Macroporous Adsorption Resin Separation Macroporous adsorption resins are a class of organic polymer adsorbent with satisfactory adsorption performance developed in the late 1970s. The resins were cross-linking polymerized with styrene or acrylate as a monomer, divinylbenzene as a cross-linking agent, and toluene or xylene as a porogenic agent, which is a polymer with a porous skeleton structure.[23] The cross-linked polymer without an ion-exchange group is generally white spherical particles with size of 20–60 meshes.

The physical and chemical properties of resin are stable. Therefore, it does not dissolve in acids, alkalis, or organic solvents, and will not be affected by inorganic salts or other strong ionic compounds with low molecular weight. For the purposes of separation, purification, decontamination, or concentration, relying on the van der Waals attraction between the adsorbed molecular (adsorbate), and working by the physical absorption of the huge specific surface, target components may be eluted with certain eluting solvents according to the adsorption power and molecular size. As for the microscopic structure, the macroporous adsorption resins consist of a many mesh pocket structure with microscopic pellets, and the total surface area of particles is very large. These properties make the resins have greater absorption capacities. On the other hand, a certain range of the aperture makes these mesh pockets possess satisfactory selectivity for compounds passing through according to the difference of molecular weight.

Recently, the macroporous resin adsorption separation technique was widely used in the extraction and separation of effective components in TCM, such as flavonoids,[24, 25] saponins,[26, 27] and alkaloids,[28, 29] as well as free anthraquinones,[30] phenol,[31] and trace elements.[32]

3.1.2.4 Integrated Extraction Separation Technology The requirement of many devices and the complexity of the technological process make separation and purification usually account for a significant proportion of the expense of the pharmaceutical engineering of TCM. The separation process of effective components is made up of a series of operation units, such as centrifugation, filtration, concentration and separation. Due to the characteristics of large volume and high turbidity, as well as the large amount of time the process takes, solid–liquid separation is generally considered to be the restrictive step of the purification process. Integrated extraction separation technology is a more effective operational unit as it effectively combines two or more operation units, which achieves the purposes of increasing yield, simplifying the purification steps, and lowering the purification and investment costs.[33]

Taking the features and requirements of obtaining the TCM chemome as penetration points, we have established a novel integrated preparation method, which uses the expanded bed adsorption separation as the core technique. This method has been successfully applied in the integrated preparation of iridoid glycosides and crocin from *Zhizi* (Gardeniae Fructus), as well as the water-soluble components from *Danshen* (Salviae Miltiorrhizae Radix et Rhizoma).[34, 35]

3.1.3 Acquisition Methods and Techniques for the Effective Compound Mixture

An effective chemome is obtained by removing the nonessential constituents without reducing the global activities, yet often there are also some unnecessary components left. In order to carry out the study of functional components,

the minimum set of effective ingredients should be obtained by further separation. The active ingredients group is the smallest set screened according to certain effects, which has eliminated the nonessential components in the effective chemome. A fine separation method is essential for the acquisition of the active ingredients group. Chromatographic separation is the most commonly used technology as of late, and high speed countercurrent chromatography (HSCCC) is one of the most promising technologies.

The HSCCC, high performance countercurrent chromatography system, developed by Professor Yiochiro Ito in 1980,[36] is a new type of liquid–liquid partition chromatography. HSCCC separation has avoided problems such as irreversible adsorption, loss and deactivation effectively, due to the fact that the separated components can be distributed in a two-phase separation, and it doesn't need solid support to retain the stationary phase. It increased the recovery rate, simplified preprocessing, and enhanced compatibility as well. For decades HSCCC developed rapidly, and exhibited unique advantages in preparation and separation. In view of the characteristics of HSCCC, researchers unified the current research on natural products and launched plenty of investigations. This emerging technology has been widely used in the preparation of effective components such as alkaloids,[37, 38] flavonoids,[39, 40] polyphenols,[41, 42] terpene,[43, 44] and saponins.[45]

3.2 CHARACTERIZATION TECHNIQUES OF CHEMOME-TCM FINGERPRINTING

A fingerprint of TCM refers to certain chromatograms or spectra of some Chinese herbal medicine after appropriate treatment and certain analysis method which can label the chemical characteristics. A fingerprint of TCM is a comprehensive, quantitative means of identification, which is based on the systematic research of TCM chemical composition, and mainly used to evaluate authenticity, quality and stability of medicinal materials, TCM formulas and its intermediate. It is characterized by holisticness and fuzziness.

3.2.1 Methodology Research of TCM Fingerprinting

Fingerprint research includes fingerprint information acquisition, processing and information mining, and access methodology research as its foundations. Analytical methods must be designed for certain analysis purposes to meet specific requirements with corresponding steps. As to TCM formulas, first, the compatible relationship of composite herbal medicines and the primary and secondary relations during the fingerprint analyzed process should be defined clearly to evaluate the effectiveness and the reliability of separation and analysis results. Second, we have to understand the major active ingredient of the composite herbal medicines, in order to assist the confirmation of analysis and examination methods. And this would guarantee the

comprehensiveness and credibility of substance foundation fingerprinting analysis. Finally, the perfect acquisition methods should be able to guarantee that the analysis result can explain the compound material compatibility relationship clearly and be consistent with basic theory of TCM at the material group level.

3.2.1.1 Sample Preparation Method of Fingerprint Analysis The objective of Chinese medicine fingerprinting includes medicinal materials (including decoction pieces), extract, and semifinished and finished products (preparations). Medicinal materials can be derived from plants, animals and minerals, and sources of medicinal plants are divided into flowers, fruits, rhizome, stems of different parts, and whole grass. Besides the original pills, powders, pastes, and sublimed preparations, the compound prescription includes capsules, granular formulations, injections, and even new controlled release preparations. Analysis results may be fundamentally different because of different sampling methods, or different sample preparation method even for the same specimen, because the chemical fingerprints that can be obtained vary greatly. Therefore, sampling methods and sample preparation in the chemical fingerprint analysis must be valued.

3.2.1.1.1 Guidelines for Sample Collection and Sample Preparation The basic principles of sample collection are that the sample must be representative and homogeneous. In terms of complex material systems, the existing state, form, and distribution of the substance will be presented as a complicated and random space spread. However, the sample can only extract a small amount from a large-scale material system for analysis. The analysis result must be able to represent the actual state of the system under test, and avoid confusion between the local feature model and the overall pattern of material system.

Take the traditional Chinese herb and the sample decoction as an example. One has to pay attention to five factors, including the sample's classification, drugging parts, production location, harvesting period, and processing methods (or processing place), which are often called the "five fixations." One has to obtain a large number of fingerprints, establish the fingerprint database of Chinese herbal medicines, and determine the representative standard fingerprint of the TCM combined with information processing methods on the basis of a large number of samples which are collected fully in different medicinal parts of different origins, different harvest periods, and different processing methods. Simultaneously, one can clarify the changes in the chemical fingerprint of drugs in different species, different areas, different harvest periods, and different processing. One has to maintain the integrity of the historic record when gathering TCM sample. The record should be mainly composed of marked varieties (preferably accompanied by identified traits and microscopic characteristics), medicinal parts, sources (including the origin or source of market) and harvest time (or purchase time), processing methods, and the

initial quality assessment of appearance. Even if the sample had already met the "five fixations," it is better to have a batch of 3–10 samples which make it more representative. In addition, one must pay attention to homogeneity when gathering a sample. Usually, the uniform processing of the Chinese herbal medicines sampling method of the "Chinese Pharmacopoeia" 2010 edition is used as the reference. As to the small-size individual herbs, they should be spread into a square shape and divided into four equal parts by drawing an X over it, and the two diagonal parts should be taken as a sample; repeat above procedures until the remaining quantity is sufficient to complete all of the necessary tests and retention samples. This is the average sample. As to large-size individual herbs, other appropriate means can be used to take an average sample.

In chemical fingerprint analysis, the basic principle of sample preparation is integrity and exclusivity. Sample preparation must reflect the characteristics of the sample sufficiently and also guarantee the integrity of sample characteristics. Fig. 3.1 shows a simple flowchart of sample preparation. First, one has to set the corresponding exclusive preparation method according to properties of ingredients such as polarity and molecular size. The selection of method should be directed at certain specialties of components and tailored to specific ingredients. It may prepare components contained in the materials system specifically. The specific preparation should guarantee that the sample does not lose its ingredients and does not change contents nor statistical proportions between the ingredients. Then the prepared samples can demonstrate the comprehensive characteristics of the material system completely, and thus make the analysis result reflect the chemical substance information of the whole material system completely and credibly.

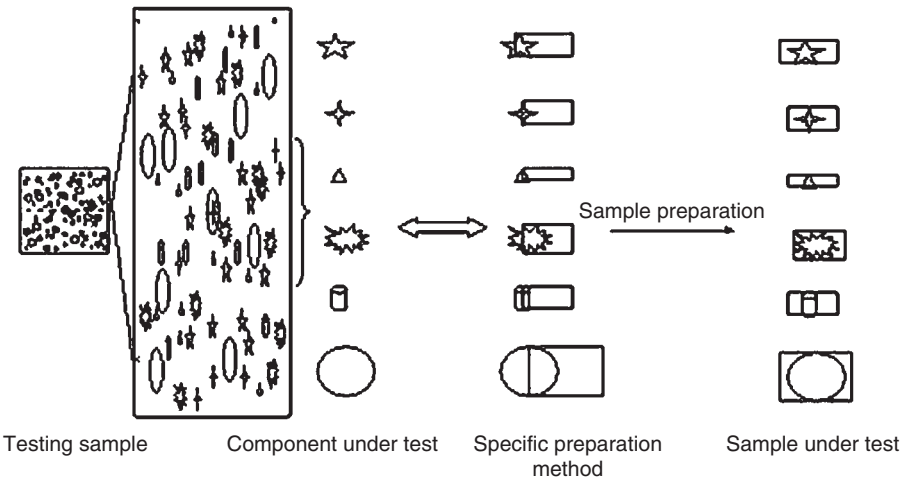


Fig. 3.1 Schematic drawing of sample preparation.

Usually, one has to choose appropriate solvent and extraction methods, try to stop the impurities from being extracted, or remove impurities as much as possible in the process of preparation of the sample of the Chinese herbal medicine during the fingerprint analysis. Samples prepared in this way show fewer impurity peaks in the fingerprint analysis, and the active ingredient peaks are more representative. Besides the above considerations, a particular solvent and extraction method could be considered to prepare the sample solution for some Chinese herbal medicines whose active ingredients have been studied thoroughly. For example, the water-soluble part (including salvia phenolic acids) and the fat-soluble part (tanshinones, etc.) of *danshen* have been shown to have pharmacological effects on the cardiovascular system. According to Chinese medicinal theory, *danshen* is a blood-activating and stasis-dispelling medicinal drug. Therefore, the two-part fingerprint of water-soluble and fat-soluble parts can be initially considered to characterize the quality of *danshen*. For most herbs, at present, we only know the active categories (such as alkaloids) and some of the active ingredients, and are constantly discovering new categories and new active ingredients. Moreover, the roles of Chinese herbal medicines in different prescriptions are various, because of the activities of different effective component groups. Therefore, the fingerprint of the samples prepared by a single solvent extraction system and a single preparation and extraction method lacks the integrity and the specificity of the material ingredient expression. For those materials whose active constituents and functions are little known, the substep extract approach can generally be considered to prepare the sample in plant-based system. General TCM formulas (that is, the final product), such as direct injection, may be considered as a sample solution, and can also be prepared by appropriate sample solution if necessary. Other solid preparations, especially the large compounds, may be considered in the TCM way, and can be extracted step-wise. But the removed impurities are different; for example, honey disturbances should be removed in the large honey pill. For some preparations, the sample preparation should be simplified according to its craft of production process. For example, the production process of certain formulations is the extraction of alkaloids, in which case it is only required to prepare the sample solution according to requirements for alkaloids preparation. The sample preparation of semifinished products and raw materials in fingerprint analysis can be determined by the features of the production process. Take injection fingerprint analysis as an example: it requires that sample preparation of the raw medicinal herbs be the production technology of the injection. Eventually, the prepared sample solution is also required to consider the request of the fingerprint determination method. For example, the sample for a liquid chromatography chromatogram should select a chromatographic elution mobile phase or a weaker solvent; the sample for thin layer chromatography (TLC) can be prepared by solvents whose polarity is similar to developing solvent. The sample for GC can be prepared by a low polarity and low boiling point solvent with the methods of steam

distillation, headspace sampling or solid phase microextraction. In short, the sample preparation process uses the exclusive extract preparation, and sample analysis results can not only fully embody the integrity of the material system, but also avoid interference by coexisting with impurities and ensure the finalized analytical results with higher characterization and accuracy.

3.2.1.1.2 Introduction of the Common Sample Preparation Methods With the development of separation science, sample preparation methods have also been substantially developed, especially the sample preparation method for complex material systems, which has gained more attention in recent years. The specific method requires special preparation methods or combination methods according to the specific nature and status of the sample preparation. As to Chinese medicine samples, the preparation method usually includes conventional extraction methods such as steam distillation, water extraction, and alcohol precipitation (water–alcohol method), alcohol precipitation of water (alcohol–water method), organic solvent extraction, fractionation, adsorption, or precipitation, and it also includes special sample preparation methods such as supercritical extraction, membrane separation, molecular distillation, resin adsorption, and microwave extraction technology.

3.2.1.1.3 Introduction of Sample Pretreatment Method Under the existing analysis conditions and equipment methods, some samples require certain pretreatments to obtain the internal information of the material system for all material components of the complex material systems analysis. Sample preparation may be considered as having two aspects: One is the purpose of the analysis, another is the analytical method. In chemical fingerprint analysis, the goal is to obtain the comprehensive integrity of chemistry information of the material system, and in light of this goal, one can select the appropriate methods for the determination. Therefore, the main objective of sample preparation is to improve the selectivity and to ensure comprehensive and intact analysis. The following gives a simple introduction according to the commonly used three chromatograph method.

As for the analysis of complex samples with TLC, the high content component and the low content component may interfere with each other, and so produce a situation where they mutually dissolve and are difficult to separate. In the case of polymer or inorganic salt in the samples, thin-layer separation performs worse. To a certain extent, TLC separation quality often depends on the experience of the operator. In sample pretreatment for TLC, conventional separation methods can be used, such as distillation, fractionation, extraction, extraction, crystallization, precipitation, distillation, filtration, and membrane separation, which are selected based on the sample and separating systems.

Gas chromatography is typically used for analysis of volatile and heat-stable substances, and needs to consider the sample ingredient comprehensively

and the nonprejudicial demonstration during the sample pretreatment. It usually needs to carry out the extraction separation in advance for TCM, and start the separation in view of volatility and weak polar parts. Conventional sample preparation methods used in TLC can be used in gas analysis. For some volatile or nonpolar substances, derivation methods are often used to make the substance volatile and easy for gas chromatographic analysis. For macromolecules, the cracking process in the sample gas chromatography is widely used. In recent years, headspace sample and solid phase microextraction methods have been widely used, which have extended the application of gas chromatography.

Eighty percent of natural product samples can be analyzed by liquid chromatography. However, the single detection method often does not fully reflect the material information of the sample for material analysis in a complex system. Besides a variety of detection methods combined for the integrated measurement, sample derivation and pre- or post-column reactions are also used during the sample processing. Recently, solid-phase extraction technology has become the most widely used treatment in the steps of sample pretreatment for liquid chromatography analysis, which was applied in impurity processing, ingredient enrichment, or the selection of preprocessing techniques for extraction. It achieved the purpose of online processing of samples and rapid analysis, but in chemical fingerprint analysis, further investigations on effects on the chemical composition caused by the solid-phase column selective adsorption should be conducted.

Sample preparation requires a combination of sample characteristics, extraction methods, analytical purposes and methods. It should be noted that composition content or composition ratio can change during the processing. Therefore, in the determination process of chemical fingerprints, one must consider the impact of all aspects; formulate a reasonable approach to prevent analytical errors which impact the representativeness, the integrity, and the credibility of the sample.

3.2.1.2 Study of the Acquisition of Fingerprints

3.2.1.2.1 Strategies for Chromatographic Analysis of Complex Material Systems In modern analytical chemistry, the analysis of complex material systems has been gradually becoming the main object of analysis. A complex system usually has a diversity of component materials, and the general differences compared with simple physical system are the following: a lot more components in the complex system, fewer components in simple system; the relationship among the components in complex system is complicated, while it is simple in simple system; there is a larger discrepancy of components in complex system, while the simple system composed of simpler contents; and there is a greater discrepancy of components in response to detection in complex system, and the linearity in the measurable scope is unclear, while the response and linearity in a simple system are clear.

Based on the details listed above, we can determine the goal of separation and analysis in complicated systems, which is to maximize the amount of information learned about the composite characteristics of the studied system. When making it specific to the complex system of TCM compound materials, the goals of chromatographic analysis are enhancing insight into the determinacy of the object; describing the responsiveness characteristics of the sample decomposition and homogenization as far as it may go; and optimizing the separation process, based on the principle of global optimization. The whole process is to transfer the unknown information in the original system of samples into known information. The goal of separation and analysis is the fuzzy model on the whole, which embodies the principle of ensuring maximal effective fingerprint peaks on the basis of comprehensive display.

On the basis of ensuring that the fingerprint meets overall similarity for Chinese herbal compounds in a complex material system, the correlation of common features in the system is analyzed, which means that the process for the preparation of medicine according to the test, intermediate goods, and finished three-way comparison of the chromatographic analysis are compared, so that the fingerprint can meet the characteristics of sample analysis.

3.2.1.2.2 Selection of Chromatographic Methods With analysis, we must find a simple and effective method to ensure the feasibility of determination methods and process. To comprehensively analyze the process, extensively analyze the relationship between units, and fully consider the correlation between a series of the determination methods are the necessary requirements for fingerprint determination. The purpose of fingerprints is to find the samples of the system itself that have a characteristic of the process, that is, the fingerprint feature. The complex composition of Chinese herb chemicals, the strong response of the interfering components, and the weak response of the components have brought some difficulties for fingerprint analysis, but rational experimental design, multisource analysis of the test and sample, a variety of analytical method combinations, and data extraction, processing, and identification of the exclusive use of the method can solve the above problems. Here, three preferred chromatographic methods in determining fingerprints of TCM were elucidated.

A selection of TLC methods includes thin-layer plate, unfolding system, and color displaying pattern, among which the thin-layer plate and color displaying pattern display the substance specificity, and unfolding system plays roles in optimization and demonstration. The selection of the unfolding system is on the basis of a Synder solvent selection system, Glajch solvent selection system, and other basic methods, which help improve the specificity of TLC.

A selection of gas chromatography methods mainly involves the sample capture method, the selection of fixative, temperature changes in the program selection, and testing methods. Capture methods and sample selection attribute an effective way to improve the GC detection method for

determination of chemical fingerprints of TCM formulas. In recent years, the development of a variety of gas–liquid, gas–solid, liquid, and solid components of the sample trap offered more options for sample capturing before separation and enrichment, and an improvement of the overall sensitivity of the method. GC/MS is the quality-oriented and general purpose detector, which provides a total ion chromatogram (TIC) or re-ion chromatograms (RIC) and each peak corresponds to the mass spectrum, and obtain more material information through the spectral libraries. Also, selection of chromatography instrument temperature, optimization, and control are used to improve separation efficiency and separation. Among them, accuracy temperature controlling, speed of approach to temperature, and the analysis of the stability of process analysis are the primary verifying indicators in GC validation method. Also, the column, stationary phase volume, different instruments, and different streaming mode and trapping methods should be served as validation parameters.

Eighty percent of the sample can be directly or indirectly separated and analyzed by liquid chromatography. Considering the character of most of the sample, the RP-HPLC is the preferred selection in TCM fingerprint access methods. At the same time, gradient elution was used in the determination process. So the primary consideration of determination method is stability and reproducibility. This means that in the determination process, the method should fully exclude any error and interference caused by measurement, so that the results objectively reflect the characteristics of the sample, and ensure the reliability of fingerprint information.

3.2.1.2.3 Principles of Optimization of Chromatographic Methods With the development and application of chromatographic methods, optimization and the intellectualization of chromatography are the research focus. Along with the strengthening of the complexity of the object analysis and requirements for comprehensive analysis, optimization of the overall chromatographic pattern of the content is still new, and needs further study.

Fingerprint determination of TCM has some specific characteristics: it should be measured within a certain time, usually 60 min based on conventional analysis and general considerations; in order to meet the requirements of fingerprint analysis, the fingerprint peaks must be well under way, with the overall dispersion characteristics; the appearance of peaks should be considered and the elution strategy should be altered if no clear target to optimize the material can be used as an indicator; the results of the evaluation and the specific test conditions, such as wave length, need to be considered after the series of measurements based on comprehensive results. To acquire the Chinese herbal compound fingerprint, the main object is to optimize the gradient elution method, which requires selection of some indicators to guide the optimization of the direction and gradient. Separation and the total separation performance are commonly used for the chromatographic separation evaluation. The choice of gradient mode is to use these indicators to establish

the optimal response function of the separation of some guidance and forecasts, and the peak width effect does not play a major role in the evaluation of separation.

According to the separation formula,

$$R = \frac{2\times(t_{R2} - t_{R1})}{W_2 + W_1}$$

separation and difference of two retention times have a positive correlation, and is positively correlated with difference of two capacity factors. In the process of pre-experiment, the stable major peaks were selected, and the sum of capacity factor variations between every adjacent two peaks was used as an indicator of separation efficiency.

Based on these factors, we can conclude that the basic principles of optimization function are as follows. Separation with time constraints: The separation is based on an overall experiment. For an unknown system, the primary response is selected to be evaluated, and the results have a certain ambiguity. Function-guided results are only a prediction, the peak should be adjusted in real time based on the actual situation.

Rules for the value of optimizing function: As related to the value, the bigger the better, indicating that the greater distinction between the major response peaks. The difference can't be negative, which means that peaks cannot be reversed. The values selected must be in order, the direction of elution is the mobile phase ratio of weak to strong, and one cannot reverse the order of elution the mobile phase, that is, the unidirectivity of the gradient. For the main response peaks or the value of capacity factors with many responsive peaks, and it is far beyond the scope of the overall capacity factors (determined by the total analysis time), one will not consider among the selected elution ratio.

3.2.1.3 Validation Study on Fingerprint Methodology An analysis should include an analysis system (analysis method, apparatus, and equipment), analysis methods, and analytical objects. A complete quality assurance system includes three levels: architecture verification, system validation, and method validation.

3.2.1.3.1 Fingerprint System Validation To validate an analysis system one must inspect the applicability of the selected analysis system by certain evaluation criteria. And an effective analytical method and process should be established ultimately so as to realize the physical properties controlling and quality assurance. As shown in Fig. 3.2, method selection, sample preparation and processing, instrument and equipment determination and calibration, as well as the data processing mode are the main analytical and appraisal contents of the system validation.

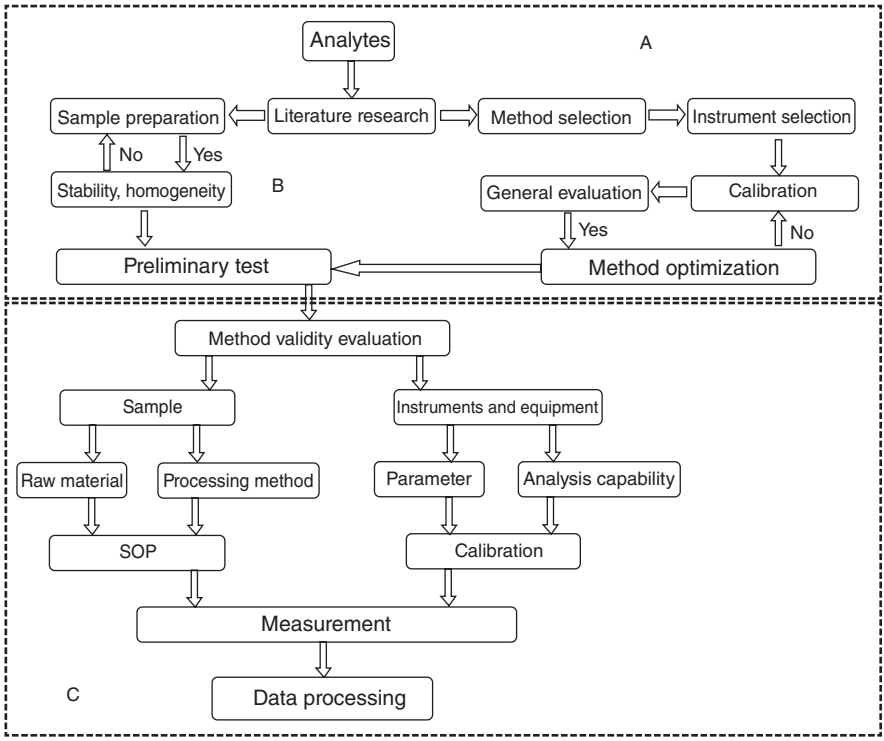


Fig. 3.2 Flow chart of analysis system validation. A: architecture; B: system; C: method.

The establishment of an analysis system is based on the analysis objects. After analysis of the related literature and study of the physicochemical properties of the object, the method is chosen and the relevant equipment property is determined in the case that the contained substance is somewhat known. The preparation of the object and procedures are standardized, and then the sample is premeasured. The routine determination is carried on and the analysis system verification finalized in the case that the methodology inspection and the determination result are credible and reasonable.

3.2.1.3.2 Verification of Fingerprinting System An analysis system takes the analysis of the basic characteristics as a start, determines the appropriate method and analytical instruments after a certain process and finally analyze the results of the tests. Analysis systems can be divided into four steps, which are analysis system establishment, process analysis, data collection, and analysis. Analysis system validation is the experimental procedure of building and remodeling experimental processes, and processes the certification mainly through the two clues. One is the analysis method, including sample source, preparation, processing, and analysis process and norms, and analysis method

determination; another is analysis process analysis and standardization; another is analysis data processing, and so on. Another one is the aspect of equipment, including equipment selection, parameter setting, instrument calibration, analytical targets setting, and analysis.

3.2.1.3.2.1 SAMPLE DETERMINATION This step of the process mainly includes sample extraction, preparation, and determination, as well as data processing. Sample preparation includes the processing and preprocessing of the raw herbs, along with the grinding of mineral medicine and natural material extraction. The most important two steps are the elimination of impurities and the purification. The main verification purpose in the preparation of TCM carries on normalization and standardized research at the effective extraction of the premise. Parameters such as time, temperature, and volume involved in the process are set. The key validation content refers to the removal of impurities, the retention of active ingredients, and new impurities occurring in the processing and their accepted value. Sample preparation is actually the pretreatment process of TCM. Samples obtained after extraction and preparation of TCM will be further purified and become suitable for measurement. The common methods are as follows: extraction, precipitation, distillation, and chromatography extraction. Extraction and chromatography are more readily used among the existing identification of TCM. In the process of sample preparation, the active ingredient recovery and transfer rates should be verified. During sample determination, one first needs to verify the effectiveness of the method, then the alterations of sample existing state in the determination process. The samples must be assured to be stable under various conditions and processing environments. Data processing for the determination of the fingerprint of TCM is a key step in the process. Therefore, it is very effective to emphasize the multisample multilevel source and specific analysis and treatment methods for the analysis and verification of data in the data processing step.

3.2.1.3.2.2 ANALYTICAL INSTRUMENT VALIDATION The process of chemical fingerprint analysis is mainly related to two categories, which are the spectral and chromatographic methods. Related instruments are associated with UV, IR, NMR spectroscopy, and mass spectrometry. Chromatographic associated technologies include thin-layer chromatography, gas chromatography, and high performance liquid chromatography. The involved detection methods include TLC, thermal conductivity detection, flame detection, electron capture, variable wavelength UV, UV diode array, fluorescence, and evaporative light scattering detection. All equipment and instruments necessary are commercially available and simultaneously include the corresponding workstation to carry on the data acquisition and the general analysis. The usual existing instrument manufacturer has integrity instrument qualification (IQ), performance qualification (PQ), and operation qualification (OQ). Instrument indicators have a strict certification and review procedure. However, the instruments must also

be calibrated to ensure that the measurement and analysis process is acceptable before the fingerprint determination. First, validation of analytical instruments is for the pretest analysis purposes to explore the corresponding indicators consistent with the analysis purposes. Second, the instrument calibration should be performed, usually, instruments adjusted and conditions of operation optimized as the base of the pretest. Finally, it refers to the SOP document reorganization, equipment failure table, maintenance records, and accessories.

3.2.1.3.2.3 EVALUATION OF SYSTEM VALIDATION Evaluation of system validation aims at evaluation of the effectiveness of the method. Others, such as process, equipment, belong to a standardization process. Corresponding evaluation must be used in the experimental process, and make judgments to ensure an effective final determination. During system evaluation, analytical methods validation is one of the key aspects, and the validation process must be established on the basis of a stable and homogeneous sample. At the initial stages of validation, it should be subject to stability evaluation of the corresponding sample.

Chinese medicine sample composition is complex. There is no good way to meet the needs for complex analytical research in modern analysis methods. Therefore, the method established in the system should carry on the multiple analysis method appraisal, provide many data, and analyze the data comprehensively. In addition, the components often exist as macro, minute, or trace amounts in Chinese medicine sample. Certain steps are required to enable the response of the most of the chemical constituents after chemical fingerprint analysis. Therefore, method development must also take into account the demands of different levels and different responses to the material composition.

3.2.1.3.3 Verification of Fingerprint Detection Methods The difference between determination methods validation and analytical methods validation is that determination methods validation includes method selection, system validation, process validation, method validation, data comparison, and evaluation, while analytical methods validation includes all of the above as well as information mining and feature extraction.

The composition of TCM formulas is complex. They include proteins, peptides, nucleotides, and other materials, such as macromolecules, inorganic salt, and inorganic metal ions. Therefore, a reasonable analysis of fingerprints of TCM must include the sample extraction, preparation, measurement, data processing, and information mining in which the extraction and preparation of the sample is the specific key stage to determine the final display. The ideal fingerprint determination process utilizes specific preparation methods to extract active ingredients or featured ingredients. The specificity of such fingerprint determination can be fully confirmed.

(1) Selection of Determination Methods: Although the chemical composition of TCM is complex, the long-term phytochemical and pharmacological studies found most of the TCMs will have an effective part and have therapeutic effects after. In particular, many parts or components of modern medicine are directly effective medicine, and usually the effective part is a class or a collection of several substances. It provides a favorable direction for the fingerprint sample preparation and determination method selection of TCM methods. We can select the specific method or combination of methods for the determination of fingerprint analysis.

(2) Verification of Determination System: The TCM fingerprint determination system is the general framework of the fingerprint determination process of TCM. It includes determination processes, determination methods, and two subsystems. According to the determination order, a determination system includes a literature search, summary of basic information, sample collection, sample preparation, sample extraction, sample preparation, the middle sample preparation, sample preparation, selection and optimization of determination method, data processing, data extraction and mining, analysis summary, audition, and verification. In the process of system assessment, it mainly studies the feasibility and credibility of the system and extent of reasonability and specificity.

(3) Verification of Determination Program: TCM fingerprint verification aims at normalization and standardization study of the extraction, preparation, process procedure, and other aspects for the determination. The main consideration includes the sample collection, preparation and process changes, and other issues associated with fingerprints.

(4) Determination Method Validation: Fingerprint determination method verification is similar to the general analysis method validation. It needs more refinement to ensure the specificity, reproducibility, and feasibility of the method. The key points of verification include specificity, accuracy, precision, measurement range and linearity, sensitivity, stability, and durability.

(5) Methods Revision and Reevaluation: According to "Experimental Technology Study Guide of Chromatographic Fingerprints of TCM Injection" (Trial), the fingerprint review contents include extraction method, solvent extraction, chromatographic columns, TLC chromatography system, and detection wavelength, as well as the precision of results, reproducibility, and stability of fingerprints for stored samples. The result can be checked by the similarity of the software, and the similarity should be over 0.9. Standardization of writing is required when submitting the abovementioned information, and the data should be complete. In general, a methodological review unit does not need needs a longer review test unless necessary. However, the review unit itself about injection still requires its own review of trial methods validation. In fact, during the fingerprint long-term determination process of TCM, it necessitates the timely summarization and adjustment of instrument parameters and examined contents. Under the influence of uncontrollable factors, it

will generate data variations, such as column aging and the remaining of sample composition residue, which should be investigated by the adaptive control system in the process of reevaluation.

3.2.2 Research on Characteristics of TCM Fingerprints

Chinese medicine has been used to cure diseases for thousands of years, using the procedures of traditional medical health care and treatment of disease. Studies on the nature of TCM require multidisciplinary theories with regards to modern science, especially to apply to the complex system science theory in the study of complex system. As for integrity, nonlinearity, fuzziness, and temporal and spatial sequences, the characteristics of TCM are complex, and new subjects and new methods adapting the complexity, mainly contain systems theory, information theory, dissipative structure, coordination theory, nonlinear science, and fuzzy catastrophe theory are all necessary. Using those theories and methods in various subjects to explore and reveal the nature of Chinese medicine is one of the core contents for modern Chinese medicine investigation.

3.2.2.1 Identification of Compositions in TCM Fingerprints TCM is a complex system with numerous ingredients. The significant difference in the content of components may obviously affect the response to the detection. It is significantly nonlinear in the measured range. Based on the above characteristics of a complex system of Chinese medicine, strategies including global analysis, active component analysis, and characteristic analysis are proposed and systems are maximally displayed using appropriate analytical tools on the basis of integrity and entirety. Fingerprints are usually used to characterize the integrity of Chinese medicine complex systems. This section will introduce how to strengthen the characteristics of the analysis in the global characterization of fingerprints, and also the identification of the ingredients in TCM fingerprints.

3.2.2.1.1 Combinatorial Analysis Strategies with Various Types of Mass Spectrometry Information and Their Application to the Elucidation of Unknown Components TCM is a very complex system with many interference factors, multitarget pharmacological effects, multilevel functions, and other characteristics. The conventional approaches to such studies involve isolating the individual components from the complex mixtures with liquid chromatography (LC), and then performing structure elucidation by nuclear magnetic resonance (NMR), mass spectrometry (MS), or other spectroscopic techniques. The compounds for analysis should be isolated and purified in order to obtain their structural information, yet it is difficult to describe the complexity of multiclass TCM. In many cases, the isolation and purification are rather difficult. To solve this problem, chromatography-based techniques such as GC/MS, LC/MS, and MS/MS (MS^n) are applied to the analysis of traditional Chinese formulas, and the advantage lies in realization of online

detection and identification of complex constituents. However, because of the lack of the standard substance, a single analysis method is not sufficient to systematically identify all the complicated components from various classes of Chinese medicine, and therefore an analytical method offering more chemical information is in great demand.

Mass analyzers of common mass spectrometers such as ion trap-mass spectrometry, Quantum MS, or time-of-flight mass spectrometry (LC-TOF/MS) have different characteristics and functions. However, it is difficult to identify the elemental compositions of unknown peaks by only one type of mass analyzer. We combined the advantages of two or more than two MS and established an analytical strategy based on multiple types of MS information.

The *Qingkailing* injection was taken as an example to describe the general strategy of screening and identification of unknown components by LC-MS fingerprint technology.

The formula is prepared with eight medicinal materials or their extracts, including *Banlangen* (Isatidis Radix), *Jinyinhua* (Lonicerae Japonicae Flos), *Zhizi* (Gardeniae Fructus), *Shuiniujiao* (Bubal Cornu), *Zhenzhumu* (Margaritifera Concha), baicalin, cholic acid, and hyodeoxycholic acid. The components of the *Qingkailing* injection are very complex, and include not only the small organic molecules group but also numerous inorganic ion and biological macromolecules, so it is a rather difficult job to quickly and accurately identify these components of the *Qingkailing* injection.

3.2.2.1.1.1 Analysis procedure for screening and identification of unknown components

a. Small molecules or extracts are preliminarily separated and analyzed by LC-TOF/MS;
b. Major unknown peaks should be matched to the possible formula and the characteristic isotope distribution should be noted specifically;
c. Look up the possible structure of unknown compounds in the SciFinder database (CA online) or ChemFinder database using precise molecular weight and possible molecular formula, as well as combining with the corresponding literature;
d. Molecular ions, fragment ions, and possible structure are identified by LC-Trap/MS^n;
e. Together with molecular ions determined by TOF-MS, the unknown components are identified by the aforementioned "double standard" (simultaneous confirmation of molecular composition and fragmentation);
f. Final confirmation by available standard substances.

3.2.2.1.1.2 Identification of unknown components in a TCM fingerprint by the combination of multiple mass-spectrometric findings

The identification of unknown compositions will be introduced by taking a unknown

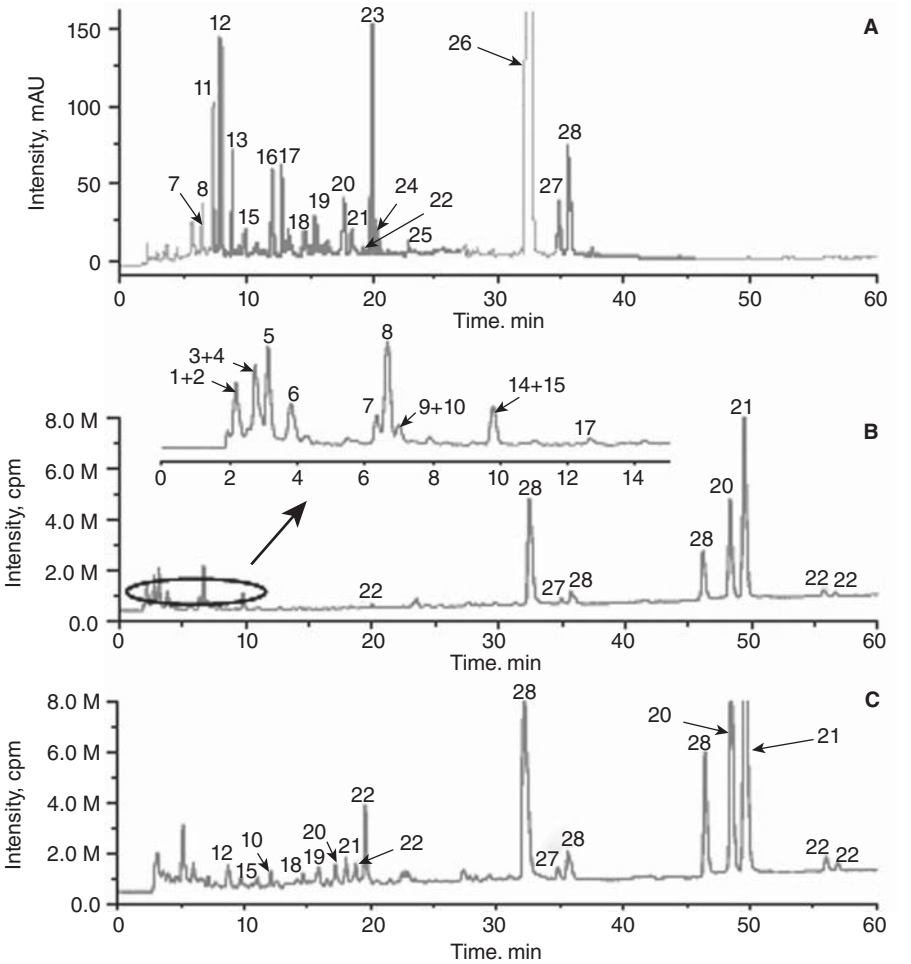


Fig. 3.3 Chromatogram of HPLC/UV. A: and total ion chromatograms (TIC) of LC-TOF/MS in positive ion mode; B: and negative ion mode; C: of *Qingkailing* injection.

component peak (peak 26 in Fig. 3.3 A and B) in *Qingkailing* injection as an example. As shown in Fig. 3.3, the retention time of peak 26 in HPLC/UV chromatogram and HPLC-MS total ion current (TIC) chromatogram is conformity, and the primary mass spectrum is relatively simple with a marked molecular ion peak and a weak fragment ion peak; peak 26 can be considered to represent a relatively pure compound. The accurate mass of this compound and matching result of possible molecules are shown in Table 3.1.

It should be mentioned that the research in natural products generally involves a large range of concentration levels (2–3 orders of magnitude), which surely affects the mass accuracy. We did notice that if the peak

TABLE 3.1 Accurate Mass Data of Baicalin and Its Characteristic Fragment Ion and the Match Results

Measured mass	Formula	Exact Mass	Error (ppm)	DBE	Comments
445.0786	$C_{22}H_{13}N_4O_7$	445.0789	–0.8371	18.5	
	$C_7H_{19}N_5O_{17}$	445.0781	1.0262	1	
	$C_9H_{21}N_2O_{18}$	445.0794	–1.9905	0.5	
	$C_{21}H_{17}O_{11}$	445.0776	2.1674	13.5	Baicalin
	$C_{24}H_{15}NO_8$	445.0803	–3.8539	18	
269.0458	$C_{15}H_9O_5$	269.0455	0.9398	11.5	Baicalin fragment ion
	$C_{16}H_5N_4O$	269.0468	–4.0307	16.5	

intensity of the studied species was high, the correct elemental composition would exceed the limit of mass accuracy, and the identification would not be successful. The probable reason for this lies in the saturation of the ADC detector in TOF instrument. The sample solution would be reanalyzed after being diluted appropriately, or we would analyze the edges of XIC peak for accurate mass.

As shown in the table, results of No. 2, $C_7H_{19}N_5O_{17}$, and results of No. 5 $C_{24}H_{15}NO_8$, seem unconventional, because of the violation of nitrogen rule (the molecular weight of this compound is 446 and the molecular composition should contain an even number of nitrogen atoms, theoretically). In order to narrow down the choices, the "show isotopic" function from the calculator is quite a helpful tool in screening empirical formulas by overlaying the theoretical isotope abundances on the actual spectrum. According to these criteria, the forth formula ($C_{21}H_{17}O_{11}$) was the only one that had a perfect match.

Subsequently, the proposed formula was searched using the "SciFinder Scholar" database combined with the corresponding literature and it inferred that this compound may be baicalin. Furthermore, the analysis on MS/MS spectra fragment from ion trap of this unknown compound showed the possible structure.

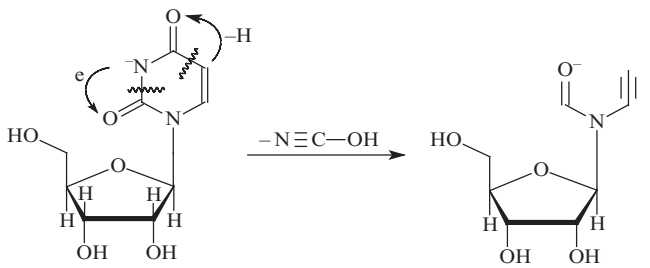
The next step was to confirm the TOF data by LC/ion trap MS^3 through investigating the characteristic fragmentation pathways of the parent compound. As can be seen in the MS/MS spectrum, the m/z 445 ion results in two major fragment ions at m/z 269 and 175, suggesting the precursor might lose neutral glucuronic acid. Furthermore, we found an m/z value of 269.0458 when checking the spectrum of LC/TOF-MS. The matching result showed that only the formula is $C_{15}H_9O_5$. Parent ion minus ions is equal to $C_6H_8O_6$—just as the glucuronide. These data give further evidence that the compound lost a molecule of glucuronide.

In this work, combined with the analysis result of LC-TOF/MS and LC-ion trap MS^n, we identified peak 26 as baicalin which was finally confirmed by the reference substance of baicalin.

According to the above screening procedures, the peaks in TIC that match the HPLC/UV spectra and had a high intensity were picked up, and the exact molecular weight was predicted and matched to the possible molecular composition. Subsequently these candidate formulas were input into the SciFinder database to be searched. The results showed that the *Qingkailing* injection may have a very complex composition. Thirty-three ingredients were determined, including several different types of isomers: leucine/isomers, chlorogenic acid/isomers, gardenoside/isomers, cholic acid/isomers, and deoxycholic acid/isomers. TOF-MS alone cannot distinguish these structurally similar compounds. Therefore these results need to be confirmed by follow-up multistage ion trap mass spectrometry fragmentation analysis combined with reference substances.

According to the peaks of TOF-MS, MS^3 analysis was conducted, so as to confirm the possible molecular composition of TOF-MS and results derived from database searching. Thirty-three compounds were finally confirmed with strong qualitative ability of the Trap-MS^n and the assisted identification with reference substances. It was found that these multistage fragmentations showed a certain specificity and regularity through analysis of MS^3 fragments, which benefits significantly its structural characterization.

Strong polar components, such as amino acids and nucleosides, were eluted in 15 min. In comparison, in this experiment, ionization efficiency of amino acids in positive mode is superior to that in negative mode. These amino acids, especially α-amino acid, are prone to have a simple rearrangement under positive ionization mode, accompanied with loss of one molecular formic acid and leading to formation of the specific fragment ion $[M\text{-}HCOO]^+$. For a nucleoside in negative mode, five-carbon sugar is removed to form the fragment ion $[M\text{-}H\text{-}rib]^-$, and uridine is also fragmented into higher abundant $[MH\text{-}HNCO]^-$, because it is prone to rearrange and lose one molecular amide in negative mode.



According to these neutral losses, 13 amino acids and three nucleosides were identified and were further confirmed by the standards.

Iridoid glycosides and organic acids are mainly eluted in 15–25 min. The five iridoid glucosides in *Qingkailing*, shanzhiside, genipin-1-gentiobioside,

geniposide, gardenoside, and scandoside methyl ester, were screened and identified. On the whole, their deprotonated molecules were easy to generate product ions of [M–H-162] and [M–H-324] via the loss of one or two molecules of glucose residue in the negative mode. Because geniposide and scandoside methyl ester are a pair of isomers glycosides, their multilevel pieces are almost identical in negative mode. Finally, geniposide was identified by standard substance.

Organic acids, including neochlorogenic acid, chlorogenic acid, isochlorogenic acid, and caffeic acid, were identified. Characteristics of the secondary fragmentation of caffeic acid were evident in negative mode and it lost CO_2 to generate the stable ion $[M\text{-}H\text{-}CO_2]^-$. However, it could be noticed that three well-separated chromatographic peaks gave an identical accurate mass (m/z 353.0878), indicating the existence of three isomers. By comparison with the literature, these three isomers were determined to be chlorogenic acid, isochlorogenic acid, and neochlorogenic acid. At the same time, their multilevel fragments had certain regularity. Finally, these three chlorogenic acids were identified through detailed analysis of multistage fragmentation, literature searching, and standard confirmation.

Ions of 353.0 to 353.5 amu were extracted to obtain three well-separated peaks in TOF-MS. Each peak corresponded to the same mass charge ratio at 353.0878 amu and matched to the same candidate formula: $C_{16}H_{17}O_9$. This suggested that there may be three isomers. Three new components are likely to be neochlorogenic acid, chlorogenic acid, and isochlorogenic acid, which are derived from the honeysuckle herb according to the literature. Trap-MS_2 also proved the hypothesis, because the base peak ions are m/z 191 in the second-order mass spectrum which is due to the loss one molecule of caffeoyl to form a stable MS^2 daughter ion $[M\text{-}H\text{-caffeoyl}]^-$. However, further exploration found that each of the three fragments of three isomers shows different regularities, and these three isomers can be distinguished on the basis of sophisticated analysis and the law of MS fragmentation.

In negative ion mode, the MS^3 spectrum that peaked at 14.3 min indicated its notable fragment ion at m/z 127 while the spectrum that peaked at 17.5 min showed three characteristic fragment ions of m/z 127, 109, and 85. Taking the probable fragmentation pathways of each compound into account, we speculated the first one was neochlorogenic acid and the last one was chlorogenic acid. The essential dissimilarity of the two compounds is due to their different MS^3 fragmentation behaviors. As can be seen, the two compounds are more inclined to generate the m/z 191 ions by losing one molecule of caffeoyl, and then losing of one molecule of CO to generate unstable intermediates marked as I and II, respectively. Soon afterwards, the intermediate I is easy to generate an epoxy structure via the loss of one molecule of H_2O because the two hydroxide radicals located at 3′ and 4′ positions of the intermediates belonged to the same plane. Besides, a stable epoxy negative ion at m/z 127 was generated by losing one molecule of H_2O which was generated by the combination of the hydroxyl at 2′ and hydrogen atoms at 5′ position. But the intermediate

II is more likely to shuck off three molecules of H_2O to form a stable epoxy negative ion at m/z 127; one is generated by the combination of the hydroxyl at 1′ and hydrogen atoms at 5′ position, and another two are generated by the two hydroxide radicals located at 3′ and 4′ positions. It can be seen that the number of H_2O lost in the fragmentation pathways was mainly determined by the different positions of the two hydroxide radicals located at 3′ and 4′ positions of the intermediates. The result was confirmed by chlorogenic acid standard (neochlorogenic acid was not obtained), its retention time and MS^3 mass spectra.

Different from the former two atlases, the MS^3 spectrum that peaked at 18.4 min presents its fragment ions at m/z 127 and 173 with relative higher intensity, and thus it can be considered an isochlorogenic acid. As shown in figure above, neochlorogenic acid is easy to generate the m/z 191 ion by losing the caffeoyl, in the negative mode. It could be observed that the hydroxide radical at 3′ position and the hydrogen atom at 4′ position are all located as axial bonds, thus the m/z 191 ion of isochlorogenic acid is more prone to generate a stable fragment ion at m/z 173 via the loss of one molecule of H_2O according to Markovnikov rule. Besides, like neochlorogenic acid, the m/z 191 ions are more inclined to lose one molecule of CO. Soon afterwards, the intermediate I was easy to generate a stable epoxy negative ion at m/z 127 via the loss of two molecules of H_2O. The result is confirmed by standard isochlorogenic acid.

As can be seen from the above examples, Trap-MS^n harbors a very powerful function in multiple fragment analysis, which helps us analyze compound structure precisely and assists in confirming the screen result of TOF-MS. It is difficult to distinguish isomers by common mass spectra. However the three isomers of neochlorogenic acid, chlorogenic acid, and isochlorogenic acid were differentiated successfully via the interpretation of their MS_3 fragmentations. It was proved that unknown isomers could be identified by mechanism of fragmentation and standards confirmation, and it accelerated the process of qualitative analysis of complex systems.

Flavonoid and steroids were mainly eluted in 30 to 60 min. Flavonoids, including baicalin, wogonoside, and a compound recently reported were identified. In the negative mode, a stable ion of $[M\text{-}H\text{-}176]^-$ is generated by losing a Glucuronide. Notably, the compound was obtained by the traditional separation method and identified by NMR, and it had never been identified with a single ion trap before. But in this experiment, TOF-MS provided us the accurate mass value of m/z 429.0827. Subsequently, the proposed formula was searched using "SciFinder Scholar 2006" database (CAS online). Furthermore, the MS/MS characteristic fragmentation of this unknown compound showed the same characteristic fragmentation patterns as baicalin and wogonoside. It means that they probably share a homologue. Only one item from database search results is consistent with this conclusion. Taking the trap fragmentation data into account, we found a unique match with the formula: chrysin 7-O-β-galactopyranuronoside. The stinasterols of hyocholic acid, cholic acid,

hyodeoxycholic acid, deoxycholic acid, and chenodeoxycholic acid were identified in *Qingkailing* in high levels. Among them, stinasterols of hyocholic acid/cholic acid and hyodeoxycholic acid/deoxycholic acid/chenodeoxycholic acid were two pairs of isomers. As we know, these compounds have very weak UV absorptions so they cannot appear in the HPLC chromatogram. Besides, these compounds usually lose one or more molecules of H_2O and become $[M\text{-}H\text{-}H_2O]^-$ and $[M\text{-}H\text{-}2H_2O]^-$ due to their relatively stable MS behavior in the soft ionization condition, which made it tough to differentiate these isomers. Ultimately, the standards were employed to distinguish them respectively.

3.2.2.1.1.3 PRELIMINARY STUDY ON THE ATTRIBUTION OF COMPONENTS With the LC/TOF-MS and LC-Trap MS^n combined analysis method, we systematically screened and identified 33 components in the *Qingkailing* injection. According to the different material composition and different chemical properties of *Qingkailing* injection (global chemome), they could be divided into six major types of effective part (subchemome). We compared formulas and single drugs or their extraction, and studied the attribution of the identified compounds as follows.

Amino acids mainly included arginine, glutamate, aspartate, glycine, proline, valine, isoleucine, leucine, tyrosine, threonine, alanine, phenylalanine, and tryptophan. They are derived from *Banlangen* (Isatidis Radix) together with the hydrolysate of *Zhenzhumu* (Margaritifera Concha) and *Shuiniujiao* (Bubali Cornu). Nucleosides mainly included uridine, adenosine, and guanosine, which are derived from *Banlangen*. Organic phenolic acids mainly included neochlorogenic acid, chlorogenic acid, isochlorogenic acid, and caffeic acid, which are derived from the *Jinyinhua* (Lonicerae Japonicae Flos). Iridoid glycosides mainly included gardenoside, genipin-1-gentiobioside, geniposide, and scandoside methyl ester, derived from *Zhizi* (Gardeniae Fructus). Flavonoids mainly included baicalein, wogonoside, derived from extracts of *Huangqin* (Scutellariae Radix). Steroidal compositions mainly included hyocholic acid, cholic acid, hyodeoxycholic acid, deoxycholic acid, and chenodeoxycholic acid, which are from cholic acid and deoxycholic acid extraction.

According to the above classification results, we basically know the compounds of the organic small molecule group in *Qingkailing* injection except the volatile components. At the same time, we also searched literature on the biological activity of these compounds, and found that these compounds did have biological activities, and there is synergistic effect in vivo after their compatibility, thus ensuring the effect of *Qinkailing* injection.

3.2.2.1.2 Quick Access of TCM Characteristic Profile and Identification of Characteristic Components Based on Tandem MS Chemical characterization of TCM focuses not only on the overall composition but also on the quality and even quantity of some featured compounds. The aforementioned chromatography or chromatography–mass spectrometry provides important solutions. In this section, we will take the analysis of ginsenosides as an

example through which to introduce a rapid analysis method based on tandem mass spectrometry technology without chromatographic separation.

3.2.2.1.2.1 QUICK ACCESS OF GINSENOSIDES' CHARACTERISTIC PROFILE BASED ON TANDEM MS Before analyzing a complex sample, a basic understanding of the sample composition is very important for the analytical method development. In Panax study, for example, we quickly established characteristic profile of glycoside in the Panax herb via our developed energy gradient neutral loss scan, and thereby obtained basic composition of ginsenosides sample.[14]

Samples of *Renshen* (Asian ginseng, Ginseng Radix et Rhizoma), *Xiyangshen* (American ginseng, Panacis Quinquefolii Radix), and *Gaolishen* (Korean ginseng, Ginseng Radix et Rhizoma) samples were purchased from Beijing Tong Ren Tang pharmacy. Energy gradient neutral loss scan: MS/MS was set to scan an energy gradient: 0–100 eV. It was realized through the following steps: RO2 increased to 111 V from 11 V in linear speed, 0.5 V gained once; while RO3 and ST3 were set to rely on RO2, and the interceptions were 2 V and 20 V respectively. IQ1 is set at 11 V, and the (RO2-Q1) substraction was defined as collision energy. So a shifty energy gradient atmosphere was produced in the collision area via electric field. In a selection of scans where neutral lost was 162 in negative ion detection mode, the sample was injected into the ion source continuously through injection pump. The mass of scanning range was set to be 400–1200, the step length was 0.3 amu, retention time was 1 ms; the pressure of collision gas was 4.8 mTorr.

Neutral loss energy gradient scan spectra of Asian ginseng (from *Jilin* Province), American ginseng, and Korean ginseng are shown in Fig. 3.4. As is shown in Fig. 3.4 A, B, and C, ginseng in medicinal materials containing glycosides was mainly distributed in the region of high collision energy. Mass spectra (Fig. 3.4 D, E, F) of these three kinds of Chinese herbal medicines were respectively extracted in the collision energy of 66 eV (near the TIC peak) and it inferred that their collision energy of glycoside distribution (due to neutral loss of 162 substances in general as containing glucose glycosides) was obtained. Combined with standards and the literature, we can infer glycosides were one of the components. As can be seen from the graph, there are more than 10 kinds of glycosides loss of glucose under the condition of the collision energy at 66 eV. Interestingly, from the three different Panax herbal extraction mass spectra, the difference was very obvious. For example, the relative abundance of 799 in Figure E was much lower than that in D and F, because the American ginseng (E) did not contain ginsenoside Rf, whereas ginseng saponins of American ginseng also have molecular ion peak of 799, but the neutral loss 162 could not be detected, so the relative abundance of 799 was lower, indicating that this method can be used for different species of ginseng herbs for rapid identification (2 min/sample).

The method is applied to the rapid analysis of Panax medicine, and shows that the development of fast classified search methods in complex samples for caring about features of the compound information is very efficient and can

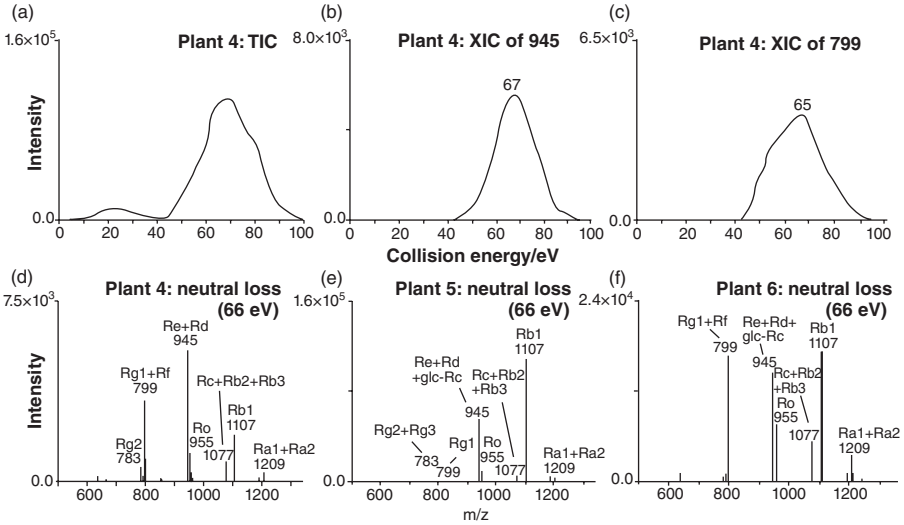


Fig. 3.4 EGNLS spectra of neutral loss 162 of Panax herbal ginseng saponin. A: TIC of Asian ginseng herb; B: XIC of m/z 945 of Asian ginseng; C: XIC of m/z 799 of Asian ginseng; the neutral loss spectra extracted at 66 Ev of D: Asian ginseng, E: American ginseng, and F: Korean ginseng, respectively.

provide abundant information as well. The method can obtain not only the composition of samples but also collision energy, which is helpful in developing follow-up methods. For the compounds we are interested in, structure analysis can be completed through tandem mass spectrometric fragmentation information. However, the phenomenon of mass spectral peaks overlapping is universal by this method which cannot distinguish similar isomers with structural fragments. It depends on the LC-MS analysis for final confirmation. Therefore, developing a variety of combined methods can systematically solve the analytical problems of complex TCM samples.

3.2.2.1.2.2 IDENTIFICATION OF GINSENOSIDES BASED ON TANDEM MS Ginseng is mainly composed of triterpenoid saponins, including glycols, triols, and saponins of oleanolic acid, while Panax quinquefolium also contains octillol-type saponin (PF_{11}). Due to the distinct characteristics and regularity of ginsenosides MS fragmentation, saponins in Panax ginseng and even unknown constituents were identified by analyzing the molecular weight and fragments of information with tandem mass spectrometry. Interpretation of ginseng saponin structure is to address two pivotal questions: one is the type of mother nucleus, and the other is the type of glycogen and the position of bond.

Main molecular ion peaks and fragment ions of ginseng saponins are summarized in Table 3.2. As can be seen from the primary mass spectrometry of ginseng saponin, there were also adductive ions of quasimolecular ion peaks

TABLE 3.2 Main Molecular Ion Peak and Characteristic Fragment Ions of Ginseng Saponin

Parent Nucleus Type[a]	Saponin Type	Accurate Molecule-Ion in First-Level Mass Spectrum[b]	Characteristic Fragment Ions Second-Level Mass Spectrum	Retention Time (min)
A	Rb_1	1126.7,1131.7, 1109.7	767.5, 587.5,487.1, 443.3, 425.3, 407.3, 360, 343, 325.1	28.0
A	Rb_2	1096.7,1101.7,1079.7	767.5, 587.5, 487.3, 443.3, 425.3, 407.3, 330,325.1,295	31.4
A	Rb_3	1096.7,1101.7,1079.7	767.5, 587.5, 487.3, 443.3, 425.3, 407.3, 330,325.1,295	32.3
A	Rc	1096.7,1101.7,1079.7	767.5, 587.5, 487.3, 443.3, 425.3, 407.3, 330,325.1,295	29.8
A	Rd	964.7,1036.7, 947.7	767.5, 587.5, 487.3,443.3, 425.3, 407.3, 325.1	35.2
A	Rd	964.7, 947.7, 1037.0	767.5, 587.5, 487.3, 443.3, 425.3, 407.3, 360, 343	36.1
A	Rg_3	785.6, 807.6	587.5, 487.3,443.3, 425.3, 407.3,325.1	37.1
B	Re	964.7,1037.0, 947.7	767.5, 587.5, 459.3, 441.3, 423.3, 405.3, 327.1, 309.1	16.5
B	Rf	801.5, 818.5	587.5, 459.3, 441.3, 423.3, 405.3, 325.1	22.5
B	Rg_1	818.5,823.5,801.5	621.5, 587.5, 459.3, 441.3, 423.3, 405.3	14.5
B	Rg_2	785.5, 807.6	767.5, 587.5, 459.3, 441.3, 423.3, 405.3, 327.1, 309.1	25.9
B	R_1	950.6, 955.6, 933.5	753.4, 587.5, 459.3, 441.3, 423.3, 405.3, 295	11.7
C	PF_{11}	801.5, 818.5	783.5,475.2, 457.3, 439.3, 421.3, 327.1, 309.1	21.6

[a]A, B, C represent saponins of glycol type, triol type and oleanolic acid type.
[b]The quasimolecular ions whose is above abundances of 5% are list, the first peak is base peak ion.

of $[M + Na]^+$ and $[M + NH4]^+$ besides the usual $[M + H]^+$ peaks. This study adopted chromatography conditions with 10 mmol/L (M) acetic acid amine added in mobile phase. Therefore, $[M + NH_4]^+$ molecules had high abundance and generally were the base peak ions.

Thus, Ginseng saponins of different parent nucleus in MS fragments had distinct characteristics and rules. The m/z 461, 443, 425, 407 could be observed in the 20(S)-protopanaxadiol type ginsenosides, because the 20 (S)-protopanaxadiol type saponin was likely to lose R_1 and R_2 molecular of glycogen and became initial parent ions at M/Z 461. This parent ion lost some molecules of water to become m/z 443, 425, 407, and so on. The m/z 477,459, 441, 423, 405 can be observed in the 20 (S)-raw ginseng triol type saponin. Because of the same rule, 20 (S)-raw ginseng triol type saponin was likely to lose R_1 and R_2 glycogen and become initial parent ions at M/Z 477, and this parent ion could further lose some molecules of water to become m/z 459, 441, 423, 405, and so on. For the 24 (R)-ocotillol type saponin (such as pseudo ginsenoside (F_{11}), the m/z 493,475, 457, 439, 421 could be observed and likewise m/z 493 was the initial parent ion after losing R glycogen group and further in order lose four molecules of water to be m/z 475, 457, 439, 421. In addition, disaccharide or monosaccharide ion peaks could be observed in secondary mass spectra, for electric charge might retain in the glycogen part during the molecular ion fragmentation process. For example, M/Z 325 ions ($[Glc\text{-}Glc (H_2O) + H]^+$) could be observed in Rb_1, Rb_2, Rb_3, Rc, Rd, Rg_3, and Rf. Fragment ions of m/z 309 could also be observed after disaccharide shrinking from Re, Rg_2, and F11. Intriguingly, high abundance fragment ions of m/z 343 and 360 (corresponding to no shrink disaccharide ions $[GlcGlc + H]^+$ and $[GlcGlc + NH_4]^+$) could be observed from Rb_1, but the characteristic ion abundance of two glycogen Glc-Glc several other saponins with the same R_1 terminal, such as Rb_2, Rb_3, Rc, Rd, and Rg_3, was not obvious. Even though Rb_2, Rb_3, and Rc had no obvious $[GlcGlc + H]^+$ and $[GlcGlc + NH_4]^+$ ions, they could be observed in abundant ions of m/z 330 (corresponding to $[Glc\text{-}Ara + NH_4]^+$ or $[Glc\text{-}Xyl + NH_4]^+$). According to the above described phenomenon, we may find an interesting pattern that the disaccharide of R_2 may easily generate a disaccharide ion without shrinking in collision induced dissociation, while the disaccharide of R_1 may be more inclined to form two glycogen ions after shrink. It is important for us to judge the position of glycogen and to identify the unknown ginsenoside structure.

For example, we found that Panax quinquefolium contained an unknown saponin. The formula of the compound is the same as ginseng saponin Rd and Re, and its RP-HPLC retention time is after Rd, but more than 10 kinds of standards of ginseng saponin did not match it; moreover, it is difficult to separate and prepare them for the moment. However, it is found that this compound in North American Native Panax quinquefolium and Panax quinquefolium cultivated in China introduction has great differences between varieties and may be used as the index component to distinguish between the two types of Panax quinquefolium, so the identification has significance.

First of all, the compound belongs to the 20 (S)-protopanaxadiol type saponin judged bythe presence of m/z 461, 443, 425, 407 and other characteristic ions; therefore its MS/MS is closer to Rd and different from Re, yet unlike Rd, the unknown saponin characteristic ions of m/z 343 and 360 are abundant, which is very similar to Rb1. It suggests the presence of disaccharide Glc-Glc exits in the end of R2. According to the literature, the structure was isolated from Panax quinquefolium by means of thin layer chromatography and named Gypenoside XVII, but reference substance or standard, and mass spectra for it had never been reported. Therefore, it is very valuable to identify the unknown compounds and analyze the structures by tandem mass spectrometry.

3.2.2.2 Extraction and Validation of Characteristic Information in Fingerprint Fingerprints can objectively reflect the information contained in the sample itself, but for the data itself, the data comparison and analysis can be conducted only after some further process, because the data itself has a certain inconsistency. For example, the determined material information response is reflected by UV absorption of material composition. The selection of wavelength only aims at the readout in an average and general sense, and it disregards response behavior of each component. Thus, it inevitably leads to discrimination of the real content caused by various response factors. Hence, standardization and unification of data are needed.

3.2.2.2.1 Pretreatment of Data The data of fingerprints can be a basis for statistics according to the integral and retention results. Usually, data processing should eliminate the effects of the determination system, that is, original data is processed relatively. Generally, in the fingerprint a reference peak is selected, and the other peak data are compared to it to get relative value for fingerprint comparison. We usually select total peak area as standard reference. First, total response of fingerprints of 10 batches of stable product samples should be in the smaller range of fluctuations, or else the product is not stable in terms of quality control; second, the total response is mixed response value, as it is the superposition of each fingerprint peaks at a certain wavelength response, the peak area percentage compared with a fingerprint response is a dimensionless quantity; Finally, as to the issue of inconsistent response, the data processing is carried out based on the general reference, so the response discrimination between the data is corrected to the same level. Hence, it is reasonable to choose peak area percentage for as comparison characteristic value.

3.2.2.2.2 Selection and Evaluation of Characteristic Wavelength As a result of the composition's large difference in Chinese medicine samples, chromatographic response behavior also differs in numerous ways, and wavelength selection is very important. The main principle is the overall presentation, to reduce the difference between the data as far as possible to

get the presentation of the average. However, in the determination process, it is difficult to average sample response at wavelength. For example, the response of scopolamine and atropine in Flos Daturae has no response in 280 nm, it is worthwhile to note whether the loss of peak or not would cause alignment error of fingerprint. We chose the maximum processing mode, so to speak, peak value under 205 nm was directly inserted into the 280 nm fingerprint data. Similarity evaluation is carried out between the general fingerprint data and 280 nm fingerprint data. The data corresponding to 280 nm is set at 0. The calculation indicated that similarity value of 10 batches of data was above 0.99, and deviation was below 1.5%, indicating the introduction or deletion of such peaks would not cause great error in fingerprint comparison. The 280 nm fingerprint data could represent the fingerprint feature of the sample. In addition, the determination of alkaloids in the Datura flower had the control of concentration measurement. Therefore, considering the integrity of the quality control, applying the 280 nm alone can meet the requirements.

3.2.2.2.3 Characteristics of Fingerprint Peaks The chromatographic fingerprint chromatogram usually includes pure substance peak, pure peak, fusion peaks, overlapping peaks, and peak groups. Gauss peak from the chromatographic elution is often identified as the pure peak, which can be divided into two types. One is the pure substance peak, which is obtained with a single pure substances response; the other is the pure peak, which shows the shape of Gauss peak and is generated by two or more substances with similar spectral and capacity factors. Fusion peak is caused by the similar retention time of the component, producing small acromion peak or tailing peak; overlapping peaks denotes different substances have the same retention behavior in the system. Compared to the pure peak, it can be scanned, analyzed, and judged by a diode array detector wavelength. Moreover, the peak group is the combination of peaks that are incompletely separated. The median retention value is usually used to represent the data, and the peak area is the sum of peaks.

The components' mutual interference and the mutual influence on chromatographic retention behavior results in noncorrelation between the data signal deviation and the magnitude of signal. The large signal may not always produce small deviation, while the response behavior of the components has dominant difference throughout the spectra. It is difficult to reach linear consensus within a certain measurement range, so the specific control of fingerprints also demands overall evaluation, and the individual peak can't provide a complete description of the overall feature.

3.2.2.2.4 Analysis of the Fingerprint Characteristic Information Fingerprint data are actually in a mingled state, and not all the peaks have the same effect on the final evaluation. We use the method of similarity evaluation, and peaks in fingerprints were distinguished; that is, the common fingerprint

peaks obtained above were again divided into general fingerprint and characteristic fingerprint peaks. Characteristic fingerprint peaks can basically represent the overall features of the fingerprint, which is used for sample evaluation.

In the classification of the fingerprint peaks, we follow the principle based on the actual measurement results of samples. We mined the characteristics from the data itself, and used the correlations between samples to explain the relevance between datum feature and sample material feature. Here, only the discovery process of data characteristics is discussed.

Fingerprinting peaks of fingerprints are classified into two types; one is classified in light of retention value. It can evaluate the variability and stability of 10 batches of fingerprints and investigate the overall dispersion in the fingerprint. The other is to take the signal magnitude to classify; because the signal magnitude is not significantly correlated with the deviation, it is reasonable to conduct classification based on the signal magnitude. Signal fluctuations will not strongly disturb its classification. During the process of searching fingerprint peaks by classifying the actual results, useful fingerprint peaks were obtained with both methods; actual results need to be analyzed to obtain reasonable conclusions. The results of the evaluation of fingerprints are comprehensive and based on stability of the method. The key of evaluation is to distinguish mixed data and to identify the feature information for a total dispersion fingerprint to request data results. Of course, the rationality of the characteristic information selection still needs to be embodied in specific applications, which should be further proved by correlation among the samples. Through extracting featured information from the overall dispersion of fingerprint data through certain means of classification which are reproduced stably, the identification result of the fingerprint is logical and rational, and thus the result can be used as the actual quality and quality control index parameter. Through verification of the fingerprint methodology research in the section, the credibility and feasibility of practical fingerprint determination is proved, and featured information is extracted using certain classification in the fingerprint data obtained. Results show that the hybrid information obtained from fingerprints can be identified on the basis of overall evaluation, and characteristic information about determination samples can be obtained.

3.2.3 New Technologies for TCM Fingerprinting

TCM and its formulas contain complex chemical compositions. How to put forward a characteristic quality evaluation system of Chinese medicine in order to solve this problem is the important guarantee of modernization of TCM. The existing Chinese medicines are usually composed of a single herb extract, and their chemical compositions are relatively simple, so it is relatively easy to establish fingerprint. But for Chinese medicines composed of many medicinal materials and with complex chemical compositions, the various

existing single determination methods are difficult to use to solve the problem, so we proposed the establishment of fingerprints of multidimensional information and multiple wavelength fingerprints to solve this problem.

3.2.3.1 Multidimensional and Multi-Informational Fingerprint The so-called multidimensional method is to use a variety of hyphenated patterns to determine fingerprints and to have clearer knowledge for complex samples of TCM compound. Currently, the commonly used technologies are HPLC/DAD, HPLC/DAD –ELSD, GC-MS, HPLC/DAD-MS/MS, and so on, which includes the chromatographic chart of HPLC or CE (the retention time of each component), the online UV spectrum generated by diode array detector, the primary mass spectrum (each component molecular weight), and the secondary mass spectrum (the spectrum characteristic fragment of a component). We used this technology to determine a variety of compounds in TCM and herbs in recent years, such as in the injection of *Qingkailing*, *Liushen* pills, *Yaotongning* capsule, *Zhizi*, *Artemisia capillaris*, *Salvia miltiorrhiza*, and so on. HPLC/DAD, HPLC/DAD-MS, HPLC-MS and other advanced combined detection methods were applied in the research.

Multi-information refers to the fact that the characteristic spectrum of TCM patterns should include both chemical and efficacy information. Among them, chemical information is mainly obtained by using multidimensional fingerprints. To obtain efficacy information, efficacy experiments are conducted on the chemome, which is obtained by modern separation means. Ultimately, the proportion of effective chemome and its effective components in the tradition Chinese formula were confirmed, and then the dose-effect relationship was calculated. Liang Qionglin et al. studied the *Shuanglong* formula using multidimensional information fingerprint technology. Multidimensional fingerprints of *Renshen* and *Danshen* were determined. Based on the efficacy study of effective components in ginseng and *Danshen*, the optimal drug ratio of two kinds of Chinese medicine in the *Shuanglong* formula was identified.

Taking the multidimensional and multi-informational characteristic spectrum of the *Qingkailing* injection, for example, we described how to create Chinese medicine's multidimensional and multi-informational characteristic spectrum. The *Qingkailing* injection with complex components was prepared by seven medicinal materials or their extracts, including *Banlangen*, Baicalinum, and cholic acid. ESI-MS fingerprint databases of the *Qingkailing* injection and a similarity comparison method were established using PE's API3000 tandem mass spectrometer to determine 50 components. The test of different batches of injections and oral *Qingkailing* from the same manufacturer revealed that different batches of the injection had a similarity of 90% compared to library, while oral *Qingkailing* only had about 40% similarity, suggesting this method could be used for identification. Based on this information, index components (material basis for efficacy) of the *Qingkailing* injection were studied. For example, in the HPLC-MS/MS analysis of cholic acid, Baicalinum determined the type and isomer of cholic acid, and the method of quantitative

determination of cholic acid and baicalin in *Qingkailing* injections. Combined with the preparation of *Qingkailing* injection, ESI-MS fingerprinting and the "quasistandard" spectrum library of formulas and parts of the single drug were established. The other three parts separated from the *Qingkailing* injection by silica gel column of the efficacy chemical experiments showed no difference, and the chemical composition showed little difference by the chemical characteristic multidimensional spectrum study. However, the other three effective parts separated by macroporous resin column showed different efficacy results, and further study showed that the three parts have distinctly different chemical compositions. The establishment of multidimensional and multi-informational chemical characteristics of HPLC/DAD-MS/MS spectrum of the *Qingkailing* injection, together with the effective part of the pharmacological efficacy experiment, finally determined the multidimensional and multi-informational characteristic spectrum of the *Qingkailing* injection.

3.2.3.2 Combination of Fingerprinting and Multicomponent Quantification Because TCM is prepared with a number of medicines, often with multiple index components (or active ingredients), it shows characteristics of the analytical object only through determination of multiple index quantitative components. In the process of quality control, a separate quantitative analysis for each component is very tedious work and requires a lot of manpower and resources; therefore, a multidimensional and multi-informational fingerprint of TCM should take the advantages of multidimensional analysis to provide quantitative information about index components.

As the index components in a compound are quite different, it is often difficult to simultaneously determine a number of index components for quantitative analysis with a single detector, particularly for the common UV detector. With UV absorption of the different index components it is difficult to have sufficient response intensity on the same fingerprint, which makes it hard to obtain quantitative information for multiple components. We have established a multiwavelength quantitative fingerprint to realize the quantitative analysis of index components in different types and with different UV absorption properties.

Water extraction of *Zhizi* mainly consists of three types of active ingredients: iridoid glycosides, organic acids, and pigments (mainly crocin). Modern pharmacological experiments show that it is not enough to make single component quantitation in Geniposide as the quantitative index for quality control of *Zhizi*: chlorogenic acid has significant antibacterial, detoxifying, and anti-inflammatory functions, Crocin is commonly used as food coloring and has the effects of being a sedative, an expectorant, a stimulant, and an antispasmodic, as well as helping with menstruation, lowering blood pressure, and so on. Therefore, it is necessary to put quantitative determination of the organic acids and pigment substances into the quality control of *Zhizi*. Like the *Zhizi*, Chinese herbal medicine is a complex system, with multiclass components in a single herb, which is difficult to separate by conventional

chromatography. Thus, according to different spectral characteristics of three main active ingredients in the water extracts of *Zhizi*, high performance liquid chromatography combined with diode array detector (HPLC/DAD) was utilized to establish a simultaneous quantitative analysis method for three types of nine components (high performance liquid chromatography multiwavelength) in three different UV–visible detection wavelengths (UV–vis). The method was used to determine each component in the *Zhizi* gleaned from five different areas.

According to full wavelength scan of the DAD detector at 200–600 nm, the results showed that iridoid glycosides had maximum absorption at 239 nm; chlorogenic acid had three peaks at the 220, 246 and 329 nm, among them, the weakest at 246 nm, the strongest at 329 nm; crocin at 250–260 nm, 320–340 nm, and 400–500 nm had three peaks of which the weakest absorption is at 250–260 nm, the strongest absorption of *cis*-crocin with lower content in *Zhizi* and saffron was at 320–340 nm, while that of *trans*-crocin with higher content, such as saffron acid and crocin 1, 2, 3, was at 400–500 nm. To achieve the goal of strong absorption and low interference between each component, iridoid glycoside was determined to be at 240 nm, chlorogenic acid at 330 nm (chlorogenic acid has absorption at 246 nm, and therefore the chromatogram of 240 nm also had chlorogenic acid peak, but the absorption was weak and it was not suitable for quantification), and crocin at 440 nm. Since iridoid glycosides are the primary active component in *Zhizi*, and according to the principles of maximization of effective information, 240 nm wavelength became the monitoring wavelength of fingerprints for comprehensive quality control of *Zhizi*, 240, 330, and 440 nm wavelengths were used for quantitative determination of different components in *Zhizi*, as shown in Fig. 3.5.

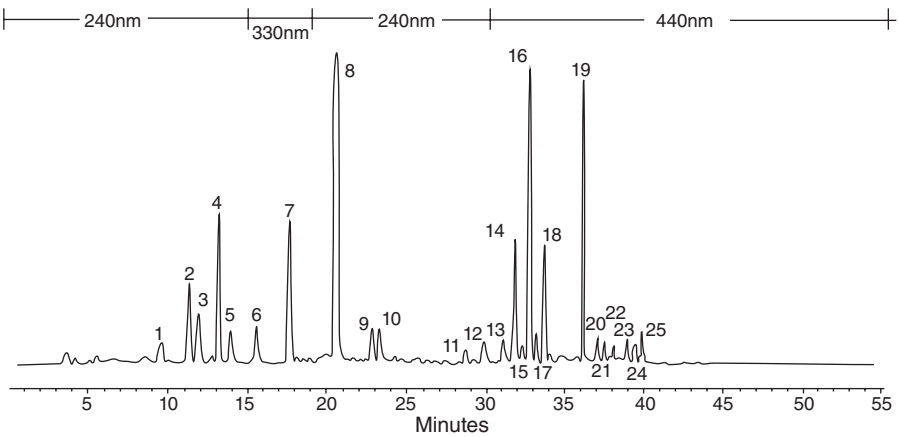


Fig. 3.5 Multiwavelength integrated fingerprint of *Zhizi*. 1: Gardenia acid; 2: gardenoside; 4: genipin-1-gentiobioside; 7: chlorogenic acid; 8: geniposide; 14: crocin 1; 16: crocin 2; 18: crocin 3; 19: croceic acid.

For more complex compounds, some of the index ingredients have no UV absorption, and a conventional fingerprint cannot offer quantitative information. For example, cholic acid of the *Qingkailing* injection has only weak absorption in the UV terminal, and a single UV detector shows almost no response, while these ingredients are major components in TCM, so the quality must be strictly controlled; therefore, it is difficult for a conventional HPLC/UV fingerprint to undergo effective quality control. However, we used HPLC/DAD-ELSD or HPLC/DAD-MS to establish a multidimensional and multi-informational fingerprint, which can provide more quantitative information about index components. In the process of studying multidimensional and multi-informational methods, appropriate detection conjugated means should be selected according to the specific properties of the relevant indicators in compound herbs, so as to take advantage of various instruments' complementary natures and maximally analyze the mass characteristics of the subject.

3.2.3.3 Two-Dimensional Liquid Chromatography Davis et al. proposed that when components containing 100 randomly distributed samples were separated using one-dimensional separation, if 82 components can be completely separated, they will require about 4 million theoretical plates. Obviously, the improvement of one-dimensional column technology is not enough to solve the problem of complete separation of sample components in complex system. According to the theory of informatics, the amount of information in a multidimensional system is equal to that of all the dimensions subtracted by the cross-information amount. The more the crossing information, the more the unavailable space, the peak area tends to focus on the diagonal area, and then the system's efficiency is reduced. Orthogonal separation of multidimensional chromatography refers to the fact that the two-dimensional separation is irrelevant, and the cross-information amount is zero and peak capacity is equal to the product of the two dimensions' peak capacity. That is, the separation mechanism is completely orthogonal, and the system can obtain maximum peak capacity, that is, $n = n_1 \times n_2$, n being the peak capacity and n_1 and n_2 being different dimensions. In order to maximize the efficiency of separation in multidimensional separation system, Giddings pointed out two simple criteria: first, different separation mechanism of two kinds of chromatographic modes, that is to meet the orthogonal requirements; second, the following separation cannot reduce one-dimensional resolution.

Besides such biodrugs as proteins, peptides are studied by multidimensional liquid chromatography techniques, and the scientists also apply it to TCM pharmaceutical analysis. The newly developed multidimensional chromatography technology has become a hot topic in the fields of applied research. Peak capacity can be substantially increased, and the ability of chromatographic separation can be improved by using a two-dimensional liquid chromatography system.

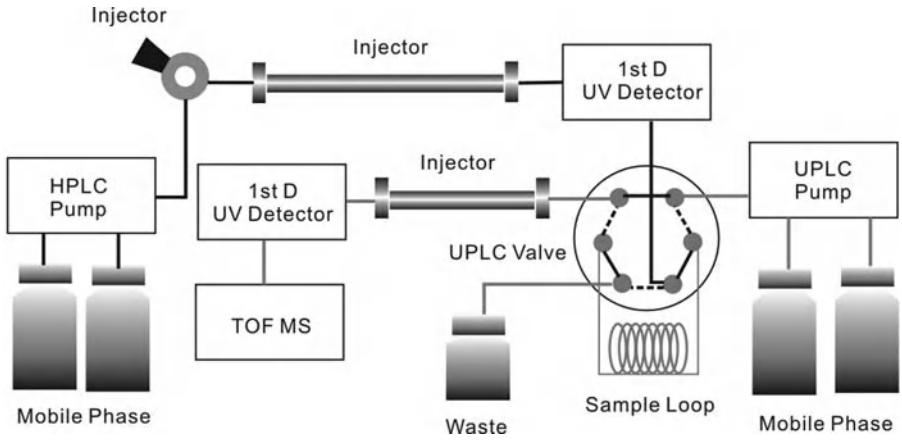


Fig. 3.6 The schematic of two-dimensional HPLC × UPLC-TOF/MS system.

As shown in Fig. 3.6, the first dimensional chromatography of an online two-dimensional HPLC × UPLC-TOF/MS system was Jsaco PU-980 HPLC Pump and Jsaco UV-97 UV Detector; the column of Toyopearl HW-40S column (200 mm × 2.0 mm [diameter]) was used in the first dimensional chromatography. The mobile phase was 10 mmol/L ammonium formate, and the flow rate was kept at 20 μl/min. Waters ACQUITY UPLC system (Waters Corp, USA) was the second dimension separation system equipped with column (100 mm × 2.1 mm ACQUITY 1.7 μm, Waters Corp, USA), the two mobile phases were phase A: water–formic acid (100:0.1, v/v); phase B: acetonitrile. The flow rate was 0.7 ml/min; column temperature was set at 80°C. LCT Premier XE mass spectrometer (Waters MS Technologies, UK) was the detector;

The interface between HPLC and UPL C adopted 100 μl quantitative loop, and UPLC automatic injection valve was a switching valve achieving the connection of HPLC and UPLC.

Matlab software was applied for data processing and mapping. The retention time of the first dimensional chromatography and the second dimensional chromatography were the *x*- and *y*-axes of the three-dimensional spectrum respectively, and peak intensity was the *z*-axis. Matlab software could draw the three-dimensional spectrum of the UV (PDA), MS (TIC, BPI). The second dimensional chromatography UPLC-MS data were normalized and retention time was corrected by MarkerLynx software, and the result could be showed in a four-dimensional spectrum. Fig. 3.7 shows *Qingkailing*'s four-dimensional fingerprints of the full two-dimensional HPLC × UPLC-TOF/MS system.

The analysis time of the first dimension HPLC column was 210 min, the peak width was 19.0 min, so the first dimension HPLC chromatographic peak capacity was 11; the analysis time of second dimension UPLC columns was

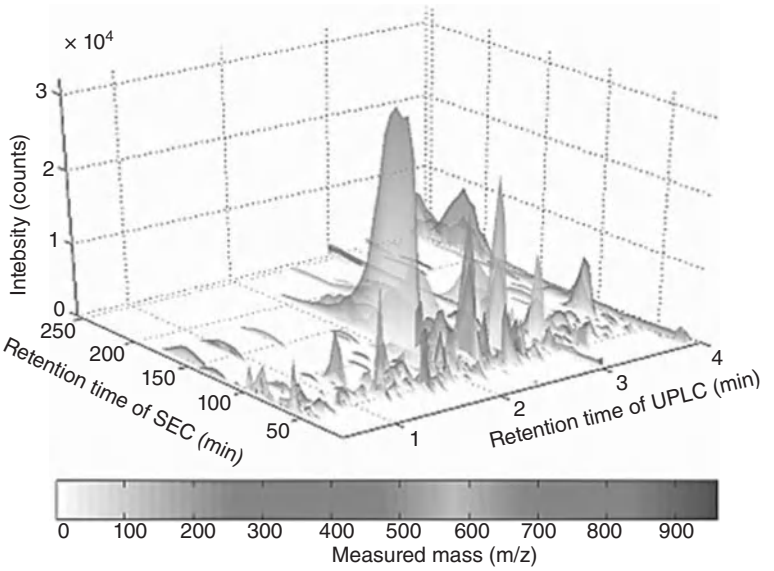


Fig. 3.7 Four-dimensional spectrum of HPLC × UPLC-TOF/ MS system. (See color insert.)

4.5 min, the average peak width was 0.045 min, so the second UPLC-dimensional peak capacity was 100. The two-dimensional HPLC × UPLC-TOF/MS system's peak capacity was 1100. Systematic analysis time was 4 h.

With the theory and principles of HPLC, UPLC covers small particle filling materials and the means of rapid detection of low system volume. Compared with conventional HPLC, UPLC's speed, sensitivity and separation were 9 times, 3 times, and 1.7 times that of HPLC respectively. Studies have shown that the general peak capacity was HPLC ≪ UPLC ≈ HPLC × HPLC ≪ HPLC × UPLC when analyzing TCM.

The analysis time of the two-dimensional liquid chromatography system compared to that of the one-dimensional liquid chromatography was longer. A key factor affecting the time of the system is the second dimension liquid chromatography analysis time. If the second dimension column uses a fast analytical column, then the full two-dimensional analysis time would be significantly shorter. Compared to HPLC, UPLC has the advantages of shorter chromatographic running time, and therefore UPLC is more suitable to be the second dimension in a two-dimensional liquid chromatography. With the development of UPLC rapid analysis column, two-dimensional HPLC × UPLC analysis system will shorten the time to 1/4 ~ 1/5.

Compared with the one-dimensional liquid chromatography in the qualitative analysis of chemical composition, two-dimensional liquid chromatography mass spectrometry system has obvious advantages in higher separation ability. As the chemical composition of TCM is very complex and the concentration

is quite different, a lot of components of small content will be hidden in the large peaks during the chromatographic separation. Thanks to the system's high separation capacity, most of the chemical composition can be completely separated, so the qualitative determination of chemical composition will not cause errors. Meanwhile, the two-dimensional liquid chromatography system uses one-dimensional and two-dimensional retention time to quantify, and chemical composition has a unique peak in three-dimensional spectrum. If combined with mass spectrometry for structural analysis, the accuracy of the qualitative determination of chemical composition will be ensured.

3.3 INFORMATION PROCESSING FOR THE STUDY OF TCM CHEMOMICS

3.3.1 Requirement of Informatics for TCM Chemomics

The aim of the study of Chinese chemomics is not only to chemically characterize the composition of TCM chemomes; the ultimate goal is to reveal the relationship between chemical composition changes of chemome (chemical composition's type, content, and compatibility mode) and the different biological effects. We should adhere to the overall system concept as a guide, and also to the strategy of "reasonable decomposition/reconstruction of integration," starting from the whole to local, and from the local to whole. Only in this way could the intrinsic law between the interaction of two complex systems in TCM and the human be deeply revealed. The efficiency of this process and the rate of success largely depend on informatics technology. The TCM chemomics study requires informatics technology included as follows.

(1) Informatics technical support for component separation and scheme design for compatibility. To use the least number of tests to obtain the maximal information and to reveal the relationship of the interaction of the components, one needs to fully absorb the ideas of systematic theory and operational research, and to use a variety of mathematic tools.
(2) Research on the relationship of composition and effectiveness needs to develop a new nonlinear and intelligent processing information technology. Effective component groups and critical effective ingredients should be quickly identified by the disturbance of chemical information matrix and the corresponding change of the drug efficacy matrix.
(3) To establish a multiparameter, compound pharmacological/toxicological evaluation or activity prediction model, and evaluate the efficacy and toxicity of TCM, it is necessary to use a variety of models, targets, and parameters so that we can obtain multilevel activity information. To integrate this activity information and establish a comprehensive efficacy/toxicity evaluation model and evaluate its scientific rationality must rely on the support of informatics technology.

(4) To elucidate the action mechanism of multicomponent compound drugs requires bioinformatics technology. The mechanism of TCM formulas should be reflected as multicomponent, multitarget, even multisystem network control mode. In order to reveal the relationship of multicomponent, multitarget, and multisystem methods effectively and depict this complex regulatory network immaterially and accurately, bioinformatics technology is essential for us to rely on.

Overall, the conventional and mature information processing tools are mostly used now, which made some achievements and improved research efficiency. At present, some of the most commonly used information-processing techniques include mathematical design methods such as orthogonal design and uniform design; research techniques of composition-effectiveness relationship also includes the reverse engineering, knowledge reduction, causal relationship discovery, sorting features, and artificial neural networks; theory and methods of multivariate statistic and discriminant analysis, including principal component regression (PCR), partial least squares (PLS), and support vector regression (SVR) methods. Other classification methods include linear discriminant analysis (LDA), partial least-squares discriminant analysis (PLS-DA), and support vector regression (SVC). We believe that with the development and maturity of the informatic technology, the study of TCM chemomics and the understanding of mutual relationship of the changes of TCM chemome composition can be deepened (chemical composition's type, content, and compatibility mode) and the different biological effects the will be more clear and comprehensive.

3.3.2 Information Processing of TCM Fingerprint

From the view of informatics, fingerprints can be summarized into three stages: information acquisition, information processing, and information mining. This section focuses on a variety of information processing methods in TCM fingerprinting.

3.3.2.1 Fusion Technique of TCM Fingerprints As Chinese herbal formulas have complex ingredients, some of the components may be not easily absorbed or even nonabsorbent in wavelength, which is more likely to affect the whole and comprehensive evaluation of the chromatographic fingerprint. If all the obtained DAD data are used for comparison, the processed data is titanic, yet it is not necessary. Because in some wavelengths some ingredients have no absorption, it contributes nothing to the similarity evaluation. For the studied *Zhizi*, it includes three types of effective components: iridoid glycosides, organic acids, and pigments. Chromatographic fingerprints using only a single characteristic wavelength cannot generally express and comprehensively evaluate the *Zhizi*, therefore, we have established the fingerprint under three characteristic absorption wavelengths for the three types of

effective parts. How to integrate information of the three-wavelength chromatographic fingerprint to achieve a comprehensive and holistic assessment of chromatographic fingerprint needs to be further addressed.

Fusion includes the simple mixture of multidimensional data and the crossing, penetration, and integration of multidimensional data as well. The integration method is a simple fusion method which transfers fingerprint data matrices of characteristic absorption wavelength into response data vectors by tandem integrated approach, and the original data will not be lost. However, immense data await processing even after integration. To reduce the amount of processed data, principal component analysis can be used to integrate multidimensional data. Under the premise of ensuring minimal loss of data, the three characteristic wavelengths of HPLC fingerprint data are integrated into a chromatographic fingerprint. Vector cosine angle, the correlation coefficient, and the distance coefficient (Euclidean distance) were applied to evaluate the similarity of integration of fingerprints based on two fusion methods of integration method and principal component analysis. Spearman's correlation coefficient was used to evaluate if the difference between the two methods would affect the order of similarity (rank).

The results showed that integrated fingerprints obtained by the two means of the integration method and principal component analysis have a high Spearman correlation coefficient, regardless of the distance factor, correlation coefficient, or the angle cosine similarity. The probability of occurrence of a hierarchical order dislocation was less than 0.05 (two-tailed test), indicating the significant correlation in hierarchy of similarity obtained by two integrations, that is, for the same batch of samples, two different fusion methods basically do not change the order of the sample and the control fingerprint.

3.3.2.2 Principal Component Analysis for Two-Dimensional Fingerprints With the development of analytical techniques, diode array detectors (DAD) have been increasingly and widely used. A DAD is generally used to test the peak purity, correct signal, and to quantitatively analyze the multicomponent indicators, but it is rarely used to construct a two-dimensional fingerprint containing chromatographic and spectral information. Among the methods to acquire multidimensional fingerprint, HPLC–MS/MS has the highest selectivity and qualitative capability. However, because the detector is expensive, it is not popular in the practical quality control process. Therefore, we developed a two-dimensional fingerprinting method based on the DAD which integrates multidimensional data and principal component analysis. In the premise of ensuring minimal loss of data, the high-performance liquid chromatographic fingerprint data with different wavelengths were integrated into a chromatographic fingerprint, which fully reflected the multidimensional chromatographic and spectral information. To verify the reliability of the two-dimensional fingerprint method, it is necessary to study the methodology, including precision, stability, and reproducibility.

We constructed a two-dimensional fingerprint of the *Qingkailing* injection, and studied the similarity of the samples. According to different similarity of two-dimensional fingerprint, samples can be divided into three categories, and this similarity feature classification is consistent with the actual classification character of the sample. The fingerprint of each sample was conducted by principal component cluster analysis, and the differences between different types of samples could be better reflected. Thus, the two-dimensional fingerprint integrated the information of different wavelengths of chromatographic analysis, and it had more featured information than the single wavelength fingerprints. Its application in the quality evaluation and quality control of TCM and TCM compound preparation can express more chemical characteristic information, and results are more accurate.

3.3.2.3 A Principal Component Analysis Method for the Generation of Reference Fingerprint As an effective means of quality control and authenticity identification of TCM, fingerprinting has become a widely accepted international quality evaluation model of TCM. When one acquires a batch of fingerprint samples, similarity analysis and evaluation of these fingerprint samples are needed for the quality control of TCM. In the process of similarity evaluation, one needs to generate the control fingerprint. The current methods of generating the control fingerprint are the typical fingerprint selection method and the common pattern method. Typical fingerprint selection method chooses a typical or representative fingerprint as a control fingerprint. However, a control fingerprint produced in this way does not contain general information about the fingerprint of the samples, and it is only the individual character of this sample, so it may not be a good represent of general character of samples' fingerprints. Moreover, the selection of a typical fingerprint also has a certain degree of subjectivity. Common model methods include the mean method and the median method. The control fingerprint generated by two the methods contains the sample information of the original fingerprint, but it is difficult to produce a quantitative evaluation of the summarized information. Therefore, using principal component analysis method to generate the control fingerprint was proposed from the angle of summarizing and integrating raw fingerprint information. Based on the principle of least squares, the control fingerprint was generated from the point of reservation and summarization of original fingerprint information, and it harbors features of optimization and could represent the overall original fingerprint of the sample. Results derived from the principal component analysis method are basically consistent with the mean method and median method. As for the presence of abnormal samples, the principal component analysis method can easily detect potential abnormal samples, and the impact of abnormal samples can be eliminated in a timely manner, so it can serve as an effective method to generate control fingerprint.

3.3.2.4 Application of Fingerprint-Based Clustering Analysis to Quality Evaluation Studies have shown that it is difficult to fully reflect differences

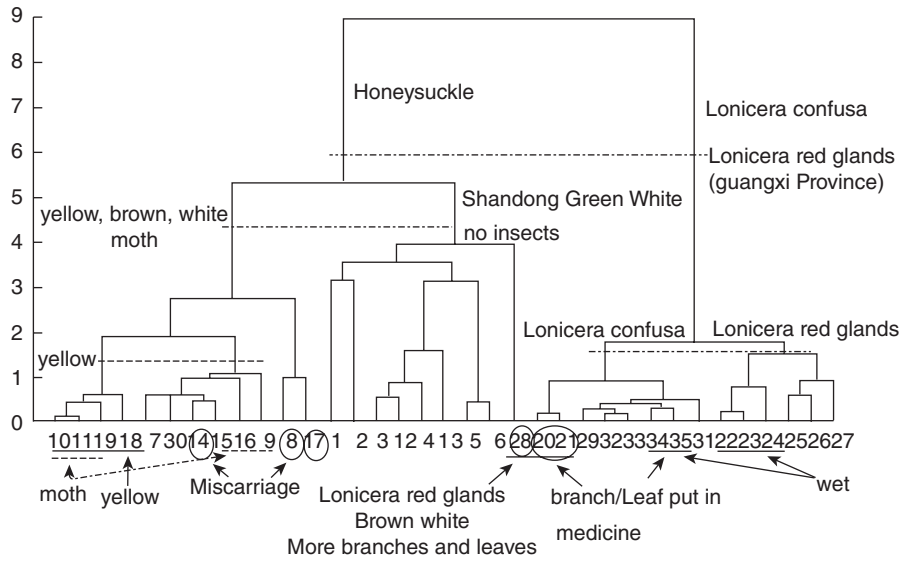


Fig. 3.8 Cluster analysis of honeysuckle fingerprints.

between different medicines only in light of similarity, so we compared original data derived from different honeysuckle fingerprints by clustering analysis (Ward method, Euclidean distance) and principal component analysis, and the results are shown in Fig. 3.8.

The cluster graph revealed the following rules.

- First, *Jinyinhua* (Lonicerae Japonicae Flos) could be divided into two categories (selected from the parameter 6) by *Lonicera japonica* Thunb. and other miscellaneous (including *Lonicera confusa* DC. and *Lonicera hypoglauca* miq.), of which there is only one wrong judge, because sample 28 (*Lonicera hypoglauca* miq.) had more leaves and the color is brownish white compared to other honeysuckles. It is reasonable that original *Jinyinhua* is divided into honeysuckle and lonicera in Chinese Pharmacopoeia (2005 ed., vol. 1).
- Selecting the distance parameter 4.5, honeysuckle category ingredients can be further divided into two categories, of which honeysuckle from Shandong Province with green and white color, no worm holes, and dry samples are clustered into one category without wrong judgment.
- Selecting the distance parameter 4.5, honeysuckle, brown and white or yellow and white, from other sources except Shandong, with worm holes and other samples clustered into one category with wrong judgment, the sample 8, 14, 17 should be attributed to Shandong honeysuckle class;
- Selecting distance parameter 1.5, honeysuckle, yellow and white, clustered into one category without wrong judgment;

– Selecting distance parameter 1.9, red glands Lonicera and Lonicera confusa were divided into two categories;
– Selecting distance parameter 1, wet sample and the other class can be separated from the sample (two samples are missing the judgment).

The above clustering analysis and fingerprints showed that the method of honeysuckle fingerprint is scientific and reliable, so it can guide the manufacturer in determining the quality of raw herbs.

3.3.2.5 Identification of Herbs Using Characteristic Chromatogram In the field of identification of TCM, identification of varieties of TCM has always been the main content. Traditional morphological identification, microscopic identification of tissue, and physical and chemical identification of Chinese herbal medicines have played a great role in the identification of TCM. However, the medicine form and medicine-given forms of TCM are changing. Traditional identification methods limit the identification of TCM produced by modern technologies. Chinese herbal medicines are gradually identified by means of modern equipment and analytical tools. Molecular biology-based molecular genetic marker identification is characterized by high sensitivity and specificity, and is less likely to be influenced by sample collection, yet it cannot identify the finely extracted herb. Fingerprint technology, established to characterize the chemical components, offers a new way to identifying varieties of TCM, and it has features of overall, macro, and fuzzy analysis. The research methods are numerous, and chromatography methods are the mainstream methods, among which high-performance liquid chromatography (HPLC) is more commonly used and plays an important role in the study of TCM fingerprinting. Identification of Chinese medicinal material fingerprints often uses the entire fingerprint. However, the contribution of peaks of the entire fingerprint in the identification of the herbs is different. Some peaks may interfere with the identification of the herbs. This section provides methods for identification of Chinese herbal medicines based on the characteristic spectra, and the data acquisition system is less demanding. Characteristic peaks were selected according to the stepwise analysis method, which has good confirmation stability, specificity, and objectivity. It is especially suitable for identification of medicinal extracts.

The implementation steps of identification of TCM by characteristic chromatography are as follows:

(1) Data acquisition: The total ion chromatography of samples is obtained by liquid chromatography–mass spectrometry.
(2) Data matching: After acquisition of the total ion chromatogram of medicines, peaks are corresponded to the extracted ion chromatograms of sample according to mass spectrometry data and the corresponding chromatographic peak retention time. The matching rules are as follows: first, chromatographies of a sample are randomly chosen as a reference

chromatogram. Then the reference chromatogram is made as a template, the peaks whose retention times are similar to the reference chromatogram of the other analysis samples were put into one group. The difference of peaks retention time of classified as a group should not be great, and it should be in a reasonable time threshold range. The threshold is determined according to the standard deviation of retention time when chromatographic methods are established, the threshold value of retention time of the current examples is 1 min. For the peaks falling into same group, those with the same mass data in the sample were aligned. If there is no peak under the certain retention time, match it with zero to ensure of the same length of the matching data.

(3) Normalization process: The purpose of the normalization process is to obtain the peak relative proportion data. Peak intensity can be characterized by the peak height or peak area; here, peak area is adopted. Selecting a stable existing chromatogram of samples as the base peak in the chromatogram of analysis samples (P_{ir}, i is ith analysis sample, r represents that the rth peak of samples analysis is the base peak), and the ratio between other peak areas and the base peak area is implemented, namely:

$$PN_{ij} = P_{ij} \Big/ P_{ir}$$

PN_{ij} (j indicates the jth peak of sample analyzed) is the relative peak area after treated, each analyzed sample containing peaks is processed by (2), the result is normalized.

(4) Selection of characteristic peaks: Based on standard data processing in Step 3, stepwise discriminate analysis (SPSS software processing) is used to choose the peaks whose area ratio has a small change in the same category and large change of area ratio across different types as the candidate peaks of feature chromatogram.

(5) Establishment of characteristic chromatogram: Characteristic chromatogram is established according to the candidate peak in Step 4; medicinal herbs are identified according to the results of similarity evaluation and cluster analysis of characteristic chromatogram.

Comparison of the original fingerprint chromatogram with the characteristic chromatogram of ginseng and the cluster analysis results is shown in Fig. 3.9.

As is shown in the original fingerprint clustering results, sample 8 was misclassified (samples 1 and 2 were American ginseng, samples 3 to 8 were ginseng), as the original fingerprint cannot completely and effectively distinguish Asian ginseng and American ginseng. After a characteristic chromatogram was used, the category boundaries were clear, the structures were definite, and Asian ginseng and American ginseng could be effectively distinguished. Clustering results were consistent with the actual classification of the samples,

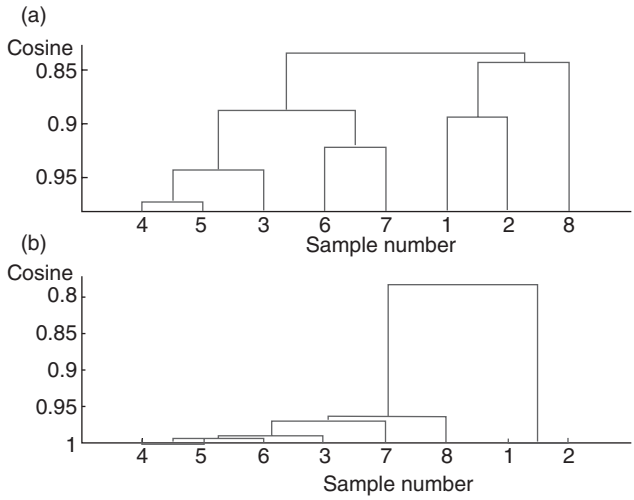


Fig. 3.9 Hierarchical cluster analysis results (cosine method). A: original fingerprint; B: characteristic chromatogram.

indicating that the herbs' distinguishability with characteristic chromatogram outperformed that of the original fingerprint.

3.3.3 Computer Assisted Technology and Software Systems of Fingerprinting

The evaluation of fingerprints is the important step and key issue for fingerprint evaluation of TCM quality and quality control. Here, we briefly introduce the developing process of chromatographic fingerprint. In the old days, chromatographic fingerprint evaluation used some peak evaluation parameters, such as strong peaks and overlap ratio. Nowadays, the degree similarity is commonly used to comprehensively characterize the similarity of chromatographic fingerprints. The quality of sample fingerprints is evaluated by comparison with the reference (standard) fingerprint.

3.3.3.1 Computer Assisted Similarity Evaluation of Chromatographic Fingerprints The chromatographic fingerprinting technology of TCM is an effective solution to the quality control modernization of TCM. Similarity is considered to objectively, comprehensively reflect the characteristics of a fingerprint, and provide effective means for the evaluation of chromatographic fingerprints of TCM.

A chromatogram is obtained to represent chemical components of TCM through the spectral or chromatographic method. Each peak of the chromatogram shall represent the corresponding material basis or part of chemical composition unity. If some Chinese material basis component unities are the

same, two spectra obtained from the experiments should be completely identical; if the majority are the same, two spectra should be very similar; if the difference is huge, the two spectra have poor similarity. Therefore, the similarity degree between spectra is a very important and effective evaluation indicator. If a chromatogram can be transformed into digits that can represent the features of the fingerprint, the similarity can be calculated based on the two digits, and the resulting value can represent the similarity of the two spectra. The concept of similarity is proposed based on this idea and is established as an important indicator for a fingerprint of TCM. As a TCM fingerprint evaluation index, similarity has changed the peak evaluation method used in the past, and treats all peaks of the spectra (or all signal points) as a vector by which to systematically characterize the spectra by digitalization. It improves the utilization of the fingerprint information, and comprehensively reflects the similarities and differences between the fingerprint patterns, and scientifically and effectively evaluates the consistency of spectra. At the same time, objective parameters were used to reflect differences between two chromatograms, which changed the past status that described the chromatograms intuitively with "like" or "unlike." Similarity calculation of TCM fingerprint uses an *n*-dimensional vector X (X_1, X_2, X_3,… X_n) to express its results; this vector can be the original data points of fingerprint, but can also be a fingerprint chromatographic peak decision result, as two pieces of fingerprint similarity between values of two fingerprint vectors have certain operations to obtain.

Using the similarity value to determine the similarity of the fingerprints to evaluate the similarity of TCM and further to evaluate the herb quality is regarded as an effective method for fingerprint technology of TCM. It fits the basic features of the integration and ambiguity of fingerprint chromatogram.

A fingerprint similarity calculation should be established for standard fingerprint matching. The reference standard fingerprint can be obtained in two ways. One is selecting the representative sample from a certain batch (10 batches of samples above), and the common peaks are obtained based on sample fingerprints. Then, mean and median values of the shared peaks are calculated, based on which reference fingerprints are established. The other method is selecting representative fingerprints from the experimental sample, such as the use of the corresponding spectra of genuine medicine. The similarity values of sample and reference fingerprints are calculated after correction of retention time, peak matching, based on which the qualification of the samples is judged.

Because the system reflected by the fingerprint is very complicated, pattern recognition is often used for research and processing. Similarity evaluation of fingerprints plays a key role in the overall characterization of component changes in the complex system of TCM chemical. According to the literature, similarity is evaluated mainly by peak overlap rate (Nei coefficient method), correlation coefficient method, distance coefficient method, vector angle cosine method, and peak overlap and common peak intensity method

(modified Nei coefficient method). These methods have their own characteristics and application scope.

Taking HPLC fingerprint of the *Tongbiding* injection, for example, the various similarity evaluation methods were comprehensively compared from the aspects of fluctuations of peak intensity, lack of certain peaks, and standardization of data by means of theoretical analysis, digital simulation, and statistical method. The results showed that the angle cosine, correlation coefficient, and Nei coefficient were not associated with the proportion of data linearity, and are suitable for the identification of TCM. If the proportion of TCM composition keeps constant, the total variation, angle cosine, correlation coefficient and Nei coefficient are difficult to reflect this change. However, distance coefficient and improved Nei coefficient can reflect it, so they are more suitable for total amount related quality analysis for TCM and Chinese herbal medicine. Distance coefficient, correlation coefficient, cosine angle, and improved Nei coefficient can evaluate the similarity of the fingerprint's intensity alterations. Among them, the correlation coefficient and cosine angle are not sensitive enough, while the distance coefficient and improved Nei coefficient are more sensitive to the fluctuation of the fingerprint. Nei coefficient and improved Nei coefficient are more sensitive to the absence of the large peak than that of the small peak. Distance coefficient shows good sensitivity in certain range regardless of the large peak or small peak. Data standardization processing doesn't greatly affect similarity evaluation by distance coefficient, correlation coefficient, and cosine, and they are more suitable for the studies of fingerprint pattern recognition.

3.3.3.2 Research and Development of "Fingerprint Workstation" In order to carry on the comparison, classification and identification of fingerprint of TCM, a "TCM fingerprint workstation" software and program were developed by Tsinghua University and The Second Military Medical University. With strong compatibility, friendly interface, complete functions, flexible online support, and report functions, the software has been applied for a copyright.

The functions of the software are as follows.

(1) It can read, store, and transfer the acquired data by analyzer, including Agilent, Waters, and customized data file.

(2) It has the function of peak selection. In some cases, the user needs to choose or delete some peaks for similarity calculation, so the function of peak selection was designed by software. The software also provides a batch selection function, such as trying to select the common peaks to calculate, using the function of "choosing participated calculation peaks" in the software can provide a convenient way to achieve this. When the mouse is placed near the interested peak, the corresponding coordinate (retention time and peak height) can be displayed, so that it is convenient for the user to observe and select.

The program takes automatic match after correction completed, and users can set the threshold of matching. The larger threshold setting allows the peaks with large deviation of retention time to fall into the same, and therefore different materials may fall into the same group of chromatographic peaks.

For the spectra with large deviations in the retention time, automatic matching sometimes cannot be fully corrected, and the author needs to match the results of adjustment according to the experience and his or her own understanding of the chromatogram. The software provides a manual adjustment function by adjusting the unreasonable matching peaks to get relatively proper matching result. Software is able to display the matching results with different brightness to show the common and uncommon peaks, and it is helpful for users to observe the common peak distribution. The software is based on the percentage area of peak, using different colors to show different percentages of peak which allows for more methods of obtaining information and allows the user to intuitively "see" the magnitude of peak and statistical information.

(3) It has the original data preprocessing functions, including data normalized vectors and weighted process. In the calculation of similarity, a sample data file can be preprocessed through the similarity parameter settings so that users can easily solve different problems with the appropriate methods to get more reliable similarity evaluation.

The weighted method is divided into two parts: automatic weight and custom weight. Automatic weight includes the variation coefficient weight and peak number weight, and the peak number weight is weighed by the peak frequency in the established sample fingerprints. Variation coefficient weight is obtained by the calculation of coefficients of variations of certain groups of chromatographic peaks, which is calculated as: $w_{RSD} = 1/(0.01 + RSD)$. *RSD* weights are set between 0 and 100 in this formula. The reason for using *RSD* weights is that the peak with small *RSD* has better identification.

The software provides a "normalized vectors" function which is an automatic weighting function. In the setting of the weights, match of the chromatographic peak group is selected to be variable, which is considered as a variable in different observation values. Mean reciprocal weight takes the arithmetic mean reciprocal of the group as weight, the group of peaks are weighted, and the mean value of all of the variables is 1. Therefore, the reference chromatogram is transformed to an n-dimensional unit vector (1, 1,…, 1), n being the total number of the match groups of peaks. The method will treat all chromatographic peaks as a measure, and it has the same effect on similarity, which tries to reflect the small chromatographic peaks impact upon the similarity difference. But due to the large measurement error of the small peak itself, the calculated results have lower stability.

When the active ingredient has a small response in the chromatogram, changes of medicinal components cannot be well reflected due to the little

contribution to the similarity from the small peaks. Therefore, experts advised that active component of chromatographic peak should undergo a weighing process. Custom weight function is used when that active component need to be weighted, but there is still lack of theory and guidance for weighting.

The abovementioned various weighting values are optional in software calculation, and they are turned off by default. If users need to use them, size and ways of the weighting can be set.

(4) The similarity evaluation system of TCM fingerprinting was established, and two ways of the angle cosine and correlation coefficients are used to calculate the peak-to-peak matching similarity of the full spectra of TCM fingerprint, which helps the overall evaluation of fingerprint of TCM. Fingerprint similarity is evaluated after similarity parameter of data document is set. In this software, similarity evaluation methods include two types: the angle cosine and correlation coefficient. If there is a standard fingerprint, this software can give the similarity evaluation of samples compared to the standard fingerprint. If there is no standard fingerprint, this software can produce standard fingerprints of the sample fingerprint data by the average and median method, and then gives the similarity evaluation of a sample compared to a reference fingerprint.

(5) The map has visualization capability, and the fingerprint can be zoomed out and in.

(6) Online support was written for the software convenient use, and multimedia instructions of the software were compiled. Software online support makes new users quickly understand the basic use of software; the user can click on the help directory to obtain the corresponding help information. The user can also use the help index to access to relevant content, allowing the user to easily understand the mechanism of software and to better grasp the usage of the software. At the same time, the software also provides multimedia instructions, which are the foundation for the popularization of the program.

(7) Software adds the statement function, including the common peaks report of TCM fingerprint and the common mode report of TCM fingerprint. During the similarity evaluation process, the user can look and preview the common peak report and the common pattern report, and can save and print the corresponding statements.

(8) It includes convenient functions such as more convenient chromatogram comparison function and the more straightforward display of common peaks. The chromatogram of several samples can be overlapped, and can also be arranged in lists to observe and compare the fingerprint easily at the user's convenience. Common peak results obtained by peak matching can use the highlight function, enabling the user to observe the matching results more intuitively.

(9) The function of point similarity computation according to the original data is added.

(10) It has the function of twin peaks correction with retention time and peak intensity correction, which allow peak automatic matching. We used the time window slope method for peak automatic matching. During the process of automatic matching, users can set reference peaks according to their needs. There are three main reference peaks, prepeak and postpeak are used for correction of retention time, and it is better to choose chromatographic peaks of well-defined substances, and internal standard peak is used to calculate the relative peak area. In the list box, chromatographic peaks are matched by default setting. If the users are not satisfied, they can change the threshold of slope to regain matched results according to the actual situation. If the individual peaks still have some problems, they can be adjusted manually. At the same time, we also offer function of saving the current matched results.

(11) Analysis module of the fingerprint pattern recognition contains the main component analysis and factor analysis algorithm. Pattern recognition research of a fingerprint can be carried on by the principal component analysis and factor analysis provided by the software. Here, principal component analysis is taken as an example to discuss. Click principal component analysis in the pattern recognition, and the sample data are automatically analyzed by principal component analysis of the software, and the analysis result is output in graphic form. The sample classification can be directly observed through the graph.

(12) Clustering analysis module of the fingerprint is newly added to achieve fingerprint cluster analysis. Cluster analysis module includes five kinds of distance (Euclidean distance, the standard Euclidean distance, Minkowski distance, block distance, Mahalanobis distance, and Hamming distance). Two kinds of similarity calculation (correlation coefficient and angle cosine) and five kinds of clustering method are commonly used (the shortest distance, the maximum distance, gravity method, average method, and deviation square method), which gives the cluster pedigree diagram, and its function is powerful.

(13) The pattern recognition module of neural networks has been newly added, which can realize the fingerprint pattern classification and prediction. According to the neural network dialog box, the user can select an appropriate transfer function, and adjust and set the corresponding parameter to realize the fingerprint pattern classification and prediction.

3.4 DEVELOPMENT OF AN INTELLIGENT QUALITY CONTROL SYSTEM IN THE PROCESS OF CHINESE MEDICINE PRODUCTION

TCM attaches more importance to integrity, and thus has different model of research and utilization ideas in comparison with Western medicine, which brings difficulties as well as opportunities into the establishment of modern

Chinese medicine quality standard system. On one hand, the establishment of Chinese native medicine quality specification must manifest the Chinese medicine medication theory, and it should reflect the medicine coordination, the medicine rulers and the ministers assist cause, the medicine nature, the medicine material base, and so on. So it must have multidisciplinary, multiangle integration in the formation process of the TCM system, which provokes the development of independent innovation of Chinese drug research and standards, and the development of the Chinese pharmaceutical research industry. On the other hand, it has brought difficulty for the establishment of a quality standard system for Chinese native medicine, which needs comprehensive consideration and assessment of its progressiveness, feasibility, verifiability, and applicability.

3.4.1 Introduction of Intelligent Quality Control System in the Process of Chinese Medicine Production

The established modern quality specification system for Chinese native medicine should be able to reflect the characteristics of TCM theory, and the material basis for Chinese herbal medicine is effective compound groups.

3.4.1.1 The Whole-Coursing, Comprehensive, and Intelligent Goal for the Construction of a Modern Chinese Medicine Quality Standardization System After original innovation on key technologies of production and quality control of TCM, and after the absorption and innovation of international advanced quality control technology, especially integrated innovation, we have accelerated the establishment of China's independent intellectual property rights which embody the essential features and quality standardization system in line with international bidirectional modernization of TCM. It includes the following: whole-process control based on optimization of medicine provision, intermediate process monitoring and comprehensive control of final products; and comprehensive control based on overall fingerprint, multiple component quantitative determination, heavy metals, and agricultural residues, and other indicators to measure biological reactive detection; and an intelligent network control system based on "quality parameter" control, near-infrared line control, automated real-time control, and remote control.

3.4.1.2 Background of Intelligent Control System in the Process of Chinese Medicine Production Fingerprint technology, especially the quantitative fingerprint technology in which fingerprint and quantitative multiple index constituents are combined, is an effective means of comprehensive characterization of the overall and partial group characteristics. In such an integrated, comprehensive, intelligent quality standardization system during construction of modern Chinese medicines, the basic model of TCM quality information expression and general quality control is bound to become the

core technology. In the past few years, fingerprints, such as HPLC, TLC, and IR, have been applied in the field of TCM identification, final product inspection, and even the analysis of some intermediate products produced during TCM production. It plays positive roles in controlling and improving the quality of Chinese herbs. However, the downside is that old fingerprints were often derived in offline analysis mode, meaning that the samples were obtained from certain production line, and after preprocessing, fingerprints obtaining, and information processing, the conclusion of quality assessment was then sent back to the control of production process. The whole process often takes time, resulting in the generation of the time difference. Therefore, online fingerprint technology and its application in online control during Chinese herb medicine production is becoming increasingly urgent and important.

In addition, China's pharmaceutical enterprises have been forced to implement GMP (good manufacturing practice) management (a pharmaceutical production quality management standard). To a certain extent, it has improved the production process of TCM quality control standards. Besides, many large Chinese manufacturers have introduced automatic control technology to the production process. At present, control of Chinese GMP production process is still very traditional, based on physical processing parameters (temperature, pressure, flow, time). Due to the diversity and variability of raw materials for TCM, and the complexity of effective material populations in Chinese medicine and production (craft), it is hard to establish definite, linear, and reproducible functional relationships or mathematical models between the fixed physical parameters and quality parameters representing efficacy of TCM. Therefore, a simple physical parameters used in automatic control based on this fact is difficult to achieve effective the product quality control in the production process. The U.S. Food and Drug Administration (FDA) proposed a dynamic pharmaceutical manufacturing practices regulation (cGMP, current good manufacturing practices). It requires that the production and logistics of the whole process must be verified, and it introduces quality control of pharmaceutical manufacturing process and process analytical technology (PAT) to the online analysis, and gradually meets, the cGMP requirements. Therefore, with the development of cGMP and other international quality control standards of pharmaceutical manufacturing processes, developing the existing physical parameter-based production control system into a system of effective substance and dynamic monitoring of drug quality information-based automatic control, namely an intelligent control system for Chinese medicine production, will be an important task and urgent need to establish modern system for quality control standardization of TCM.

3.4.1.3 Basic Conceptions of Intelligent Control System in the Process of Chinese Medicine Production

The so-called intelligent control of the production process of TCM is to effectively integrate the automatic control

system, online monitoring system, and intelligent analysis system. It transforms the online monitoring signal into quality information representing the efficacy of substances through intelligent analysis. The decision-making information is generated in the expert system and real-time feedback to the automation and control systems, which realizes the optimization of the inherent quality and efficiency within the herbs.

The so-called intelligence of the system has a triple meaning. First, transforming the online spectral information (currently near-infrared spectroscopy is mainly used) into the fingerprint of TCM that represents the efficacy material group composition information very well or into the contents of a multiple indicator component. Second, the intelligent control algorithm is used to transform conventional automatic control into intelligent control containing expert decision-making systems. Third, the intelligent quality control model needs to be realized, and it transforms the process parameter-based control (temperature, pressure, flow, time, etc.) into TCM inherent quality (ingredients, fingerprint, extraction rate, purity, etc.) and optimized comprehensive benefit-based control.

It can be predicted that, with the development of the Chinese medicine industry and the increasing requirements for drug quality and safety, an intelligent control system of the TCM production process will vigorously develop and be widely applied. In order to facilitate a comprehensive understanding of TCM production processes, and to provide better guidance and reference for subsequent development and application, we summarized the basic concept of an intelligent control system of Chinese traditional medicine production processes into a new "1, 2, 3, 4" system.

- One system
  Intelligent control system of Chinese traditional medicine production process
- Two combinations
  Combination of online detection of near-infrared spectroscopy and quantitative multiple index constituents of HPLC fingerprints of TCM
  Combination of online quality analysis and intelligent control
- Three keys
  NIR/HPLC conjugation model
  Automatic control
  Intelligent control
- Four effects
  Waste product elimination and quality assurance
  Acquirement rate increase, and resource conservation
  Energy consumption reduction, and energy conservation
  Pollution reduction, and environment protection

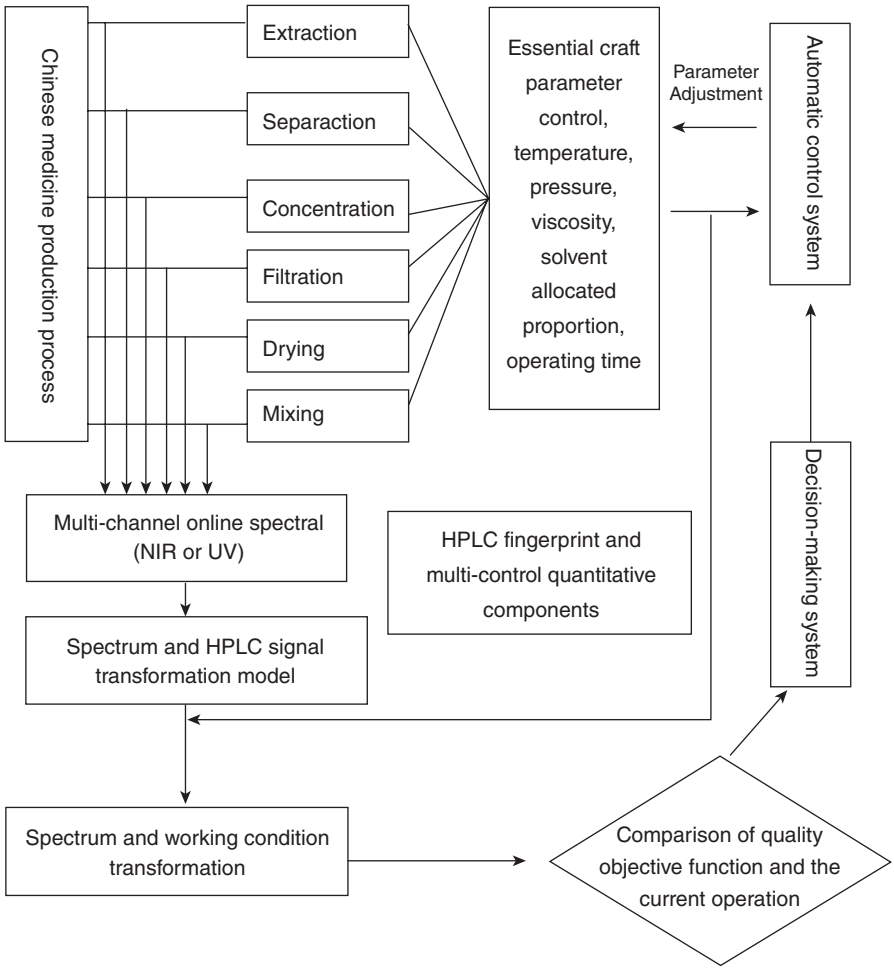


Fig. 3.10 Map of the intelligent control system in TCM production process.

3.4.2 Implementation and Key Techniques of Intelligent Control System in the Process of Chinese Medicine Production

The overall technical roadmap of intelligent control system in the process of Chinese medicine production is shown in Fig. 3.10.

3.4.2.1 Qualitative Control Mode for the Quality of TCM Considering the multicomponent, multitarget and multichannel synergetic therapeutic effects of Chinese medicine, the quality control mode of TCM should primarily reflect the integrity. Fingerprints of TCM can reflect the overall chemical compositions and speciality, so it is the preferred technology for global qualitative control of Chinese medicine.

3.4.2.2 Quantitative Control Model for the Quality of TCM Quantitative fingerprints should be established by combining multidimensional and multi-informational technologies, so as to realize the comprehensive control of Chinese medicine qualitatively and quantitatively.

3.4.2.3 Offline Control Mode for the Qualitative and Quantitative Control of TCM Chromatography and coupling techniques can provide chemical information for the qualitative and quantitative analysis of effective components owing to their strong separation and analysis abilities. But due to the long analysis duration, it can only be achieved offline at present.

Qualification and quantitative control of TCM preparations can be fulfilled offline by the combination of multicomponent quantification and general qualification with fingerprint.

3.4.2.4 Online Control Mode for the Qualitative and Quantitative Control of TCM Online NIR spectroscopy can complete the sampling and analysis within milliseconds, so it qualified the online analysis. The same NIR spectra reflect the same chemical composition, so the global similarity and discriminatory analysis based on the spectrum can be used for the qualitative analysis in TCM quality control, which is similar to fingerprinting. Online NIR spectroscopy combined with intelligent computing technology can be used for the real-time prediction of general fingerprints and contents of multiple components. It can fulfill the online diagnosis of the production process and detect some integrated values such as total nitrogen, physical quantities, and density. The detection scope is much wider than that of single chromatography analysis.

3.4.2.5 Online NIR Detection Technique

(1) NIR can detect liquid, solid (including powder), extracts, and other forms of Chinese medicine.
(2) NIR can flexibly use transmission, reflection, through reflection, diffuse reflection, and so on.
(3) Sample pretreatment is not necessary.
(4) Spectral scanning speed can be up to milliseconds or seconds-level, and it is suitable for online testing during production.

3.4.2.6 NIR Spectral Modeling Method

(1) Outlier detection: It detects spectrum abnormalities, chemical anomalies, and the exceptive modeling association between the spectrum and chemical values by statistics.
(2) Pretreatment: It eliminates spectral noise and extracts useful information. The developed pretreatment methods mainly include first order

derivative, two-order derivative, multiplicative scatter correction (MSC), spectra normalization (SNV), and wavelet denoising.

(3) Wave length selection: It is used to choose the wavelength containing useful information, and the method includes subwavelength selection and multiwavelength combination selection.

(4) Dimensionality reduction: The dimension of spectral data is reduced by linear or nonlinear methods, including PCA and KPCA.

(5) Regression analysis: The spectrum is correlated with the chemical and physical information of Chinese medicine, including PLS and SVR.

(6) Discriminating analysis: the target parameters and process conditions are judged through spectra signal, and the methods include SIMCA and SVC.

3.4.2.7 Automatic Control System

(1) Process parameter detection: Flow, liquid level, temperature, pressure, vacuum, valve position, concentration, density, pH, moisture content, and so on;

(2) Automatic control during production process: Single-point, unit equipment, important process, whole-course automation of production line;

(3) Video monitoring of production status: Monitor the field situation to ensure that equipment is working and to supervise technological process, production operation, and so on;

(4) Online analysis of production process: Extraction process modeling and simulation, online analysis of important process parameters;

(5) Online management of production process: Condition recorder, efficiency analysis, material balance, energy cost accounting and management form;

(6) Production optimization scheduling: Plan, schedule, and automatic-plan according to production tasks;

(7) Process parameter optimization: The process is optimized via accumulated process data and offline analysis data.

3.4.3 Application and Implementation Progress of Intelligent Control System in the Process of Chinese Medicine Production

The online NIR spectroscopy technology realizes the advent of a new model, in which efficacy-associated chemical component groups can be detected online and intelligently controlled during the production process. It ensures the quality is controllable in the production process. We believe that this novel quality control technique of TCM production will be a revolution. It will have a great impact on the quality control of Chinese medicine. And the application

of online NIR spectroscopy will be very broad, almost covering the whole process from raw material source to the final production; for example:

3.4.3.1 Raw Material Testing and Quality Evaluation

(1) Origin identification of Chinese *materia medica*
(2) Authenticity identification
(3) Qualitative identification
(4) Grade estimation and determination
(5) Moisture determination

3.4.3.2 Extraction (Water Extraction, Alcohol Extraction): Optimization and Endpoint Determination

(1) Static extraction
(2) Dynamic extraction
(3) Continuous countercurrent
(4) Percolation, ultrasonic, microwave-assisted extraction

3.4.3.3 Refinement

(1) Alcohol precipitation
(2) Water precipitation
(3) Ultrafiltration

3.4.3.4 Purification

(1) Column chromatography (constant, medium, or high pressure)
(2) Simulated moving bed (SMB)
(3) Expanded bed

3.4.3.5 Condensation/Vacuum Concentration The condensation process is mainly used for the evaporation of water or solvent. And the endpoint of concentration can be controlled by determining the moisture (solvent) content, the concentration, or the density of target components.

3.4.3.6 Evaporation Evaporation is used in processes such as vacuum drying, spray drying, and fluidized bed drying, to determine the moisture (solvent) content and control the related endpoint of operation and drying.

3.4.3.7 Mix For powder, extract, and other semimanufactured products of Chinese medicine, NIR reflection or transmission-reflection is used to monitor the uniformity of mixing.

3.4.3.8 Preparation Monitor the coating, tableting, encapsulation and other processes.

In recent years, we successfully developed several sets of intelligent control systems used in the process of Chinese medicine production with the financial support of the State Development and Reform Commission (SDRC) and National Science and Technology Ministry in China according to the quality control and production requirements of related companies. And some have been implemented in related companies as follows: online quality analysis and intelligent process control in the column chromatography process of Salviol B (Shanghai Green Valley Pharmaceutical Co. Ltd, China), quality standards improvement and intelligent control in the production process of *Qingkailing* (Hebei Shineway Pharmaceutical Co., China), online quality analysis and intelligent control in the extraction process of *Anshen bunao* liquor and *Xuefuzhuyu* liquor (JiLin AoDong Medicine Industry Group Co., Ltd.), and online quality analysis and intelligent control of the mixing procedure of *Yaotongning* capsule (Chengde Jingfukang Pharmaceutical Co., China).

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CHAPTER 4

PHARMACOKINETIC INVESTIGATION ON TCM FORMULAS BASED ON GLOBAL SYSTEMS BIOLOGY

The pharmacokinetics of TCM, an extension and development of Western pharmacokinetics, is a complicated system. Taking the system-to-system idea in systems biology and the formula theory in TCM as the basic guiding principles, and with the help of informatics, data mining technologies, and modern analysis techniques as basic research methods, it shows the pharmacological chemical substance basis of formulas and the principles of prescription formulas, offers guidance for rational use of drugs in clinic, and promotes the innovation in dosage forms of TCM as well as research and development of new drugs. It plays a significant role in the modernization and internationalization of TCM.

4.1 PHARMACOKINETIC CHARACTERISTICS OF TCM FORMULAS

Investigating pharmacokinetic characteristics of TCM formulas offers another perspective on the underlying formula theory and mechanisms of TCM prescriptions. This mode of study can be used to guide formulation innovation, optimize medicine regimen, and suggest dosage-response relationship, providing the theoretical and practical basis for deep investigation of TCM formulas. However, the nature of TCM is complex, involving, for example, the compatibility of *Jun*, *Chen*, *Zuo*, and *Shi*, the diversity of ingredients, and the

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interaction of the drugs, which determines the complexity of pharmacokinetic investigation.

Due to extensive ingredients in TCM, current investigations focus on single ingredients only. Currently, investigations of pharmacokinetics of TCM focus on single ingredient. It's easier to investigate single ingredients, since they have a clear composition and can be easily detected. However, the characteristics of single ingredients are far different from those of TCM formulas, and the pharmaceutical characteristics of single ingredients cannot represent those of TCM formulas. For example, the main ingredients in *Renshen* (Ginseng Radix et hizoma) are ginseng saponin and ginseng polysaccharide. There are three classes of saponin, the main effective ingredient in *Renshen*: protopanxadiol (such as ginseng saponin Rb1, Rb2, Rc, Rd, Rh2, M-Rb1, and M-Rd), protopanxatriol (such as ginseng saponin Re, Rf, Rg1, Rg2, and Rh1), and oleanolic acid (such as ginseng saponin Ro and Ri). According to preliminary studies, more than 80 different types of saponin extracted from different parts of the *Renshen* have been found. The main sugars in *Renshen* are glucose, oligosaccharides, and polysaccharides, which have some biological activity. The polysaccharides that show biological activity and are isolated from ginseng pectin include SA, SB, PA, and PN. There are also more than 12 alkaloids, oligopeptides, and polypeptides that show biologic activity.[1, 2] Therefore, the investigation of any single kind of ginseng saponin cannot represent the overall characteristics of *Renshen*. Therefore, the pharmacokinetics of single ingredients cannot represent those of TCM formulas.

The metabolism of ingredients increases the difficulties of pharmacokinetic investigation. When the ingredients of TCM enter the gastrointestinal tract (pH and flora) of the human body, they go through metabolism, such as oxidation, reduction, hydrolysis, and isomerism. However, the metabolites are always the same as the ingredients contained in TCM. Therefore, it is hard to identify whether the detected compounds are the ingredients contained in TCM or the metabolites, and the relative proportions of these compounds from different sources. These factors increase the difficulties of pharmacokinetic investigation.

For instance, when people are orally administered ginseng saponin Rg1, which turns into ginseng saponin Rh1 via metabolism in the intestinal tract, ginseng saponin Rh1, the metabolite, is absorbed into blood and excreted through urine.[3] Salvianolic acid A is condensed with one molecule of prapanoid acid and two molecules of caffeic acid, salvianolic acid B is condensed with three molecules of prapanoid acid and one molecule of caffeic acid, and salvianolic acid C is condensed with two molecules of prapanoid acid, all of which are degraded to prapanoid acid.[4] The actual assayed content of prapanoid acid cannot reflect the pharmacokinetic characteristics of every ingredient and prapanoid acid itself definitely, so the parameters should be corrected. For example, the pharmacokinetic characteristics of total salvianolic acid and every ingredient can be identified after the pharmacokinetic investigation of

single ingredients is conducted first and the individual pharmacokinetic pattern has been identified.

Multiple responses of multi-ingredient effects make constructing correlation between ingredients and effects difficult; the choice of pharmacokinetic indicator cannot represent the overall characteristics of a TCM formula. The extensive and complex ingredients contained in TCM and the resulting diverse pharmacologic effects make constructing correlations between them difficult. The choice of pharmacokinetic indicator cannot represent the overall characteristics of TCM formulae. For instance, ginsenoside possesses the effect of anti-anoxia.[5] *Renshen* can reduce blood viscosity, improve the metabolism of glycolipid and sex hormones, protect the cardiac muscle from ischemia and anoxia, and improve cardiac kinetic energy.[6] Research has also shown that ginseng saponin Rb1 and Re can significantly reduce the apoptosis of ischemia-reperfusion cardiac cells and alleviate cardiac ischemia-reperfusion injury.[7] Conducting pharmacokinetic investigations of multiple ingredients in TCM and identifying the typical ingredients in TCM pharmacokinetic research are difficult.

4.2 METHODOLOGY OF PHARMACOKINETICS OF TCM FORMULAS

Currently, the research methods for pharmacokinetics of TCM formulas have two classifications: blood/urine concentration method and biology effect method. Additionally, reports about utilizing pharmacokinetics-pharmacodynamics (PK-PD) are increasing.

4.2.1 Blood/Urine Concentration Methods

4.2.1.1 Blood Concentration Method This method fits the drug concentration–time curve, determines the pharmacokinetic model, and calculates various pharmacokinetic parameters by assaying the concentrations of one or several known ingredients of TCM or TCM formulas in body fluids, aiming to reflect the pharmacokinetic law of TCM or TCM formulas.

4.2.1.2 Urine Concentration Method It is difficult to use the blood concentration method for some drugs. When determining the possible effect or toxicity of the drugs, when the drug dose cannot be very high or the assay method is not easy to establish, the urine concentration method can be used. The urine concentration method is easy to establish because the samples can be obtained conveniently. For example, the dosage amount of norcantharidin is small, the blood concentration is low and the individual differences of physiological disposition are large, so it is suitable to adopt the urine concentration method for norcantharidin pharmacokinetic research in humans. The samples can be easily prepared or conveniently obtained, and the quantitative analysis method is highly precise. The urine concentration method estimates the

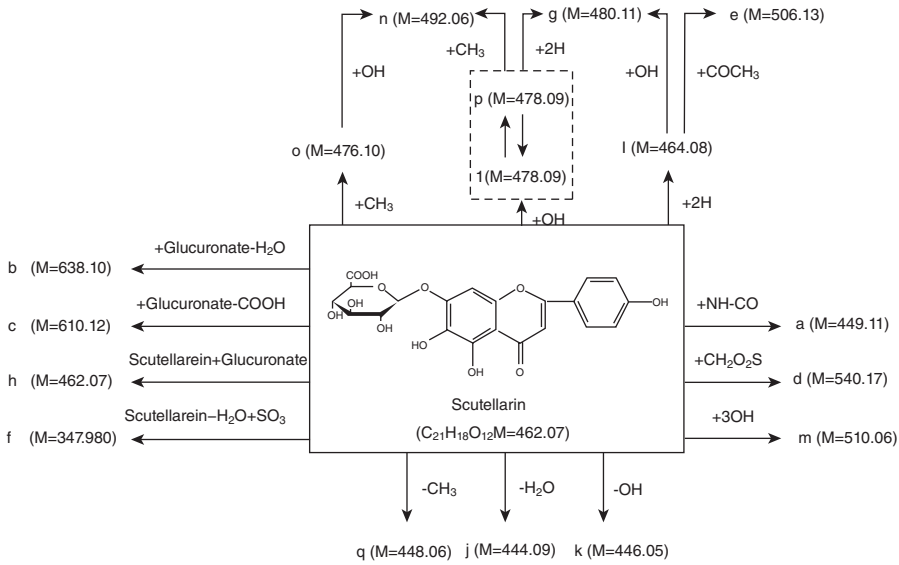

Fig. 4.1 Metabolites in rat urine after scutellarin being orally administrated as a single dose. Compounds: a: $C_{20}H_{19}O_{11}N$; b: $C_{27}H_{26}O_{18}$; c: $C_{26}H_{26}O_{17}$; d: $C_{22}H_{20}O_{14}S$; e: $C_{23}H_{22}O_{13}$; f: $C_{15}H_8O_8S$; g: $C_{21}H_{20}O_{13}$; h: $C_{21}H_{18}O_{12}$; i: $C_{21}H_{20}O_{12}$; j: $C_{21}H_{16}O_{11}$; k: $C_{21}H_{18}O_{11}$; l: $C_{21}H_{18}O_{13}$; m: $C_{21}H_{18}O_{15}$; n: $C_{22}H_{20}O_{13}$; o: $C_{22}H_{20}O_{12}$; p: $C_{21}H_{18}O_{13}$-iso; q: $C_{20}H_{16}O_{12}$.

pharmacokinetic parameters of norcantharidin, such as elimination half-life, and provides the basis for adjustments of norcantharidin clinical individualization dosage regimens.[8]

In recent years, there has been much literature focusing on the pharmacokinetics of TCM using the urine concentration method. Our study has shown that five metabolites of scutellarin in rat blood and bile were discovered, including scutellarin aglycone, two sulfated products of scutellarin, 6-methyl-scutellarin, and 6-methyl-scutellarin aglycone. Four metabolites of scutellarin in human urine were discovered, including parent, two isomers, and methylating scutellarin.[9, 10] Seventeen metabolites in rat urine were discovered after oral administration, and the trace metabolites could be quantitatively analyzed.[11] Fig. 4.1 shows the metabolite profile of scutellarin. Fig. 4.2 shows the excreted amount–time curves of scutellarin and its metabolites, respectively.

Average excretion rate is used to calculate urine excretion rate instead of instantaneous excretion rate, and the time intervals of urine collections are no more than or equal to twice the drug's half-life. Otherwise, large error would occur, which is unfeasible for drugs with short half-life. Using the deficiency method can reduce the influence of the data fluctuation, and is more feasible for the estimation of the pharmacokinetic parameters of drugs with a two-compartmental model. However, the time for urine collection would require seven half-lives.[11, 12]

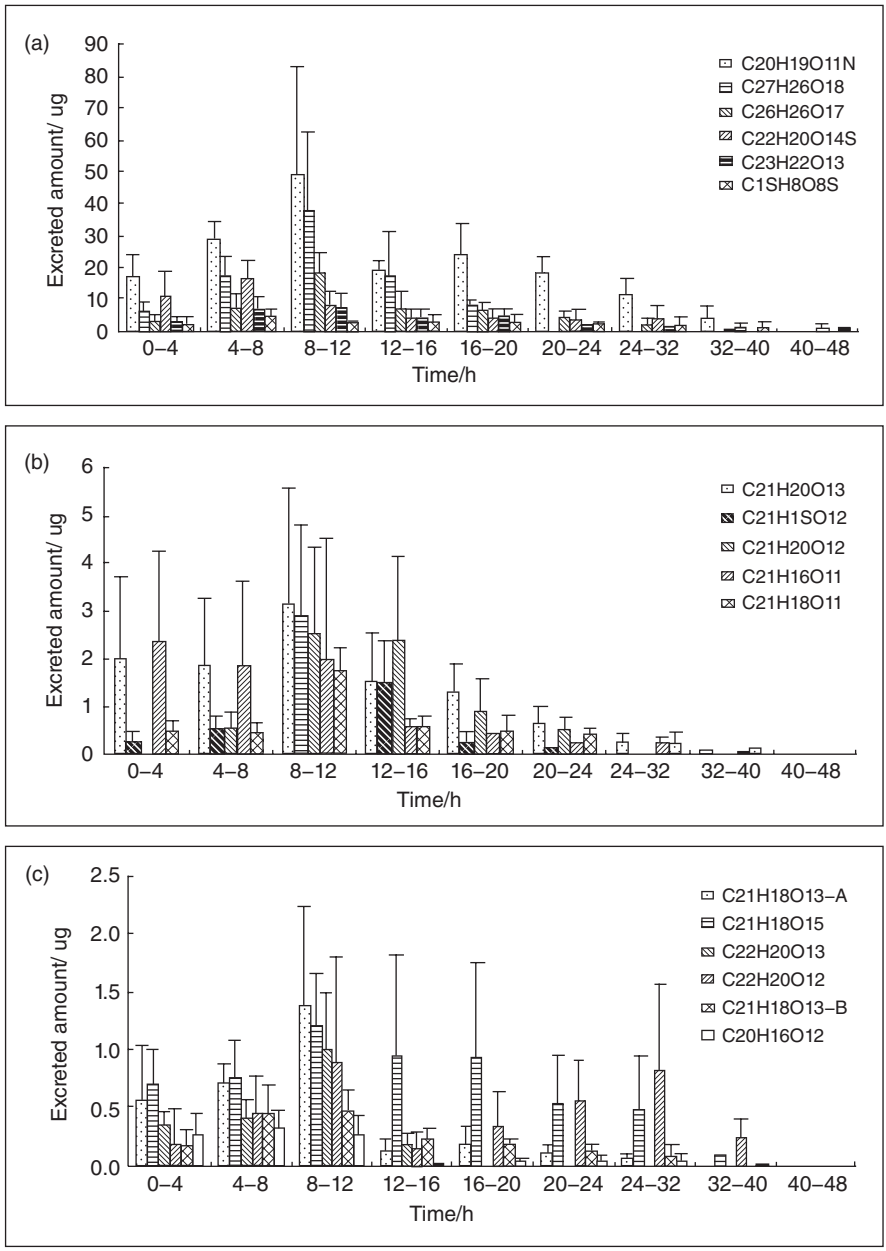

Fig. 4.2 Excreted amount–time changes of scutellarin and its metabolites in rat urine after scutellarin being orally administrated as a single dose (80.8 mg/kg, n = 6). A: compounds: $C_{20}H_{19}O_{11}N$, $C_{27}H_{26}O_{18}$, $C_{26}H_{26}O_{17}$, $C_{22}H_{20}O_{14}S$, $C_{23}H_{22}O_{13}$, C15H8O8S; B: compounds: $C_{21}H_{20}O_{13}$, $C_{21}H_{18}O_{12}$, $C_{21}H_{20}O_{12}$, $C_{21}H_{16}O_{11}$, $C_{21}H_{18}O_{11}$; C: compounds: $C_{21}H_{18}O_{13}$, $C_{21}H_{18}O_{15}$, $C_{22}H_{20}O_{13}$, $C_{22}H_{20}O_{12}$, $C_{21}H_{18}O_{13}$-iso, $C_{20}H_{16}O_{12}$.

4.2.2 Biology Effect Method

It is difficult to choose the definite indicator for pharmacokinetic research because the chemical ingredients of TCM formulas are complicated, the effective ingredients are not clear or the ingredients are difficult to detect, and the internal effect mechanism is not distinct. Therefore, the biology effect method has been used in the early stages of TCM pharmacokinetic investigation to estimate the internal process of TCM formulas in human body, which may provide more insight for clinical studies.

4.2.2.1 Pharmacological Effect Method The basic principle of the pharmacologic effect method is the assumption that the drug presents a linear disposition in the body, that there is a functional relationship between the concentration of the drug in the site of action, $Q(t)$, and effect intensity, E. $Q(t) = f[E(t)]$, whereas, at some time after the oral administration, $Q(t)$ is proportional to the dosage D. The functional relationship in this time between $Q(t)$ and E can be demonstrated using the functional relationship between D and E, as $D = f[E(t)]$. Afterwards, model analysis and calculations of pharmacokinetic parameters can be carried out.[13]

The pharmacological effect method reflects the global pharmacodynamic process of formula investigation, demonstrating that the compatibility of TCM formulas is global, and the result parameters could provide more insight for clinical studies.

4.2.2.2 Toxicological Effect Method This method combines the principles of blood concentration multipoint dynamic determination in pharmacokinetics and the method of determining drug accumulation using animal acute mortality, in which drugs were administered to different kinds of animals under different time intervals. The percentage of drug in the body is calculated at different time intervals, and the percentage of drug in the body versus time curve is fitted to estimate the toxicological effect kinetic parameters.

4.2.2.3 Microbial Index Method and Other Bioassay Method This method focuses on some TCM formulas with antibacterial activity. The concentration of biological sample can be determined based on the fact that antibacterial medicine diffuses in the agar plate containing bacteria, generating bacteriostatic annulus, and the size of the diameter of bacteriostatic annulus has a logarithmic linear relationship with concentration of the sample in some concentration ranges. The pharmacokinetic parameters could then be calculated. The microbial indicator method is easy and feasible, and the determined indicator can reflect the pharmacological effect directly. For instance, the pharmacokinetic parameters of ligustrazine essential oil can be determined by taking the bacteriostatic effect as the indicator.[14]

4.2.3 PK-PD Model

When studying blood drug concentration and pharmacological effect, we found that the relationship between blood drug concentration and pharmacological effect is not linear.[15] The peak of pharmacological effect significantly lags behind the peak of blood drug concentration, and when we mapped the pharmacological effect-blood drug concentration, we obtained a counterclockwise lag ring curve. To account for this phenomenon, Sheiner et al. proposed a hypothetical effect compartment that links to the plasma compartment (central compartment) based on the classic pharmacokinetic model, and built a novel PK-PD model combining traditional pharmacodynamics, which successfully explained the phenomenon that the pharmacological effect of tubocurarine lags behind its blood concentration.

4.2.3.1 Characteristics of PK-PD Model The PK-PD model is a comprehensive study of the pharmacokinetic process and pharmacological effect and concentration indicators in the body. PK-PD study provides important research methods to clarify the effect mechanism of TCM, develop a dynamic design of drug dosage form, and provide insight for the clinical administration of TCM. It also helps to resolve individual differences of clinical response to the drug, explain the influence of environmental factors on the internal process in the body both *in vitro* and *in vivo*, and simulate clinical tests.

4.2.3.2 Classification of PK-PD Model

(1) Direct link and indirect link: For direct link models, the pharmacological effect is directly linked to the effect site concentration, which means the drug immediately exerts an effect after it reaches the effect site with no lag time. For indirect link models, there's lag time in the observed blood concentration and the pharmacological effect. There are two ways to link models: linking the blood concentration in a peripheral compartment to PD, and linking the effect compartment to PD.

(2) Direct response and indirect response: For direct response models, the drug exerts pharmacological effect through directly interacting with target tissue in the effect compartment. For indirect response models, the drug exerts the desired pharmacological effect through stimulating or inhibiting some physiological processes.

(3) Soft link and hard link: These links are established using the clinical and external testing and evaluated information. For a soft link, the construction of PK-PD model is connected by a set of "effect compartments," and the data of PK and PD are connected by the two-way information flux method adopted in the fitting process of the model. For a hard link, the PK-PD model is constructed using the data from the *in vitro* receptor binding test and the free drug concentration in steady state to predict the pharmacodynamic results.

(4) Time-dependent link and time-independent link: For a time-independent link, the effect intensity always depends on the drug concentration in the effect site for most PK-PD models, with constant pharmacodynamic parameters versus time. Changes in the effect intensity would only occur when the drug concentration in effect site changes. For a time-dependent link, some of the pharmacodynamic parameters change with the time. So even if drug concentration in the effect site remains constant, the effect intensity would still change with time.

4.3 APPLICATION OF PK-PD MODEL IN THE TOXICOLOGICAL RESEARCH OF *LIUSHEN* PILLS (LSP)

In recent years, researchers have adopted the PK-PD model to investigate TCM pharmacodynamics. Pharmacodynamic research on sophorcarpidine and ammothamnine using the model have shown that the effect is not directly related to blood concentration, but the relationship between pharmacological effect and drug concentration in the effect site corresponds to the S type E_{max} model.[16] Zhou et al.[17, 18] have investigated the pharmacodynamic correlation of coculine preparation and obtuse leaf erycibe stem injection using the blood concentration method, the toxicological effect method, and the PK-PD model. The study suggests the testing results of blood concentration method and toxicological effect method have shown correlation in some time range. The PK-PD model combines the drug concentration, effect, and time, and can therefore exactly evaluate the dynamic process of the drug *in vivo* and the dynamics of the produced pharmacologic effect. However, investigations using PK-PD are basically restricted to the point-to-system model between single ingredients and formulas.

In order to discover the concentration effect and concentration–time–toxicity relations of LSP, we have studied the pharmacokinetics, toxicokinetics, and influences of dosage and administered time on the toxicological effect in various pharmacologic and toxicological levels in animals, tissues, and blood biochemistry.[19]

This investigation has adopted HPLC-TOF/MS to establish the quantitative analysis method for three bufadienolide compounds in LSP, and has obtained the pharmacokinetic parameters for two of these compounds, resibufogenin and bufalin. The average blood concentration–time curves of arenobufagin diene lactone compounds, resibufogenin, and bufalin are shown in Fig. 4.3, and the pharmacokinetic data are shown in Table 4.1. Cinobufogenin has been detected in blood after beagle dogs were administered LSP. However, cinobufogenin metabolizes very fast, and its concentration is too low to be detected at 30 min, so the pharmacokinetic parameters cannot be obtained using the traditional one-compartmental or two-compartmental model. This method has provided the pathway of LSP dynamics in the body, and can be used in PK-PD investigation of LSP.

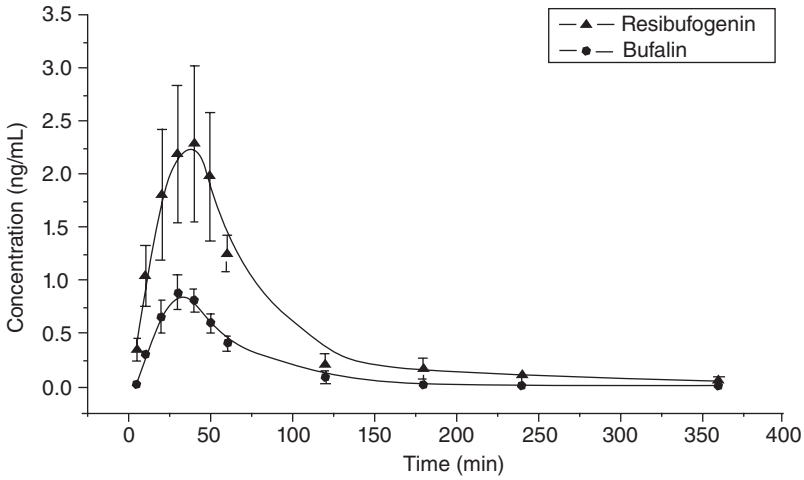

Fig. 4.3 Concentration–time curves of resibufogenin and bufalin after beagle dogs were intragastrically administered *Liushen* pills (48.6 mg/kg).

TABLE 4.1 The Pharmacokinetic Parameters of Resibufogenin and Bufalin after Beagle Dogs Were Intragastrically Administrated LSP (48.6 mg/kg) ($\bar{x} \pm s$, $n = 6$)

Property	Resibufogenin	Bufalin
AUC_{0-t}(ng/mL·min)	160.72 ± 21.97	55.55 ± 7.55
$AUC_{0-\infty}$(ng/mL·min)	163.33 ± 21.98	55.72 ± 7.47
C_{max}(ng/mL)	2.35 ± 0.71	0.91 ± 0.15
t_{max}(min)	38.33 ± 4.08	33.33 ± 5.16
$t_{1/2\alpha}$(min)	20.74 ± 5.89	25.45 ± 13.28
$t_{1/2\beta}$(min)	35.30 ± 12.65	32.11 ± 11.99

Based on the pharmacokinetic results of the acute toxicity test of LSP in beagle dogs, this part of the test used the same serum containing the drug and selected the time points according to the biology effect method for the sample collection points, in which pathologic and physiologic reactions have occurred. Meanwhile, the pathologic and physiologic indicators were determined as well as the internal blood concentration of bufadienolide compounds (resibufogenin and bufalin), the active and toxic ingredients in LSP, in the blood. Furthermore, correlation analysis was carried out taking the concentrations of bufogenin and bufalin as well as every pathologic and physiologic indicator, and the results suggested that there is a correlation between the many pathologic and physiologic indicators and the concentration–time changes of bufadienolide compounds after beagle dogs were administered LSP. Pathologic and physiologic indicators obtained from correlation analysis versus time have been profiled, giving dynamic curves of every indicator from different compatibilities and dosages, as shown in Tables 4.2 and 4.3.

TABLE 4.2 Linear Regression Results of Blood Drug Concentration and Physiological Indicators after Beagle Dogs Were Administrated 0.2 mg/kg (left) and 48.6 mg/kg (right) of LSP: Hematology Indicators

	Low Dosage (0.2 mg/kg)			High Dosage (48.6 mg/kg)		
Indicator	Correlation	Coefficient	Comment	Correlation	Coefficient	Comment
MPXI	Negative	>−0.7	–	Negative	>−0.7	–
CH	Positive	~0.7	Fluctuation recovering quickly	Positive	~0.7	Recovery speed significantly slow down
HWD	Positive	0.67~0.94	–	Positive	0.67~0.94	–
MPV	Positive	~0.7	Regular fluctuation recovering quickly	Positive	~0.7	Regular fluctuation recovering quickly
MCHC	–	–	Increase first and then recover	Negative	~−0.63	–
MCH	–	–	–	Negative	~−0.7	–
ECO	Positive	~0.75	–	Positive	~0.75	–
LUC	Positive	>0.9	–	Positive	>0.9	–
MONO	Negative	−0.9~−0.7	Decrease first and then increase, peak valley appears at 40 min	Positive	−0.9~−0.7	Decrease first and then increase, peak valley appears at 40 min

MPXI: mean neutrophilia MPO activity index; CH: cholesterol; HWD: hemoglobin concentration distribution; MPV: mean platelet volume; MCHC: mean corpuscular hemoglobin concentration; MCH: mean corpuscular hemoglobin; ECO: eosinophil; LUC: large unstained white blood cell; MONO: monocyte percentage.

TABLE 4.3 Linear Regression Results of Blood Drug Concentration and Physiological Indicators after Beagle Dogs Were Administrated 0.2 mg/kg (left) and 48.6 mg/kg (right) of LSP: Plasma Chemistry Indicators

	Low Dosage (0.2 mg/kg)			High Dosage (48.6 mg/kg)		
Indicator	Correlation	Coefficient	Comment	Correlation	Coefficient	Comment
BUN	–	–	Increase first and then recover to normal status	Negative	–0.62	Increase first and then recover to normal status
TBIL	Positive	~0.6	–	Positive	~0.6	–
ALP	Positive	0.73	–	Positive	0.73	–
CK	Positive	~–0.65	Increase first and then recover to normal status	Negative	~–0.65	Increase first and then slowly recover to normal status

BUN: urea nitrogen; TBIL: total bilirubin; ALP: alkaline phosphatase; CK: creatine creatase.

(1) Hematology Indicators:
Hematology indicators obtained from correlation analysis versus time are shown in Table 4.2.

(2) Plasma Chemistry Indicators: Plasma chemistry indicators obtained from correlation analysis versus time are shown in Table 4.3.

For the LSP high and low dosage groups and the venenum bufonis group, the values of CK keep in the normal range (the level before administration).

(3) Electrocardiogram Indicators: The big dip in heart rate appeared at approximately 40 min in arenobufagin groups, whereas fluctuations in heart rates were minor in LSP and LSP without arenobufagin (de-arenobufagin) groups, except there was a slight increase at approximately 40 min. It has been reported that a remarkable decrease in heart rhythm and increase in systole appeared after the beagles were injected with arenobufagin, which corresponds to the results of our research. However, the remarkable decrease in heart rhythm did not happen in the LSP group, suggesting the compatibility of LSP might have the effect of reducing toxicities. Second, the change tendency of heart rate in the de-arenobufagin group at low dosage is similar to that at the dosage of 48.6 mg/kg, whereas the change tendency of heart rate in LSP and arenobufagin groups keeps conformity at both of the two dosages, which again proved that the decreasing effect in heart rate with LSP was induced by arenobufagin. The attenuation of LSP weakened at high dosage, and the heart rate was obviously slowed down. At the dosage of 48.6 mg/kg, heart rate presents negative correlation with the concentration–time curve of bufadienolide compounds, and the correlation coefficient is approximately 0.7.

The change tendency of Q wave after LSP is administered, arenobufagin and de-arenobufagin keeps conformity at the dosage of 0.2 mg/kg. However, Q wave value in the de-arenobufagin group is significantly higher than that in the LSP and arenobufagin groups at the dosage of 48.6 mg/kg, suggesting that arenobufagin may have the effect of increasing Q wave value through antagonizing some kind of formula in LSP. The results of correlation analysis show that the blood concentration of two bufadienolides compounds in LSP presents a positive correlation with Q wave value, with the correlation coefficient being 0.76.

In conclusion, after the beagles were administered LSP, the change curves of the following items with time have linear correlations with the concentration–time curves of the two bufadienolide compounds (resibufogenin and bufalin). The following items conclude: alkaline phosphates (ALP), creatine (CK), and total bilirubin (TBIL) in serum chemistry test items; cholesterol concentration, hemoglobin concentration distribution, mean corpuscular-hemoglobin concentration (MCHC), mean corpuscular-hemoglobin amount (MCH), and mean neutrophilic MPO activity indicator (MPXI) in blood test items; the percentages of eosinophile granulocyte (Eos) and non-staining macro-

leukocyte (LUC) in leukocyte classing items; and physiologic indicators such as heart rate and Q wave in electrocardiogram testing items.

On the basis of the correlation analysis and through interpretation of the pharmacokinetic data, the results have suggested that the creatine creatase (CK) indicators in the arenobufagin and LSP groups are significantly different from those in the de-arenobufagin group. The change tendencies in the arenobufagin and LSP groups are in line with the data range, and the abovementioned change regularity is same at the low dosage (0.2 mg/kg) and high dosage (48.6 mg/kg). The results have manifested that the arenobufagin in LSP may have the effect of lowering CK, therefore exhibiting the pharmacologic action of protecting cardiac muscle cells. The correlation analysis has shown that the concentration–time curve of bufogenin presents negative correlation with CK value–time curve, suggesting that bufogenin may be the active ingredient in arenobufagin with myocardial preservation. The correlation coefficient of the concentration–time curves of resibufogenin and bufalin and the CK value-time curve is less than 0.6, for which one of the reasons is the activity difference and the other reason is that the natural abundance of bufalin in LSP is lower than that of resibufogenin.

Our research has correlated the chemical information with physiologic and toxicological information by PK-PD method, and screened the physiologic indicators related to the effect or toxin of LSP from the amount of complex physiologic indicators by the correlation analysis of concentration–time curve and effect (or toxin) –time curve, in order to reveal the effective or toxic effect of LSP and to provide useful references for investigation into the mechanism of action and compatibility attenuation of LSP.

4.4 PROSPECT

Pharmacokinetic research of TCM formulas should demonstrate the concepts of holism and systematology. Treatments based on syndrome differentiation and formula compatibility are the essence of TCM pharmaceutics. The pharmacologic effect of TCM formulas is the comprehensive effect produced by the synergism and antagonism of the various compounds contained in TCM. Thus, the concept of holism is a characteristic of the pharmacokinetic investigation in TCM formulas and is a guide that should be followed. Single ingredients or TCM formulas can be taken as an organic whole, and the research method must therefore reflect the "holistic" characteristics. The interaction between the formulas and the body can be regarded as a complex interaction among two systems, and for this reason, investigation of TCM formulas can take ideas from systems biology.

Systems biology based on complicated network systems must be developed at full speed in the future. The complexity of the ingredients in TCM formulas decides the complexity and variety of the formula's effect. Therefore, multiple research approaches should be positively adopted to develop chemomic technologies for TCM formulas in pharmacokinetic research, which combine

research technologies at different levels that contain multicomponent quantification methods of drugs in the body, multicomponent quantification pharmacology, network pharmacology, quantitative metabonomics, genomics, and proteomics. By establishing a network model through intelligent computation, the correlations and inherent connections between the pharmacokinetic characteristics and complicated pharmacologic effects, "PK-PD system," of TCM formulas can be systematically reflected, which will reveal the internal behavior of drug in deep level, and reflect the complicated network system characteristics of TCM formulas on a whole systematical level.

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CHAPTER 5

APPLICATION OF GENOMICS IN THE RESEARCH OF TCM

Genomics is an important component of systems biology. A summary of genomics and its applications in the research of TCM is introduced in this chapter. The research approach and the technologies of genomics used in TCM system research are introduced in Section 5.1. The application prospect of genomics in TCM system research is introduced in Section 5.2. The cases of the application of genomics in TCM system research are introduced in Section 5.3. We used genomics to research the mechanism of *Shexiangbaoxin* pills. Another case is genomics research of the mechanisms of three TCM formulas, *Shuanglong* formula, *Shexiangbaoxin* pills and the *Danhong* injection for myocardial infarction. There are nine commonly differentially expressed genes in the three TCM formulas, and the functions of the nine genes are explained. In the process of treatment of myocardial ischemia with the three TCM formulas, the expression of the genes in the myocardium of rats was significantly affected, but the metabolic pathways regulated by the three TCM formulas were not completely the same.

5.1 GENOMICS AND TCM SYSTEM RESEARCH

5.1.1 Introduction

Genomics is a branch of science that refers to genome mapping of all the genes of an organism, analyzing the sequence of nucleotides, localizing

Systems Biology for Traditional Chinese Medicine, First Edition. Guoan Luo, Yiming Wang, Qionglin Liang, and Qingfei Liu.

genes, and analyzing genetic functions. Genomics research mainly includes two aspects: structural genomics and functional genomics.[1] Structural genomics generally identifies the gene structures by measuring genetic sequences or determining genetic mutation loci (SNP chip). Functional genomics interprets the biological functions and physiological pathology mechanisms of the genes from their expression changes by methods of microarray (cDNA chip, miRNA chip, DNA methylation chip, and PCR). Recently, genomics research has been focusing on the following three aspects: genome sequencing for diseases such as cancer to explain the developmental mechanisms of the disease, and provide the theoretical basis for personal therapy in the future; improving drugs' therapeutic effects and reducing their adverse reactions and side effects as well as discovering precise applications of a drug according to genotypes of drug-related proteins; and in epigenetic mechanism and function research, explaining the molecular mechanisms of the signal, the epigenetic regulation, and the development and adaptation of an individual.

5.1.2 Methodology and Technology Platform of Genomics for TCM

Because of the complexity of the components and functions of TCM, the methodology and techniques to research the pharmacological activities and active mechanisms of TCM have not been established in China. Therefore, we propose that the combination of the whole and the parts is needed in the research of TCM systems. In other words, the active mechanisms of TCM will be qualitatively analyzed as a whole first, and then quantitatively evaluated locally in detail. The development of genomics provided new ideas and methods for the research of TCM. Moreover, genomics corresponds with our theoretical system that emphasizes the combination of the whole and parts in TCM research. The TCM characteristics of multiple ingredients, mutiple targets, and mutiple pathways could be connected to the functional genes by the vast genetic information that could be detected with high-throughput gene expression profile chips. For example, the gene targets would correspond to different compatibilities of TCM formulas by the differences in gene expression profiles, and the administration dosage would be related to the TCM compatibility theory of *Jun*, *Chen*, *Zuo*, and *Shi* by the expression level and the organ specificity of these genes. Furthermore, the close relationship of each component of TCM would be clarified according to the analysis of the interaction of gene targets corresponding with the different compatibilities of TCM formulas, which is also very helpful for clarifying the pharmacological material basis and compatible theory of TCM. After the active mechanism of TCM is qualitatively analyzed on the global situation, the significant differentially expressed genes screened above will be validated by real-time fluorescence quantitative polymerase chain reaction (PCR) to enable the therapeutic and mechanistic evaluation of TCM quantitatively.

5.2 PROSPECT OF GENOMICS IN TCM RESEARCH

In The 21st Century Innovation Medicine Forum, Dr. Ferid Murad, a Nobel prize winner and "the father of Viagra," stated that "in the process of TCM modernization, the key is not getting bogged down in a composition of the molecular formula, but finding out the effective components." The important thing that we have to do now is scientifically prove the efficacy of TCM. Some concepts of TCM are similar to those of modern medicine. For example, personalized medicine in Western medicine is consistent with "treatments based on syndrome differentiation" in TCM; and the theoretical basis of both is based on patients but not specific diseases. If the concept of TCM diagnosis and treatment was introduced to genomics research, the essence of the genetic relationship could be dialectically analyzed from the complex network of genes, then functional genomics could achieve the level of "cause for (gene expression changes and the changes of genes) type (epigenetics)." Could the pharmacology, components and curative effect of TCM be quantitatively detected by the technology of genomics? And could the genetic changes also be quantitatively detected by this technology? The research of TCM will break through the limitation of "quantity" when this problem is solved. Thus it can be seen that the integration of TCM and genomics is reasonable and necessary.

There are a lot of similarities between genomics and the holistic theory and dialectic approach of TCM. On the basis of single gene research, the complexity of genetic interaction is fully understood by the research of genomics. In other words, the development of a disease may be due to changes besides one specific gene, but at the same time, the altered expression of one gene could cause multiple diseases. The developmental trend of systematic research was decided by the characteristics of TCM multiple ingredients, multiple targets, and multiple pathways. So we can see the similar characteristic approaches of genomics and TCM, and that infiltration of research ideas and methods between the two is possible.

Gene chip technology is an important high-throughput genomics research technology. Its advantages of high-throughput, parallel- and high-content enable it to be introduced to TCM research, and provide a technological way to analyze the abundant information in TCM research. So this technology is a bridge between genomics and TCM research. Considering the characteristics of gene chip and the complexity of TCM, using gene chips to study the genetic effects of TCM might resolve the "black box" of the mechanisms of TCM.

5.3 CASES OF THE APPLICATION OF GENOMICS IN TCM RESEARCH

In this section, two cases of the application of genomics in TCM system research are introduced. One is the research on the mechanism of *Shexiang-baoxin* pill treatment mechanism, a well known TCM formula for myocardial

infarction. In this study, the activity mechnism of *Shexiangbaoxin* pills for myocardial infarction was explored on the gene expression level by gene chip and real-time PCR. Another case is a comparative study of three TCM formulas for myocardial infarction. In this study, we used gene chip technology to compare the mechnisms of three TCM formulas, *Shuanglong* formula, *Danhong* injection, and *Shexiangbaoxin* pills, on the gene expression level. If common differentially expressed genes result from the treatments of three TCM formulas, the genes will be the treatment targets of this disease. This is a new perspective in the research of TCM treatment mechanism.

5.3.1 Research of *Shexiangbaoxin* Pills Treatment Mechanism

5.3.1.1 Introduction of* Shexiangbaoxin *Pills *Shexiangbaoxin* pills are a TCM formula composed of *Chansu* (Bufonis Venenum), *Suhexiang* (Styrax), *Shenxiang* (Moschus), *Niuhuang* (Bovis Calculus), *Renshen* (Ginseng Radix et Rhizoma), *Rougui* (Cortex Cinnamomi), and *Bingpian* (Borneolum Syntheticum). It has a remarkable curative effect in the treatment of cardiovascular diseases.

In the study we used mRNA expression profile array to analyze the molecular mechanism of *Shexiangbaoxin* pills for myocardial infarction treatment. The genetic expression changed after the *Shexiangbaoxin* pills were used as treatment in rats with coronary artery ligation. Three interested genes were obtained from the results of the array: Fn1, Fndc1, and Tpm1. Real-time PCR was used to verify the results for these three genes. Finally, further molecular biology experiments were conducted on these genes to examine their roles in the process of myocardial infarction development and *Shexiangbaoxin* pill treatment. *Chansu*, one of the components of *Shexiangbaoxin* pills, is very good in strengthening the heart, but it is also toxic to the heart. However, the toxicity is significantly reduced when it is composed in the formula of *Shexiangbaoxin* pills. Thus mRNA expression profile array and quantitative PCR were conducted to explain the mechanisms of compatibility and toxicity reduction of *Shexiangbaoxin* pills.

5.3.1.2 Experimental Front left artery ligation SD rats were divided into six groups in this experiment as follows: *Shexiangbaoxin* pills high dose group (60 mg/kg), *Shexiangbaoxin* pills middle dose group (30 mg/kg), *Shexiangbaoxin* pills low dose group (15 mg/kg), Western medicine treatment group (isosorbide mononitrate), a placebo group, and a sham surgery group (open chest not ligation). The ischemic tissues in the myocardial infarction edge were used for gene chip screening. Each group contained no fewer than eight samples. *Shexiangbaoxin* pills high dose group, the placebo model group, and the sham surgery group were selected for gene chip screening. There were 27 whole genome oligonucleotide microarrays (Bio Company) used in this experiment.

In order to study the toxicity of *Chansu* to the mammal's heart and the compatibility mechanism of *Shexiangbaoxin* pills, four administration dosages of *Chansu* were used in the experiment. The minimum dose of *Chansu* (LD group) is the maximum administration dose in clinical practices, and the *Shexiangbaoxin* pills group is the aforementioned *Shexiangbaoxin* pills high dose group. The compatibility and toxin attenuation mechanisms of *Shexiangbaoxin* pills could be explained through comparing the different gene expressions between the LD group and the *Shexiangbaoxin* pills group.

5.3.1.3 Results

5.3.1.3.1 Result of Pathological Biopsy The photomicrographs of the *Shexiangbaoxin* pills group, the placebo group, the sham surgery group and the positive control group are shown in Figure 5.1.

Myocardial tissue biopsies were dyed by hemotoxylin and eosin (HE) staining. The following phenomena could be observed with the microscope: severe myocardial cell edema in the myocardial infarction area caused by coronary artery ligation in the model group, stove shapes, flake necrosis, fibrosis,

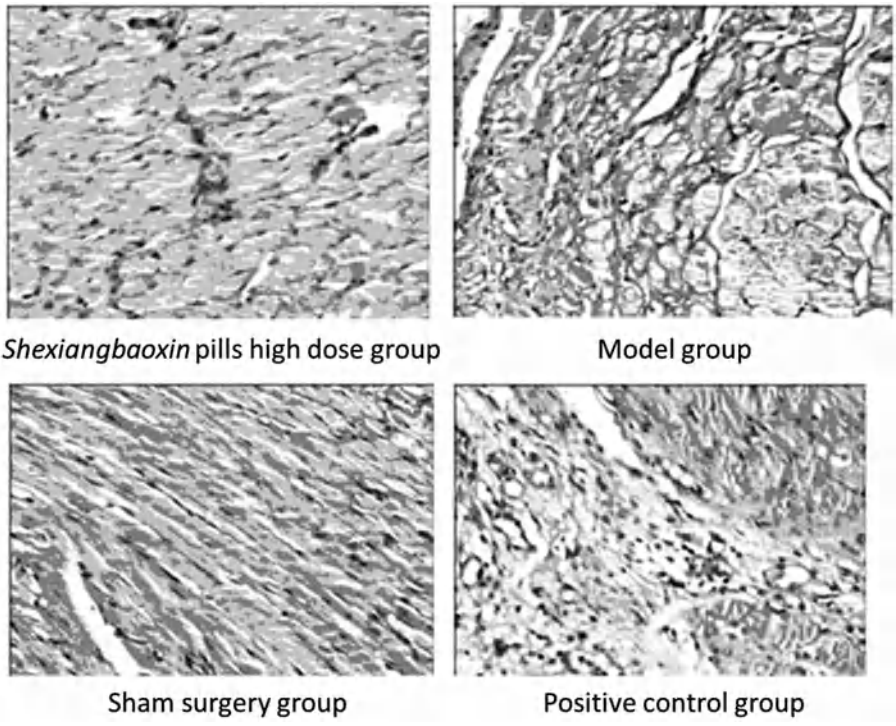

Fig. 5.1 Photomicrographs of myocardial tissues. The myocardial tissue biopsies were dyed by HE red. (See color insert.)

calcification, inflammatory cells infiltrating edema, dilated vessels, and interstitial bleeding. The results proved the model successful. The myocardial cells' horizontal grains arranged in order, and the muscle and nuclei of the myocardial cells were normal in the sham surgery group, but myocardial cell mild edema, a little stove necrosis, mesenchyme edema, and inflammatory cell infiltration were still visible. The myocardial arrangement was recovered in the high dose *Shexiangbaoxin* pills group, but a few myocardial cells still had mild edema, a little stove necrosis, mesenchyme edema, and inflammatory cell infiltration. Compared with the model group, the positive control group were restored and improved to a high level, coagulation necrosis, fibrosis, and scar tissue were not visible, but a few myocardial cells still had mild edema; there was a little stove necrosis and mesenchyme edema, and an average amount of inflammatory cells infiltration, vascular dilatation and congestion, and interstitial bleeding. The photomicrographs showed that *Shexiangbaoxin* pills could significantly reduce myocardial cell degeneration, the area of necrosis, and inflammation reaction. This result indicated that *Shexiangbaoxin* pills obviously relieved myocardial infarction.

5.3.1.3.2 Differentially Expressed Genes Clustering and Function Analysis First, the differentially expressed genes were clustered; the results are shown in Fig. 5.2. In general, the genes that were upregulated in the myocardial ischemia model were downregulated after the *Shexiangbaoxin* pill treatment, and vice versa. The results showed that *Shexiangbaoxin* pills could improve the differentially expressed genes in the myocardial ischemia model.

Function enrichment analysis and pathway analysis were carried out for the differentially expressed genes. The gene ontology (GO) nodes of molecular functions of the differentially expressed genes in the model group and the *Shexiangbaoxin* pill group mainly belonged to seven classes: NAD combination, acyl transfer enzyme activities, protein binding, insulin growth factor combination, protein transfer enzyme activities, the whole united protein binding, and pyruvate dehydrogenase activity. Protein binding is an especially important function in these classes. The metabolic pathways of the differentially expressed genes in the model group and the *Shexiangbaoxin* pills group were basically as follows: the Krebs cycle, valine, leucine, and isoleucine degradation, ECM receptor reaction, sugar solution and difference, Alzheimer's disease, acetone acid metabolism, and fatty acid metabolism. All of the six metabolic pathways were related with myocardial energy metabolism.

Further analysis was conducted on the changes of differentially expressed genes and the differentially expressed genes whose expression levels were restored or close to normal after *Shexiangbaoxin* pill treatment in the model group. These genes might play an important role in the process of myocardial infarction and restoration. Nine genes were obtained by this analysis (Table 5.1).

The key factors in determining myocardial functionality are the generation, transfer, storage, and application of myocardial cells. Myocardium is at an

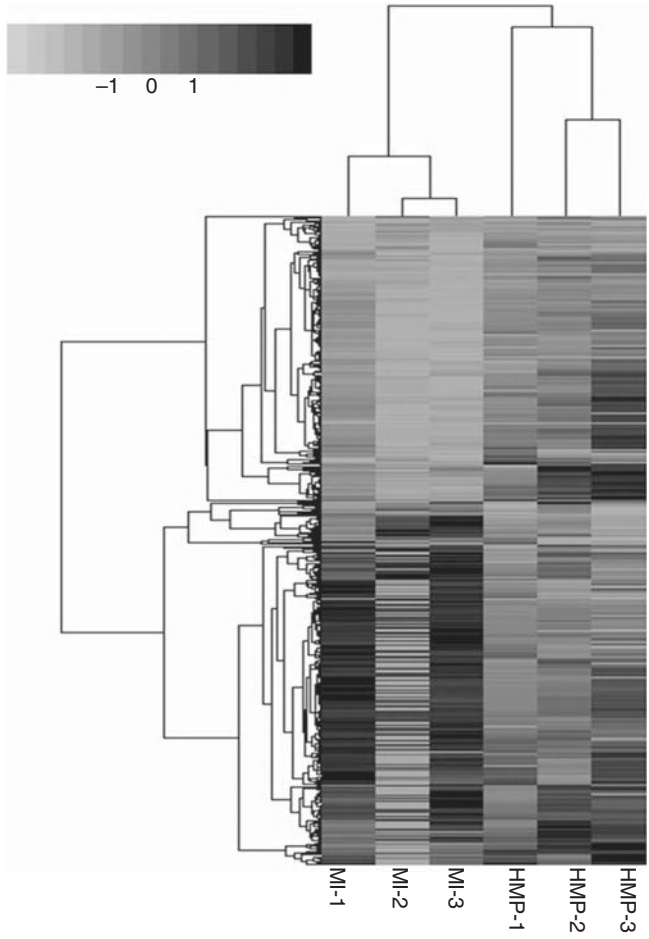

Fig. 5.2 Differentially expressed gene clustering of myocardial ischemia rat model after *Shexiangbaoxin* pill treatment. MI-l–3: the differentially expressed genes of myocardial ischemia rat model; HMP-l–3: the different expression genes of myocardial ischemia rat model after *Shexiangbaoxin* pill treatment. The value of grayscale shows whether the gene was regulated up or down.

"energy hungry" state when heart failure happens. Myocardial energy metabolism is the material basis of protecting normal heart function and renewing structures ceaselessly. So the myocardial metabolic disorder is an important factor for heart failure. Combining pharmacology experiments and the metabolism measurement results, the differentially expressed genes were further screened; two genes (Tpm1, Fndc1) were selected for PCR validation, and their functions in the process of acute myocardial ischemia and treatment were analyzed.

TABLE 5.1 Reverse Adjust Genes Treated with *Shexiangbaoxin* Pills

Gene	Description
Uqcrc2	The alcohol cytochrome C reductase protein
Postn_predicted	Periostitis, specific cells into film factor
Hadhb	Acetyl coa transfer dehydrogenase
Acaa	Acetyl co A acyl transfer enzyme 2
C7	Complement composition C7
Fn1	Fiber connection protein family members
Fndc1	Fiber connection protein family members
Arf1	ADP the DNA base
Tpm1	Original myosin

5.3.1.3.3 Real-Time PCR Measurement and Analysis of Tpm1 and Fndc1
The experimental groups are shown in Fig. 5.3. Total RNA were extracted from the sample tissues, and then these total RNA were reverse transcribed to cDNA, and at the end, real-time PCR were conducted on the cDNA templates. Tpm1 and Fndc1 primer information are shown in Table 5.2.

GAPDH was selected as the housekeeping gene. The external standard method was used to quantify the amount of the samples. The expressions of targeted genes were defined as the ratio from the targeted gene to the internal standard GAPDH. In each group, t-test was used to analyze the data. Significance was defined as $p < 0.05$.

As shown in Fig 5.3, the expression of Tpm1 was low in the sham surgery group, and its expression was upregulated in the model group. After *Shexiangbaoxin* pill treatment, its expression was close to the sham surgery group. Also, the expressions of Tpm1 in the three *Shexiangbaoxin* pill groups with different dosages were consistent, and were the same to the isosorbide mononitrate group.

The expression of fiber connecting protein Fndc1 in the normal state (the sham surgery group) was lower than its expression in the myocardial ischemia state (the placebo model group). There was a significant difference ($p = 0.025$) between them. After the high dose *Shexiangbaoxin* pill treatment, the expression of Fndc1 lowered, and there was a significant difference ($p = 0.062$) compared with the sham surgery group. This result could be explained as that *Shexiangbaoxin* pills play a regulatory role in the expressions of fiber connecting proteins.

The statistical results showed (Table 5.3) that the model group was significantly different compared with the sham surgery group; the *Shexiangbaoxin* pill high, middle, and low dose groups; and the positive medicine group, but there was no significant difference between the model group and the other groups. The expression of Tpm1 was high in the myocardial ischemia state, and it was restored after the *Shexiangbaoxin* pill treatment. However, the gene

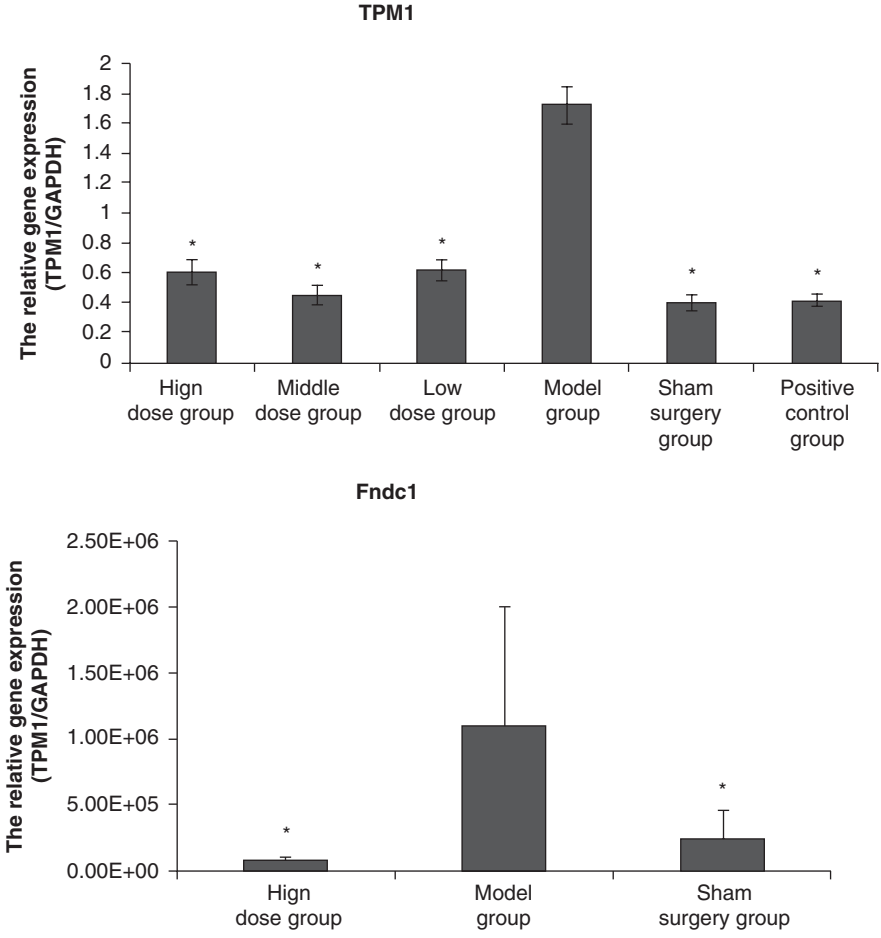

Fig. 5.3 Expressions of TPM1, Fndc1 quantified by real-time PCR.

TABLE 5.2 Primers Design of Tpm1, Fndc1, and GAPDH

Gene	Sequence	Length
Tpm1-F	5′ ATT GCT GAA GAT GCT GAC CG 3′	153bp
Tpm1-R	5′ CAA GTT GTT CGT CAC CGT TT 3′	
Fndc1-F	5′ TCA GAC CTC GTC AGT GAC C 3′	
Fndc1-R	5′ CAA CCA GCA GAG GAT TGT C 3′	152bp
GAPDH-F	5′ GCA TCC TCG GCT ACA GTG AG 3′	
GAPDH-R	5′ TCC ACC ACC ATG TTG CTG TA 3′	163bp

TABLE 5.3 Significance Analysis of Tpm1 Affected by *Shexiangbaoxin* Pills

	Low Dose	Positive Control	Medium Dose	Model	Sham Surgery
Positive control	0.205				
Medium dose	0.213	0.876			
Model	0.012	0.006	0.006		
Sham surgery	0.155	0.890	0.770	0.003	
High dose	0.496	0.389	0.408	0.020	0.336

expression could not distinguish the degree of recovery for myocardial ischemia after the *Shexiangbaoxin* pill treatment.

5.3.1.4 Discussion Tpm1 is an important member of the original myosin family (α-original myosin). It is an important regulatory protein for muscle contraction, and can regulate the interactions among actomyosins. In muscle cells, this gene can affect calcium regulation during muscle contraction; in nonmuscle cells, it can stabilize the cytoskeleton of actin filament.[2]

Tropomyosin also plays a very important role in the process of myocardial development. Its genetic mutation is related to cardiomyopathy. Some studies have shown that two mutations of Tpm1, Asp175Asn and Glu180Gly, are closely related to the autonomic adjustment control of cardiac pressure, and helpful for preventing stroke.[3] Other studies have found that this gene is closely related to loss of actin or cell transformation. The inhibitory function of TGF-β to tumors is changed when this gene becomes silenced.[4] In this study, the expression of Tpm1 was restored after the *Shexiangbaoxin* pill treatment. This result shows that *Shexiangbaoxin* pills have an active function in muscle contraction, and play an important role in protecting myocardium through regulating cardiac pressure. Therefore, this also explains the mechanism of *Shexiangbaoxin* pills as a "heart saving formula" in a different point of view.

Comparing the physiological processes of *Shexiangbaoxin* pill treatment and low doses of *Chansu* treatment indicates that the processes related to toxicity, such as damaged ionic stable state[5] or injured actins,[6] did not appear, and the functions of the extracellular matrix and fiber collagen were reserved. The extracellular matrix (ECM) is very important for cells' integrity, because it forms a stress-tolerating network through the linkage of myocardial cells and the capillaries of the ventricular wall.[7] Abnormality in the constriction and relaxation function of the heart would be induced by the destruction of ECM structure.[8] The collagenous ECM is a structure that supports the heart in its normal form. The constriction and relaxation functions of the heart would be affected by the structure of the collagen and the ratios among different types of collagen.[9] Thus, ECM is very important for the normal function of cardiac cells and the heart's structural repair.[10] In

addition, the modulation function of *Chansu* to blood pressure was also increased after compatibility. This is the same as the functions of *Shexiangbaoxin* pills, as *Shexiangbaoxin* pills improve the extension of coronary artery and blood flow,[11, 12] reduce myocardial infarction, and promote angiogenesis. This result reflected the compatibility and toxicity-reducing functions of TCM.

The result of KEGG pathway enrichment showed that arachidonic acid metabolism and linoleic acid metabolism were related in the process of *Chansu* treatment. Given that fatty acids are the main energy source for the heart,[13] we speculated that low doses of *Chansu* increased the fatty acid metabolism by accelerating energy consumption through interfering the irritable response, such as cardiac constriction, anti-apoptosis regulation, and regulation of cell proliferation. But the fatty acid metabolism was not involved in *Shexiangbaoxin* pills that contained the *Chansu* treatment. In addition, the pathway of ECM-receptor interaction involved in the *Shexiangbaoxin* pill treatment was the same with the physiological process of ECM building affected by *Shexiangbaoxin* pills. The results showed that the toxicity of *Chansu* was decreased when used along with other components, different from when it is used by itself.

5.3.2 Comparison of the Mechanism of Three TCM Formulas for Myocardial Infarction

Coronary artery disease is a common and frequently occurring disease. It is a heart disease caused by myocardial ischemia when the vessels are clogged by coronary atherosclerosis. Research has shown that the *Shuanglong* formula has a significant therapeutic effect for myocardial ischemia. *Shexiangbaoxin* pills and the *Danhong* injection are TCM formulas commonly used in clinical practice for treating heart diseases. In this study, the myocardial ischemic rat model and gene chip technology were used to research these three TCM formulas. The treatment mechanisms of these three TCM formulas would also be revealed at the genetic level.

The *Shuanglong* formula is a experimental prescription from Lianda Li, an academician in China College of Engineering. It is composed of *Renshen* and *Danshen* (Salviae Miltiorrhizae Radix et Rhizoma), two commonly used TCM materials, and has a curative effect toward *Qi* deficiency and blood stasis syndrome. *Shexiangbaoxin* pills are a TCM formula that has a remarkable curative effect in the treatment of cardiovascular diseases. The *Danhong* injection is a TCM treatment composed of the extracts of *Honghua* (Carthami Flos) and *Danshen*. Clinically it is used to treat coronary heart diseases, angina pectoris, myocardial infarction, ischemic encephalopathy, and cerebral thrombus.

These three TCM formulas all have curative effects for myocardial infarction. Their compositions also have overlapping components, such as *Renshen*, which is contained in both *Shuanglong* formula and *Shexiangbaoxin* pills, and

Danshen, which is contained in both *Shuanglong* formula and *Danhong* injection. So we proposed that these three TCM formulas should have similar molecular mechanisms in the treatment of myocardial infarction. These molecular mechanisms might be the treatment targets of TCM on myocardial infarction. In this study, the molecular mechanisms of these three TCM formulas would be explained by gene chip technology through a gene expression level in addition to the research on common regulatory targets of these TCM formulas.

5.3.2.1 Experimental In rats, the dosage of the *Shuanglong* formula was 7 g/kg; the dosage of the *Shexiangbaoxin* pill was 60 mg/kg; the *Danhong* injection group was directly injected in the tail veins; the model group was given 10 ml/kg of 0.5% CMC-Na solution; the sham surgery group had open thoracic rats; and the control group had normal rats. All groups were given 10 ml/kg of 0.5% CMC-Na solution. The first time to fill the stomach with medicine for each group was after the rats awakens from anesthesia. Medication was given once per day for 7 days. The specific experimental procedure and quality control processes were the same as in the description in Section 5.3.1.

5.3.2.2 Data Analysis of Myocardial Infarction Model Double channel chips (Bio Co.) were used in this experiment. The sample fluorescence ratio (ratio = cy5/cy3) was used as the output in one chip. The specific information of the chips as follows: the model group = the sham surgery group/the model group, the *Shuanglong* formula group = the *Shuanglong* formula group/the model group, the *Shexiangbaoxin* pills group = the *Shexiangbaoxin* pills group/the model group, the *Danhong* injection group = the *Danhong* injection group/the model group.

5.3.2.2.1 Screening of Differentially Expressed Genes and Analysis of Gene Function There were 795 differentially expressed genes in the model group after myocardial ischemia, and most differentially expressed genes were downregulated (11% were upregulated, 89% were downregulated). In other words, expressions of the genes were increased in the model group. The heart can be injured after acute myocardial ischemia and reperfusion. Myocardial infarction is caused by this state; and then the related stress responses in the body are enabled; at the end, the genetic expressions would have significant changes.

The GO term enrichment analysis of the differentially expressed genes was carried out. In the model group, the molecular functions term was 14.6%, the cell components term was 25.3%, and the physiological processes term was 60.1%. The result of GO node enrichment analysis showed that the GO nodes of molecular functions involved in myocardial infarction were mainly from 23 classes (Table 5.4). The significance level of the GO node was $p < 1.0\text{E-}5$.

TABLE 5.4 Molecular Function Enrichment of Differentially Expressed Genes in the Model Group

GO Term	Genes	*p*-Value	*q*-Value
Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) activity	72	1.4E-111	1.1E-108
NAD binding	74	4.2E-107	1.6E-104
Protein binding	154	4.3E-59	8.08E-57
GTP binding	34	7.35E-26	6.91E-24
GTPase activity	18	4.01E-19	3.02E-17
Nucleotide binding	69	8.64E-19	5.91E-17
ATP binding	48	5.41E-14	2.71E-12
Calcium ion binding	29	5.67E-12	2.03E-10
Oxidoreductase activity	32	2.09E-11	7.16E-10
Transcription factor activity	25	3.11E-09	8.36E-08
Triose-phosphate isomerase activity	4	3.11E-08	7.37E-07
Actin binding	13	7.6E-08	1.68E-06
Ubiquitin-protein ligase activity	7	3.27E-07	5.85E-06
Heparin binding	6	4.39E-07	7.68E-06
Nitric-oxide synthase regulator activity	3	6.56E-07	1.1E-05
Unfolded protein binding	8	7.55E-07	1.2E-05
Peptidyl-prolyl cis-trans isomerase activity	6	7.65E-07	1.2E-05
Integrin binding	4	2.59E-06	3.75E-05
Acyltransferase activity	8	4.26E-06	5.52E-05
RNA binding	15	4.26E-06	5.52E-05
Iron ion binding	9	5.98E-06	7.5E-05
Potassium ion binding	7	6.98E-06	8.2E-05
Protein C-terminus binding	5	7.67E-06	8.87E-05

The disorder of metabolic function is the main factor causing myocardial ischemic. The GO function enrichment analysis of differentially expressed genes showed that the genes involved in physiological processes were mainly involved in the sugar metabolism. Further analysis of metabolic pathways that the differentially expressed genes were involved in was carried out with the KEGG database. There were 15 signal transduction pathways caused by myocardial ischemia in the model group (Table 5.5). The significance level of the GO node was $p < 1.0$ E-4.

5.3.2.2.2 Discussion The current opinion is that the barriers for mitochondrial energy synthesis, the disorder of Ca^{2+} steady-state, and the generation of a large number of free radicals are induced by myocardial ischemia and reperfusion, which eventually leads to cell apoptosis or death, in addition to irreversible damages such as myocardial infarction.

Under anoxia conditions, the state of oxidation and reduction in cells will be disturbed and there will be a generation of a large number of free radicals.

TABLE 5.5 Metabolism Pathways of Differentially Expressed Genes in the Model Group

Pathway	Genes	*p*-Value	*q*-Value
Glycolysis / Gluconeogenesis	67	5.3242E-108	7.7E-106
Alzheimer's disease	70	4.8733E-77	3.51E-75
Citrate cycle (TCA cycle)	10	7.99325E-13	1.28E-11
Gap junction	11	7.58135E-09	7.28E-08
Focal adhesion	12	1.8882E-06	9.38E-06
MAPK signaling pathway	14	2.01688E-06	9.68E-06
Valine, leucine and isoleucine degradation	6	1.16981E-05	4.11E-05
Ubiquitin mediated proteolysis	9	1.39941E-05	4.69E-05
Axon guidance	9	1.49211E-05	4.88E-05
Tight junction	9	1.58999E-05	5.09E-05
Antigen processing and presentation	8	2.10673E-05	6.45E-05
Wnt signaling pathway	9	3.45163E-05	9.94E-05
Beta-alanine metabolism	4	8.47259E-05	0.000207
Adipocytokine signaling pathway	6	9.79655E-05	0.000235
Fatty acid metabolism	5	9.94639E-05	0.000235

This is one of the reasons for cell apoptosis or death. It can be seen from the results of GO term enrichment of differentially expressed genes that oxidation, reduction enzyme activity, and catalytic activity were notably affected after myocardial ischemia.

The disorder of Ca^{2+} steady state is another major cause for myocardial injury. Studies reported that when myocardium is under an anoxic condition, an abundance of Ca^{2+} is deposited into the mitochondria of myocardial cells, the process of oxidative phosphorylation is interfered with, and energy metabolism is impaired [14]. The result of this study showed that ion combinations such as these involving calcium ions were abnormal in a week after myocardial ischemia.

Besides sugar metabolism, metabolic processes related to Alzheimer's disease are the main metabolic pathways involved. Alzheimer's disease is a serious neurological disease. The intramyocardial energy metabolism, focal adhesion, and migration of white blood cells through the endothelium were abnormal after myocardial ischemia and reperfusion. These metabolic pathways are also perturbed in Alzheimer's disease. Physiological processes such as focal adhesion, gap junctions, and tight junctions play an important role in maintaining normal physiological function.

5.3.2.3 Data Analysis of Shuanglong *Formula*

5.3.2.3.1 Screening of Differentially Expressed Genes and Analysis of Gene Function There were 629 differentially expressed genes in the *Shuanglong* formula group (18% were upregulated, 82% were downregulated). In

TABLE 5.6 Molecular Function Enrichment Result of Differentially Expressed Genes in the *Shuanglong* Formula Group

GO Term	Genes	*p*-Value	*q*-Value
Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) activity	58	5.73E-89	3.68E-86
NAD binding	59	2.01E-84	6.45E-82
Protein binding	90	3.7E-24	3.96E-22
Calcium ion binding	36	2.35E-20	2.16E-18
Pheromone binding	10	5.05E-17	3.24E-15
GTPase activity	12	3.7E-12	1.32E-10
GTP binding	18	3E-11	1.02E-09
RNA binding	16	5.33E-08	1.22E-06
Acyltransferase activity	9	6.76E-08	1.5E-06
ATP binding	32	7.66E-08	1.64E-06
Carnitine O-palmitoyltransferase activity	3	8.69E-08	1.76E-06
Copper ion binding	7	4.47E-07	8.21E-06
Zinc ion binding	34	6.55E-07	1.14E-05
Integrin binding	4	1.12E-06	1.89E-05
Unfolded protein binding	7	2.26E-06	3.63E-05
Transcription factor activity	18	2.48E-06	3.79E-05
Heparin binding	5	3.69E-06	5.28E-05
Protein heterodimerization activity	6	6.58E-06	9.01E-05

the model group, 89% of differentially expressed genes were upregulated. It could be seen that the genetic expression of the *Shuanglong* formula group was the opposite of that of the model group. It could be explained by the fact that the *Shuanglong* formula might have a reverse recovery function for myocardial infarction.

The GO term enrichment analysis of the differentially expressed genes was carried out. In the *Shuanglong* formula group, the molecular functions term was 13.2%, the cell components term was 27.6%, and the physiological processes term was 59.2%. The results of GO node enrichment analysis showed that the GO nodes of molecular functions involved in myocardial infarction were mainly from 18 classes (Table 5.6). The significance level of the GO node was $p < 1.0E\text{-}5$. Most of the molecular functions from the enrichment results were the same as the results of the model group.

Further analysis of metabolic pathways with the differentially expressed genes was carried out in the KEGG database. The analysis process was the same as that for the model group. There were 17 signal transduction pathways involved in the *Shuanglong* formula group (Table 5.7). The significant level of the GO node was $p < 1.0$ E-5.

5.3.2.3.2 Discussion The ratio of the model group data represented the sham surgery group/the model group. In this group, 89% of the differentially

TABLE 5.7 Metabolism Pathways of Differentially Expressed Genes in the *Shuanglong* Formula Group

Pathway	Genes	*p*-Value	*q*-Value
Glycolysis/gluconeogenesis	46	1.67E-66	1.1E-64
Alzheimer's disease	50	1.57E-50	5.2E-49
Base excision repair	14	8.6E-17	1.42E-15
Graft-versus-host disease	12	4.27E-13	4.7E-12
Allograft rejection	12	6.53E-13	6.16E-12
Antigen processing and presentation	14	1.21E-12	9.95E-12
Type I diabetes mellitus	12	2.57E-12	1.88E-11
Autoimmune thyroid disease	12	3.08E-12	2.03E-11
Complement and coagulation cascades	11	5.72E-11	3.43E-10
Cell adhesion molecules (CAMs)	14	2.75E-10	1.39E-09
Natural killer cell mediated cytotoxicity	13	1.52E-09	5.91E-09
PPAR signaling pathway	10	1.77E-09	6.49E-09
Fatty acid metabolism	8	5.22E-09	1.72E-08
Adipocytokine signaling pathway	9	1.36E-08	3.45E-08
MAPK signaling pathway	13	1.85E-06	3.75E-06
VEGF signaling pathway	7	5.93E-06	9.55E-06
Amyotrophic lateral sclerosis (ALS)	7	6.51E-06	1.02E-05

expressed genes were downregulated. The expressions of genes related to metabolism and ionic combination were raised; and the expressions of these genes in the model group were higher than those in the sham surgery group. One week after treatment with the *Shuanglong* formula, 82% of the differentially expressed genes were downregulated. It indicated that a part of the highly expressed genes was restored to the sham surgery group after treatment with the *Shuanglong* formula.

Through chip data analysis, it was shown that the *Shuanglong* formula mainly exerts its effects by affecting physiological processes such as energy metabolism, ionic combination, immune response, and redox reactions after treatment for one week. The main efficacy of the *Shuanglong* formula for myocardial ischemia was illustrated by these data at the genetic level. In the ion binding-related processes, besides calcium binding, the rarely reported zinc binding was also involved. Zinc binding is very important physiologically. Zinc is closely related to enzymes, and the activities of more than 80 enzymes are dictated by zinc. Zinc is also crucial to the repair of trauma and immune response. In addition, it is the necessary material to maintain normal immune and defense responses. Zinc plays an important role in the maintenance of the structure and function of cell membranes. Zinc can combine with mercaptan to block the strong catalytic reaction of free radicals with iron caused by mercaptan combining with iron. It can also inhibit lipid peroxidation and stablize

the structure and function of cell membrane to enhance a cell's resistance to ions and free radicals.

In summary, the *Shuanglong* formula has remedial effects for myocardial injury caused by acute myocardial ischemia. Its main effects are on energy metabolism, regulation of oxidation and reduction, immune response, and ion bindings (calcium ions and zinc ions).

5.3.2.4 Data Analysis of Shexiangbaoxin *Pills*

5.3.2.4.1 Screening of Differentially Expressed Genes and Analysis of Gene Function There were 190 differentially expressed genes in the *Shexiangbaoxin* pill group (40% were upregulated, 60% were downregulated). The number of differentially expressed genes in the *Shexiangbaoxin* pills group was less than that in the *Shuanglong* formula group.

The GO term enrichment analysis of the differentially expressed genes was carried out. In the *Shexiangbaoxin* pill group, the molecular functions term was 12.6%, the cell components term was 28.7%, and the physiological processes term was 58.7%. The result of GO node enrichment analysis showed that the GO nodes of molecular functions involved in myocardial infarction were mainly from eight classes (Table 5.8). The main molecular function was a structural constituent of ribosome (GO: 0003735), which is different from the results for the model group and the *Shuanglong* formula group. Because the differentially expressed genes selected in the *Shexiangbaoxin* pills group were much fewer than in the model group and the *Shuanglong* formula group, the significance level of the GO node was $p < 1.0$ E-3.

Further analysis of metabolic pathways with the differentially expressed genes was carried out in the KEGG database. The analysis process was the same as that of the model group. The main metabolic pathway in the *Shexiangbaoxin* pill group was base resection, but sugar solution/difference and the Alzheimer's disease-related pathways that had high enrichment in the *Shuanglong* formula group did not appear in the *Shexiangbaoxin* pill group. The

TABLE 5.8 Molecular Function Enrichment Results of Differentially Expressed Genes in the *Shexiangbaoxin* Pills Group

GO Term	Genes	*p*-Value	*q*-Value
Structural constituent of ribosome	48	1.12E-58	1.25E-56
GTPase activity	5	2.6E-06	3.64E-05
GTP binding	7	1.11E-05	0.000121
Glycine-tRNA ligase activity	2	1.24E-05	0.000121
Protein binding	22	5.57E-05	0.000341
ATP binding	12	0.000239	0.001254
Nucleotide binding	15	0.00028	0.001254
Protein heterodimerization activity	3	0.000445	0.001781

TABLE 5.9 Metabolism Pathways of Differentially Expressed Genes in the *Shexiangbaoxin* Pills Group

Pathway	Genes	*p*-Value	*q*-Value
Base excision repair	13	1.23E-21	1.26E-19
Ribosome	6	1.76E-07	5.97E-06
Gap junction	5	1.1E-05	0.00014
T cell receptor signaling pathway	4	0.000457	0.003328

significance level of the GO node was $p < 1.0E\text{-}3$. The result of the metabolic pathways of differentially expressed genes in the *Shexiangbaoxin* pills group is shown in Table 5.9.

5.3.2.4.2 Discussion From the present pharmacological studies of the *Shexiangbaoxin* pill, it is known that its main functions are related to the concentration of calcium ions in the cells, collagen, nitric oxide synthase, superoxide dismutase SOD, angiotensin, myocardial nutrients, fatty acid metabolism, neutrophils infiltrating, and so on.[15–18]

It can be seen from the enrichment result of the molecular functions and the physiological processes by GO term analysis that the differentially expressed genes were clustered around protein synthesis and the main metabolic pathway was DNA base excision and repair. Myocardial ischemia occurred after myocardial infarction for one week. It was shown through chip analysis that the energy metabolism and ion concentrations in the myocardial cells were disturbed in the model group. These aspects were also mainly regulated after the *Shuanglong* formula treatment. However, after *Shexiangbaoxin* pill treatment, the biological process of protein synthesis was mainly regulated, although energy metabolism and calcium binding were also affected. The molecular mechanisms were different between *Shuanglong* formula and *Shexiangbaoxin* pills, although both of them had a significant curative effect on myocardial infarction caused by myocardial ischemia. The genes related to energy metabolism were regulated after the *Shuanglong* formula treatment, and the genes related to protein synthesis were regulated after the *Shexiangbaoxin* pill treatment.

5.3.2.5 Data Analysis of Danhong *Injection*

5.3.2.5.1 Screening of Differentially Expressed Genes and Analysis of Gene Function There were 171 differentially expressed genes in the *Danhong* injection group (29% were upregulated, 71% were downregulated). In other words, 71% of the differentially expressed genes were downregulated after the *Danhong* injection treatment. The number of differentially expressed genes in the *Danhong* injection group was similar to that in the *Shexiangbaoxin* pill group.

GO term enrichment analysis of the differentially expressed genes was carried out. In the *Shexiangbaoxin* pill group, the molecular functions term was 19.9%, the cell components term was 31.3%, and the physiological processes term was 48.8%. The result of GO node enrichment analysis showed that the GO nodes of molecular functions involved in the myocardial infarction were mainly from 12 classes (Table 5.10). The significance level of the GO node was $p < 1.0E\text{-}3$.

Further analysis of metabolic pathways with differentially expressed genes was carried out in the KEGG database. The main metabolic pathways in the *Shexiangbaoxin* pill group were for circadian rhythm, sulfur metabolism, and notch signaling pathway. The *p*-value was very small and the difference between the *Danhong* injection group and the model group was significant, but the enrichment degree of the differentially expressed genes was low. The significance level of the GO node was $p < 1.0E\text{-}3$. The result of the metabolic pathways of differentially expressed genes in the *Danhong* injection group is shown in Table 5.11.

TABLE 5.10 Molecular Function Enrichment Results of Differentially Expressed Genes in the *Danhong* Injection Group

GO Term	Count	*p*-Value	*q*-Value
Lipoprotein transporter activity	2	0	0
Protein binding	26	1.96E-12	1.62E-11
Transcription factor activity	11	1.95E-09	9.19E-09
Calcium ion binding	10	2.75E-08	1.14E-07
Alcohol sulfotransferase activity	2	4.08E-06	1.35E-05
Structural molecule activity	8	2.36E-05	5.18E-05
Phosphatidylinositol 3-kinase activity	2	1.42E-04	2.12E-04
Sequence-specific DNA binding	5	3.59E-04	3.59E-04
Hormone activity	3	4.75E-04	4.61E-04
SH3 domain binding	2	7.49E-04	6.87E-04
Phosphatidylinositol-4-phosphate 3-kinase activity	1	8.29E-04	6.93E-04
Fatty-acyl-ethyl-ester synthase activity	1	8.29E-04	6.93E-04

TABLE 5.11 Metabolism Pathways of Differentially Expressed Genes in the *Danhong* Injection Group

Pathway	Genes	*p*-Value	*q*-Value
Circadian rhythm	4	3.53E-09	2.29E-07
Sulfur metabolism	3	5.36E-07	1.16E-05
Notch signaling pathway	3	7.86E-05	5.11E-04
Complement and coagulation cascades	3	2.09E-04	9.70E-04
Urea cycle and metabolism of amino groups	2	7.48E-04	0.00286

5.3.2.5.2 Discussion The main active ingredients come from *Danshen* and *Honghua*. *Danshen* is also one of the components of the *Shuanglong* formula. It has the functions of activating blood circulation, eliminating stasis, and dredging collaterals. *Honghua* has the functions of promoting blood stasis, stasis, and relieving pain.

The main mechanisms of myocardial injury and infarction caused by myocardial ischemia are the obstruction of mitochondrial energy synthesis, disorder of Ca^{2+} steady-state, generation of a large number of free radicals, and release of some cytokines. By the analysis of differentially expressed genes screened from the gene expression profiles of the *Danhong* injection group, it can be seen that protein binding, release of transcription factors, and calcium ions were regulated in the *Danhong* injection group. Myocardial injury and infarction caused by myocardial ischemia could be treated through these pathways.

5.3.2.6 Comparison of Shuanglong *Formula,* Shexiangbaoxin *Pills, and* Danhong *Injection*

5.3.2.6.1 Screening of Differentially Expressed Genes The data of 12 gene chips of *Shuanglong* formula, *Shexiangbaoxin* pills, and *Danhong* injection treatment were jointly analyzed. A total of 53 differentially expressed genes were analyzed and output by Matlab. These genes are clustered as shown in Fig. 5.4A.

It can be seen from the clustering result in Fig. 5.4 that the repeatability of three samples of the *Danhong* injection group was the best. Genes under the probe Rn30005061 were clustered in one group and their expressions were basically the same. The expressions of genes of the *Shuanglong* formula group were more similar to those of the model group than the other two groups. This result showed that the expressions of genes after *Shuanglong* formula treatment were most similar to the sham surgery group. In other words, the curative effect of *Shuanglong* formula was the best in the three TCM formulas. According to the clustering results, the ratios of the genes whose expressions were different in each group were analyzed, and the functions of the genes whose ratios had similar trends were further analyzed. This study played an important role in the research of heart function recovery and regulation of global metabolic level after TCM treatment.

5.3.2.6.2 Analysis of the Functions of the Genes Whose Expressions Had a Consistent Trend The 12 mRNAs selected by clustering analysis were retrieved in the NCBI database, and 10 candidate genes were selected for further research. The information of these 10 genes is shown in Table 5.12 (the gene whose probe number is Rn30026733 was not found in the NCBI database, and the gene detected by the probes R002464_01 and Rn30013271 was the same).

The expressions of all of the genes were significantly changed in the model, and were close to the normal level after the three TCM formula treatments.

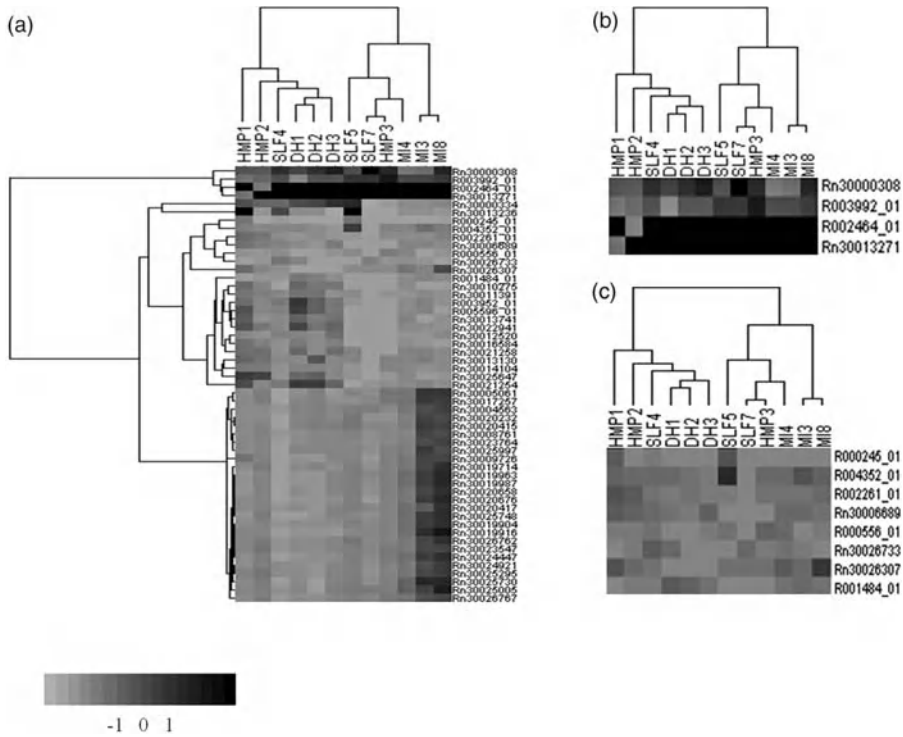

Fig. 5.4 Result of different expression genes clustering. A is the hot pots picture of different expression genes, B and C are the genes which have the same changing trend in clustering analysis in each group (four genes were regulated up and 8 genes were regulated down, compared with the model group). MI-l–3: differentially expressed genes of myocardial ischemia rat model; HMP-l–3: differentially expressed genes of myocardial ischemia rat model after *Shexiangbaoxin* pills treatment; SLF 4, 5, 7: differentially expressed genes of myocardial ischemia rat model after *Shuanglong* formula treatment; DH-l–3: differentially expressed genes of myocardial ischemia rat model after *Danhong* injection treatment. The value of grayscale shows whether the gene was regulated up or down.

The expressions of Cfb (complement factor B), Nfil3 (also called E4BP4, E4 promoter combining protein-4), and Arntl (aromatics receptor nuclear transfer factor 2) in the samples of the sham surgery group were high. They were downregulated in the model group. After the three TCM formula treatments, they were upregulated to close to the expression level in the sham surgery group. The expressions of Nppa (urinary sodium excretion peptide precursor A), Hamp (hepcidin antimicrobial peptides), Dbp (albumin promoter D sites combining protein), Per1 (stage gene congener 1), Nppb (urinary sodium excretion peptide precursor B), and Emp1 (skin membrane protein 1) were downregulated in the model. After the three TCM formula treatments, they

TABLE 5.12 Biological Functions of Candidate Genes

No.	Probe	Gene	mRNA ID	Biological Process Involved
1	Rn30000308	Cfb	NM_212466	The activity of Serine internal peptides enzyme
2	R003992_01	Nfil3	NM_053727	DNA transcription regulation and signal transduction
3	R002464_01	Arntl	NM_024362 NM_012612	DNA transcription regulation and signal transduction
4	R002261_01	Dbp	NM_012543	DNA transcription regulation and signal transduction
5	Rn30006689	Per1	NM_001034125	DNA transcription regulation and signal transduction
6	R000245_01	Nppa	NM_012612	Regulate the process of blood vessel size
7	R000556_01	Nppb	NM_031545	Regulate the process of blood vessel size
8	R004352_01	Hamp	NM_053469	Iron ions balance
9	R001484_01	Emp1	NM_012843	Cell growth

were downregulated to close to the expression level in the sham surgery group. The functions of these nine genes are mainly related to regulation of gene transcription, signal transduction, blood vessels, and development of cell growth. Among these functions, cell circadian rhythm might play an extremely vital role in the process of myocardial infarction and the recovery of cardiac function after treatments. Some studies found that Nfil3, Arntl, Cfb, Nppa, and Nppb were very important in cardiovascular physiological and pathological processes. Per1 has important effects in cancer, endocrine disorders, and mood disorders, and the diseases closely relate to abnormalities in biological clock. When myocardial infarction occurs, the metabolic state is significantly changed, and metabolism is disordered. After the three TCM formula treatments, the global state of the rat model turned better and metabolic disorders was alleviated. These processes were closely related with the biological clock. Therefore, we speculated that the genes may be the therapeutical targets of TCM for myocardial infarction.

It can be seen that the metabolic pathways regulated by the three TCM formulas were different from the metabolic pathways of the differentially expressed genes. Energy metabolism and ion binding were mainly influenced by the *Shuanglong* formula. Base repair was mainly influenced by *Shexiangbaoxin* pills. Rhythm adjustment was mainly influenced by the *Danhong* injection, and the genes enriched were fewer. There were also overlaps in the metabolic pathways, such as base repair, which was the main pathway regulated by both *Shuanglong* formula and *Shexiangbaoxin* pills. It can be explained that the three TCM formulas not only had independent actions, but also had some common regulation mechanisms.

From the global analysis of all the chips of the *Shuanglong* formula, *Shexiangbaoxin* pills, and the *Danhong* injection, it can be seen that the energy metabolism, the ability of protein synthesis and combination, oxidation and reduction, and calcium ion binding were very important in the process of myocardial injury caused by myocardial ischemia and the drug treatments. In this study, the three TCM formulas exerted their effects through these pathways, but the regulatory emphasis of each was different. Although the molecular mechanisms of the three TCM formulas were not completely the same, they had very good effect in treating myocardial ischemia. It suggested that the common pathways induced by the three TCM formulas were likely the targets of the pathogenic mechanism of myocardial ischemia and drug treatment. At the same time, the Chinese medicine theory of "different governance" to disease mechanisms was explained. Therefore, it was very important to find the common mechanism of the three TCM formulas by comparing the three formula treatment groups with the model group.

We used genomics, combined with gene chip technology, to study the influence of the expressions of genes of the myocardial infarction rats treated by the *Shuanglong* formula, *Shexiangbaoxin* pills, and the *Danhong* injection. The results showed that all three TCM formulas had great influence on the expressions of genes in the myocardial tissue of the myocardial infarction rats, but the main regulatory pathways related were not completely the same. The treatment mechanisms of the *Shuanglong* formula work mainly through affecting energy metabolism, cell oxidation and reduction, immune response, and ion binding (including calcium ions and zinc ions). The treatment mechanisms of *Shexiangbaoxin* pills mainly focus on base repair and regulation of protein synthesis. The treatment mechanisms of the *Danhong* injection mainly focus on regulating protein binding, transcription factor activity, and calcium binding.

There are nine common differentially expressed genes by the joint analysis of the gene chips data of the three TCM formulas. Previous research has confirmed that Nfil3, Arntl Cfb Nppa, and Nppb are important in the cardiovascular physiological and pathological processes. Per1 has important effects in treating cancer, mood disorders, endocrine disorders, and diseases closely related to an abnormality of the biological clock. Therefore, the nine genes might be the common targets of TCM for myocardial infarction. Moreover, this was an important piece of information for further research on the drug mechanisms. This study provided data support and research direction for the further research of the mechanism of TCM, and provided a new idea for TCM pharmacology research.

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CHAPTER 6

PROTEOMICS STUDY OF TCM

6.1 PROTEOMICS IN TCM RESEARCH

6.1.1 Overview of Proteomics

Proteomics is used to investigate the law of biological activities and the nature of important physiological and pathological phenomena through studying the expression levels of global proteins.[1, 2] As a supplement to the protein products from an organism's genome, proteomics provides methods of identification and functional analysis, as well as the research strategy for studying proteins. Nowadays, proteomics has become a major area of rapid development in molecular biology, because of its systematic, large-scale analysis of proteins. It has also formed combined analysis platforms with the genome, transcriptome, metabolome, and so on. Proteomics has become a major part of systems biology research.[2]

For a long time, we have observed things through their homogeneity and simplicity, which belong to the most basic level. However, many things show diversity and complexity by the composition of homogeneity and simplicity. For example, such things include physical activity and organism activities. New features are generated through combination, and call for analysis by overall technical methods and strategies. In the system, the measuring methods include genomics, transcriptomics, proteomics, and metabonomics; and the experimental database is built through high-throughput, large-scale test platform

Systems Biology for Traditional Chinese Medicine, First Edition. Guoan Luo, Yiming Wang, Qionglin Liang, and Qingfei Liu.

corresponding with the omics technologies. Then the system simulator of actual biological or physiological processes is generated by the experimental database and the theoretical information database. This simulator can establish the system analysis model by bioinformatic analysis. Then the system profile database and structure database are formed from the above model. After parameter optimization and visualization, further tests or feedback trials are carried out to verify assumptions about the physiological process. Consequently, a forecast of actual physiological processes and developmental trends is obtained. On the whole, systems technologies are the key to system analysis in the basic research framework of systems biology. The final model validation and knowledge discovery can be achieved only through omics techniques to obtain results of physiological processes. Proteome information acquisition is the main content of establishing system structure and network units.

6.1.2 Main Content of TCM's Proteomics Research

TCM is composed of complex compounds, involving chemical components (such as alkaloids, saponins, terpenoids, and glycosides), macromolecules (proteins, peptides, and DNA molecules), and ionic components (inorganic salt and inorganic metal ions). Comprehensive application of various analytical methods is necessary for systematic quality control of TCM to determine the quality control unit, which is composed of key indicators from the preliminary material information.[3–5] Compared with Western medicine, TCM has unique advantages in treating diseases that are caused by multiple genes and factors. However, the deficiency of quality control and efficacy evaluation of TCM has seriously affected its application in modern society. Because both TCM and proteomics have systematic, periodic, spatial, and temporal characteristics, introducing proteomics into TCM research is conducive to revealing the variability of an object in different periods and is more in line with a syndrome's own characteristics. Generally, in systems biology research, the TCM proteomics technology platform is mainly applied in two aspects, as follows: one is on animal drugs, herb attribution, and quality control of medicinal materials; the other is the study of the changes of functional proteins and relative metabolites in vivo after TCM treatments. In recent years, the omics research platforms (e.g., genomics, proteomics, and metabonomics) have been built based on modern systems biology and provided the operational practice platforms for systematically studying complex TCM. In medical research, the main research contents of proteomics mainly reduce to several aspects, as follows.

Proteomics research in pharmacology: The core of TCM pharmacology research is TCM mechanisms. In the post-genomic era, the mechanism of TCM may be revealed on the molecular level. Protein fingerprinting was obtained through biochip or similar technologies. Then comparative analysis of protein expression profiles will show the regulating targets and the affected processes required to clarify the mechanisms of TCM in protein expression.

Furthermore, through choosing the appropriate animal model, the study of TCM compatibility mechanisms can reveal the rationality of syndrome differentiation and attenuation synergy.

Syndrome proteomics: Comparative proteomics was carried out between TCM syndromes and modern diseases. The main research content of syndrome proteomics is the changing trend of differentially expressed proteins and discovery of protein markers. Syndrome differentiation in TCM emphasizes the coordination of whole body function. So TCM treatment is not directly against the causative agents, but rather focuses on adjusting the body's function to enhance its resistance to diseases. Syndrome proteomics research could map protein fingerprints in different stages of syndrome development to provide the drug targets and the scientific basis for TCM treatment.

Model animal proteomics: Building diseased animal models that are consistent with symptoms observed in clinical practices of TCM is important in the research of new TCM drugs. But for a long time, the research ideal was based on anatomical and physiological simulation, and it could indicate very well the dialectical unity of disease and symptom in the animal models. Corresponding to proteomics research of clinical diseases, proteomics research of animal models could provide the material basis to scientifically reflect pharmacological efficacy. However, the establishment of diseased animal models and evaluation criteria is still the main difficulty in the proteomics research of TCM syndrome animal models.

Natural herb proteomics: The research focuses on the protein expression changes in the process of plant cultivation and growth, as well as the process differences and indicator selection of proteins in plant optimization and disease research. By natural herb proteomics research, an authentication system of famous-region drugs and a standard protein map of superior strains can be set up; therefore, the quality of TCM or Chinese herbal medicine can be controlled. Finally, with the accurate identification of famous-region drugs, improvement on the production of active substances and reduction of harmful ingredients could come true.

Information mining of proteomics: Information mining is the important downstream technology of omics research. It is a way to provide relative network biological information for TCM through informatics tools and algorithms.

Our main focus is on proteomics technology that can be applied in pharmacological research on TCM. With experience over a long period of time, we proposed the overall process of TCM's systematic proteomics research (shown as Fig. 6.1). The proteomics study approach is an organic process with parallel analysis and cycle validation. It includes a sample total analysis about proteins, peptides, and metabolites. Metabolites include drug metabolites and endogenous metabolites, which are also the research contents. In the flow map, metabolites are used to illustrate that the purpose of the analysis is to construct the network, which is formed by interactions of protein and small molecules.

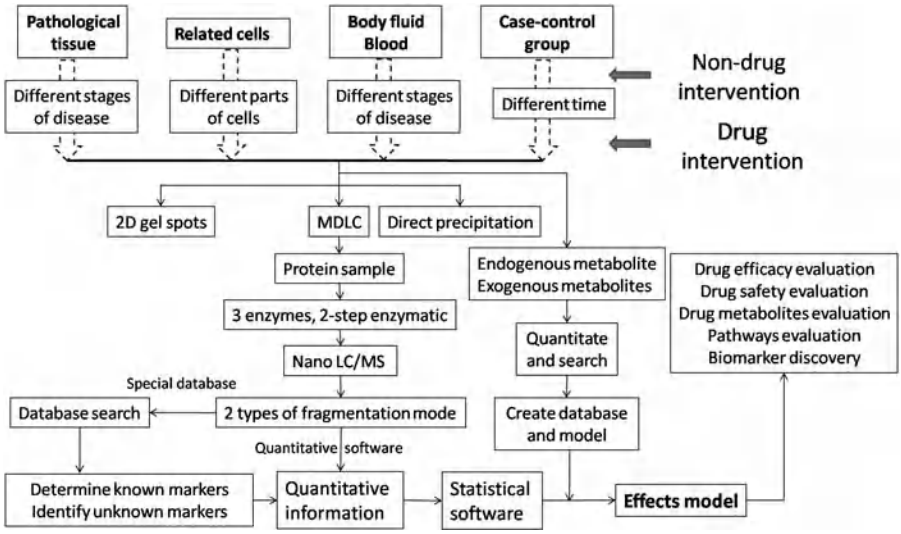

Fig. 6.1 Map of TCM proteomics research.

6.2 A CASE STUDY OF PROTEOMICS IN TCM

Proteomics is an important research tool in the post-genomics era, and has been widely applied to various fields of life sciences, such as drug discovery, and cancer and stem cell research. Proteomics has been initially applied to the research of cardiovascular diseases and was successful in finding some related proteins.[6–10] These proteins may help clarifying the pathogenesis of cardiovascular diseases, and some of them could also serve as markers for disease diagnosis, therapy evaluation, and disease prognosis. Myocardial infarction (MI), a cardiovascular disease, is associated with ischemic necrosis of cardiac muscles due to a decrease in the supply of blood to a portion of the myocardium below a critical level necessary for viability and proper physiological function. *Sanqi* (Notoginseng Radix et Rhizoma, PN), a precious medicinal herb, has the ability to promote blood circulation, remove blood stasis, and promote tissue regeneration. It has also been verified that PN plays an important role in regulating cardiovascular functions,[11, 12] such as protecting from myocardial ischemia,[13] lowering blood lipid content, and preventing atherosclerosis. Panax notoginseng saponins (PNS) are extracted from PN roots and are the main biologically active ingredients of PN. Therefore, we first built a rat MI model to analyze the protein expressions between infarcted myocardial tissue and normal myocardial tissue, and then investigated the effect of PN and PNS on the MI model using proteomics technology. Finally, the pathogenesis mechanism of MI and the treatment mechanism of TCM could be revealed at the protein expression level.

6.2.1 Comparative Proteomics Analysis in the Rat Myocardial Infarction Model

The myocardial infarction model (male Sprague-Dawley rats, 180–200 g) was carried out by ligation according to reports in the literature.[14] The levels of serum enzymes (when MI occurred, LDH, CK, and MDA values were significantly increased; SOD value was significantly reduced) and histopathology (cardiac infarct size) were used to evaluate the efficiency of the model. The infarcted tissue was separated from the MI rat's heart 24 h after surgery for future proteomics analysis. Comparing the protein expression profile between the infarcted and normal tissue, the pathogenesis of MI could be understood and the potential protein markers of MI would be found.[15, 16]

6.2.1.1 Sample Preparation and Protein Separation Please refer to the previous report for the detail methods of extracting protein from tissue, the condition of two-dimensional gel electrophoresis (2-DE) separated protein, the protein image analysis, and the method of differential expressed protein detected by a time-of-flight delayed extraction MALDI mass spectrometer.[17] In order to achieve optimum separation conditions, three different pH range IPG strips (pI 3–4.5, pI 8–10, and pI 5–8) and four staining gel methods (silver staining, CBB-R250, colloidal CBB-G250, and improved colloidal CBB G-250) were studied. Finally, the pI5–8 gel and silver staining were chosen to analyze the protein expressions of myocardial tissue.

6.2.1.2 Analysis and Confirmation of the Differentially Expressed Proteins After PDQuest 7.1 software analysis, the differentially expressed proteins between normal myocardial tissue and myocardial infarction tissue were displayed (Fig. 6.2). Compared to normal tissue, the two groups shared 38 proteins; 22 of them increased in the myocardial infarction tissue, and the others decreased. Meanwhile, 34 proteins only existed in the normal tissue, and 12 only in the myocardial infarction tissue.

Based on the difference of protein profiles, the differentially expressed protein spots in all three parallel experiments were cut from the gels and subjected to trypsin digestion, followed by peptide extraction for MS analysis and peptide fingerprinting analysis. The peptide mass fingerprinting (PMF) method was used to identify 13 proteins via relevant databases on the internet, and related data are shown in Table 6.1.

In the identified proteins, HSP27 and HSPB6 belong to the heat shock protein family. HSP27 is mainly expressed in myocardial cells, smooth muscle cells, and endothelial cells. HSP27 plays an important role in the process of actin polymerization, cytoskeletal stability, and preventing apoptosis under stress. The heat shock proteins have protective effects for cardiovascular tissues under stressful conditions such as ischemia.[18] It has been confirmed that HSP27 can be used as a potential atherosclerosis marker.[19] In the myocardial infarction tissue, two different spots were identified to be HSP27, and

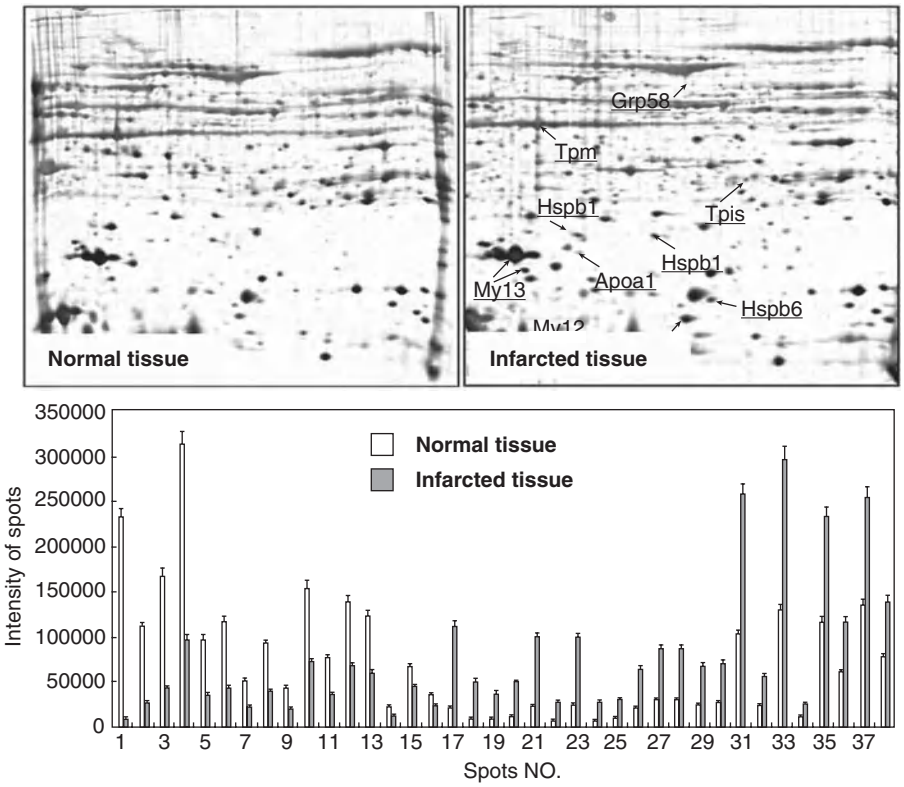

Fig. 6.2 Two-dimensional gel maps and significant differentially expressed proteins in rat myocardial tissue.

their expression changes were opposite. One of them increased 2.8-fold, and the other decreased 1.8-fold. This result suggested that the phosphorylation of HSP27 was changed when MI occurred; and the change might have a protective effect of HSP27 against the ischemic myocardial injury.

Apolipoprotein A-I (apoA-I) is a major protein of high density lipoproteins, and is expressed in myocardial tissue.[20] Its serum concentration can be used as a test marker for fatal heart disease.[21] In this study, the expression of apoA-I was downregulated in the myocardial tissue, as was its expression in dilated cardiomyopathy.[22]

Superoxide dismutase [Cu-Zn] (Cu.Zn-SOD), an enzyme associated with cardiovascular diseases,[23] can catalyze the disproportionation reaction of superoxide anion radical and block the tissue damage from radicals and NO degradation. In the MI model, the Cu.Zn-SOD expression was upregulated to more than threefold its original level.

Combined with the gene expression analysis, the expressions of proteins and genes had good correlations in the process of MI development (shown in Table 6.2).

TABLE 6.1 List of Identified Proteins

NO.	Protein Name (Gene Name)	AC for Databases	MW/pI	Score	Matched/ All Peptides	Seq. Cov.	Infarcted/ Normal
RC01	apolipoprotein A-I (Apoa1)	gi|2145143 P04639	29.87/5.51	85	12/42	32%	0.14
RC02	Glucose regulated protein, 58 kDa (Pdia3)	gi|38382858 P11598	57.04/5.88	67	10/40	24%	0.12
RC03	Heat shock protein 27 (Hspb1)	gi|8248633 P42930	22.86/6.12	138	14/59	63%	0.66
RC04	Tropomyosin alpha chain, striated muscle	gi|92921	32.75/4.71	171	17/40	40%	0.95
RC05	Heat shock protein 27 (Hspb1)	gi|8248633 P42930	22.86/6.12	90	12/68	61%	3.17
RC06	Tpi1 protein (Tpi1)	gi|38512111 P48500	27.21/7.07	64	7/40	32%	3.04
RC07	Heat shock protein, alpha- crystallin-related, B6 (Hspb6)	gi|20302069 P97541	17.55/6.05	81	8/52	54%	2.57
RC08	Superoxide dismutase [Cu-Zn] (Sod1)	gi|818029 P07632	15.74/5.88	64	7/61	40%	1.85
RC09	Myosin, light polypeptide 3 (Myl3)	gi|6981240 P16409	22.26/5.03	74	5/9	29%	1.14
RC10	Myosin, light polypeptide 3 (Myl3)	gi|6981240 P16409	22.26/5.03	153	10/16	55%	5.20
RC11	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform (Myl2)	gi|56683 P08733	18.87/4.86	91	12/40	50%	2.08

TABLE 6.2 The Expression Correlation of Proteins and Genes

No.	Protein Name	Accession	Protein Expression	Gene Expression
RC01	Apolipoprotein A-I	NM_012738	0.14	0.95
RC02	Glucose regulated protein, 58 kDa	NM_017319	0.12	0.43
RC03	Heat shock protein 27	NM_031970	0.66	0.18
RC06	Triose-phosphate isomerase 1	NM_022922	3.04	1.24
RC07	Heat-shock protein beta-6	NM_138887	2.57	/
RC08	Superoxide dismutase [Cu-Zn]	NM_017050	1.85	1.24
RC10	Myosin light polypeptide 3	NM_012606	5.20	1.24

TABLE 6.3 Component Analysis of PNS Fingerprint

No.	RT. (min)	[M-H]-	[M+HCOO]-	Component	Conc. (mg/g)
1	12.27		977.51	NR1	11.83
2	13.75		991.54	Re	0.6445
3	13.85	845.49		Rg1	30.99
4	24.55	1107.59		Rb1	29.24
5	27.07		683.44	Rf1 or Rh1	1.352
6	29.80		991.54	Rd/Isomer	1.881
7	31.79		991.54	Rd/Isomer	0.4269

6.2.2 Comparative Proteomics Analysis of the Effect of Panax Notoginseng in Myocardial Infarction Treatment

Based on the abovementioned MI proteomic analysis, the comparative proteomics research on the PN treatment for MI was carried out. First, the main composition of PN was identified.

6.2.2.1 The Identification of PN Components and Administration in the Rat Myocardial Infarction Model To scientifically clarify the efficacy of TCM, the combination of chemical constituent research and pharmacological study was necessary. If the chemical composition of TCM is unclear, it is impossible for pharmacological studies to elucidate the mechanism and efficacy of TCM. Following chemomics research, the components of PNS were verified and semiquantified by LC/ESI-MS. Table 6.3 shows the analytical results.

The experimental rats were divided into normal, MI, PN, and PNS groups. Thirty minutes after successful MI modeling, rats of the PN group were administrated PN (2 g/kg) and those of the PNS group were administrated PNS (equal to the amount contained in 2 g/kg PN). After 24 h, the rats were scarified and the infarct border tissues of left ventricles were obtained for further proteomics analysis.

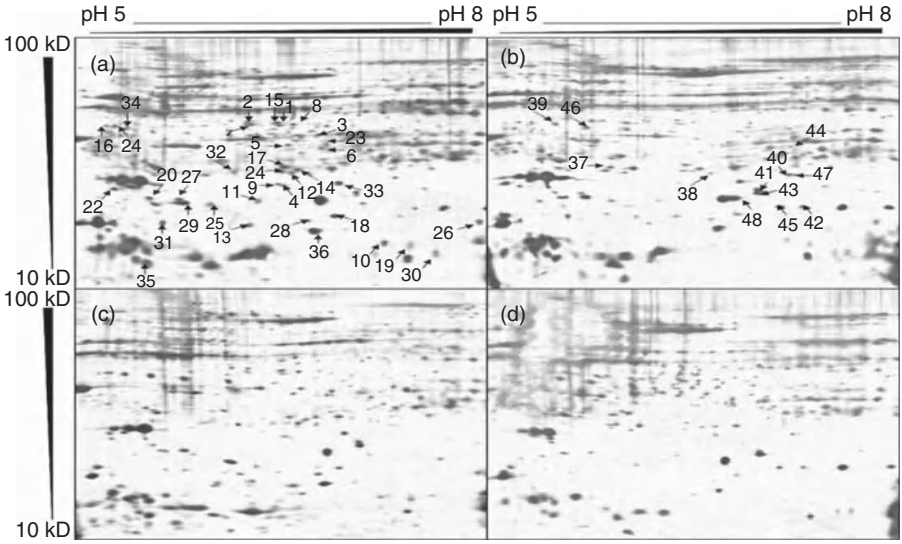

Fig. 6.3 Two-dimensional gel maps of rat myocardial tissue. (a) normal group; (b) MI group; (c) PN treatment group; (d) PNS treatment group.

6.2.2.2 Sample Preparation and Protein Separation The separation and analysis of the protein samples were carried out as described in Section 6.2.1. Fig. 6.3 displays the protein expression. According to the gray values corresponding to proteins in different groups, the significant differentially expressed protein spots were obtained in the treatment processes of PN and PNS.

The above analysis revealed that in 29 differentially expressed protein spots between MI and normal groups, three spots were only expressed in normal tissues and two were only expressed in MI; 20 protein spots were significantly reduced in infarct tissue and four protein spots were significantly increased (change-fold > 2).

The aforementioned significant differentially expressed proteins were adjusted in the treatment groups. In the PN treatment group, three spots that were only expressed in normal tissues were detected, and there was not any significant difference between the normal and PN treatment groups. The two protein spots that were only expressed in the infarcted tissues, were not detected. Among the 20 downregulated protein spots in the MI group, the expressions of 16 proteins increased after administration of PN. Like the four upregulated protein spots, two of them were downregulated to close to normal by PN. Moreover, the expressions of some protein spots, whose expressions had slight variations between the MI and normal groups, were close to normal after administration of PN. The results showed that PN had a good therapeutic effect against MI. The overall protein expression was regulated close to normal in the PN treatment group.

TABLE 6.4 List of Identified Proteins

NO.	Protein Name (Gene Name)	AC for Databases
18	Heat shock protein, alpha-crystallin-related, B6 (Hspb6)	gi|20302069/P97541
20	Myosin, light polypeptide 3 (Myl3, Mlc1v)	gi|6981240/P16409
32	Apolipoprotein A-I (Apoa1)	gi|6978515/P04639
35	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform (Myl2)	gi|56683/P08733
36	Superoxide dismutase [Cu-Zn] (Sod1)	gi|818029/P07632
37	Heat shock protein 27 (Hspb1, Hsp27)	gi|204665/P42930
38	Heat shock protein 27 (Hspb1, Hsp27)	gi|204665/P42930
44	Triosephosphate isomerase 1 (Tpi1)	gi|12621074/P48500

In the PNS treatment group, some of the 29 differentially expressed protein spots were also regulated to their normal expression levels, but the amount of these proteins and the degree of recovery by PNS were not as good as those for the PN treatment.

Most of the differentially expressed protein spots in the MI group displayed the trend of adjusting to normal after administration of PN. However, a few of the protein spots showed the opposite trend, as more obvious changes happened after TCM treatment compared to the normal group. This phenomenon might be directly associated with the protein functions. The damaged proteins in MI, such as skelemin, would be recovered by TCM treatment. Also, the expression changes of some proteins were more deviated from the normal group after TCM administration. The deviation might be temporary, as this temporary deviation could promote the recovery of other relative proteins.

Based on the difference in protein profiles, the protein spots of interest in the differentially expressed proteins were cut from the gels and subjected to trypsin digestion, followed by peptide extraction for MS analysis and peptide fingerprinting analysis. The PMF method was used to identify eight proteins via relevant internet databases; the related data are shown in Table 6.4.

6.2.2.3 Functional Analysis of Differentially Expressed Proteins Induced by PN Research on the mechanism of myocardial ischemic injury mainly focuses on changes in a variety of protein components, such as cytoskeleton, energy metabolism, material transport, and signal transduction. When MI occurred, the expressions of many cytoskeletal proteins changed significantly, including myosin light chain (MLC-1v and MLC-2v). Myosin is the major cardiac structural protein, and is also the main contractile protein. One of the important pathology symbols of heart failure is abnormal expression of the contractile protein. In the present study, the development of MI was accompanied by reduced expression of MLC, but

the MLC2 expression recovered after PN was administrated. However, there was no significant improvement for basic functional chain MLC2 with PN treatment. Increased expression of MLC2 could play an important role in increasing ATP activity and calcium sensitivity. In addition, PNS did not seem to regulate MLC2.

The heat shock proteins HSP27 and HSP-B6 are important for promoting actin polymerization. In the myocardial infarction tissue, two different spots were identified to be HSP27, and the expressions of both of them increased. It indicated that the phosphorylation level of HSP27 was changed to activate protective actions against ischemic myocardial injury when MI occurs. PN had an obvious therapeutic effect for MI. When the MI rats were administrated with PN, the expression of HSP27 was decreased. Additionally, the adjusting effect of PNS for HSP27 was weaker than that of PN. Protein HSP-B6 is strongly expressed in heart, skeletal, and smooth muscle, and is the receptor of cAMP and cGMP protein kinase. It has been suggested that cardiovascular injury may be associated with the participation of HSP-B6 in these two metabolic pathways.[24, 25] The expression change of HSP-B6 was also detected as MI occurred in this study.

In proteomics research between the normal and MI groups, we found that apoA-I expression decreased in myocardial infarcted tissue, similarly to how its expression changes in dilated cardiomyopathy. In the PN treatment group, the expression of apoA-I increased to more than its expression in the normal group. According the result, we speculated that the therapeutic approach of PN might be due to improvement in apoA-I expression to increase the excretion of cholesterol, which eventually relaxes blood vessels and ameliorates myocardial ischemia and hypoxia. Same as its effect on MLC2, PNS did not have a significant effect on the expression of apoA-I.

In myocardial ischemia and certain cardiovascular diseases, Cu.Zn-SOD protects DNA, proteins, and cell membranes from damages induced by reactive oxygen species. In myocardial infarction tissue, the expression of Cu.Zn-SOD increased slightly, but its expression was down to near the normal level in the PN treatment group. It explains the fact that the vasodilator effect of PN partly relieves myocardial ischemia and reduces the production of reactive oxygen species.

Triosephosphate Isomerase (TPI) can catalyze the interconvertible reaction of the two isomers, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, an important step in glycolysis.

When a body is in a relatively hypoxic condition (such as myocardial ischemia), anaerobic glycolysis occurs to produce lactic acid and energy. As MI occurred, the level of glycolysis increased; and obviously, the related expression of TPI progressively increased. This expression of TPI was consistent with its expression in the DCM. In the PN treatment group, the expression of TPI decreased. The reason might be that anaerobic respiration gradually replaces aerobic respiration after the administration of PN, which leads to reduced

glycolysis and demand for TPI. Finally, the degree of myocardial ischemia and hypoxia was ameliorated by PN administration.

The chemical composition of PN has four parts, as follows: volatile components, PN saponins, dencichine, and other components. Although PNS has the main biological active ingredients for PN, PNS alone cannot represent all the effects of PN. The overall effects were reflected by a combination of all the parts of PN. To understand the relationships between different components of PN and their individual efficacies, we need in-depth analysis including using traditional molecular biology methods.

6.3 THE APPLICATION OF HIGH CONTENT SCREENING IN TCM RESEARCH

The emergence of drug high content screening (HCS) technology is an innovation in the systems biology era; it is also a research technology based on whole cellomics. It can be based on comprehensive features, such as the living body and dynamic and multiple indices. For these reasons, HCS technology is very applicable to the studies of complex composition and multiple targets of TCM. It is able to reflect the whole changes of cells and reveal the most essential connotations for life and disease in a microcosmic level. The continued development and application of HCS will bring revolutionary changes for systems biology and even the TCM field.

6.3.1 Overview of HCS and Its Application in TCM Research

HSC can simultaneously detect cell morphology, growth, differentiation, migration, apoptosis, signal transduction, and metabolic pathways under the effect of screened samples, while maintaining the integrity of cell structures and functions. In a single experiment, information relevant to many genes, proteins, and other cellular components can be obtained, and biological activity and potential toxicity of the screened samples can also be determined. HCS is a diverse and functional screening platform for detecting multiple targets on the cellular level. The multidimensional and real-time biological effects that screened samples have on cells can be obtained through high-resolution fluorescence digital imaging systems.[26, 27]

TCM (both formulas and single herbs) is precisely a complex and multicomponent system. The effects of effective components (or effective ingredients) on a whole body include multiple targets and are system-to-system. The application of HCS, a technology of multiple targets and multiple indices, has inherent advantages in the mechanism research of TCM. First, for the specific cell lines selected according the effects of TCM, HCS technology can be used to discover the cellular functions, while a cell is emphasized as a whole in order to obtain more objective and comprehensive results. Second, HCS technology can carry out high content screening for the "top-down" level

in the chemomics research system. The top-down system contains three levels, including overall chemome (formula), effective chemome, and active ingredients group. Simultaneously detecting multiple indicators on the cellular level for the three levels of TCM can quickly and effectively find effective components or ingredients and compare the pharmacodynamic effects with formulas and single ingredients using multiple indices, especially in the drug toxicity and efficacy screening. Finally, HCS technology simultaneously researches the different roles of multiple targets induced by the three parts of TCM on the cellular level. It is not only in line with the characteristics of TCM multiple target adjusting, but also enables us to study the effect of TCM on cell signal transduction pathways. The application of HCS technology is crucial for understanding the mechanism and clarifying the effects of TCM.

6.3.2 Case Studies of HCS Applied in TCM Research

In this study, HCS was used in the TCM chemeomics research system. The effects of the *Delisheng* injection, its active components, and ingredients were investigated on HepG2 (human hepatocellular liver carcinoma cell line) apoptosis.

The *Delisheng* injection, an anticancer formula injection of TCM, was the first drug that obtained the Class 2 TCM new drug certification. The injection is composed of *Hongshen* (Ginseng Radix et Rhizoma Rubra), *Huangqi* (Astragali Radix), *Chansu* (Bufonis Venenum), and *Banmao* (Mylabris), and is produced with a production process like that of modern drugs. In recent years, pharmacological research and clinical applications of the *Delisheng* injection showed that it could inhibit tumor cell growth, promote tumor cell differentiation, induce tumor cell apoptosis, protect bone marrow function, and enhance immune function. Therefore, the injection has a therapeutic effect on the treating lung cancer, liver cancer, pancreatic cancer, rectal cancer, and so on.[28–31] But the chemical basics of *Delisheng* injection, as well as the mechanism research of *Delisheng* injection according to the multilevel theory of the chemeomics system, are still rarely reported.

6.3.2.1 Chemeomics Research of DLS Injection Using LC-MS, the chemical compositions of the *Delisheng* injection, *Hongshen* intermediate, *Huangqi* intermediate, *Chansu* intermediates, and *Banmao* extract were analyzed. A total of 81 components of the *Delisheng* injection were identified by mass spectrometry. Except sucrose and 10 unknown ingredients, among the remaining 70 ingredients, 20 came from *Huangqi*, 22 came from *Hongshen*, 26 came from *Chansu*, and two come from *Banmao*.

Most of the existing studies were only for single compounds in the *Delisheng* injection. But as a formula, we need to know whether the efficacy of the formula is better than that of a single ingredient, whether there is any interaction among the various components, and which signal pathways were altered

TABLE 6.5 Administration Information of Different Groups

Drug		Concentration	Time
DLS injection		10, 50, 100 μL/mL	24 h
Hongshen	Total saponins	10, 100, 1000 μg/mL	
	Rh_2	10, 50, 100 mg/L	
	Rb_1	10, 50, 100 mg/L	
Huangqi	Astragalus saponins	10, 50, 100 mg/L	
	Astragaloside IV	5, 10, 20 mg/L	
Banmao	Cantharidin	1, 5, 10 mg/L	
Chansu	Bufogenins (BGs)	1, 10, 100 mg/L	
	Cinobufagin (CBG)	0.5, 1, 2 mg/L	
	Bufalin (BL)	0.5, 1, 2 mg/L	
	Bufogenins (RBG)	0.5, 1, 2 mg/L	

in the process of cancer treatment by the *Delisheng* injection. Before the mechanism of the injection is clearly explained, these questions must be answered.

Application of HCS in chemeomics research of the *Delisheng* injection would provide a quick and efficient way to solve the abovementioned questions.

6.3.2.2 Experimental The HepG2 cell was chosen as a model to study the effects of the *Delisheng* injection and its components on hepatoma cell apoptosis, in order to determine the cancer-treating mechanisms of the injection.

HepG2 cells were cultured as stated in the previous report.[32] Cells with a passage number after 3 were inoculated to 96 well plates. After 20 h, the cells were divided random into different groups and exposed to the drugs. Table 6.5 shows detailed information about the drugs. Integrity of the nucleus, cell membrane permeability, and mitochondrial membrane potential were detected for cell apoptosis 24 h after drug administration (Multiparameter Apoptosis 1 Kits, Thermo cellomics® Arrayscan HCS system).

6.3.2.3 Effect of Delisheng Injection on HepG2 Apoptosis Fig. 6.4 shows the results of HCS. We find that the cell number was significantly reduced, nuclei appeared to be scattered, cell membrane permeability increased, and mitochondrial membrane potential decreased in the toad and cantharides groups compared with the negative control group. However, in the Hongshen and Huangqi groups, there was not any significant change.

The capacity of promoting cell apoptosis by *Delisheng* injection was significantly higher than that of the other groups. Among the ingredients of the TCM formula, toad and cantharides were the main active components for promoting

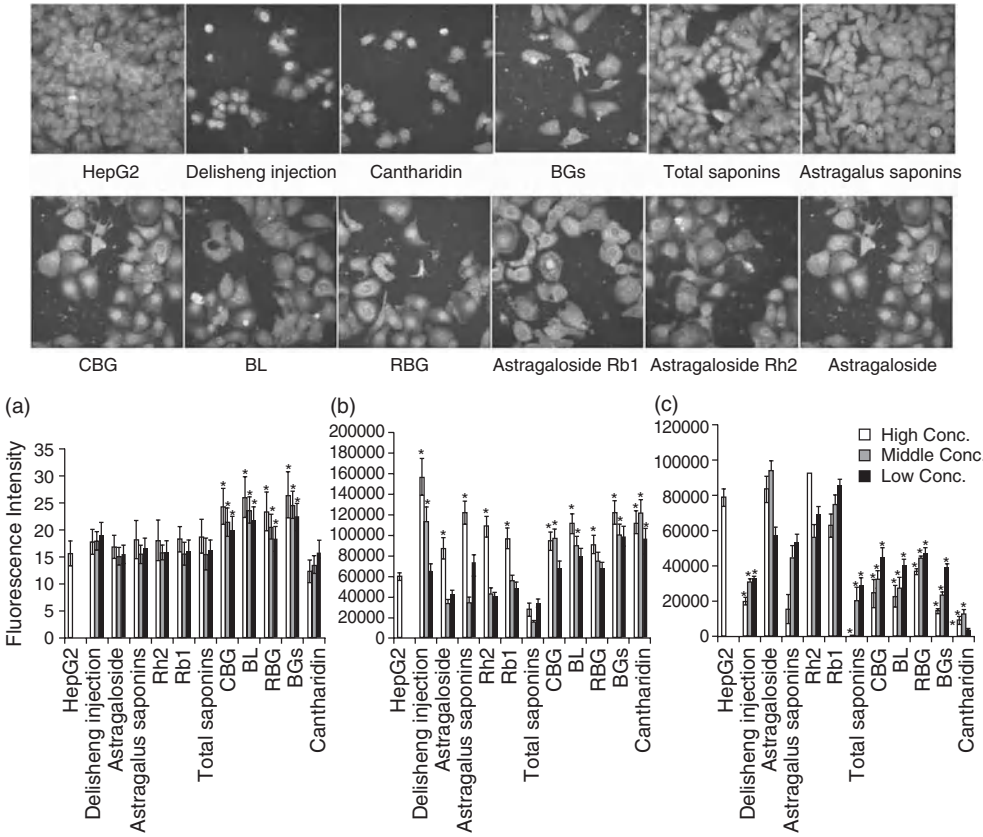

Fig. 6.4 High content screening results of *Delisheng* injection and its components on HepG2 apoptosis. A: the integrity of the nucleus; B: the cell membrane permeability; C: mitochondrial membrane potential. The blue represents the nucleus. If the cell was healthy, the shape of nucleus was a regular circle. The green color shows the cell membrane permeability, and more green represents greater membrane permeability and more cell apoptosis. The red represents the mitochondrial membrane potential. The higher mitochondrial membrane potential showed better activity of the cells. $P < 0.05$ versus the negative control group. (See color insert.)

cell apoptosis, but *Hongshen* and *Huangqi* had relatively mild effects, and in some extent they could moderate the cytotoxicity of *Banmao* and *Chansu*. Four herbs in the *Delisheng* injection all had their unique pharmacodynamic mechanisms, including providing synergy, reinforcing healthy *qi*, and removing pathogenic factors, all of which combined leads to a good therapeutic effect. This is also fitting with TCM theory. From this study, we speculated that the *Delisheng* injection first affected membrane permeability, and then resulted in the loss of hepatoma cell membrane protection that reduced mitochondrial

membrane potential and curbed the energy supply for cell proliferation. All of these quickly promoted apoptosis of hepatoma cells to ultimately achieve an anticancer effect. The application of HCS will have a good prospect in screening research of TCM.

6.4 LIMITATIONS AND PROSPECT OF TCM PROTEOMICS

6.4.1 Limitations

Currently, proteomics research technology has been applied in many aspects of TCM research, such as the mechanism of TCM symptoms, the efficacy mechanism of TCM, and pharmacological research. But in general terms, TCM proteomics research is still in the initial research stage. Most of the current studies only focus on a single sample to describe the differences in proteomics profile. There is only a simple up or down description for the expressions of proteins, but no research on the biochemical and physiological effects of the differentially expressed proteins has been conducted. In addition, some studies are only literature reviews of the differentially expressed proteins. In the future, more in-depth research on protein modification and protein interactions will need to get more attention. On the other hand, the application of proteomics focuses on the mechanism research of TCM. The association of proteomics and the mechanisms on the biochemical and molecular levels for pharmacological efficacy is lacking. Research on the nature of TCM syndromes and viscera-stale theory of TCM are still blank. Moreover, proteomics technology itself has deficiencies. For example, the main research technology is still focused on two-dimensional gels. Multidimensional separation techniques and mass spectrometry are not well utilized. The abilities of isolation, identification, and recovery have to be improved. The research on isolation and identification of the highly hydrophobic, small and large molecular weight, strong acidity or alkaline, and other trace proteins needs to be better. In the overall omics research of TCM, the relationships among the model, syndrome, disease, and TCM have not yet formed a complete research pathway. The research methodology still lacks practical ideas.

6.4.2 Prospect

Proteomics research of TCM is still in its initial stage, so it needs to construct general research pathways and platforms based on practices. According to the current research state, considering the demand of metabonomics and proteomics research built on modern chromatography and mass spectrometry technologies, we believe that the key technologies that need to be further developed and improved in TCM proteomics research include: filling technology of nanocolumn and hybrid filler technology, online

extraction and preparation techniques for trace proteins, the data collection method for multienzyme and multimode mass spectrometry fragmentation, nonisotope labeling quantitative methods of trace proteins, the LC/MS rapid medical diagnostics database, and the efficacy evaluation library of TCM.

One of the important objectives of proteomics research is through screening the biomarkers of diseases to find the relationships and pathways between drugs and proteins. Building the related rapid diagnostics database and the efficacy evaluation library of TCM will be a new bright spot in the development of TCM.

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CHAPTER 7

APPLICATION OF METABONOMICS IN RESEARCH ON TCM

7.1 CURRENT RESEARCH SITUATION OF METABONOMICS

Metabonomics, the scientific research of endogenous metabolic responses of living systems to stimuli, serves as a source not only of qualitative but also of quantitative data of metabolites essential for the description of the metabolic cycle.[1] The emergence of metabonomics aroused great interest from scientists from various countries; and it has enjoyed rapid development during the last few decades. As an application-driven science, metabonomics has been extensively applied in the fields of clinical pathology and physiology, disease diagnosis, medicine toxicology, safety assessment, early diagnosis of serious diseases, and personalized treatment. It also has extensive and significant prospects of use in the modernization process of traditional Chinese medicine.

7.2 INTEGRATION OF QUANTITATIVE METABONOMICS PLATFORM TECHNOLOGY (QMPT)

The objective at the highest level of metabonomics is to determine the quality and quantity of all the metabolites in living organisms, which is not easy to determine due to current limits in analytical technology. Metabolic fingerprinting, which can provide a macroscopic mode, is the most commonly used

Systems Biology for Traditional Chinese Medicine, First Edition. Guoan Luo, Yiming Wang, Qionglin Liang, and Qingfei Liu.

method in the present research, but it has several problems. First, limited by the analytical technology, the biomarkers found often concentrate on some low-pole or high content metabolites, such as lipids; and changes in strong polarity or metabolites with low concentrations are omitted, resulting in loss of information. Second, current research methods on metabonomics often adopt a linear method in processing information, but the responses of many metabolites are nonlinear, which also leads to a loss of information. Third, the current quantitative research on metabonomics is rather semiquantitative, as the results are not accurate enough. Finally, there is a lack of specificity in different diseases and metabolic networks. It can be argued from the above aspects that metabolic fingerprinting is just a blurry macroscopic mode. To solve these problems, the development of quantitative metabonomics needs to be emphasized. However, most of the research in this field simply mixes some standards and makes qualitative and quantitative analyses on the mixture by different instruments and methods, thus only covering a narrow range. At present, the best work in this respect was the study on 91 mixed standards, which leaves the issue of specificity unsolved as before. In addition, the problem of quantitative limits also restricts the development and application of quantitative metabonomics. Thus, there is so far no analytical technology that is widely recognized and accepted for research in quantitative metabonomics. Based on the current situation, Luo et al.[2] proposed a combination of the macroscopic mode and microcosmic mode that focuses on specific pathways and conducts research from the aspects of biology and chemistry. The former sets up different metabolic pathways for research objects according to a priori knowledge, and the latter adopts metabolic fingerprinting. As an analogy shown in Fig. 7.1A, the microcosmic mode enables one to distinguish a certain part of the elephant by having blind men touch the elephant. It leads to varied results because there is a lack of recognition for the whole, though every local feature is grasped. While the macroscopic mode aims to observe the entire elephant, which gives rise to a favorable understanding of the whole situation, a partial opinion is also being generated since it is short of in-depth understanding of local details. Aiming at solving the problem, an integrated method combining the macroscopic and microcosmic mode is proposed, which is called quantitative metabonomics platform technology (QMPT). On one hand, a metabolic fingerprint is applied to make an overall analysis and to grasp the outline as a whole and discover unknown markers. On the other hand, multiple metabolic cycles can be quantified and focused on, key biomarkers can be confirmed, and changing spatiotemporal laws can be studied in detail (Fig. 7.2). The two models are complementary. This complementary characteristic makes the research of metabonomics in complex systems more comprehensive and precise.

Diabetic nephropathy (DN) is a disease with abnormalities in multiple genes and metabolic cycles, and involves various metabolic pathways. The application of quantitative metabonomics platform technology is indispensable for such a complicated disease. In research on the macroscopic mode,

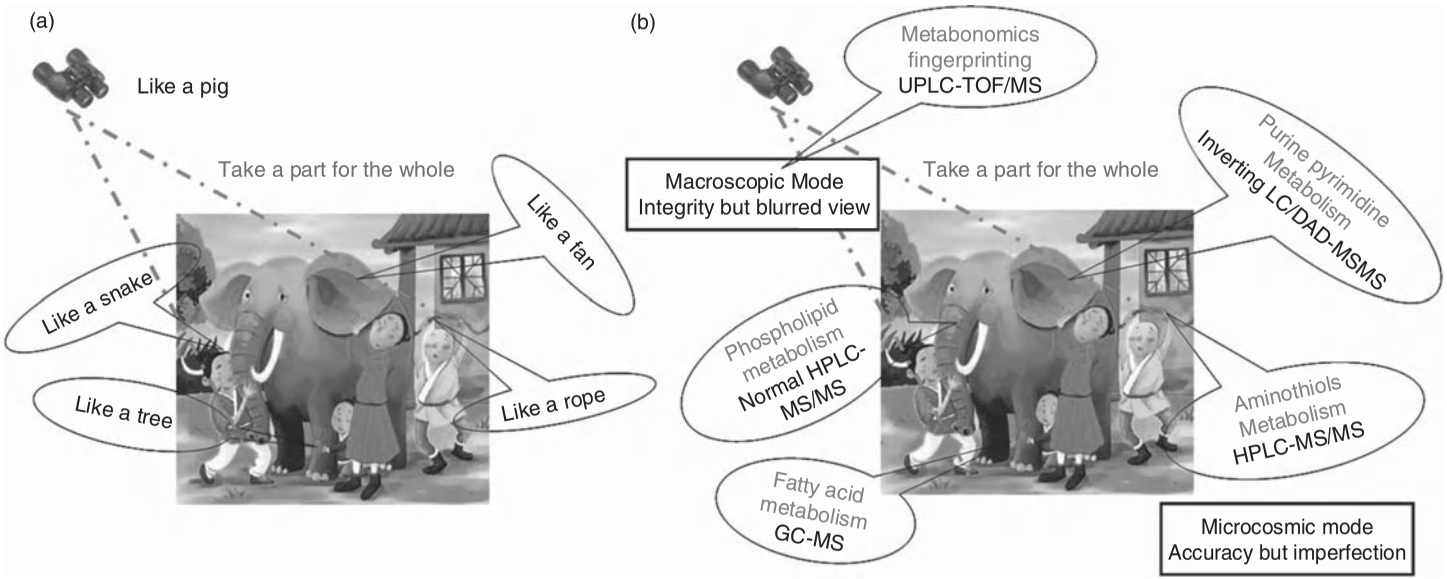

Fig. 7.1 Sketch map for "Take a part for the whole" (a) and quantitative metabonomics platform technology for research on diabetic nephropathy (b). (See color insert.)

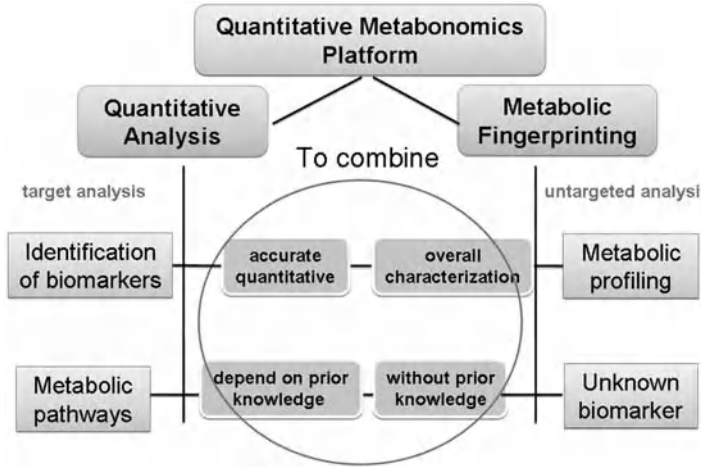

Fig. 7.2 Quantitative metabonomics platform technology: integration of macroscopic and microcosmic modes.

ultra performance liquid chromatography tandem quadrupole/time of flight mass spectrometry (UPLC–Q/TOF MS) is employed to create an analytical method of metabolic fingerprints, reflecting the overall information of metabolites. With the microcosmic mode, the focus on idiosyncratic metabolic pathways is shown in Fig. 7.3.

Accurate quantitative methods are created for four pathways, including phospholipid, fatty acid, purine pyrimidine, and thiol amino acid pathways, to analyze important metabolites. Again, by the analogy of blind men touching an elephant as shown in Fig.7.1B, the microcosmic mode comprises phospholipid, fatty acid, thiol amino acid, and purine pyrimidine, while the macroscopic mode refers to metabolic fingerprinting. The QMPT platform integrates accurate quantitative methods and metabolic fingerprinting, and combines a variety of instruments and techniques including normal-phase HPLC/TOF-MS, reverse-phase HPLC/DAD-MS/MS, and HPLC-MS/MS, as well as GC-MS, in order to comprise an overall and concrete profile on DN, or the "elephant."

7.3 APPLICATION OF METABONOMICS IN THE FIELD OF MEDICINE

7.3.1 Metabonomics and Research on Clinical Diagnosis and Disease Mechanisms

Diseases lead to changes in organisms' pathological and physiological processes, and eventually cause corresponding alterations in metabolites. Some of the metabolites are analyzed and compared with those of a healthy person to determine potential biomarkers of the disease so that a better disease diagnostic method can be provided.[3] Methylmalonic acidemia is a disease with

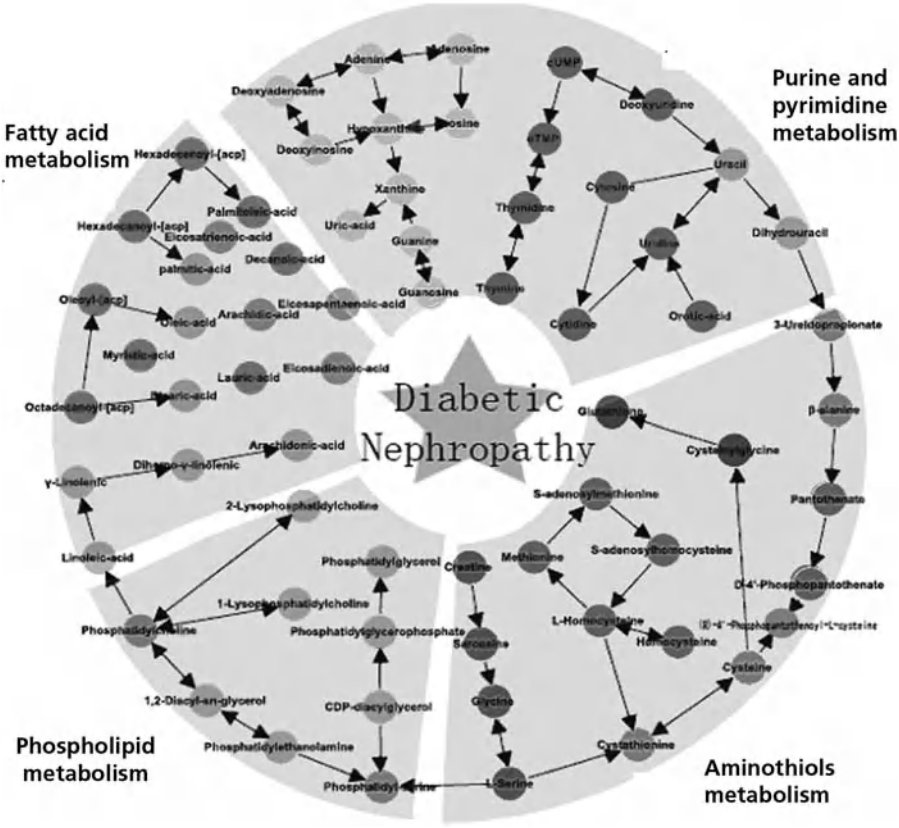

Fig. 7.3 Metabolites and metabolic cycles related to diabetic nephropathy.

higher morbidity among the inherited metabolic disorders. Zhao Jiyuan et al.[4] developed a technique to examine methylmalonic acidemia by combining GC/MS and ESI/MS/MS, in which the methylmalonic acid is determined quantitatively. Meanwhile, GC/MS rule can also satisfy the need of examination among the high risk group.

Metabonomics can realize the qualitative and quantitative research on all metabolites in a specific biological constitution and identify unknown metabolites. The overall metabolic pathways can be described on the basis of substrates, products, intermediates, and key enzymes. Xia et al.[5] made both qualitative and quantitative analysis on major chemical compounds involving metabolic pathways of purine in plasma of patients suffering from diabetes mellitus (DM) and DN by means of LC-MS/MS. It was discovered that adenosine, inosine, uric acid and zanthine may play an important role in monitoring the development of DM, which can be used for assessment of clinical therapeutic methods. By using RPLC-MS/MS, Pang et al.[6] set up a rapid analytical technique to simultaneously fix the quantity and determine the nature of seven

major phospholipids. After analyzing the blood samples of the control group and DN patients in different stages, we found that the phospholipid concentration for healthy people is higher than that of DN patients. With the progression of DN, phospholipid content reduces. This method is likely to provide the methodological foundation for the diagnosis of DN and determination of its stage of development. Jiang et al.[7] developed a method for simultaneous determination of aminothiols among the Hcy metabolic cycles, and such a method is expected to discover other potential biomarkers. A high-performance liquid chromatography–electrospray tandem mass spectrometric (HPLC–ESI-MS/MS) method was established for the simultaneous quantitation of eight aminothiols in plasma with N-(2-mercaptopropionyl)-glycine as the internal standard. Then, the method was applied in the case–control study of patients with DM and DN. S-Adenosyl-L-homocysteine (SAH) and S-adenosyl-L-methionine (SAM) were suggested as better potential biomarkers of DM and DN. Zhang et al.[8] measured metabolites in plasma related to neural tube defects (NTDs) by applying the newly developed LC/MS/MS technique, and detected that they may act as potential pathogenic factors for NTDs' early diagnosis, provide more information on NTDs pathology, boost current measures of nutritional intervention, and reduce pregnancy risks of NTDs. Wang et al.[9] developed a technique to determine simultaneously 11 compounds in maternal serum involving one-carbon metabolism by HPLC-MS/MS. On one hand, it may be explained that NTDs result from multiple factors rather than a single factor. On the other hand, it provides a methodological foundation for diagnosis and prevention of NTDs and other diseases involving one-carbon metabolism. Liang et al.[10] established a method to simultaneously determine 16 compounds in three metabolic pathways by HPLC-MS/MS that can quantify three types of metabolites whose polarities are very different, simultaneously. The method is likely to provide a new approach for study of NTDs' mechanism and detection of new potential biomarkers. Chen et al.[11] set up a solid-phase extraction method applicable to analyze steroid hormones in tissue samples and developed a GC/MRM technique based on a secondary mass-spectrometric technology that is employed to measure the content of testosterone and dihydrotestosterone in rat prostate samples. The two methods prove the metabolic influence on testosterone and dihydrotestosterone in the prostate.

7.3.2 Correlational Study on Metabonomics and TCM Formulas

The complexity of effective compound mixture in TCM formulas cannot be revealed and quantified by prior scientific and technological means. It not only makes modern medicine query the definite curative effect of traditional Chinese medical science, but also restricts the development of traditional medicine therapy itself. Research on the overall influence of a prescription on an organism by means of metabonomics aims to seek the effective compound mixtures playing major roles in the prescription, which is similar to looking for biomarkers, and disproves the rationality of prescribed components.

Doubtless, it can significantly enhance clinical effects and reveal the scientific essence of the traditional Chinese medical sciences.[12]

The following is the related applications of metabonomics in chemical research of TCM formulas. First is related to the safety assessment of TCM formula. Metabonomics, as an independent technology, has been widely applied to toxicity appraisal of candidate drugs. The U.S. FDA has accepted the research results regarding metabonomics as an important reference index for declaration and registration of new drugs.[13] Research on metabonomics has greatly shortened the cycle of safety research on TCM formulas, since it can analyze various target metabolites and metabolic pathways in a rapid and effective way, help orient the target tissue and determine the extent of toxic side effects, deduce the mechanism of action and channels of toxicity, and detect biomarkers during the occurrence, development, and disappearance of damage. Wu et al.[14] applied the coupling technique of HPLC-ICP-MS, under the guidance of chemomics, to establish analytic methods for arsenite, arsenate, monomethyl arsenic, and dimethyl arsenic in *Liushen* pills, and to confirm the restraining role of several simplex drugs in *Liushen* pills on arsenic dissolution in realgar, which further infers that it is one of the possible ways to reduce the toxicity of realgar. Pseudoephedrine plays a certain role in treatment and also has toxic side effects. Ai et al.[15] adopted HPLC to simultaneously determine the contents of ephedrine hydrochloride and pseudoephedrine hydrochloride in the *Yaotongning* capsule; such a method can be used in quality control of Chinese ephedra in its preparation.

The second way metabonomics is applied in chemical research of TCM formulas is in personalized medicine. Personalized medication is a significant progressive step in the application of modern medicine.[16] In today's modernized research on TCM, we should draw lessons from the methods of both Western medicine and TCM, conduct research on the personalized medicine aspect of TCM formulas for clinical uses, and complete research on TCM formula and clinical system, so that TCM formulas can be accepted by the medical field of the whole world. Zhang et al.,[17] taking compound *Liushen* pills and Japanese *Jiuxin* pills as the objects of study, made a quantitative analysis on their volatile components by adopting a GC/MS technique. In terms of the material of *Liushen* pills and *Jiuxin* pills, volatile components are primarily from musk and borneol, whose key components are camphol and isoborneol. An external standard method is applied to measure the content of camphol and muskone in the *Liushen* pill and the *Jiuxin* pill. A single standard substance is employed to replace the material with similar structure to measure the content of isoborneol. The method is simple, feasible, stable, and reliable, which provides a precise, ingenious, and effective method for the quantitative analysis of volatile components in TCM.

Chemical compounds of TCM formulas constitute a complex system, which requires complex science to come up with solutions, rather than relying on the traditional linear thinking model. According to Luo et al.[18, 19] the chemical composition plays the most important role in TCM. They suggested a modern

research system of prescription chemistry, namely "one combination, two basic elucidations, three chemical layers, and four pharmacological levels," which is different from the old research idea of TCM formulas. Metabonomics has characteristics of integrity, systematization, and comprehensiveness. These characteristics coincide with the concept of holism, the dynamic concept and dialectic approach of the TCM theory. The application of metabonomics into the research of TCM formulas can not only determine the components involved in all functions and activities of an organism, but can also analyze the results comprehensively. It facilitates the acquisition of related theories of TCM and a deep understanding of functional mechanism of TCM.

7.4 EXAMPLES OF METABONOMIC RESEARCH ON TCM

7.4.1 Metabonomics Study on Effect of *Baixiangdan* Capsule on Premenstrual Syndrome (PMS) with Liver-*Qi* Invasion in Rats

7.4.1.1 Research Background Emotions in traditional Chinese medical science include the seven emotions (i.e., joy, anger, anxiety, thought, sorrow, fear, and fright) and five minds (i.e., joy, anger, anxiety, thought, and fear) as well as five spirits (i.e., spirit light, ethereal soul, corporeal soul, reflection, and mind) covering various aspects of modern psychology, such as mood, feeling, passion, will, spirit, ideology, mentality, and state of mind. Emotional disease refers to a type of disease wherein emotional factors play a major role in the process of occurrence and development. It is comprised of mental disease, psychosomatic disorder, psychological illness, neurological disability, and all functional diseases. PMS can be divided into two types: anxiety and depression.[37] The *Baixiangdan* capsule, a TCM formula, contains *Baishao* (Paeoniae Radix Alba), *Xiangfu* (Cyperi Rhizoma), and *Mudanpi* (Moutan Cortex). It is a new TCM drug that tranquilizes the liver and regulates vital energy, eliminates distension and relieves pain, assists in harmonizing the stomach, and treats the PMS liver-*qi* invasion. PMS involves both physical and psychological syndromes. According to modern Chinese medical theory, the pathogenesis of PMS is closely related to the liver failing to course freely. There are two tendencies for failure of the liver's free coursing: one is sthenic disperse of liver-*qi*; the other is stagnation of liver-*qi*, so liver diseases can easily lead to liver-*qi* invasion syndrome and liver-*qi* depression syndrome. The PMS patients of the liver-*qi* invasion are often restless and irritable with breast pain during the premenstrual period; for the liver-*qi* depression, the patients are often sullen and gloomy with stuffiness of the chest during the premenstrual period. The theories and technologies of metabonomics are applied into TCM syndromes and intervention research of TCM formula on these syndromes. Rats with liver-*qi* invasion acted as animal models for PMS. The *Baixiangdan* capsule was employed to intervene in the process. UPLC/TOP-MS was used to establish metabolic fingerprinting for the rat urine samples. The metabolic fingerprinting changes of the rat urine samples of different groups were investigated.[20] The

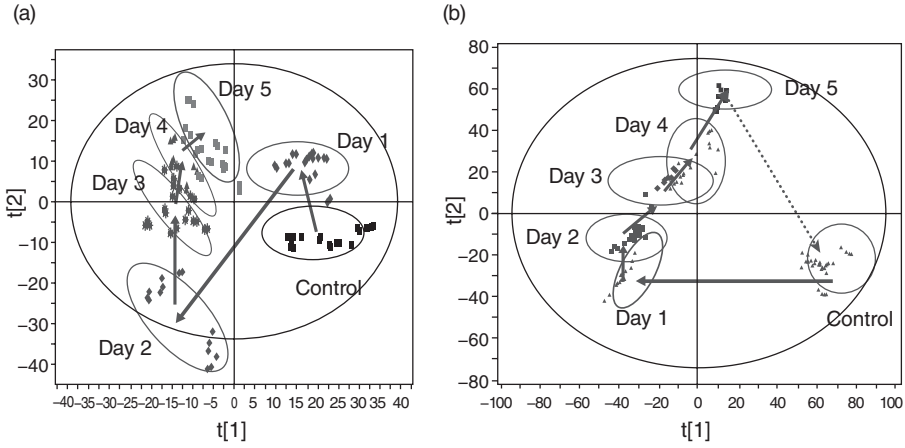

Fig. 7.4 Score plot of PCA of model group with PMS liver-*qi* invasion (a), and score plot of PLS-DA of *Baixiangdan* capsule groups in the first five days of rat urine fingerprint performed on the UPLC–TOF/MS profiles (b).

metabolic model and potential biomarkers closely related with the diseases were explored. All these provide clinical diagnosis and treatment for PMS.

7.4.1.2 Metabolic Profiling of Rat Urine Sample with PMS Liver-Qi Invasion Thirty healthy female rats (SPF) in estrous cycle were randomly divided into three groups: normal control group (control group), PMS molding group (model group), and PMS dose group (dose group) for collection of urine. UPLC/Q-TOF-MS was employed to determine and acquire metabolic fingerprinting of the representative rat urine samples after parameter optimization.

Multivariate statistical analysis was performed using the software package SIMCA-P 11.5 (Umetrics AB, Ume., Sweden). PCA was used first to investigate the general interrelations as shown in Fig. 7.4A, in which the rats' variation trends in metabolic track during the 5 days of molding was indicated. The first 5 days in molding can be distinguished from the 0th day in control; and the distance became closer on the third, fourth and fifth days, showing that the model stabilizes.

7.4.1.3 Metabolic Profiling of Rat Urine after Intervention of Baixiangdan Capsule To evaluate the intervention effects of the *Baixiangdan* capsule on the rats with PMS liver-*qi* invasion, the partial least squares discriminant analysis (PLS-DA) model was adopted to make a multivariate statistical analysis on the medicated rat urine sample for 5 days (Fig. 7.4B). It can be observed from the figure that the intervention of the *Baixiangdan* capsule on rat pathological and physiological conditions has the tendency to go from normal to damaged, then back to normal. In addition, the trend of recovery will be more obvious if there is a continuous dose intervention. Then, PLS-DA was applied

to maximize the difference of metabolic profiles of the control group, model group, and dose group in 5 days. Meanwhile, PLS-DA was made for the normal group, model group, and dose group on every single day, revealing that the three groups within every day can be well separated. All the evidence indicated that medicine intervention changed the rats' metabolic model.

The VIP (variable importance in the projection) value of each variable in the model was ranked according to its contribution to the classification. The VIP list of retention time to exact mass pairs was obtained from PLS-DA by the software MassLynx v4.1 (Waters, UK). Finally, 14 compounds were identified as potential biomarkers (Table 7.1).

7.4.1.4 Potential Biomarkers Related to PMS The potential biomarkers found to be related to PMS primarily include 2-aminoadipate, genistein, hippuric acid, 5-(2-hydroxyethyl)4-methyliazole, 11-epi-prostaglandin F2a, 4-hydroxyglutamate, 2,3-diaminopropionic acid, shikimate-5-phosphate, and melatonin, and can be roughly divided into three classifications: compound that are involved in amino acid metabolism, compounds that are involved in signal transmission, and compounds that lead to physiological activators.

First is amino acid metabolism and emotional disease. Amino acid metabolism has a close relationship with the occurrence and progression of emotional disease. Glutamic acid (Glu) is the main excitatory neurotransmitter in the brain, and can participate in protein metabolism and glycometabolism in the brain, promote oxidation process, and improve the function of central nervous system.[21] In the process of brain development and in general, the glutamic acid system plays a role in the synaptic strength and functions of neurons. It was discovered that, for rats with PMS liver-*qi* invasion, glutamic acid in the hypothalamus and marginal convolution drops remarkably while glutamic acid in the cortex and hippocampus rises notably.[22] It proves that PMS liver-*qi* invasion is closely related to the level of glutamic acid. 2-aminoadipate is a metabolite produced by the main metabolic pathway of lysine, and is controlled by receptors of glutamic acid to withstand neutral excitational activities. Additionally, the normal metabolism of lysine also relies on the regulation of glutamic acid as shown in Fig. 7.5. 4-Hydroxyglutamate and glutamic acid can transform into each other. Therefore, the increase of 4-hydroxyglutamate can bring about the rise of glutamic acid. Biomarkers, such as hippuric acid (benzoyl glycine) and 4-hydroxyglutamate, can be found in the construction of metabolic pathway centered on glutamic acid, as shown in Fig. 7.5. Also, 4-hydroxyglutamate is one of the important intermediate products of hydroxyproline. The reaction of glycine and α-oxoglutarate generates glyoxylic acid and glutamic acid, and is reversible. Furthermore, glycine and benzoic acid can react in the body to produce hippuric acid, demonstrating that hippuric acid is likely to be one of the biomarkers for PMS.

Second are the neurotransmitters and emotional disease. It was found that PMS is to some extent related to a systematic imbalance of

TABLE 7.1 Potential Biomarkers Identified in Urine According to the Results of PLS-DA

NO.	T_R (min)	Exact Mass	Elemental Composition $[M+H]^+$	Metabolite Identification	Metabolite Pathway
1	5.75	162.0759	$C_6H_{12}NO_4$	2-Aminoadipate	Lysine metabolism
2	6.42	130.0869	$C_5H_7NO_3$	5-oxoproline	Glutathione metabolism
3	19.33	355.2639	$C_{20}H_{34}O_5$	11-epi-Prostaglandin F2α	Arachidonic acid metabolism
4	10.54	255.0659	$C_7H_{11}O_8P$	shikimate-5-phosphate	Phenylalanine, tyrosine and tryptophan biosynthesis
5	5.30	164.0805	$C_5H_9NO_5$	4-Hydroxyglutamate	Arginine and proline metabolism
6	19.33	373.2744	$C_{26}H_{28}N_4O_4S$	Biocytin	Vitamin digestion and absorption
7	12.47	271.0607	$C_{15}H_{10}O_5$	Genistein	Isoflavonoid biosynthesis
8	8.27	252.1595	$C_{14}H_{21}NO_3$	Deoxyadenosine	Purine metabolism
9	19.19	371.2589	$C_{20}H_{38}N_2S_2$	6-Keto-prostaglandin F1a	Prostaglandins
10	4.60	180.0719	$C_9H_9NO_3$	Hippuric acid	Phenylalanine metabolism
11	5.78	144.0601	C_6H_9NOS	5-(2-Hydroxyethyl)4-methyliazole	Thiamine metabolism
12	4.69	105.0683	$C_3H_8N_2O_2$	2,3-Diaminopropionic acid	–
13	6.30	233.0919	$C_{12}H_{13}N2O_3$	Melatonin	Tryptophan metabolism
14	4.60	118.0921	$C_5H_7NO_2$	5-amino-valerate	Arginine and proline metabolism

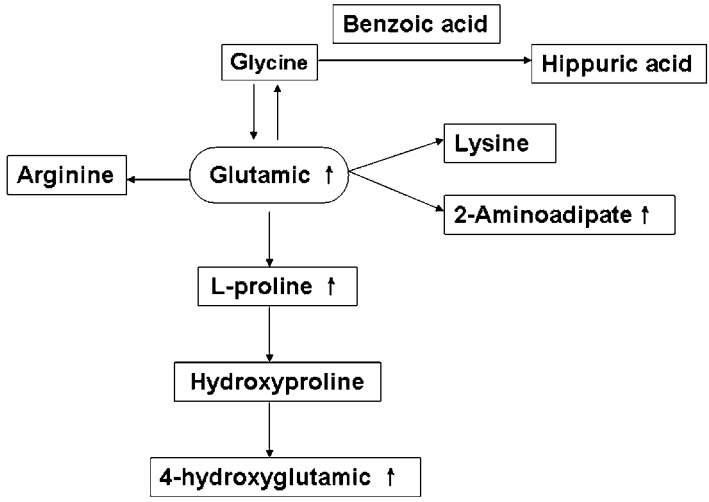

Fig. 7.5 Metabolic pathway of glutamic acid.

the neurotransmitter 5-hydroxyl tryptamine (5-HT).[23, 24] The overlapping occurrence of PMS symptoms has a distinct correlation with a drop of 5-HT level.[25] Melatonin is involved in the metabolic pathway of 5-HT. A decrease in 5-HT level leads to the rise of melatonin. It is widely recognized that melatonin has extensive physiological activities. Its daily and seasonal rhythm has a close relation to the functional regulation of immunity, the neuroendocrine system, and the reproductive system. The composition and secretion of melatonin is manifested with nycthemeral rhythms, seasonal rhythms, and life periodic variations. PMS liver-*qi* invasion itself is a complex syndrome involved with periodicity, immunity, neuroendocrine, and reproductive systematic functions. Hence, the rise of melatonin may attribute to the premenstrual moods of dysphoria and irritability.

The *Baixiangdan* capsule, as the new TCM drug for treating PMS liver-*qi* invasion, can improve the expressions of neurotransmitters such as catecholamines, adrenergics, and norepinephrine to approach the normal level[26] for patients with PMS liver-*qi* invasion. The same is true of the level of serum estrogen and progesterone of the patients with PSM liver-*qi* invasion after the treatment by taking *Baixiangdan* capsules.[27] The study found that there is an increasing trend for 2-aminoadipate and 4-hydroxyglutamate in the model group, which may be owing to the rise of glutamic acid. Although the situation is the same in the dose group, every measurement of 2-aminoadipate is lower than that of the model group. It indicates that the *Baixiangdan* capsule intervenes with glutamic acid metabolism. In addition, the capsule can decrease melatonin and increase 5.7.4-genistein and 11-epi-prostaglandin F2a. All these results provide a new foundation for revealing the functional mechanism of *Baixiangdan* capsule.

7.4.2 Metabonomics Study on Impaired Glucose Tolerance (IGT) Volunteers Intervened with by the *Tianqijiangtang* Capsule

7.4.2.1 Research Background DM is a common endocrine metabolic disease and is a serious noninfectious chronic disease following cardiocerebrovascular disease and cancer. IGT is known as the early stage of DM, which may be experienced by a normal person who later develops into a diabetic of another variety.[29]

The *Tianqijiangtang* capsule originates from many years of clinical effective empirical formulas and has been improved by numerous experts. The capsule, composed of *Huangqi* (Astragali Radix), *Tianhuafen* (Trichosanthis Radix), *Nvzhenzi* (Ligustri Lucidi Fructus), *Renshen* (Ginseng Radix et Rhizoma), *Digupi* (Lycii Cortex), *Huanglian* (Coptidis Rhizoma), *Shanzhuyu* (Corni Fructus), *Mohenlian* (Ecliptae Herba), and *Wubeizi* (Galla Chinensis), is efficient in clearing heat and engendering liquid, boosting *qi* and nourishing *Yin*, and fortifying the spleen , as well as supplementing the kidney and containing essence. Animal and clinical experiments indicate that *Tianqijiangtang* capsule has the following curative effects: it can enhance the sensibility of the body to insulin, effectively protect islet β-cells, intervene with IGT, prevent and postpone the occurrence of DM, improve diabetics' TCM syndromes and dyslipidemia comprehensively, and prevent the emergence of complications.[30, 31] Research on the change of endogenous metabolites of patients with IGT by treatment with *Tianqijiangtang* capsules enables us to find the effects of the *Tianqijiangtang* capsule on metabolic levels.[28]

7.4.2.2 Analytical Results of Metabonomics Patients with IGT were randomly divided into the treatment group and control group, both of which are intervened by having the same lifestyles, namely dieting and basic kinesitherapy. Participants in the control group took placebo capsules (the empty capsule) orally, while subjects in the treatment group took *Tianqijiangtang* capsules. In this study, the UPLC-TOF/MS-based metabonomic approach was used to acquire initial metabolic profiles of the serum. Changes took place in metabolic condition of the participants with IGT after their treatment by *Tianqijiangtang* capsule. PCA was first used to investigate general interrelation among the groups, including clustering and outliers among the samples. It was found that differences were more obvious 6 months after treatment than 3 months after. Then, PLS-DA was used for maximizing the difference of metabolic profiles between patients with IGT before their treatments and after three and six months of treatments (Fig. 7.6A). The figure reflects that there are remarkable differences in the patients' metabolic conditions in diverse treatment periods, and presents a trend of deviation. Comparisons between the treatment group with *Tianqijiangtang* capsule and the control group showed that the patients' metabolic levels of serum after treatment for six months deviated from levels before treatment, as shown in Fig. 7.6B. It

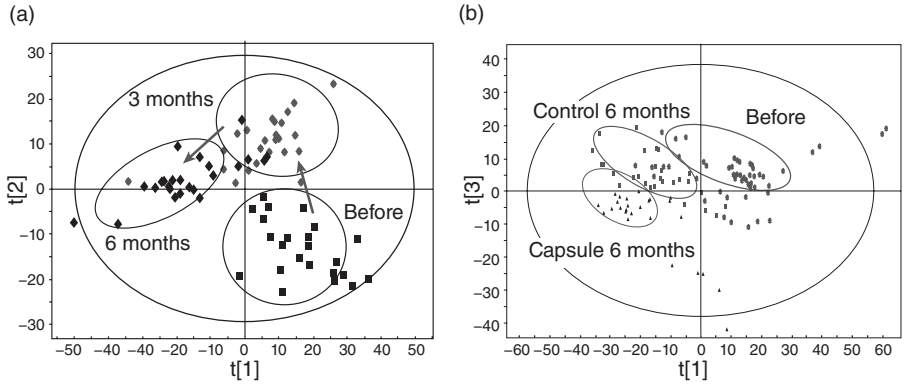

Fig. 7.6 PLS-DA plots of serum fingerprint from patients with IGT. Before treatment and being treated for 3 and 6 months (a) and before treatment, control group, and *Tianqijiangtang* capsule in treatment for 6 months (b).

indicated that the treatment group showed a more significant effect than the control group.

7.4.2.3 Potential Biomarkers Related to IGT According to the groupings of pretherapy, treatment for 3 months, and treatment for 6 months, the analytical results are generated by data processing of multivariate statistics as shown in Fig. 7.6A. These endogenous metabolites include phospholipids, glycolipids, glucosides, nucleosides, and carnitine. When treated for three or six months, the contents of most of the endogenous metabolites in the participant's body are endowed with biological significance compared to the contents before treatment ($P < 0.05$). In addition, in comparison to the control group in the same period, the difference in content also presents biological significance ($P < 0.05$) (Table 7.2).

The identified potential biological metabolites present a remarkable significance in biology, since phosphatidylcholine (PC), phosphatidyl ethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidyl glycerol (PG) all belong to the phospholipids group. All these materials can transform into each other and participate in human lipid metabolism.[32] Diglyceride, the hydrolysate of PC, as the secondary messenger of lipid, can activate protein kinase C and facilitate the progress of DM. In addition, PC can also regulate lipid peroxidation initiated by human superoxide anion radicals and improve the hemorheology of aging organisms.[33] Lipid metabolism disorder participates in the entire process from the emergence of DM to the development of chronic complications. When insulin resistance appears, disorders in the patient's lipid metabolism develop. The PC, PS, PI, and PE in diabetics' red cell membranes decrease obviously.[34]

Eicosane acylcarnitine is a long-chain fatty acylcarnitine formulated by the combination of long-chain fatty acyl groups. Acylcarnitine, whose energy is

TABLE 7.2 Identification Results of 19 Potential Biomarkers (mean ± SD)

NO	T_R (min)	Exact Mass	Identification Results	Control Group			Dose Group		
				Before	3 months	6 months	Before	3 months	6 months
1	5.70	497.5166	Eicosane acylcarnitine	7.86 ± 4.89	8.02 ± 5.7	7.26 ± 5.19	8.07 ± 5.4	8.44 ± 5.45	10.98 ± 5.6*△
2	7.02	1050.6746	Trihexosylceramide	0.31 ± 0.11	0.28 ± 0.09	0.18 ± 0.09	0.16 ± 0.08	0.72 ± 0.27△	1.07 ± 0.06*△
3	12.48	1149.8586	Ganglioside GA2 (d18:1/22:0)	2.51 ± 1.78	3.07 ± 2.02	3.96 ± 1.45	3.41 ± 1.96	5.09 ± 2.65△	11.02 ± 9.77*△
4	12.53	1172.8493	Vitamin D2 3-glucuronide	9.83 ± 7.17	7.37 ± 7.85	11.7 ± 8.85	13.14 ± 8.02	11.82 ± 8.21△	30.61 ± 18.69*△
5	14.82	772.0833	Diguanosine diphosphate	61.11 ± 46.76	69 ± 59.27	58.44 ± 45.62	64.5 ± 52.25	53.02 ± 41.48*△	38.52 ± 27.49*△
6	7.57	811.669	PC (18:2/P-18:0)	12.48 ± 9.97	8.56 ± 6.32	10.73 ± 7.06	15.66 ± 10.15	17.68 ± 15.99△	0.26 ± 0.36*△
7	7.73	854.5594	PC(22:6/20:4)	23.33 ± 14.06	28.24 ± 11.92	26.12 ± 15.35	28.89 ± 16.82	35.88 ± 15.84	9.84 ± 7.39*△
8	7.65	700.4569	PE(15:0/18:3)	10.94 ± 8.04	14.45 ± 6.58	16.23 ± 14.51	16.7 ± 6.95	24.82 ± 19.75△	4.61 ± 2.85*△
9	8.01	839.5705	PI(18:0/16:0)	123.53 ± 101.12	128.03 ± 98.61	91.23 ± 87.51	162.25 ± 127.77	164.44 ± 119.73△	46.21 ± 64.29*△
10	8.18	812.5452	PS(20:4/18:0)	117.6 ± 104.09	245.86 ± 237.87*	164.23 ± 134.82	181.12 ± 174.03	189.05 ± 183.14△	55.08 ± 17.95*△
11	8.20	797.553	PG(16:0/22:5)	0.71 ± 0.17	0.82 ± 0.96	1.36 ± 0.08	1.49 ± 1.31	0.94 ± 0.66	0.08 ± 0.41*△

*In comparison with before treatment $p < 0.05$.

△Compare treatment group with control group in the same period $p < 0.05$.

supplied by tricarboxylic acid, is a kind of special amino acid existing extensively in organisms and is an indispensable material for human long-chain fatty acid metabolism to generate energy.[35] Trihexosylceramide is a type of glycosylsphingolipid, linked by bimolecular galactos, monomolecular glucose, and ceramide, and plays positive roles in signal transmission, cell adhesion and factor regulation and protein transport.[36] Ceramide, as the secondary messenger of lipid, mainly regulates growth inhibition, polarization, senescence, and apoptosis of cells. In recent years, it was found that ceramide plays an important role in the occurrence and evolution of type 2 DM and its complications, and it can reduce insulin resistance, enhance the sensibility of insulin, and control diabetic complications. Additionally, ganglioside is a kind of glycolipid, possessing a strong immunological competence in addition to signal transmission. Diabetic peripheral neuropathy (DPN) is a common complication of DM and also causes diabetes. There are a few effective therapeutic methods for DPN. Ganglioside has the function of strengthening nutritional factors in the body, retarding or interrupting myelinoclasis of peripheral nerve, and promoting the growth of nerves and repair of myelin sheath. Ganglioside is universally used for the treatment of diabetic neurogenic complications, and can provide an experimental foundation for the efficacy of the *Tianqijiangtang* capsule in controlling diabetic complications.

Metabonomics research on the intervention of *Tianqijiangtang* capsules in IGT indicates that the metabolic conditions of participants with type 2 IGT have been dramatically improved after the treatment by *Tianqijiangtang* capsules, and the medication's effect is better compared with that of the control group treated with only a placebo. The study supports scientifically the curative effect of the treatment by *Tianqijiangtang* capsules in IGT and preventive treatment of type 2 DM, and helps the recognition of its functional mechanism on the molecular level.

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CHAPTER 8

APPLICATION OF CHEMOMETRICS AND BIOINFORMATICS IN TCM RESEARCH

The output of metabonomics is usually multidimensional and complex data and needs to be analyzed with multiple chemometrics methods. Besides, with the development of metabonomics and the overlap of various omics technology, bioinformatics is becoming more and more important.

8.1 INTRODUCTION OF CHEMOMETRICS

8.1.1 Current Progress of Chemometrics

The development of chemometrics can be broadly divided into the following four periods: (1) before the 1960s, many statistical and mathematical methods were gradually being applied in chemistry and analytical chemistry; (2) in the 1970s, when the word "chemometrics" was first proposed, chemists and analytical chemists not only used the developed data and signal analysis methods in some adjacent areas, like statistics, mathematics, and many engineering subjects, for chemical research, but also developed many new analytical methods in consideration of the characteristics of chemical science; (3) chemometrics became a popular technology in the 1980s; and (4) in recent years, chemometrics has become a general analysis means for chemists. After more than 30 years, the application of chemometrics in environmental chemistry,[1, 2] food chemistry,[3–7] medicine and clinical chemistry,[8–11] geochemistry,[12] and

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especially in process analytical chemistry[13–14] makes modern analytical chemistry not only useful in providing simple chemical measuring data, but also in providing chemical information and directly participating in chemical practice and the solution of chemical problems. This is another rise and revival of analytical chemistry.

8.1.2 Main Research Content of Chemometrics

Chemometrics studies areas[15] include the following nine categories: (1) data processing: classical statistical analysis, nonparameter statistical analysis, regression and correlation analysis, multiple statistical analysis, variance analysis; (2) experimental design and optimization: factorial design, uniform experimental design, orthogonal experimental design, instrument parameter optimization; (3) analysis sampling: theory sample type and sampling method, group sampling and the estimation of minimum sampling volume, quantitative inspection, dynamic sampling and continuous sampling, count test; (4) analytical signal processing: filtering, smooth and derivation, curve fitting, diversity spectrum technology, Fourier transform; (5) signal discrimination: principal component analysis, factor analysis, signal differentiation, deconvolution of overlapping signals, curve fitting; (6) correction techniques: linear correction, nonlinear correction, standard addition method, fuzzy correction; (7) chemical pattern recognition: dimension reducing and display technique, feature extraction, decision, and classification, linear learning machine, *k*-nearest neighbor algorithm, soft independent modeling of class analogy (SIMCA) method, cluster analysis; (8) quality control: quality control and assurance system, method reliability evaluation, sensitivity and limit of detection, reproduction and stability, selectivity; and (9) artificial intelligence and expert system: database and spectrogram search, computer-assisted instruction, artificial neural network, chemical expert system, and smart laboratory.

8.2 CHEMOMETRIC TECHNIQUES AND THEIR APPLICATIONS IN TCM RESEARCH

Chemometrics techniques mainly include multivariate statistical analysis, signal processing, pattern recognition, database management, and nonlinear calculations. Also, a large part of these has been used in the modernization research of traditional Chinese medicine. Multivariate statistical analysis techniques are commonly used in data processing of instrument analysis and pharmacological experiments, quality evaluation of TCM, effective compound screening, and many other aspects. Pattern recognition techniques are often used for TCM component-effectiveness relationship identification, chemical analytical spectrogram recognition, quality assessment of raw herbs, optimization of TCM formulas, and so on.

8.2.1 Data Preprocessing Methods for Metabonomic Studies

Data pretreatment methods include obtaining the original data matrix, screening independent variables, data standardization, and filtering. Here are the two commonly used pretreatment methods in metabonomics.

8.2.1.1 Orthogonal Signal Correction In orthogonal signal correction (OSC), it is useful to remove extraneous variance from spectral arrays that are unrelated to concentration arrays before establishing the quantitative calibration model. OSC does this by finding directions in spectral arrays that describe large amounts of variance while being orthogonal to concentration arrays. Then, models are built on the corrected data. After these processes, OSC can reduce principal components in building models, simplify models, and improve a model's predictive ability and robustness. Fig. 8.1 shows three groups of human plasma samples which were mainly studied on the relationship between coronary heart disease and hyperlipidaemia. After being preprocessed with OSC and when the noise was effectively filtered out, the cluster and classification performance was improved.

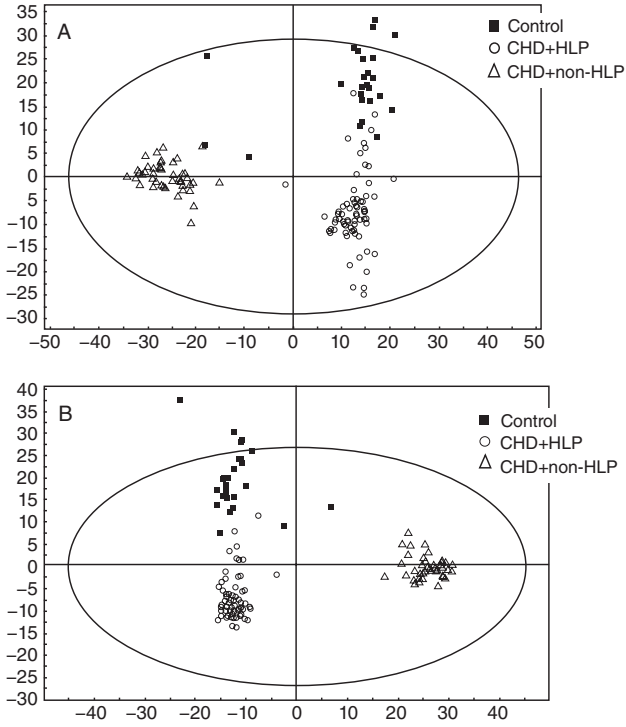

Fig. 8.1 PLS-DA analysis of human plasma fingerprints before and after using OSC. A: before using OSC; B: after using OSC. CHD, coronary heart disease; HLP, hyperlipidaemia.

8.2.1.2 XCMS Software Peak extraction, nonlinear retention time alignment, and relative quantification can be done by various forms (X) of chromatography mass spectrometry (XCMS). XCMS can be used to process HPLC-MS data obtained from metabonomics and proteomics; and by adding new algorithms, it also can be applied in processing other mass spectrometric data such as GC-MS and CE-MS data.

8.2.2 Pattern Recognition Methods and Applications for Metabonomic Studies

Pattern recognition methods mainly include PCA, PLS, artificial neural networks (ANN) and *k*-nearest neighbor algorithms (KNN). PCA is the first and one of the most widely used multivariable pattern recognition methods, the purpose of which is to reduce the data dimensions in order to remove overlapped information under coexistence of numerous chemical information, while PLS is essentially a regression method that is based on feature vectors. Both of these techniques are linear methods and are used quite commonly, but their limitation lies in that they cannot handle nonlinear problems, which often exist in data processing. In this case, ANN is a better solution, which consists of a large number of processing units through appropriate means of interconnection, and is a new data and knowledge information processing system that simulates human brain function. KNN is a type of instance-based learning. An object is classified by a majority vote of its neighbors, with the object being assigned to the class most common amongst its *k* nearest neighbors (parameter *k* is usually determined by cross-validation).

8.2.2.1 Application of ANN in DN Metabonomic Research Through our partner laboratory in the Institution of Pharmaceutical Innovation (University of Bradford) and consulting references, we obtained software called FormRules that integrates fuzzy logic and neural network, as well as ANN software called INForm.[16] These two models were used to establish a disease prediction model in order to analyze the underlying mechanisms of DN.

All samples were divided into five groups, including control, DM, DN phase III, DN phase IV, and DN phase V. The total numbers of samples and variables involved in quantitative research were 160 and 15, respectively. After establishing the model, all the variables were found to have important contributions. Then after adjusting the parameters, four variables were obtained, which were cytosine, uric acid, xanthine, and thymidine. All the samples were randomly separated into the training set ($n = 128$) and the test set ($n = 32$). First, all of the 15 variables were used for prediction for the test set, then the linear regression analysis was constructed by plotting the true values versus the predicted values; finally, the R^2 value (prediction accuracy) of the fitting line was 0.8981. When using the reduced variables, the prediction accuracy was 0.8875, which was similar to the accuracy when all the variables

were used. This indicated that the concentrations of these four compounds could represent the concentration information of all 15 compounds. However, the prediction accuracies of these two models were both below 90%, showing that the concentrations of these compounds could not completely represent the development of DN.

The total number of samples in metabonomic research was 133 and the total number of variables was over 4,000. The first 149 variables were selected by PCA for model establishing and 22 were obtained as the reduced variables. All the samples were randomly separated into the training set ($n = 107$) and the test set ($n = 26$). The prediction accuracies both exceeded 90% when using all of the 149 variables (0.9363) and the reduced 22 variables (0.9264), indicating that these variables could represent the development of DN and could be used for the prediction of unknown samples. Furthermore, it also showed that the reduced 22 variables could represent most of the information contained in 149 variables and could be used as potential biomarkers to aid diagnosis. Although the variables had been reduced to 22, there were still too many for clinical diagnosis. Therefore, in order to keep reducing variables, the C-value in the fuzzy neural network was changed and the whole fuzzy neural network model was simplified to keep it from overfitting. At last, 9 important variables were obtained and the prediction accuracy was 0.9315, which was also over 90% and indicated that these 9 variables could represent the information contained in 149 variables. However, when continually processing this kind of procedure, the prediction accuracy decreased to below 90%. Consequently, the variables could no longer be reduced by using this C-value method because the model could no longer represent the relationship between input and output variables.

Some coinciding variables were found when comparing the three groups after reduction. Because all the models based on these three groups of variables were able to achieve good predictive results, the coinciding variables might be the most important ones for model establishing. By using these four variables, the prediction accuracy was 0.9281 and still exceeded 90%, which meant that these four variables could represent the total information contained in 149 variables and could also represent the development of DN. Moreover, when variables were continually being reduced, the prediction accuracy would decrease rapidly if any one variable was excluded. Therefore, the variable set consisting of these four variables includes the final potential biomarkers.

8.2.2.2 Application of KNN in the Prediction of Cerebral Infarction The requirements for the KNN algorithm are very strict. The sample number of each group in training set should be roughly the same, otherwise the result will lean toward the group with more samples. In order to improve the prediction accuracy, the samples with vague classification or within the outlier range should not be introduced into the training set. The number of feature variables

in the algorithm should be far smaller than the number of samples. Besides, metabonomics often produces thousands of variables, so appropriate feature extraction methods must be used to reduce the data dimensions. Compared with PCA, PLS has components that would maximize the discriminative power of the classification in KNN. Consulting references,[17] an approach combining PLS and KNN was adopted to explore the cerebral infarction-related prediction model.

All the samples were randomly divided into the training set (n = 103) and the test set (n = 26). Since the first principle component denoted more than 90% of the all variables after PLS, it was input into the KNN algorithm. In KNN, the Euclidean distances of an unknown sample to all members of the training set were calculated and ranked in order. The three smallest distances (k = 3) were chosen, and the classes to which the unknowns were closest were examined. The "majority judgment" was used for classification. To reduce possible bias and variability, a random division of the training set and test set was carried out for twenty trials, and the means and standard deviations of prediction accuracy were calculated. As a result, the PLS-KNN method obtained an average prediction accuracy of 100%; therefore, it is a reliable method for prediction and diagnosis of clinical cerebral infarction patients.

8.2.3 A Model Validation Method for PLS-DA Analysis

Many parameters can be used to judge the effectiveness of the PLS-DA model, including the Q^2 value, number of misclassifications, true/false positive rates, true/false negative rates, and the area under curve of a receiver operating characteristic curve (AUROC). However, these parameters do not give a measure for statistical significance (such as p-value) between the original model and the hypothetical model (after changing class labels of the samples). Cross-validation can be used to validate a classification model for the low number of samples obtained. The total data were divided into a training set, a validation set, and a test set. A model was established and optimized by the training and validation set, while the test set was used to test the model performance. A permutation test was carried out to evaluate if the original classification of the samples in the two designed groups is significantly better than any other random classification in two arbitrary groups. After hundreds of permutation tests, the results of the original classification and random classification were shown as a H_0 distribution. If the p value was less than 0.05, then there was significant difference between these two classifications and the established model would be reliable. Consulting references,[17] a method combining cross-validation with a permutation test was used for the PLS-DA result validation of cerebral infarction samples.

A permutation test of the class labels should in theory lead to an average of 65 misclassifications. To verify this hypothetical prediction error as well as

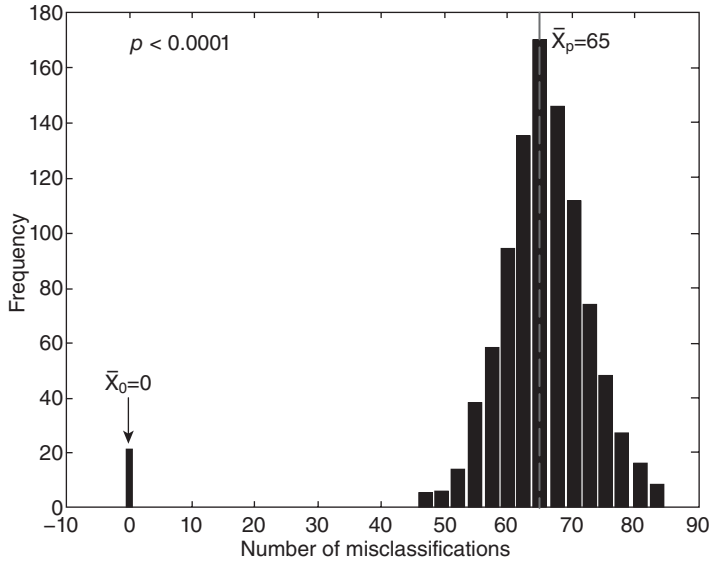

Fig. 8.2 Comparison of the mean cross-validation error of original PLS-DA model ($\bar{x}_o$) and the H0 distribution of no effect ($\bar{x}_p$) based on the cross-validation error of 1000 permutations.

the distribution width, the cross-validation prediction errors from 1,000 different permutation tests were collected. The method to determine the cross-validation prediction error in each permutation test is the same as the one for the original classification model. The distribution of prediction errors was obtained from the 1,000 permutation tests. The prediction error of the original model was 0; and its p value was much smaller than 0.0001 when comparing the cross-validation prediction error of the original model with the permutation test under the H_0 distribution (Fig. 8.2). Finally, the experimentally obtained H_0 distribution exactly matched the requirements, so the PLS-DA model is reliable enough for screening and identifying potential biomarkers.

8.3 INTRODUCTION OF BIOINFORMATICS

8.3.1 Current Progress of Bioinformatics

Early bioinformatics research work mainly focused on the genome, studying the acquisition, processing, storage, distribution, analysis, and interpretation of related biological information. Today, the development of various omics technologies greatly expands the application area of bioinformatics. New challenges that the bioinformatics field is facing include complex data integration

through a set of omics technology platforms, as well as direct links between traditional genetics and biological phenotypes through genome, transcriptome, proteome, and metabonome. The main objective of bioinformatics on metabonomics is to build and perfect the metabonomic database of undetermined biological systems, so that it can be linked with the existing database of genes and proteins, and promote and improve systems biology.

8.3.2 Main Research Content of Bioinformatics

The main research content of bioinformatics research includes genomics, proteomics, biological networks and gene microarrays.

In genomics, bioinformatics mainly studies sequence alignment, structure prediction, and biological evolution. Sequence alignment is the basis of bioinformatics and includes double sequence alignment and multiple sequence alignment. There are already mature dynamic programming algorithms and an alignment software package BLAST for double sequence alignment. However, multiple sequence alignment still needs a fast and effective algorithm. Gene prediction includes the prediction of gene location and gene function. Currently, gene location prediction has changed from simple exon prediction to entire gene structure prediction. Comparison of sequence homology is often the first step in function prediction of a new gene. After homology search of the sequence in the DNA and protein sequence database, a series of genes or gene segments with high homology can be obtained to predict the function of the new gene.

In proteomics, bioinformatics mainly focuses on the prediction of protein structure and function. Structure and function of proteins are considered to be closely related. Generally, proteins with similar structures always have similar functions. Protein structure prediction now includes protein prediction through amino acid composition, protein secondary structure and tertiary structure prediction, prediction of physical properties of proteins through protein sequence, and some special local regional structure prediction. Among these, the methods of secondary structure prediction mainly cover stereochemistry, graph theory, statistics, nearest neighbor decisions, rule-based expert systems, molecular dynamics, and artificial neural network methods. One of the more successful theoretical methods for spatial structure prediction is the homologous modeling method.

In network biology, bioinformatics mainly studies protein networks, gene expression networks, signal transduction, transcription regulatory networks, and metabolic networks. Researchers used massive biological data to build different types of networks, and linked these networks with one another to form more complex networks, in order to complete a variety of biological functions. Bioinformatics applied various models, such as the hidden Markov model, the Bayesian model, and the artificial neural network model, in extracting biological data to build new biological networks, or to integrate and expand new biological information on known biology networks, and to study the

structural characteristics of these networks to interpret complex biological problems.

Bioinformatics is required from the design of gene microarray probes to data processing and data mining of chip expression profiles. One of the hot and key topics of bioinformatics is gene expression data analysis, and its related methods include statistical analysis, hierarchical clustering in pattern recognition, self-organized neural networks in artificial intelligence, meta-analysis, and module analysis. Furthermore, biochip technology improves drug screening, target gene identification, and new drug testing, and rapidly decreases the cost. Biochip technology can be widely used in the diagnosis and treatment of diseases, pharmacogenomic profile research, drug screening, TCM species identification, optimization of crop nurturing and selection, judicial expertise, food and health supervision, environmental monitoring, and national defense.

8.3.3 Bioinformatics-Related Database

The National Center of Biotechnology Information (NCBI) first founded the GenBank database and then developed the Entrez database in 1991, which combines and provides sequence information from GenBank, EMBL, PIR, and SWISS-PROT databases as well as sequence-related literature information from the MEDLINE database. In addition, NCBI also provides other databases, including Online Mendelian Inheritance in Man (OMIM), Molecular Modeling Database (MMDB) containing three-dimensional protein structures information, UniGene database integrating human gene sequence information, GMHG database containing human genome gene mapping information, and toxonomy database containing biological category information.

The KEGG (Kyoto Encyclopedia of Genes and Genomes) database is a pathway database based on existing resources, and is an integration of molecular interaction information, gene and protein information, and biochemical substance information. Protein networks, gene networks, and compound networks are integrated to a collection of pathway maps, enabling researchers to study visually the relationships among genes, proteins, and compounds. KEGG consists of 16 main databases, broadly categorized into systems information, genomic information, and chemical information. It also contains seven categories of pathway information, about metabolic pathways, genetic information pathways, environmental information pathways, cellular process pathways, organism system pathways, human disease pathways, and drug development pathways.

DIP (Database of Interacting Proteins) combines a variety of experimental information of protein interactions, and it mainly consists of three parts, including proteins, interactions, and experiments, which store protein information, interaction information, and related experimental information, respectively. The protein interaction data not only can help the studies on particular interactions among proteins, but can also improve the organization and

complexity study on signal transduction, protein interaction, and cellular networks.

STITCH (Search Tool for Interactions of Chemicals) is a synthesis database based on the integration of various resources, such as literature, pathway data, drug-target relationships, and binding sites. The relationships between proteins and small molecules are shown in the form of networks, enabling researchers to visually understand the relationships between compounds and target protein. STITCH contains interactions for over 74,000 small molecules and over 2.5 million proteins in 630 organisms.

TTD (Therapeutic Target Database) provides information about existing therapeutic target proteins and genes, disease phenotypes, pathways, and corresponding drugs/ligands. Recent updates include information about 1,894 protein targets (348 successful, 292 clinical trial, and 1,254 research targets) and 5,126 drugs (151 approved, 1,279 in clinical trial, and 2,332 experimental drugs).

8.4 BIOINFORMATICS TECHNIQUES AND THEIR APPLICATIONS IN THE RESEARCH OF TCM

8.4.1 The Necessity of Bioinformatics in TCM

After thousands of years, the clinical efficacy of TCM proves that it is an indispensable part of world's medicine. One of the most important theories in TCM is that human physical factors, environmental factors, and different statuses of diseases should be accounted as a system altogether. However, the limitation of TCM is that it always interprets the mechanisms using its own words, which are hard to understand. As the research of bioinformatics and systems biology develops rapidly, more and more researchers are aware that the essence of TCM can only be revealed by systems biology in complex TCM theory studies as well as integrating and analyzing a variety of clinical and biological experimental data. Applying a system integration theory of bioinformatics (such as networks and pathways) in TCM theory study and applying molecular networks in TCM mechanism and diagnostic method study[18–21] are more scientific and more life phenomenon essence-based, and will improve the modernization research of TCM.

8.4.2 Main Research Content of Bioinformatics in TCM

8.4.2.1 Bioinformatics for the Metabonomic Study of TCM Formulas

One of the important obstacles encountered in the process of modernization of TCM is that for a long time, people only knew the clinical efficacy of some specific raw herbs, but had no idea about the main ingredients or active ingredients of these herbs. An effective way to solve this problem is to establish a TCM metabonomic database after qualitative and quantitative analysis of the

main ingredients in TCM by using a variety of high-throughput techniques, such as mass spectrometry and nuclear magnetic resonance. This approach will also aid mechanism research and clinical application of TCM. In addition, metabonomics is another important tool for clinical diagnosis. There are already some metabonomics-related databases, as follows: ArMetdatabase, storing experimental data of plant metabonomics; DOME database, a management system storing metabonomic, transcriptomic, and proteomic data[22]; Metabonomic Society, committed to the data standardization research of metabonomics; Metabonomic Standards Initiative, studying the challenges and problems in the process of metabonomic standardization; and Armet[23] and SMRS[24] databases, giving details of the storage, translation, and experimental type of metabonomic data.

Varieties of TCM metabonomic databases can help people understand clearly the main components in raw herbs and the connections and differences between different ones. They will also benefit follow-up studies on the effective ingredients and their compatibility with TCM and TCM formulas.

8.4.2.2 Bioinformatics for the Systems Biology Research of TCM Due to the application of high-throughput experiments and computer techniques, the emphasis of TCM research shifts to networks and pathways of system biology[25–30] instead of studies on single drugs and single targets. This is why we proposed that we should study TCM from the perspective of systems biology, that is, traditional Chinese medicine systems biology. Methods based on this new research concept will help to overcome many of the limitations[31–33] existing in current studies on complex diseases and drug development.

Recently, modern metabonomic techniques have been used more and more in the research of complex disease diagnosis and metabolic biomarker identification. For instance, in the research of type 2 diabetes, diabetic nephropathy, and neural tube defects, some metabolites were found to be useful for clinical diagnosis and pathological typing by analysis of the changes of pathway-related metabolites in patients' blood and urine samples.[34–38] Another example is that we tried to use high performance liquid chromatography mass spectrometry method in the analysis of in vivo metabolism of scutellarin in rat[39] in order to find the related pathways and evaluate its efficacy and toxicity. This whole strategy of using metabonomics in disease research matches with some TCM theories, such as the holistic view and syndrome differentiation-based treatment. Therefore, their combination may effectively promote the modernization of TCM theories.

Furthermore, the strategy integrating data from genomics, transcriptomics, proteomics, and metabonomics; biological function annotation systems; biological pathways, and network modeling can be used for biomarker identification in disease diagnosis and classification, such as for cancer and immune and nervous system diseases, in efficacy evaluation of experimental treatment, and in therapeutic target discovery in drug development.[40–44] Additionally, because

systems biology can figure out the overall relationships among various biological levels, this strategy is beginning to affect the research process of cardiovascular diseases and metabolic diseases.[45–47]

8.4.2.3 Bioinformatics Database of TCM Nowadays, the constantly accumulating data about Chinese herbal medicine makes it easier for scientists to conduct related research, such as predicting the active ingredients in natural herbs through searching the compound structural information, or seeking potential molecular targets by using different algorithms in predicting the interaction between compounds and genes.

There are currently many TCM databases that can provide a variety of related information, but only a few are already published and free to use, such as the TCM–ID database, which contains prescriptions, herbs, and their ingredients, and some of the 3D structure information about herb ingredients[48]; the TCM toxicology database, describing the details about the scientific evidence of TCM toxicity[49]; the database of 3D structures of compounds separated from traditional Chinese herbs, not only providing the basic molecular information and optional 3D structure information, but also providing information about classification, effective component groups, and clinical efficacy[50]; the TCM database, including basic information on chemical compositions, molecular structures, and active ingredients[51]; the TCMGeneDIT database, providing relevant information about TCM, genes, diseases, curative effects and ingredients, and related information, as well as also integrating protein interaction and biological pathway information in order to find the regulated relationships between TCM and genes.[52]

In addition, since TCM is a compatible medical system with Western medicine, it can not only provide a steady flow of resources for new drug research and development, but can also provide effective personalized treatment according to the differences between individuals by using various prescriptions and compatibilities of Chinese medicinal crops. With these strengths, there is still a weak point, which is the approach to interpreting this unique strategy of diagnosis and treatment with scientific and reasonable evidence. This will be the principal problem in the modernization process of TCM. Therefore, we need to collect the accumulated clinical data from the thousands of years for which TCM has existed and experimental data from modern scientific research, integrate these data by various bioinformatic methods, discover the underlying relationships among those data, and eventually build a database for scientific diagnosis and TCM formulas.

8.4.3 Inverse Molecular Docking and Its Application in TCM Research

Molecular docking, a recognition process between or among two or more molecules, is an important molecular simulation method involving space and energy

matching among molecules. This method is widely used in drug and material design. DOCK, AutoDock, and Flexx are examples of commonly used software. Instead of screening drugs, the main purpose of inverse docking is to explore the mechanisms of activity, toxicity, and side effects of drugs. To fulfill these objectives, INVDOCK software is used to automatically identify potential target proteins and nucleic acids of small molecules, such as candidate drugs, natural ingredients, or other chemical constituents. One of the key roles of inverse docking is aiding to find potential target proteins for drugs with known activity but ambiguous mechanisms, such as some raw herbs and prescriptions. For those with already known mechanisms, the other importance of using inverse docking is to discover their secondary target proteins and to provide some helpful evidence for possible new uses. As described above, inverse docking has the ability of finding multiple targets of one compound; and because TCM itself has the characteristic of targeting multiple genes and targets, this method is especially appropriate for active substance research in TCM.

Shuanglong formula (SLF) is a TCM formula constituted of *Renshen* (Ginseng Radix et Rhizoma) and *Danshen* (Salviae Miltiorrhizae Radix et Rhizoma), and is applied to the treatment of myocardial infarction, angina, and coronary heart disease. In order to study the probable therapeutic mechanism of SLF, first, inverse molecular docking was used to predict the target proteins of 68 important active compounds, while annotation software was used to annotate these target proteins into corresponding pathways (shown in Fig. 8.3). The results showed that SLF is a multicomponent prescription with multiple targets. Second, through literature and database search, a predicted "disease–function–pathway" network (shown in Fig. 8.4) was set up based on the related pathways and functions of myocardial infarction treatment with SLF. Integrating these target proteins, pathways, and related functions can help to explain the possible mechanisms of SLF in treating myocardial infarction and design continuing validation tests. In addition, since the target proteins may be involved in some side effects, this prediction network model may also contribute to further safety evaluation and dose design, enabling scientific explanation of clinical effects and related mechanisms of TCM.

8.5 CONCLUSIONS

The amount of data obtained from proteomics, metabonomics, and other omics (including chemomics) is enormous and needs to processed and mined properly. In addition, all of these omics techniques have been applied to TCM systems biology, which is a system-to-system interaction that is different from simple system or point-to-system research and needs to be solved with continually developing data processing and analysis methods. In this chapter, we summarized the commonly used chemometrics and bioinformatics methods and related databases, and utilized some examples to illustrate the advantages of using these techniques, such as OSC, XCMS, artificial neural networks, KNN

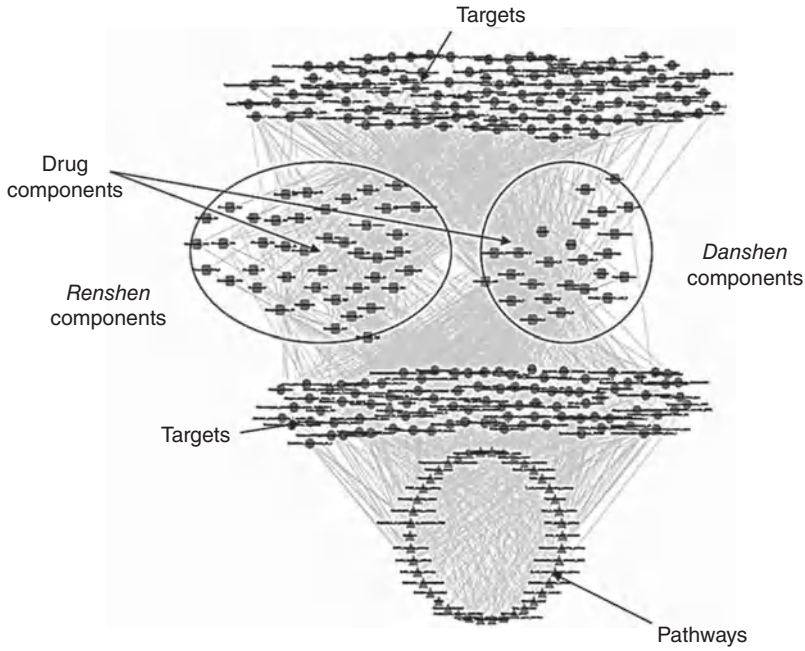

Fig. 8.3 Predicted "component–target–pathway" network map of SLF. Rectangles represent small molecule components; circles represent target proteins; triangles represent pathways; lines represent a protein and can be a target of the component or a target protein can be annotated to the pathway.

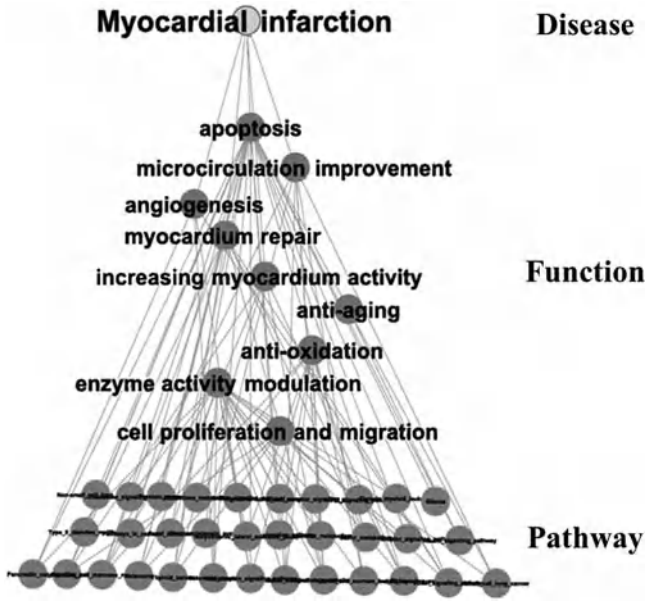

Fig. 8.4 Predicted "disease–function–pathway" network map of SLF treatment.

algorithm, cross and permutation tests, and inverse molecular docking for TCM modernization research. Thus, data processing and analytical methods are essential for TCM systems biology research. We not only need to take advantage of existing techniques, but we also need to develop some new techniques that fit the characteristics of TCM.

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CHAPTER 9

STUDY OF INTEGRATED BIOMARKER SYSTEM OF DIABETIC NEPHROPATHY

9.1 INTRODUCTION OF DIABETIC NEPHROPATHY

Diabetes mellitus (DM) is a major global health issue. Among all the nations, India, China, and the United States have the highest morbidity rates of DM. On "United Nations Diabetes Day" in 2007, the International Diabetes Federation (IDF) claimed for the first time that diabetes, a noncommunicable disease, is spreading to the whole world in a fashion almost similar to that of AIDS, and is a serious threat to human health. The IDF reported that there are over 246 million adults with diabetes worldwide, but this figure could reach 380 million by 2025, most of which would be in the developing countries. It was also reported that two people develop diabetes and one person dies of diabetes every 10 sec around the world. In China, 3,000 new diabetes patients emerge every day, or about 1.2 million every year according to a survey from the Ministry of Health. In 2007, the IDF estimated that the number of diabetes patients would be about 46.1 million in China in 2025 according to data in 2002. But it was also pointed out that the projected data may understate the current situation of diabetes development in China. With improvements in the economy and standard of living, especially in the last 10 years, changes in lifestyle and increasing life expectancy caused a rapid increase in diabetes in China. The Diabetes Society of the Chinese Medical Association National Diabetes Survey Collaborative Group found that the current prevalence of diabetes in China is 11.6%, and is 13.3% for men, 10.6% for woman. The

Systems Biology for Traditional Chinese Medicine, First Edition. Guoan Luo, Yiming Wang, Qionglin Liang, and Qingfei Liu.

survey, which lasted from June of 2007 to May of 2008, covered 14 cities and provinces, including provinces with large populations such as Sichuan, Henan, and Shandong, and cities such as Beijing, Shanghai, and Guangzhou. The prevalence of diabetes increases with age, and its chronic complications are a major threat to human health. Of the chronic diabetic complications, vascular complications are the most dangerous ones. Diabetic nephropathy (DN) is one of the most important microvascular complications, and is one of the major causes of death for diabetes patients. The epidemiological research of domestic and international data shows that 30% of diabetic patients develop DN.[1–3] Modern Western medicine recognizes DN to be caused by kidney damage from diabetes, as evidenced by diabetic glomerulosclerosis. DN is a metabolic disease involving a variety of complex metabolic disturbances. The basic pathological lesions are glomerular basement membrane thickening and mesangial matrix proliferation, and the clinical manifestations include proteinuria, edema, hypertension, and renal damage.

9.1.1 Diabetic Nephropathy-Related Mechanisms

DN is more prevalent in patients who have had diabetes for more than 10 years. Proteinuria is the earliest manifestation of DN. Because the pathogenesis of DN is rather complex, the pathogenic mechanism of the disease has not yet been fully elucidated. Current research data show that the pathogenesis of DN is multifactorial, the majority of which are as follows.

9.1.1.1 The Toxicity of Hyperglycemia

9.1.1.1.1 Advanced Glyclation End-Products (AGEs) Sustained high glucose can alter the chemical structure of glycation products, forming olamine bond structures, and further result in AGEs through dehydration and rearrangement. AGEs that accumulate in protein and cell walls can lead to vessel wall thickening, decreased flexibility, and glomerular basement membrane thickening. They also combine with specific receptors, causing increased release of IL-1 and tumor necrosis factor-α (TNF-α). In addition, AGEs stimulate endothelial cells to produce and release collagenase and other proteolytic enzymes, resulting in the increase of their clearance from the body. Chronic hyperglycemia can cause continued accumulation of AGEs, release of cytokines, and increased permeability of blood vessel walls. During hyperglycemia, glucose combines with proteins in circulation and tissue without the involvement of catalytic enzymes, which can promote free radicals and participate in an oxidative stress response, accelerating the development of the chronic disease.[4]

9.1.1.1.2 The Metabolic Activation of Polyalcohol Pathway Sustained high glucose can activate aldose reductase (AR), and change sugars in cells to sorbitol and fructose through polyalcohol pathway metabolism. AR is rich in

a variety of renal cells, including glomerular basement membrane, mesangial cells, and epithelial cells. It is difficult for AR to travel through the cell membrane, and its accumulation in cells can cause cell swelling, destruction, and aldose increase. Increased aldose induces enhanced nonenzymatic glycation of extracellular matrix collagen and increased collagen. Increased hydration of collagen, basement membrane thickening, and reduced cell inositol interfere with Na^+-K^+-ATP activity, and increasingly impair cell metabolism and function. Sorbitol pathway activation increases the consumption of reduced coenzyme II and reduced glutathione, decreasing antioxidant capacity and free radical scavenging ability. Thus, activation of polyol pathway may be the inducing factor of chronic hyperglycemia damages.

9.1.1.1.3 Protein Kinase C (PKC) Under the effect of hyperglycemia, increased PKC activity causes glomerular hyperperfusion, hyperfiltration, and increased glomerular extracellular matrix synthesis. Recent studies have shown that PKC activation can upregulate the expressions of cell adhesion molecules in mesangial cells and promote leukocyte adhesion in glomerular accumulation, accelerating glomerular injury.[5]

9.1.1.2 Changes in Renal Hemodynamics Changes in renal hemodynamics are directly involved in the development of diabetic nephropathy, and are mainly dictated by glomerular hyperfiltration. It is generally presumed that the continued glomerular filtration leads to glomerular damage through the following two aspects.

Sustained glomerular hyperfiltration and glomerular hypertension can damage glomerular endothelial cells, increase glomerular filtration, increase the exudates of plasma mesangial area, reduce the ability to remove macromolecules in mesangial cells, and induce mesangial obstruction. In addition, the macromolecules that are accumulated in the mesangial area, which can stimulate mesangial cell proliferation and promote mesangial matrix production, can result in mesangial expansion and accelerated glomerular sclerosis.

Sustained high glomerular filtration and pressure may stimulate collagen synthesis of glomerular filtration membrane of epithelial cells, induce not only glomerular capillary basement membrane thickening, but also mesangial matrix production of mesangial cells, and ultimately promote glomerulosclerosis.[6, 7]

9.1.1.3 Action of Cytokine The action of cytokines in the pathogenesis of DN involves glomerular hemodynamics, extracellular matrix metabolism, cell proliferation, and cell hypertrophy. According to the characteristics of cytokines, cytokines can be broadly categorized as follows: cytokines related to the changes of glomerular hemodynamics, such as insulin-like growth factor (IGF-1) and platelet derived growth factor (PDGF); cytokines that are involved in cell hypertrophy, including transforming growth factor (TGF-β) and IGF-1;

cytokines that are involved in extracellular matrix metabolism, including TGF-β, IGF-1, and PDGF; cytokines that participate in cell proliferation, which are PDGF and fibroblast growth factor (FGF); cytokines that mediate insulin signaling, including tumor necrosis factor (TNF-α) and IGF-1; and cytokines that are involved in apoptosis, including TNF-α and TGF-β1. In the case of diabetes, the expression of cytokines are regulated by factors including insulin and glucose levels, AGE, PKC activity, hemodynamics, vasoactive factors, and lipoprotein and protein intake.[8]

9.1.1.4 Oxidative Stress Reactive oxygen species (ROS), such as O_2^- and H_2O_2, are generated by a wide range of aerobic tissues and organs. Similar to NO, large amounts of ROS are generated by specialized immune cells, while a few are generated by the second messengers in mesangial cells. ROS is related to cell proliferation, apoptosis, and growth factor activation. Radical free radicals directly act upon lipids and proteins of cell membrane, causing lipid peroxidation and protein denaturation.

The damages of oxidative stress on renal tissue are as follows:

(1) ROS attack unsaturated fatty acids, resulting in lipid peroxidation and leading to physiological damage in the cell membrane of renal tissue and increase of the vascular permeability.
(2) SOD, GSH-PX, CAT, and other antioxidant enzymes in renal cells promote glycation or oxidation, and reduce the antioxidant capacity of the kidneys, as well as inactivate intracellular key enzymes and transport proteins of Na^+-K^+-ATP enzyme.
(3) DNA damage in renal cells and mitochondria.
(4) Lipid peroxidation reduces membrane fluidity of red cells, and increases adhesion of endothelial cells.
(5) H_2O_2 and O_2^- pass cell membranes and ion channels, leading to oxidative damage by a chain reaction. Decreased hyaluronic acid in connective tissue decreases viscosity, destroys cell adhesion between the filling materials, and increases vascular permeability.[9, 10]

9.1.1.5 Genetic Factors Genes play very important roles in the pathogenesis of DN. Most patients with diabetes will develop DN, and DN also shows family aggregation. DM patients with a family history of hypertension have a significantly higher chance of having DN than patients without a family history of hypertension.

Angiotensin-converting enzyme (ACE) and GLUT-1 gene polymorphisms are found associated with DN. DD type of ACE gene and XBa-I (-) allele gene of GLUT1 are found to be related to the development of DN. The genetic polymorphisms of enzymes in heparan sulfate proteoglycan metabolism (N-deacetylase) and gene insertion/deletion polymorphisms of angiotensin I-converting enzymes are also involved in the pathogenesis of DN.[11]

9.1.2 Translational Medicine and Diabetic Nephropathy

Translational medicine is an innovative way to quickly translate the results of basic research into clinical technology, which emphasizes interdisciplinary studies and will revolutionize medical advances.[12, 13] The purpose of the current conventional Western medical diagnosis is to treat the human disease by targeting organ/tissue or molecular targets. Western medicine focuses on the pathological changes of diseases and has the advantage in describing specific pathological biology. Western medicine utilizes modern equipment and imaging techniques to examine the biochemical indicators and uses quantitative targets to diagnose disease, so it has significant advantages in target organs such as specific parts of the lesion. However, it has numerous disadvantages in detecting multifactorial complex diseases. On the other hand, the purpose of conventional TCM diagnosis is to treat the sick person (syndrome), which aims at improving overall symptoms of patients by treating both the disease and the symptoms. TCM diagnosis is not only focused on the pathological changes in patients, but also the physiological changes. The TCM diagnostic processes, using such methods as "inspection, listening and smelling examination, inquiry, and palpation," depends on the subjective experiences of the doctor. Research on quantitative indicators of TCM diagnosis is ongoing, but it still lacks scientific data support. Take DN, for example; Western medical diagnosis relies on renal functions, and proteinuria is used as a diagnostic gold standard, but it does not efficiently diagnose patients who do not have significant proteinuria.[14] TCM diagnoses the symptoms of the patients, describes the global change using *Qi*, *Xue*, *Yin*, and *Yang*. TCM is useful in the early diagnosis of diseases, but the current diagnosis process lacks quantifiable indicators and research evidence.

9.1.3 Systems Biology Study of Diabetic Nephropathy

With the progression of science and technology, systems biology emerged with cross-integration between disciplines; and because of its comprehensive and unique advantages, it has become an important approach for research on diseases. Combined with clinical research studies, it will be efficiently used in early diagnosis and mechanistic studies of diseases. In addition, the overall strategy of systems biology research is consistent with the theory of TCM. Therefore, systems biology is a good structure for the standardization of complex theoretical TCM, and the most suitable for the multiple target and multiple system methods of TCM philosophy. It is very interesting to introduce systems biology to the study of syndromes, as it can improve the scientificity and quantitation of disease diagnosis and treatment.[13, 14] Systems biology also contributes to the chemical substance basis of TCM and is the best way to reveal the nature of the syndromes.

Translating the progress in molecular medicine to new therapies has many hurdles and remains a very fragmented process, so a new professional figure

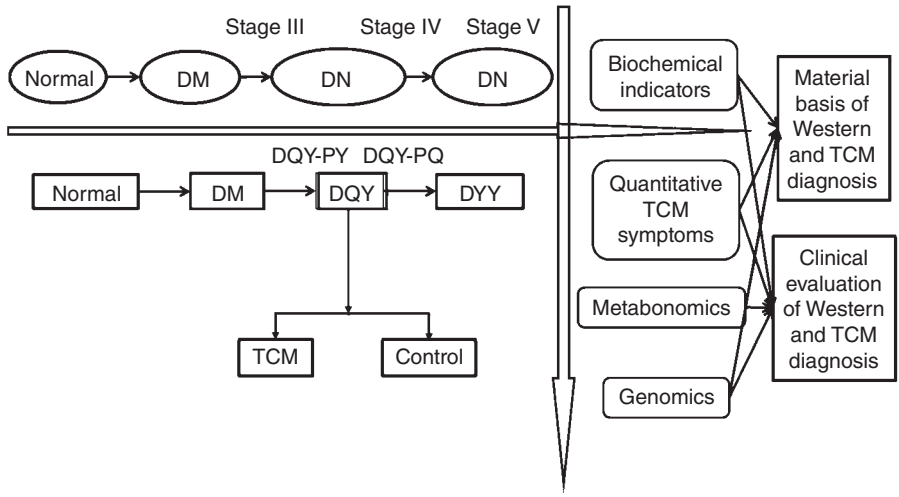

Fig. 9.1 Research map of diabetic nephropathy. DM, diabetes mellitus; DN, diabetic nephropathy; DQY-PY, deficiency of both *qi* and *yin*, particularly *yin* deficiency; DQY-PQ, deficiency of both *qi* and *yin*, particularly *qi* deficiency; DYY, deficiency of both *yin* and *yang*.

with specific skills is needed to navigate the whole itinerary of translational medicine.[15] Research on diseases also has a similar fragmented tendency. Translational medicine will lead to revolutionary changes in Western medical diagnosis, from genetic diagnosis to biological networks (clinical systems biology), including genes, proteins, and metabolites.[12, 13] Since "the most striking innovations often come from combining knowledge across disparate domains . . . [in order] to stimulate innovation, we must intentionally catalyze the mixing of scientists and clinicians from different disciplines,"[13] such as western medicine and TCM, knowing that new approaches will emerge from their interactions. We proposed the integrative biomarker system (IBS), integrating "macroscopic representation" and "microcosmic features," to explore the systematic characteristics of patients and symptoms. IBS is composed of three aspects: clinical biochemical index (combined with molecular imaging technology, if necessary), multilevel biomarkers found in clinical systems biology (including genes, proteins, and metabolites), and quantitative index of TCM syndrome differentiation typing reflected in the overall symptoms. The roadmap of the research is shown in Fig. 9.1.

DN is the most common complication of DM, and is the leading cause of death for patients with diabetes. Epidemiological research data show that about 30% of diabetic patients are associated with DN. DN is kidney damage caused by diabetes, and the specific indication is diabetic glomerulosclerosis. The basic pathological lesions of DN are glomerular basement membrane thickening and mesangial matrix proliferation. Clinical manifestations of the

disease are proteinuria, edema, hypertension, and renal damage, so early diagnosis of DN is extremely important. Previous studies on DN include the relevant functional genes, such as lipid genes[16, 17] and TGFβ[18]; related proteins, such as lipoprotein[19] and apolipoprotein[20]; and related metabolic pathways, such as glycosylation end product[21] and lipid metabolism.[22] Here, we try to establish an integrated biomarker system (IBS) for the diagnosis of DN based on Western Mogensen staging[57] and TCM syndrome typing of syndromes combined with potential biomarkers of genes and metabolites (using metabonomics and genomics). Such an approach is also used in evaluations of efficacy of TCM drugs for DN.

9.2 MOGENSEN STAGING AND TCM TYPING OF DN

Cases considered in the research were not only examined against the criteria of Western staging, but also the standards of TCM typing of DN. Among more than 100 cases, the oldest patient was 88 years old and the youngest was 36 years old. There was a well balanced distribution between male and female, with the ratio of male to female being 1:0.92.

The patients' heights and weights were measured or collected through inquiry, and the BMI value was calculated by the ratio of weight (W) to height squared (H^2). The blood pressure was measured using a standard sphygmomanometer with the patients in the normal sitting posture. The urinary protein excretion was carried out three times within 6 months, and the average was taken as the final result. Other clinical data, such as glycated hemoglobin, fasting glucose, high density lipoprotein, and low density lipoprotein were obtained according to clinical criteria. As the patients' blood and urine samples were to be used in the study of systems biology, and metabonomics study requires high quality samples, in order to improve the quality of obtained samples, samples of urine and blood were collected from patients with empty stomachs in the morning. Degradation is another key issue that has to be solved in dealing with biological samples, as metabonomics study requires the samples to maintain their original characteristics. So it was important to establish a standard of fast, clean, and sterile techniques for handling the specimen for the requirements of the experiment.

9.2.1 Mogensen Staging of DN

Referring to the standards in "The Practical Nephrology" (Shanghai Science and Technology Press, December 1999), patients whose symptoms are consistent with the following requirements can be diagnosed as having DN: the exact history of diabetes; urinary albumin excretion rate being more than 20 μg/min; and repeated detection of urine protein being more than 5 mg/dL. Currently, DN is clinically divided into five phases: patients in stage I, II, and part of stage III belong to the subclinical stage, mainly showing increased glomerular

filtration and hypertrophy, increased renal volume, thickening glomerular basement membrane, mesangial proliferation, and temporary microalbuminuria. Most diabetes patients in the aforementioned stages do not develop into clinical stages of DN. The last three stages have significant practical significance: early stage DN (stage III), also called early stage DN, is signified by urinary albumin excretion rate of 20–200 μg/min with mildly elevated blood pressure and abandoned glomerular; and clinical DN (stage IV), which is characterized by urinary albumin excretion rate of more than 200 μg/min (> 300 mg/24 h), and urinary protein excretion of more than 0.5 g/24 h. Patients with severe clinical DN have daily urine protein excretion of more than 2.0 g, which can be associated with mild microscopic hematuria. The pathological lesion of kidney results in an increased number of glomeruli, decreased renal function, and the emergence of hypertension. Serum creatinine of most patients is not high. Advanced DN (stage V), also called end-stage renal failure, is characterized by widespread capillary occlusion, renal glomerular hyalinosis. During stage V, the glomerular filtration rate becomes very low, and patients develop azotemia, aggravated hypertension, hypoalbuminemia, proteinuria, increased edema, anemia, hyperkalemia, and metabolic acidosis. Patients in stage V often have other microvascular complications, such as retinopathy, peripheral neuropathy, cardiomyopathy, coronary heart disease, cerebrovascular disease, and peripheral vascular disease. These serious complications are often the fatal factors of diabetic uremic patients, and also make it difficult for the replacement therapy in end-stage renal failure patients.

In this study, related professionals selected nearly two hundred hospitalized cases suffering from DN with complete information (gender, age limited) under strict quality control from May 2007 to February 2006 in four hospitals, Beijing University of Chinese Medicine Dongzhimen Hospital, Beijing Sino-Japanese Friendship Hospital, Beijing Medical University Oriental Hospital, and Beijing Guanganmen Hospital. In addition, all the patients were divided into stage III, stage IV, and stage V, according to the Mogenson stages.[57]

9.2.2 TCM Syndrome Typing of DN

In this study, Professor Ping Li from the Sino-Japan Friendship Hospital developed a diabetic nephropathy syndrome questionnaire by reviewing "Diagnostics of Chinese Medicine," TCM diagnosis symptoms, and also previous retrospective studies and expert survey findings, and studied semiquantitative integration of DN with respect to the four diagnostic symptoms. Under strict quality control, the patients were selected according to TCM symptom typing. The TCM syndrome characteristics and evolution principles of patients with DN were summarized using the traditional method, along with cluster analysis, principal component analysis, maximum likelihood factor analysis, and correlation analysis. TCM experts found 10 main symptoms of DN: fatigue, edema, dry mouth, backache, increased urination at night, anorexia, cold intolerance and cold extremities, nausea, dizziness, and urinary turbidity. Symptoms with

high frequencies in the early stages are fatigue, dry mouth, backache, five feverish centers, polyuria, edema, and polydipsia. Symptoms with high frequencies in the middle stages are fatigue, edema, backache, urinary turbidity, dry mouth, dizziness, anorexia, and nocturia. Symptoms with high frequencies in the advanced stage are edema, fatigue, nausea, cold intolerance and cold extremities, vomiting, anorexia, and oliguria.

Most experts believe that the basic pathogenesis of DN is deficiency in origin and excess in superficiality. The deficiency in origin of DN TCM syndrome differentiation includes deficiency of *qi* and *Yin*, deficiency of *Yin* and *Yang*, *qi* deficiency of the spleen and kidney, *Yang* deficiency of the spleen and kidney, and *Yin* deficiency of the liver and kidney. Other symptoms usually occur once to twice, such as *qi* deficiency of the lung and kidney, *Yang* deficiency of the liver and kidney, *Yang* deficiency of the lung and kidney, *Yin* deficiency, *Yang* deficiency, *Yin* deficiency of the heart and spleen, *qi* deficiency of kidney and spleen, and *Yin* deficiency of lung and stomach. The symptoms of excess in superficiality are blood stasis, turbid damp, humid heat, and turbidity. Eventually, 14 experts were involved in syndrome differentiation of the types of DN and diagnosing the patients according to different types of DN. All the samples were divided into the following groups: normal group, DM, *Yin* deficiency with both *qi* and *Yin* deficiency (DQY-PY), *qi* deficiency with both *qi* and *Yin* deficiency (DQY-PQ), and deficiency of *Yin* and *Yang* (DYY).

The pathogenesis of DN is the deficiency of *qi* and *Yin* throughout the disease phases, and eventually develops into the deficiency of *yin* and *Yang*.[23–25] In this study, DN patients were divided according to syndrome differentiation, and classified with 34 symptoms in 9 categories (scored as 0 through 6, where 0 is the mildest, 6 is the most severe), including *yin xu nei re* (*Yin* deficiency with internal heat, YDIH), *shen yin xu* (deficiency of kidney-*Yin*, DK*Yin*), *gan shen yin xu* (deficiency of liver-kidney-*Yin*, DLKY), *pi wei qi xu* (deficiency of spleen-stomach-*qi*, DSSQ), *fei qi xu* (deficiency of lung-*qi*, DLQ), *qi xue xu* (deficiency of both *qi* and blood, DQX), *tan shi* (phlegm-damp, PD), *yu xue* (stagnated blood, SB), *shen yang xu* (deficiency of kidney-*Yang*, DK*Yang*; Table 9.1). Table 9.2 lists the score standard of 21 key symptoms.

This research was approved by the ethics committee. There were 30 healthy adults, 22 diabetes patients, and 61 DN patients selected in the research. In the DN group, 18 patients belonged to stage III, 14 patients were in stage IV, and 29 patients were in stage V (dialysis patients) according to the Mogensen staging. Additionally, with the TCM typing, 17 patients belong to DQY-PY, 21 patients were DQY-PQ, and 23 patients were DYY.

Western diagnostic indexes are primarily based on pathological changes, while TCM diagnosis is mainly based on the overall physiological and pathological changes. For example, urinary protein is the main index of DM and DN, and has significant differences in different Mogensen stages (Table 9.3), but there is no significant difference in different TCM types (Table 9.4). Similarly, serum creatinine and blood urea nitrogen have significant differences at different stages in Western medical diagnostics, while these two can only

TABLE 9.1 Nine Categories of Symptoms in TCM Typing

Category	Symptom
YDIH	*Yan gan kou zao* (pharyngoxerosis, YGKZ); *shou zu xin re* (feverish palms and soles, SZXR); *niao shao se huang* (oliguria and cloudy yellow, NSSH); *pa re hanchu or dao han* (protect against heat or night sweating, PRHC or DH), *xin fan* (fidgetiness, XF); *duo shi shan ji* (Polyphagia with frequent hunger, DSSJ); *kou gan* (xerostomia, KG)
DK*Yin*	*Yao xi suan ruan* (soreness and weakness of waist and knees, YXSR); *jian wang* (forgetfulness, JW); *yao tong* (lumbago, YT)
DLKY	*Tou yun* (dizziness, TY); *tou tong* (headache, TT); *bian mi* (constipation, BM); *kou ku* (bitterness in the mouth, KK)
DSSQ	*Shi shao na dai* (anorexia, SSND); *da bian bu shi* (dry, hard, and infrequent stools, DBBS); *wan fu zhang man* (epigastric distension, WFZM)
DLQ	*Juan dai fa li* (lassitude, JDFL); *qi duan lan yan* (lack of *qi* and no desire to speak, QDLY); *Yin huan gan mao* (easily suffer from common cold, YHGM)
DQX	*Mian se wu hua* (lusterless complexion, MSWH); *chun jia se dan* (pale lip and fingernails); *shou zu chi zong* (tetany, SZCZ); *chou chu jing jue* (convulsions, CCJJ)
PD	*E xin* (nausea, EX); *ou tu* (vomiting, OT); *zhi ti kun zhong* (drowsy body, ZTKZ); *kou zhong nian ni* (sticky mouth, KZNN)
SB	*Ji fu jia cuo* (squamous and dry skin, JFJC); *zhi ti ma mu* (numbness in limbs, ZTMM); *mian se hui an* (gloomy complexion, MSHA)
DK*Yang*	*Wei han zhi leng* (cold intolerance and cold extremities, WHZL); *fu zhong* (edema, FZ); *ye niao pin* (nocturia, YNP)

distinguish the lightest DQY-PY and the most severe DYY in TCM symptom typing.

Yin xu nei re (*Yin* deficiency with internal heat, YDIH) got the highest score in the DQY-PY group. The main symptom of YDIH is *yan gan kou zao* (pharyngoxerosis, YGKZ). *Fei qi xu* (deficiency of lung-*qi*, DLQ) got the highest score in DQY-PQ, and the main symptoms of it are *juan dai fa li* (lassitude, JDFL) and *qi duan lan yan* (lack of *qi* and no desire to speak, QDLY). Additionally, *shen Yang xu* (deficiency of kidney-*Yang*, DK*Yang*) got the highest score in DYY, and the main symptoms of it are *wei han zhi leng* (cold intolerance and cold extremities, WHZL) and *fu zhong* (edema, FZ) (Table 9.5). The score for YDIH, especially for the symptom *Shou zu xin re* (feverish palms and soles, SZXR), was significantly lower, and the score for DLQ, especially for symptoms JDFL and QDLY, was significantly higher in DQY-PQ when compared to DQY-PY. The score of DK*Yang* was significantly higher in DYY than the other two groups, especially for symptoms WHZL and FZ. These results indicated that the developmental trend of DN was consistent with TCM theory—DN begins with deficiency of *Yin*, develops into deficiency of *qi*, and

TABLE 9.2 Typing Standard for 21 Main Symptoms

Category	Symptom	Score: 0	Mild (Score: 2)	Middle (Score: 4)	Severe (Score: 6)
YDIH	YGKZ	Negative	Occasional	Preference for water	Polydipsia
	SZXR	Negative	Occasional	Sometimes	Continuous
	PRHC or DH	Negative	Protect against heat slightly, sweating slightly, or occasional night sweating	Protect against heat, sweating sometimes, or night sweating frequently	Protect against heat greatly, sweating at intervals, or night sweating every night
	XF	Negative	Occasional	Irritability, restlessness and inquietude, still capable of self-control	Irritability, restlessness and inquietude, hardly capable of self-control
DK*Yin*	YXSR	Negative	Occasional	Sometimes	Everyday
DLKY	BM	Negative	Defecate with effort	Stool induration, every 2 or 3 days	Stool induration, once in more than 3 days
DSSQ	SSND	Negative	Eating decrease slightly	Eating decrease obviously	Almost aphagosis
	WFZM	Negative	Occasional	Sometimes	Continuous
DLQ	JDFL	Negative	Vigor and toleration decrease, without affecting daily life	Lassitude, feel lack of energy after common activities	Lassitude, feel lack of energy even after rest
	QDLY	Negative	Short breath after activities, no desire to speak in normal time	Sometimes short breath at rest, no desire to speak	Continuing short breath at rest, low tone, weak to talk
	YHGM	Negative	Having a cold more than 6 times every year	Having a cold more than 10 times every year	Having a cold more than 12 times every year

DQB	MSWH	Negative	Pale white	Pale white without luster	Pale white or etiolate
	CJSD	Negative	Lip and fingernail less rosy	Pale lip and fingernail without luster	Pale white lip and fingernail without luster
PD	EX or OT	Negative	Occasional	Sometimes feeling nausea, vomiting occasionally	Constant nausea, vomiting sometimes
	ZTKZ	Negative	Slightly tired, lassitude and lack of energy after activities	Obviously tired, lack of energy at rest	Heavy cumbersome limbs, constant lack of energy, with no relief
	KZNN	Negative	Feel slightly sticky in mouth	Feel sticky in mouth and a little malaise, taste abnormal flavor occasionally, want to drink	Feel sticky in mouth and extraordinary malaise, gargling at intervals
SB	JFJC	Negative	Localized asperity on muscle and skin, aridity, lack of luster	Asperity, aridity, cornification, desquamation on different part of muscle and skin	Aridity, cornification, desquamation on all parts of skin
	ZTMM	Negative	Dead limbs	Numbness of limbs	Numbness of body
DK*Yang*	WHZL	Negative	Feel a little chilly, limbs cool	Feel obviously chilly, limbs cold	Feel utmost chilly, limbs cold as ice
	FZ	Negative	Slight hydrops in palpebra and ankle	Obvious hydrops on facial surface and ower extremity	Hydrops on facial surface and ower extremity, or ascites in chest
	YNP	Negative	Nocturia a little frequently, 2 times every night	Nocturia frequently and much in volume, 2 or 3 times every night	Nocturia frequently and much in volume, more than 3 times every night

TABLE 9.3 Basic Information and Clinical Biochemistry Indexes of DM and DN Patients

Index	DM	DN	DN III	DN IV	DN V
Gender (male/female)	22 (11/11)	61 (37/24)	18 (7/11)	14 (7/7)	29 (23/6)
Age (year)	59.27 ± 3.80	58.41 ± 9.05	57.06 ± 3.43	58.14 ± 4.73	59.37 ± 3.68
BMI	24.14 ± 1.13	24.88 ± 3.45	25.98 ± 1.62	23.44 ± 1.90	24.88 ± 1.15
DM history (year)	10.09 ± 2.02	13.41 ± 7.35*	12.33 ± 2.80	10.36 ± 3.50	15.55 ± 2.88°
Contractive pressure (mmHg)	131.1 ± 5.9	143.9 ± 23.2**	128.1 ± 5. 9	135.9 ± 7.0	157.6 ± 8.8$^{\circ\circ}$
Diastolic pressure (mmHg)	77.64 ± 3.38	78.80 ± 10.73	77.67 ± 2.91	77.64 ± 4.36	80.07 ± 4.98
Urine protein mg/24 h	0.11 ± 0.16	2343 ± 690**	118.1 ± 35.6	2376 ± 988$^{\triangle\triangle}$	4177 ± 997$^{\triangle}$
Urea nitrogen (mmol/L)	5.50 ± 0.54	15.45 ± 10.42**	5.85 ± 1.03	10.23 ± 2.64$^{\triangle\triangle}$	23.93 ± 3.05$^{\circ\circ}$
Serum creatinine (μmol/L)	79.75 ± 7.09	386.1 ± 356.9**	84.07 ± 5.75	180.8 ± 120.4	672.6 ± 105.2$^{\circ\circ}$
Glycosylated hemoglobin (%)	8.06 ± 0.47	8.86 ± 2.34	9.55 ± 1.11	8.61 ± 1.41	8.24 ± 0.64
Fasting blood glucose (mmol/L)	8.05 ± 1.18	7.30 ± 2.46	8.13 ± 1.56	7.19 ± 0.83	6.84 ± 0.75
Cholesterol (mmol/L)	5.34 ± 0.64	5.15 ± 1.57	4.90 ± 0.41	5.74 ± 0.90	5.02 ± 0.65
Triglyceride (mmol/L)	2.52 ± 1.40	2.28 ± 1.60	2.16 ± 0.64	2.24 ± 0.83	2.36 ± 0.64
High density lipoprotein (mmol/L)	1.11 ± 0.11	1.19 ± 0.39	1.16 ± 0.16	1.29 ± 0.15	1.16 ± 0.16
Low density lipoprotein (mmol/L)	3.26 ± 0.35	2.88 ± 1.21	2.82 ± 0.27	3.27 ± 0.70	2.74 ± 0.51

*$p < 0.05$,
**$p < 0.01$ between DM and DN;
$^{\triangle}p < 0.05$,
$^{\triangle\triangle}p < 0.01$ between DN IV and DN III;
$^{\circ}p < 0.05$,
$^{\circ\circ}p < 0.01$ between DN V and DN IV.

TABLE 9.4 Basic Information and Clinical Biochemistry Indexes of Different TCM Types

Index	DM	DQY-PY	DQY-PQ	DYY
Gender (male/female)	22 (11/11)	17 (9/8)	21 (14/7)	23 (14/9)
Age (year)	59.27 ± 3.80	58.00 ± 3.64	57.20 ± 3.30	60.28 ± 3.56
BMI	24.14 ± 1.13	24.54 ± 1.34	24.74 ± 1.39	25.47 ± 1.34
DM history (year)	10.09 ± 2.02	12.16 ± 4.45	12.48 ± 2.88	14.38 ± 2.75
Contractive pressure (mmHg)	131.1 ± 5.9	134.7 ± 6.4	136.3 ± 7.6	147.4 ± 8.8*
Diastolic pressure (mmHg)	77.64 ± 3.38	77.53 ± 3.40	79.24 ± 4.23	76.17 ± 3.49
Urine protein mg/24 h	0.11 ± 0.16	1104 ± 860	2801 ± 1356	2474 ± 883
Urea nitrogen (mmol/L)	5.50 ± 0.54	10.06 ± 3.37	14.00 ± 3.77	17.01 ± 3.98*
Serum creatinine (μmol/L)	79.75 ± 7.09	240.6 ± 135.6	323.5 ± 129.6	451.8 ± 133.1*
Glycosylated hemoglobin (%)	8.06 ± 0.47	8.72 ± 1.27	9.12 ± 0.92	7.29 ± 0.76$^{\triangle}$
Fasting blood glucose (mmol/L)	8.05 ± 1.18	7.59 ± 1.15	7.51 ± 1.10	7.21 ± 0.77
Cholesterol (mmol/L)	5.34 ± 0.64	5.25 ± 0.76	5.12 ± 0.47	5.10 ± 0.35
Triglyceride (mmol/L)	2.52 ± 1.40	2.10 ± 0.77	1.98 ± 0.45	2.13 ± 0.52
High density lipoprotein (mmol/L)	1.11 ± 0.11	1.25 ± 0.13	1.18 ± 0.15	1.26 ± 0.16
Low density lipoprotein (mmol/L)	3.26 ± 0.35	2.95 ± 0.49	3.03 ± 0.41	2.79 ± 0.40

*$p < 0.05$ between DYY and DQY-PY;
$^{\triangle}p < 0.05$ between DYY and DQY-PQ.

TABLE 9.5 Comparison of Symptom Scores Among Different TCM Types in DN Patients

Symptom		DM	DQY-PY	DQY-PQ	DYY
YDIH	YGKZ	2.55 ± 0.61	2.56 ± 0.91	2.09 ± 0.72	2.07 ± 0.74
	SZXR	0.97 ± 0.50	2.00 ± 0.81	0.35 ± 0.40**	0.97 ± 0.40$^{\triangle,\circ}$
	NSSH	0.66 ± 0.30	0.61 ± 0.27	0.39 ± 0.24	0.57 ± 0.22
	PRHC or DH	0.59 ± 0.36	1.06 ± 0.45	0.56 ± 0.32	0.47 ± 0.22$^{\triangle}$
	XF	0.31 ± 0.26	0.89 ± 0.34	0.83 ± 0.36	0.67 ± 0.30
	DSSJ	0.45 ± 0.30	0.33 ± 0.34	0.26 ± 0.28	0.40 ± 0.29
	KG	0.76 ± 0.37	1.50 ± 0.54	1.26 ± 0.46	1.47 ± 0.44
	Normalized total score	0.90 ± 0.18	1.28 ± 0.34	0.82 ± 0.23*	0.94 ± 0.20$^{\triangle\triangle}$
DK*Yin*	YXSR	0.69 ± 0.52	1.56 ± 0.72	2.17 ± 0.78	2.53 ± 0.73
	JW	0.38 ± 0.31	0.39 ± 0.35	0.70 ± 0.29	0.37 ± 0.29
	YT	0.45 ± 0.23	0.78 ± 0.29	0.61 ± 0.32	0.93 ± 0.25
	Normalized total score	0.50 ± 0.26	0.91 ± 0.30	1.16 ± 0.34	1.28 ± 0.31$^{\triangle}$
DLKY	TY	0.59 ± 0.30	0.72 ± 0.40	0.89 ± 0.38	0.80 ± 0.34
	TT	0.28 ± 0.26	0.44 ± 0.38	0.35 ± 0.40	0.60 ± 0.38
	BM	0.93 ± 0.38	0.83 ± 0.47	0.83 ± 0.42	1.03 ± 0.46
	KK	0.24 ± 0.23	0.50 ± 0.23	0.30 ± 0.23	0.23 ± 0.15
	Normalized total score	0.51 ± 0.20	0.62 ± 0.20	0.59 ± 0.22	0.67 ± 0.21
DSSQ	SSND	0.07 ± 0.14	0.11 ± 0.21	1.39 ± 0.76**	0.80 ± 0.52
	DBBS	0.21 ± 0.20	0.22 ± 0.25	0.22 ± 0.24	0.50 ± 0.31
	WFZM	0.10 ± 0.11	0.17 ± 0.17	0.26 ± 0.22	0.37 ± 0.33
	Normalized total score	0.13 ± 0.09	0.17 ± 0.11	0.62 ± 0.29*	0.56 ± 0.22$^{\triangle}$
DLQ	JDFL	1.79 ± 0.56	1.22 ± 0.63	3.22 ± 0.77**	3.20 ± 0.67$^{\triangle\triangle}$
	QDLY	1.59 ± 0.49	0.89 ± 0.70	2.09 ± 0.58*	1.60 ± 0.58
	YHGM	0.28 ± 0.31	0.22 ± 0.19	0.09 ± 0.12	0.17 ± 0.21
	Normalized total score	1.22 ± 0.39	0.78 ± 0.40	1.80 ± 0.38**	1.66 ± 0.35$^{\triangle\triangle}$

DQX	MSWH	0.28 ± 0.22	0.61 ± 0.41	0.70 ± 0.45	0.87 ± 0.35
	CJSD	0.34 ± 0.24	0.50 ± 0.35	0.78 ± 0.39	1.20 ± 0.34$^{\triangle}$
	SZCZ	0.21 ± 0.22	0.33 ± 0.31	0.22 ± 0.21	0.80 ± 0.37$^{\bigcirc}$
	CCJJ	0.14 ± 0.21	0.33 ± 0.31	0.17 ± 0.16	0.20 ± 0.14
	Normalized total score	0.24 ± 0.14	0.44 ± 0.24	0.47 ± 0.18	0.77 ± 0.21$^{\bigcirc}$
PD	EX	0	0.11 ± 0.14	0.22 ± 0.21	0.53 ± 0.31
	OT	0	0	0.04 ± 0.08	0.17 ± 0.16
	ZTKZ	0.28 ± 0.19	0.39 ± 0.31	1.04 ± 0.42*	1.00 ± 0.35$^{\triangle}$
	KZNN	0.17 ± 0.14	0.28 ± 0.21	0.30 ± 0.26	0.30 ± 0.19
	Normalized total score	0.11 ± 0.06	0.19 ± 0.11	0.40 ± 0.16	0.50 ± 0.16
SB	JFJC	0.28 ± 0.19	0.61 ± 0.38	0.43 ± 0.30	0.97 ± 0.32$^{\bigcirc}$
	ZTMM	0.48 ± 0.21	0.72 ± 0.30	0.83 ± 0.26	0.90 ± 0.24
	MSHA	0.62 ± 0.38	1.11 ± 0.63	1.83 ± 0.60	2.00 ± 0.65
	Normalized total score	0.46 ± 0.20	0.81 ± 0.30	1.03 ± 0.24	1.29 ± 0.33
DK*Yang*	WHZL	0.72 ± 0.44	0.44 ± 0.49	0.52 ± 0.44	2.67 ± 0.63$^{\triangle\triangle, \bigcirc\bigcirc}$
	FZ	0.93 ± 0.50	0.78 ± 0.45	1.30 ± 0.68	1.73 ± 0.59$^{\triangle}$
	YNP	0.83 ± 0.38	0.89 ± 0.40	1.09 ± 0.32	0.80 ± 0.24
	Normalized total score	0.83 ± 0.32	0.70 ± 0.22	0.97 ± 0.32	1.73 ± 0.31$^{\triangle\triangle, \bigcirc\bigcirc}$
Total score	Normalized total score	4.90 ± 1.21	5.91 ± 1.04	7.86 ± 1.19*	9.39 ± 1.26$^{\triangle\triangle}$

Normalized total score: Sum of symptom scores for each category/number of symptoms;
$^{*}p < 0.05$,
$^{**}p < 0.01$ between DQY-PQ and DQY-PY;
$^{\triangle}p < 0.05$,
$^{\triangle\triangle}p < 0.01$ between DYY and DQY-PY;
$^{\bigcirc}p < 0.05$,
$^{\bigcirc\bigcirc}p < 0.01$ between DYY and DQY-PQ.

finally leads to *Yin* damage that extends to *Yang*, causing deficiency in both *Yin* and *Yang*. Additionally, the significantly increased total score of nine categories indicated that the disease was getting more severe.

DLQ got the highest score in DM and its main symptoms are JDFL and QDLY. YDIH and DK*Yang* got the two highest scores in DN III, and its main symptoms are YGKZ, WHZL, and *ye niao pin* (nocturia, YNP). *Shen Yin xu* (deficiency of kidney-*Yin*, DK*Yin*), DLQ, and DK*Yang* got the three highest scores in DN IV. The main symptoms of them are *yao xi suan ruan* (soreness and weakness of waist and knees, YXSR), JDFL, WHZL, and FZ. DLQ, *yu xue* (stagnated blood, SB), and DK*Yang* got the three highest scores in DN V, and its main symptoms are JDFL, *mian se hui an* (gloomy complexion, MSHA), WHZL, and FZ (Table 9.6). These previous results showed the same tendency with TCM typing: from *Yin xu* (deficiency of *Yin*, DY), *Qi xu* (deficiency of *qi*, DQ), to *Yang xu* (deficiency of *Yang*, DY). For example, SB (MSHA and *zhi ti ma mu* [numbness in limbs, ZTMM]), DK*Yin* (YXSR and *yao tong* [lumbago, YT]), and DK*Yang* (WHZL) scores showed significant differences between DM and DN. These five symptoms may be used for auxiliary support in diagnosis of DM and DN. However, TCM symptoms could not distinguish the three different stages well. Even the scores of these three categories increased as the disease processed, there was no significant difference, which indicated the existence of a difference between Western medical and TCM diagnostic methods. TCM focuses on global symptoms and states, emphasizing syndrome differentiation, while Western medicine focuses on pathological characteristics, emphasizing the target organs.

9.3 THE METABONOMICS STUDY OF DN

Since DN is affected by multiple factors and multiple metabolic pathways, our laboratory developed an integrated approach combining the macroscopic and microcosmic modes, called integrative metabonomics platform technology (IMPT). IMPT was described in Chapter 7. The macroscopic mode is represented by a metabolic fingerprint, which can generally illustrate the overall outline used to find the unknown biomarkers. The microcosmic mode is represented by the accurate quantification of multiple metabolic pathways, which mainly focuses on the important metabolic cycles and verifies the key biomarkers and study of time and space variations. The combination of these two modes can achieve more comprehensive and accurate data of metabonomics in complex systems.

9.3.1 Integrated Metabonomics Study Based on Mogensen Staging of DN

The important metabolites from metabolic fingerprints and four pathways, including phospholipids, fatty acids, purine and pyrimidine, and aminothiols metabolisms were detected and analyzed in the study.

TABLE 9.6 Comparison of Symptom Scores Among DM and Different Stages of DN

Symptom		DM	DN	DN III	DN IV	DN V
YDIH	YGKZ	2.55 ± 0.61	2.18 ± 0.44	2.75 ± 0.74	1.70 ± 0.82	2.06 ± 0.69
	SZXR	0.97 ± 0.50	1.01 ± 0.31	1.58 ± 0.74	0.80 ± 0.44	0.72 ± 0.38
	NSSH	0.66 ± 0.30	0.50 ± 0.13	0.46 ± 0.26	0.55 ± 0.26	0.50 ± 0.20
	PRHC or DH	0.59 ± 0.36	0.63 ± 0.18	0.96 ± 0.34	0.55 ± 0.36	0.44 ± 0.23
	XF	0.31 ± 0.26	0.75 ± 0.18*	1.00 ± 0.29	0.55 ± 0.36	0.69 ± 0.30
	DSSJ	0.45 ± 0.30	0.39 ± 0.18	0.50 ± 0.35	0.50 ± 0.39	0.25 ± 0.23
	KG	0.76 ± 0.37	1.36 ± 0.26*	1.63 ± 0.33	0.85 ± 0.41△△	1.47 ± 0.50
	Normalized total score	0.90 ± 0.18	0.98 ± 0.14	1.27 ± 0.24	0.78 ± 0.23△△	0.87 ± 0.22
DK*Yin*	YXSR	0.69 ± 0.52	2.29 ± 0.43**	2.25 ± 0.68	2.60 ± 0.90	2.12 ± 0.68
	JW	0.38 ± 0.31	0.47 ± 0.17	0.42 ± 0.29	0.55 ± 0.30	0.47 ± 0.29
	YT	0.45 ± 0.23	0.80 ± 0.16*	0.92 ± 0.26	0.85 ± 0.36	0.69 ± 0.24
	Normalized total score	0.50 ± 0.26	1.17 ± 0.18**	1.19 ± 0.27	1.30 ± 0.40	1.07 ± 0.29
DLKY	TY	0.59 ± 0.30	0.82 ± 0.20	1.04 ± 0.36	0.75 ± 0.31	0.69 ± 0.35
	TT	0.28 ± 0.26	0.47 ± 0.22	0.92 ± 0.47	0.30 ± 0.43	0.25 ± 0.23
	BM	0.93 ± 0.38	0.86 ± 0.25	0.67 ± 0.40	0.75 ± 0.45	1.06 ± 0.44
	KK	0.24 ± 0.23	0.32 ± 0.11	0.67 ± 0.22	0.35 ± 0.21	0.03 ± 0.06○○
	Normalized total score	0.51 ± 0.20	0.63 ± 0.12	0.82 ± 0.22	0.55 ± 0.23	0.52 ± 0.17
DSSQ	SSND	0.07 ± 0.14	0.79 ± 0.39**	0.42 ± 0.41	1.00 ± 0.72	0.94 ± 0.56
	DBBS	0.21 ± 0.20	0.39 ± 0.17	0.29 ± 0.22	0.50 ± 0.39	0.41 ± 0.29
	WFZM	0.10 ± 0.11	0.29 ± 0.15	0.21 ± 0.17	0.20 ± 0.18	0.41 ± 0.33
	Normalized total score	0.13 ± 0.09	0.50 ± 0.14**	0.30 ± 0.15	0.60 ± 0.28	0.58 ± 0.26
DLQ	JDFL	1.79 ± 0.56	2.79 ± 0.43*	2.33 ± 0.61	2.60 ± 0.90	3.25 ± 0.70
	QDLY	1.59 ± 0.49	1.55 ± 0.36	1.50 ± 0.59	1.20 ± 0.60	1.81 ± 0.62
	YHGM	0.28 ± 0.31	0.14 ± 0.10	0.17 ± 0.15	0.15 ± 0.16	0.12 ± 0.19
	Normalized total score	1.22 ± 0.39	1.48 ± 0.22	1.33 ± 0.32	1.24 ± 0.46	1.74 ± 0.37

(*Continued*)

TABLE 9.6 (*Continued*)

Symptom		DM	DN	DN III	DN IV	DN V
DQX	MSWH	0.28 ± 0.22	0.78 ± 0.22*	0.38 ± 0.33	0.85 ± 0.46	1.03 ± 0.30
	CJSD	0.34 ± 0.24	0.89 ± 0.21**	0.12 ± 0.14	0.85 ± 0.43$^{\triangle\triangle}$	1.50 ± 0.68$^{\bigcirc}$
	SZCZ	0.21 ± 0.22	0.47 ± 0.18	0.21 ± 0.16	0.55 ± 0.30$^{\triangle}$	0.62 ± 0.37
	CCJJ	0.14 ± 0.21	0.22 ± 0.11	0.21 ± 0.20	0.25 ± 0.19	0.22 ± 0.17
	Normalized total score	0.24 ± 0.14	0.60 ± 0.12**	0.23 ± 0.13	0.66 ± 0.24$^{\triangle\triangle}$	0.85 ± 0.19
PD	EX	0	0.33 ± 0.15*	0.25 ± 0.24	0.15 ± 0.16	0.50 ± 0.29
	OT	0	0.10 ± 0.08	0.08 ± 0.11	0.10 ± 0.13	0.13 ± 0.15
	ZTKZ	0.28 ± 0.19	0.86 ± 0.21**	0.79 ± 0.31	0.80 ± 0.39	0.94 ± 0.37
	KZNN	0.17 ± 0.14	0.32 ± 0.12	0.17 ± 0.15	0.50 ± 0.27$^{\triangle}$	0.31 ± 0.20
	Normalized total score	0.11 ± 0.06	0.40 ± 0.09**	0.32 ± 0.13	0.38 ± 0.16	0.47 ± 0.17
SB	JFJC	0.28 ± 0.19	0.72 ± 0.20*	0.46 ± 0.26	0.75 ± 0.34	0.91 ± 0.37
	ZTMM	0.48 ± 0.21	0.82 ± 0.15*	0.83 ± 0.25	0.85 ± 0.36	0.78 ± 0.21
	MSHA	0.62 ± 0.38	1.63 ± 0.36**	0.92 ± 0.53	1.60 ± 0.67	2.19 ± 0.59
	Normalized total score	0.46 ± 0.20	1.08 ± 0.18**	0.74 ± 0.24	1.12 ± 0.31	1.31 ± 0.30
DK*Yang*	WHZL	0.72 ± 0.44	1.41 ± 0.39*	1.50 ± 0.72	1.15 ± 0.66	1.50 ± 0.63
	FZ	0.93 ± 0.50	1.39 ± 0.35	1.00 ± 0.53	1.60 ± 0.73	1.56 ± 0.58
	YNP	0.83 ± 0.38	0.89 ± 0.17	1.17 ± 0.30	0.85 ± 0.29	0.72 ± 0.27
	Normalized total score	0.83 ± 0.32	1.24 ± 0.20*	1.22 ± 0.32	1.23 ± 0.39	1.27 ± 0.33
Total Score	Normalized total score	4.90 ± 1.21	8.07 ± 0.74**	7.43 ± 0.90	7.86 ± 1.53	8.69 ± 1.31

Normalized total score: Sum of symptom scores for each category/number of symptoms;
*$p < 0.05$,
**$p < 0.01$ between DN and DM;
$^{\triangle}p < 0.05$,
$^{\triangle\triangle}p < 0.01$ between DN stage IV and DN stage III;
$^{\bigcirc}p < 0.05$,
$^{\bigcirc\bigcirc}p < 0.01$ between DN stage V and DN stage IV.

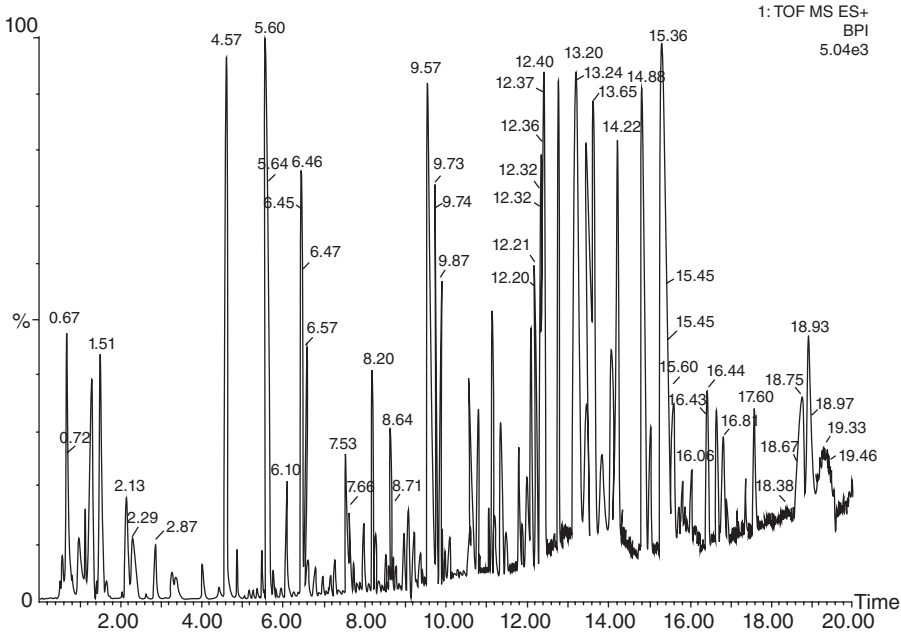

Fig. 9.2 Representative metabolic fingerprint of plasma sample using UPLC-TOF/MS.

9.3.1.1 Metabolic Fingerprint Research of DN Metabolic fingerprints of samples from controls, DM, DN III, DN IV, and DN V groups were analyzed using UPLC-TOF/MS. Chromatography obtained by Waters Acquity BEH C_{18} column (100 × 2.1 mm, 1.7 μm, Waters, MA, USA), and the column temperature was maintained at 30°C with a flow rate of 0.4 mL/min. Autosampler temperature was set to 4°C, and 10 μL was injected into the column. The injection was repeated three times per sample. The mobile phase was contained 0.2% formic acid in phase A and acetonitrile in phase B.[26] The representative chromatography of samples is shown in Fig. 9.2.

9.3.1.1.1 Pattern Recognition by PCA After peak matching, peak alignment, peak extraction, and normalization using MarkerLynx software (Waters), UPLC-MS data of all the plasma samples were processed with SIMCA-P software for PCA analysis, and the score plot is shown in Fig. 9.3.

It can be seen from the score plot that there is a clustering tendency among these groups. The metabolites in patients had significant changes as the disease prolonged, which can be seen from the form of data clustering. In addition, the plot also showed that the control, DM, DN III, and DN IV groups were relatively close, while the samples in DN V were much scattered, which indicated that various complications were different and the individual differences of the patients increased with the development of the disease.

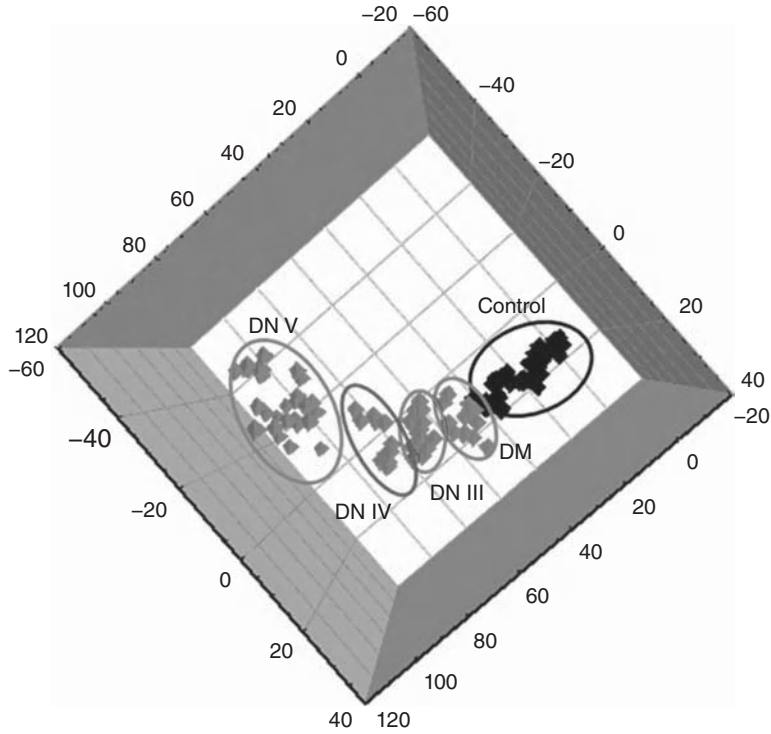

Fig. 9.3 PCA score plot of all the plasma fingerprints.

According to the loading plot, the metabolites with important contributions to the classification can be found. Metabolites that are farther from the center have greater contributions to the classification. These metabolites can be considered to be potential biomarkers associated with the disease. As the compounds 496.34, 251.06, 331.22, 520.34, 524.37, 522.36, 347.22, 130.05, 209.12, 544.34, 546.35, and 568.34, located far from the origin, these compounds may be of great significance in the development of the disease.

The significant metabolites after analysis were examined for their possible formulas using the i-FIT function in the MassLynx software. Then compounds with good isotopic matching and small deviations of exact mass values may be the correct compounds. The main databases used in the metabonomics study were: http://www.pubchem.ncbi.nlm.nih.gov, http://www.genome.jp/kegg/ligand.htmL, and http://redpoll.pharmacy.ualberta.ca/ ~ aguo /www_hmdb_ca /HMDB/.

Take m/z = 496.34 as an example to illustrate the process of potential biomarker identification in the metabonomics study. First, an accurate exact mass (m/z was 496.3409) was obtained, and then the possible elements were obtained using the i-Fit function. The results showed that C31H47NO2P had the lowest value of i-Fit and minimum deviation. Then the MS/MS analysis was carried

TABLE 9.7 List of Potential Biomarkers from PCA

NO.	m/z	Elemental Composition $[M+1]^+$	Biomarker Identification
1	496.34	$C_{24}H_{51}NO_7P$	Lyso-PC(16:0)
2	251.06	UN	UN
3	331.22	$C_{21}H_{31}O_3$	Deoxycorticosterone
4	520.34	$C_{26}H_{51}NO_7P$	Lyso-PC(18:2)
5	524.37	$C_{26}H_{55}NO_7P$	Lyso-PC(18:0)
6	522.36	$C2_{26}H_{53}NO_7P$	Lyso-PC(18:1)
7	347.22	$C_{21}H_{31}O_4$	Corticosterone
8	130.05	$C_5H_8NO_3$	Pidolic acid
9	209.12	$C_{10}H_{13}N_2O_3$	L-kynurenine
10	544.34	$C_{28}H_{51}NO_7P$	Lyso-PC(20:4)
11	546.35	$C_{28}H_{53}NO_7P$	Lyso-PC(20:3)
12	568.34	$C_{30}H_{51}NO_7P$	Lyso-PC(22:6)

UN: Unknown compound.

out. The main peak of 184.08 fragments was obtained here. Later, m/z = 496.34 was input into the database (http://redpoll.pharmacy.ualberta.ca/ ~ aguo / www_ hmdb_ca/HMDB/), and Lyso-PC (16:0) with the elemental composition of C24H51NO7P was the only compound among 69 compounds that was also consistent with the features of neutral loss of m/z 184.08 (choline moiety) in MS/MS.[27]

As described above, compounds of significant difference among the groups were identified, and the potential biomarkers are listed in Table 9.7.

9.3.1.1.2 Pattern Recognition by PLS-DA All the data were analyzed using a supervised method to screen for potential biomarkers. Therefore, the data processed by MarkerLynx would be imported into the SIMCA-P software for the PLS-DA analysis, and the score plot is shown in Fig. 9.4. It can be seen from this figure that samples in the DN V group can be completely separated from the other four groups, but the separation of DM, DN III, and DN IV was not very good. Thus, these three groups and the control groups were analyzed, and the result is shown as follows (Fig. 9.5).

According to the VIP values from PLS-DA and t-test ($p < 0.05$), compounds of significant differences were screened out among groups. Table 9.8 lists the compounds identified with the method described above.

9.3.1.1.3 Biological Significance of the Potential Biomarkers LysoPhosphatidylcholine (Lyso-PC) is a group of phospholipids containing only one fatty acid. Its concentration in vivo is very low, but its function is very important. Different from phospholipids, which contain two fatty acids and some lipophilic phospholipids, Lyso-PC is balanced between its lipophilicify and hydrophilicify. Lyso-PC also has a balance between water and nonwater layers.

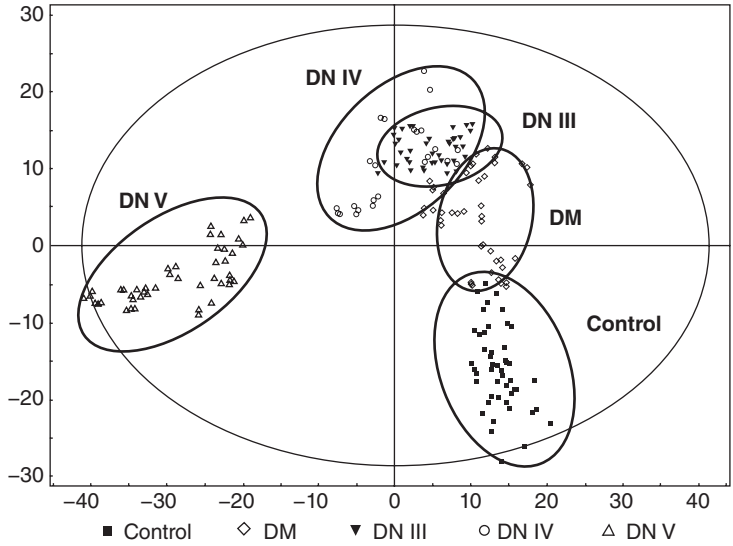

Fig. 9.4 Score plot of all the plasma fingerprints using PLS-DA.

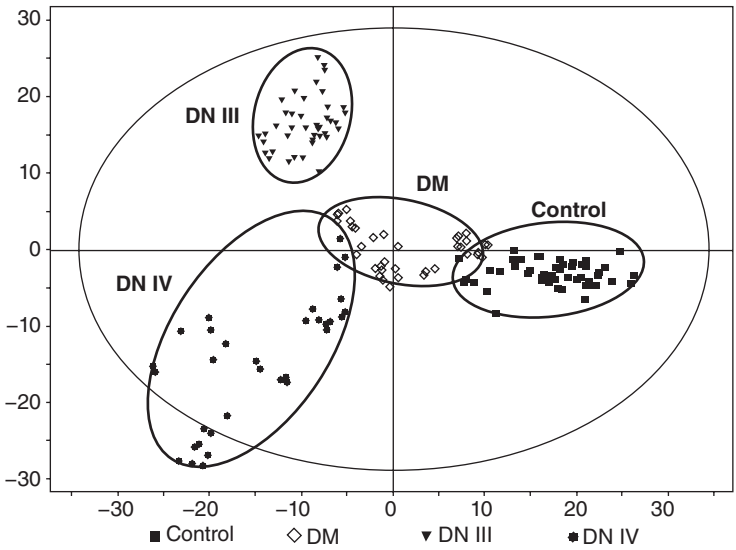

Fig. 9.5 Score plot of plasma fingerprints of DM, DN III, DN IV, and control groups using PLS-DA.

TABLE 9.8 List of Potential Biomarkers from PLS-DA

NO.	m/z	Elemental Composition $[M+1]^+$	Biomarker Identification
1	118.08	$C_5H_{11}NO_2$	L-Valine
2	327.20	UN	UN
3	130.05	$C_5H_8NO_3$	Pidolic acid
4	464.31	UN	UN
5	132.08	$C_6H_{14}NO_2$	Leucine / iso leucine
6	205.09	$C_{11}H_{13}N_2O_2$	L-tryptophan

These special characteristics increase the solubility and purification of phospholipids and also accelerate the rate of exchange between cytoplasm and membrane. Therefore, Lyso-PCs will damage red blood cells in vivo.

Lyso-PC can augment inflammation in many diseases. Lyso-PCs inhibit glucose transport by binding with the glucose membrane transporters. Moreover, Lyso-PCs inhibit the insulin-stimulated glucose metabolism through the activation of PKC-α pathway to promote the formation of peroxide ions in blood, resulting in increased oxidative stress. Studies[10, 28] have shown that damages of oxidative stress on renal tissues include: attack of the body's fatty acids, resulting in the formation of lipid peroxidation products and physiological damage of renal tissues; damage of kidney cells and DNA in the mitochondria; formation of lipid peroxidation products in the red blood cell membrane, reducing fluidity of the membrane and increasing adhesion of endothelial cells; and decreased levels of hyaluronic acid in the connective tissue and loss of viscosity, as well as increased vascular permeability.

Lyso-PC has been reported to inhibit tyrosine phosphorylation by activating JNK or PKC pathways, thereby inhibiting insulin signaling. Increased activity of PKC causes glomerular hyperperfusion, hyperfiltration, and increased synthesis of glomerular extracellular matrix. Recent studies have shown that PKC activation could upregulate the expression of cell adhesion molecules in mesangial cells and promote leukocyte adhesion that causes glomerular accumulation, resulting in accelerated glomerular injury.[5]

Lyso-PC can also inhibit Na^+, K^+-, and ATPase activity, thereby leading to abnormalities in cell structure and function, and ultimately leading to hemodynamic disorder and direct impact on the glomerular and tubular function. Na^+, K^+-, and ATPase are ubiquitous in the cell membrane and are involved in many biochemical processes. Its activity may be associated with the occurrence of many diseases. The transformation trend of Lyso-PC levels was consistent with the quantitative results reported in the literature.[29]

Deoxycorticosterone is a typical kind of mineralocorticoid hormone, while corticosterone acts as both mineralocorticoid and glucocorticoid. Mineralocorticoid contributes to renal tubular reabsorption of sodium and potassium in order to maintain their appropriate concentrations in the plasma. Excess secretion of deoxycorticosterone may lead to edema, because sodium and

water that are excessively retained in the body result in increased blood volume, blood pressure, high blood sugar, and low potassium.

Studies[6, 30] have reported that sustained glomerular hypertension can damage glomerular endothelial cells and filtration membrane permeability, resulting in increased number of mesangial macromolecules and reduced ability to remove macromolecules in vivo. In addition, accumulation of the macromoleculars in the mesangial cell stimulates the proliferation of mesangial matrix products, resulting in mesangial expansion and acceleration of glomerular sclerosis. The continued glomerular filtration pressure can also stimulate the epithelial cells and increase collagen synthesis, causing not only glomerular capillary basement membrane thickening, but also increased mesangial matrix products. Glucocorticoid is a counter-regulating hormone for insulin, which can affect insulin resistance. Moreover, glucocorticoids may also act directly on pancreatic β cells to inhibit the release of glucose-stimulated insulin.

Because of the lack of insulin, the metabolism of three substances (sugar, lipids, and proteins) turn from anabolism into catabolism, making a large number of proteins break down into amino acids. Branched-chain amino acids (valine, leucine, and isoleucine) provide energy for the body mainly through oxidative metabolism in skeletal muscle, and such a process is enhanced by stimulation with insulin. The relative or absolute deficiency of insulin promotes protein degradation and inhibits the absorption of branched-chain amino acids. With the development of the disease, increased kidney damage and disrupted protein synthesis lead to degradation of branched-chain amino acids. The levels in vivo have similar trend compared to Lyso-PCs, and the results are shown as follows.

L-tryptophan and kynurenine levels in DN V patients were found to have significant differences, while other groups had no significant difference (Fig. 9.6).

L-tryptophan is metabolized to L-kynurenine through the kynurenine metabolic pathway (Fig. 9.7). L-tryptophan-2,3-dioxygenase is the major enzyme in the kynurenine metabolic pathway. It was reported that renal dysfunction could enhance the activity of L-tryptophan-2,3-dioxygenase and inhibit the kynurenine enzymes.[27] Enhanced activity of L-tryptophan-2,3-dioxygenase results in more L-tryptophan being metabolized to L-kynurenine, while decreased activity of the kynurenine enzymes inhibit the L-kynurenine catabolism, resulting in lower levels of L-tryptophan and increased levels of L-Kynurenine.

9.3.1.2 Quantitative Study of Phospholipid Metabolism Liquid chromatography tandem ion trap mass spectrometry (HPLC-ion trap/MSn) was used to identify more than 70 phospholipids in the seven classes, and liquid chromatography tandem time of flight mass spectrometry (HPLC-TOF/MS) was used to quantify these phospholipids. The chromatogram of phospholipids in seven classes is shown in Fig. 9.8. There were two parts in the quantitative

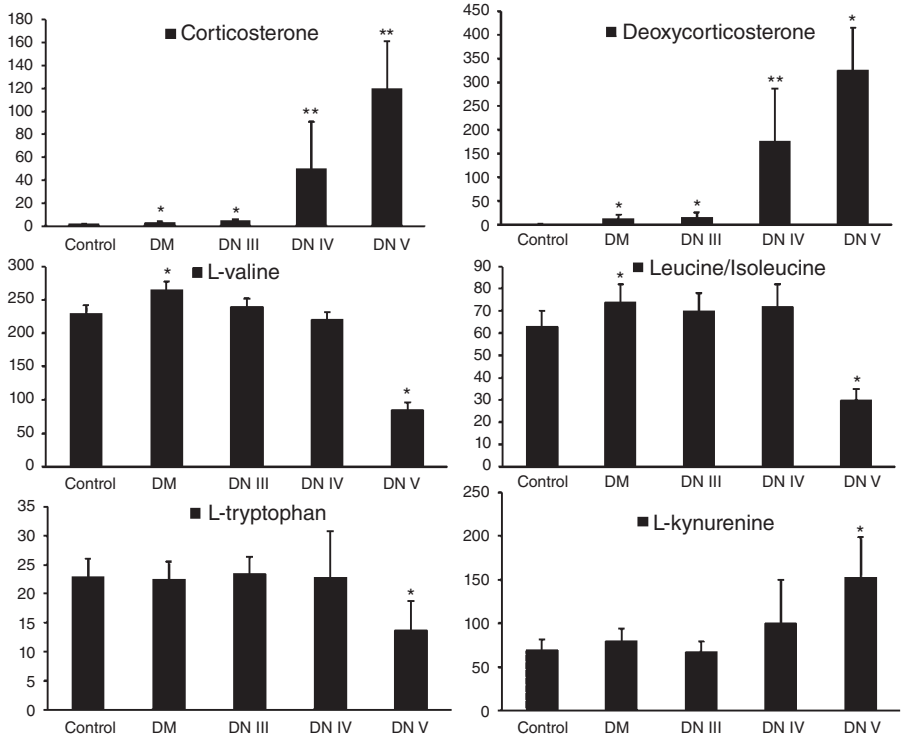

Fig. 9.6 Levels of potential biomarkers in each group (*p < 0.05, **p < 0.01 when compared with control group).

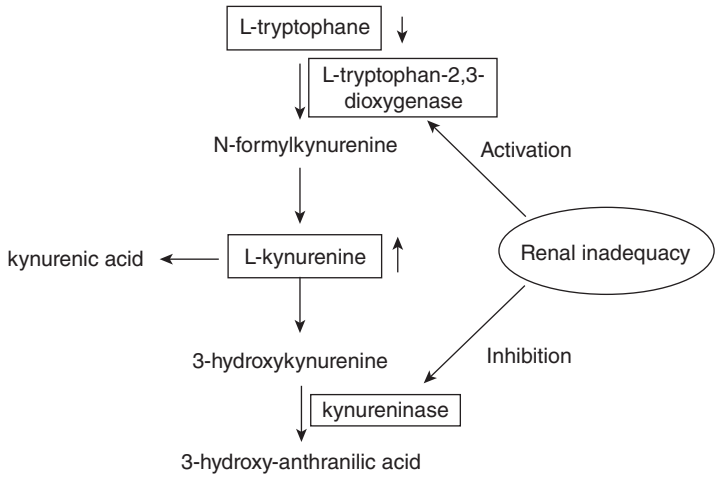

Fig. 9.7 Kynurenine metabolic pathway.

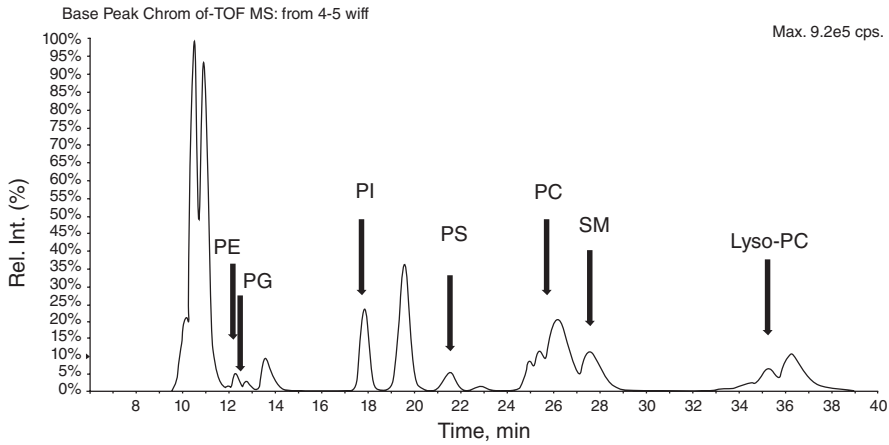

Fig. 9.8 Chromatogram of seven classes of phospholipids using normal HPLC-TOF/MS.

analysis: one is the semiquantification of seven major classes of phospholipids, including phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), sphingomyelin (SM), and lyso-phosphatidylcholine (LPC); the other one is the precise quantification of 20 phospholipids.[31]

9.3.1.2.1 Metabolic Fingerprint of Phospholipid Metabolism The metabolic fingerprint data of phospholipids were obtained with the Waters Markerlynx software, and the data was processed by PCA. Nine phospholipid biomarkers in the five categories related to DN were found, containing PC (m/z = 792, m/z = 826, m/z = 830, m/z = 854, m/z = 802), PE (m/z = 750), PI (m/z = 885), SM (m/z = 747), and Lyso-PC (m/z = 540).

The metabolic fingerprints of nine potential biomarkers are closely related to the development of DN, and provide a good regularity to understand the mechanism of the disease and to evaluate the efficacy. The phospholipids and the other key phospholipids reported were further quantitatively researched to identify the potential biomarkers of DN.

9.3.1.2.2 Quantitative Study of Phospholipid Metabolism A total amount of seven classes of phospholipids and the contents of 20 compounds were analyzed. Analysis showed that the total amount of total phospholipids, PE, PG, PC, Lyso-PC, and PE m/z750, PG m/z747, PC m/z854 had regularity according to Western staging of DN. The results are shown in Table 9.9.

The details of various phospholipids are discussed as follows. Phosphatidylethanolamine (PE), also known as cephalin, contains a polar ethanolamine group. Additionally, PE is an important component of cell membranes. With the development of the disease, the levels of PE m/z 750 significantly decreased

TABLE 9.9 Quantitative Results of Phospholipids (Concentration: μg/mL)

Class	m/z	Normal	DM	DN III	DN IV	DN V
PE	766	16.20 ± 3.07	14.30 ± 4.26	14.57 ± 4.39	17.49 ± 3.48	18.14 ± 3.11
	750	55.18 ± 8.52	45.36 ± 11.82	42.05 ± 13.14	30.48 ± 10.02	29.23 ± 5.38
	742	11.66 ± 1.55	13.46 ± 2.66	12.31 ± 2.49	12.48 ± 3.37	15.95 ± 2.68
PG	747	13.31 ± 1.80	11.68 ± 2.95	9.39 ± 2.79	7.42 ± 2.19	6.50 ± 1.14
	745	4.66 ± 0.60	3.64 ± 0.65	4.14 ± 0.83	3.69 ± 0.56	4.41 ± 0.57
PS	788	13.06 ± 1.11	11.18 ± 1.62	11.44 ± 1.77	10.74 ± 2.08	11.19 ± 1.17
	810	23.99 ± 1.73	21.84 ± 3.33	21.08 ± 3.24	19.72 ± 3.38	20.68 ± 2.49
	834	13.56 ± 2.46	10.54 ± 2.14	10.20 ± 1.96	11.20 ± 3.29	11.01 ± 1.68
PI	885	37.32 ± 8.21	31.84 ± 7.67	40.32 ± 8.82	38.02 ± 10.73	27.82 ± 6.40
	833	9.08 ± 2.98	8.53 ± 3.28	9.23 ± 3.59	9.23 ± 4.34	8.54 ± 2.76
	861	16.38 ± 4.15	13.95 ± 4.10	15.90 ± 4.52	16.84 ± 6.70	13.36 ± 3.50
PC	834	11.21 ± 0.75	8.66 ± 1.14	9.56 ± 1.06	9.60 ± 1.10	8.09 ± 1.01
	802	639.04 ± 34.15	549.49 ± 44.67	591.28 ± 62.21	534.7 ± 108.75	508.70 ± 44.34
	854	132.68 ± 10.49	114.91 ± 11.87	113.73 ± 13.72	109.35 ± 19.42	105.93 ± 12.77
SM	775	26.52 ± 1.66	25.63 ± 2.57	31.11 ± 5.72	30.55 ± 4.25	23.66 ± 2.24
	747	190.72 ± 10.77	173.92 ± 22.15	209.29 ± 37.95	213.46 ± 38.96	93.37 ± 19.94
	845	48.63 ± 3.18	42.34 ± 4.92	47.85 ± 7.86	54.96 ± 9.65	43.66 ± 3.69
Lyso-PC	540	30.18 ± 3.08	35.89 ± 7.23	33.11 ± 7.82	36.65 ± 9.03	23.33 ± 2.80
	568	12.57 ± 1.54	13.69 ± 3.51	12.85 ± 3.45	13.72 ± 3.23	10.47 ± 1.49
	588	6.89 ± 1.06	7.75 ± 1.93	7.18 ± 1.85	7.84 ± 1.99	6.26 ± 1.00
Total		1311.65 ± 164.35	1122.44 ± 272.30*	1248.52 ± 341.26	1182.02 ± 353.49*	1082.46 ± 246.42*

*$p < 0.05$, compared with control group.

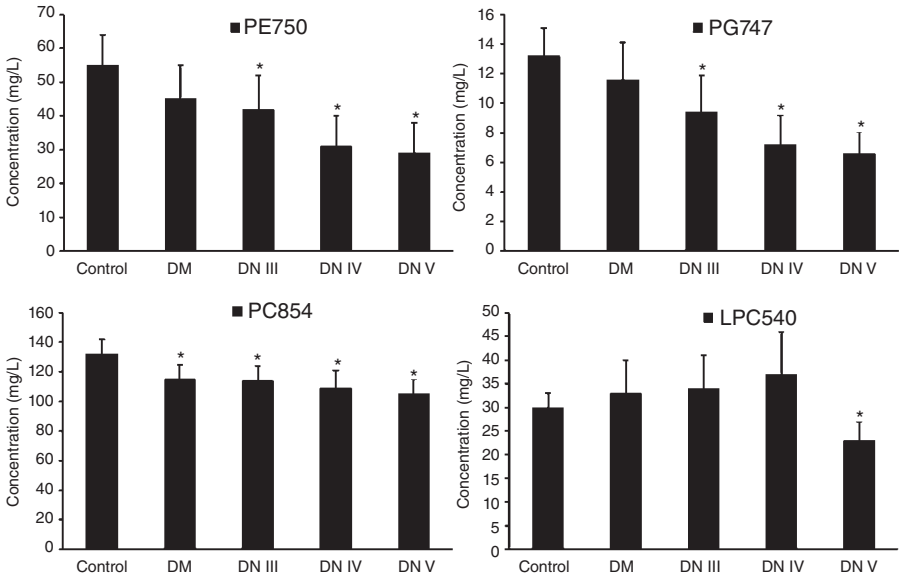

Fig. 9.9 Comparison of four phospholipids concentration in five groups (*$p < 0.05$ when compared with control group).

among the groups (Fig. 9.9). The results from the qualitative analysis showed that the molecular composition of m/z = 750 was PC16:0/C22:4, which is arachidonic acid that contains a fatty acid chain (C22:4). Usually, arachidonic acids in tissues are mainly stored as fatty acyl groups in the plasma membrane phospholipids, especially in PI and PC. There is also almost no free arachidonic acid in the cell. In the case of diabetes, there was de novo synthesis of diacylglycerol (DAG) from high blood sugar, which activates the PKC pathway. DAG further activates phospholipase A2 and accelerates the hydrolysis of membrane phospholipids so that the release of arachidonic acid is increased. From the above experiment, decreased concentration of phospholipids m/z = 750 gradually transformed them into more free arachidonic acid. Arachidonic acid in platelet plasma membrane separates and generates thromboxane under the action of cyclooxygenase and thromboxane. It destructs the metabolic balance of the original platelet after a series of transformations and plays an important role in the development of DN.

Arachidonic acid is created by three pathways: the prostaglandin cyclooxygenase pathway; the lypoxygenase pathway, which eventually produces leukotrienes, 5-hydroxy-eicosatetraenoic acid (HETE), 8-HETE, 12-HETE, and 15-HETE; and the pathway that produces 19-HETE and 20-HETE by cytochrome P450 monooxygenase. Numerous studies have shown that arachidonic acid has an important regulatory role on renal transport of ions. Therefore, disturbed arachidonic acid metabolism severely damages proper kidney functions.

The total amount of PE phospholipid concentration in the patients was much lower than normal, as demonstrated in Fig. 9.10, and it gradually decreased with the development of the disease, which verifies that PE concentration decreases due to the activation of phospholipase.

PC contains polar choline head. Fig. 9.9 shows that PC m/z = 854 decreased with the development of the disease. The total amount of phospholipids in the patients was much lower than normal, especially in the DN V group (Fig. 9.10).

PC class is the direct supplier of glycerol, fatty acid, phosphoric acid, choline, and amino alcohols in the body, and can synthesize acetylcholine by directly moving through the blood-brain barrier. PC can also emulsify and decompose fats, improving blood circulation and removing peroxides. It has been reported that deficiency of PC decreases the function of the pancreas, resulting in ineffective transport of glucose into cells, which is one of the causes of diabetes. PC content in a human body is very high, and it is the main component of membrane phospholipids. PC deficiency can seriously affect the structure and permeability of cell membranes. The study found that PC m/z = 854 decreased with the development of the disease, so it may serve as the potential biomarker for diagnosis and treatment evaluation.

PG contains glycerol polar heads. The total amount of two PG phospholipids significantly decreases with the course of the disease ($p < 0.05$). The amount in patients was much less than normal, and there was no significant difference between DN IV and DN V. The decrease of PG was mainly due to excessive activation of phospholipase and accelerated hydrolysis of membrane phospholipids. The results suggested that sharply decreased concentration of PG phospholipid might be closely related to early progression of DM and DN.

Lyso-PC contains only one fatty acid chain, which comes from PC by breaking down a long chain of fatty acids under the action of phospholipase. It can be seen from Fig. 9.10 D that the concentration of Lyso-PC m/z = 540 was much higher in DM, DN III, and DN IV than normal, but it was significantly decreased in DN V.

The change tendency of the total amount of the three Lyso-PC phospholipids was similar to that of the compounds Lyso-PC m/z = 540 (Fig. 9.10). The concentration of Lyso-PC in DM, DN III, and DN IV was much higher than the normal group, while it was significantly reduced in DN V. Therefore, it can be used to distinguish the patients in DN V.

The pathogenesis of DN may be related to the PKC pathway. Activation of the PKC pathway causes increased activity of phospholipase A_2 (PLA_2), and phospholipase A_2 specifically catalyzes the glycerol C-2 position of phospholipids during hydrolysis of free fatty acids. In the early period of DN, increased activity of phospholipase catalyzes the formation of Lyso-PC, which leads to elevated concentrations of Lyso-PC. The solubility characteristic of Lyso-PC phospholipid accelerates the purification of the membrane and the rate of exchange in cytoplasm of cells (especially red cells), which causes exacerbated

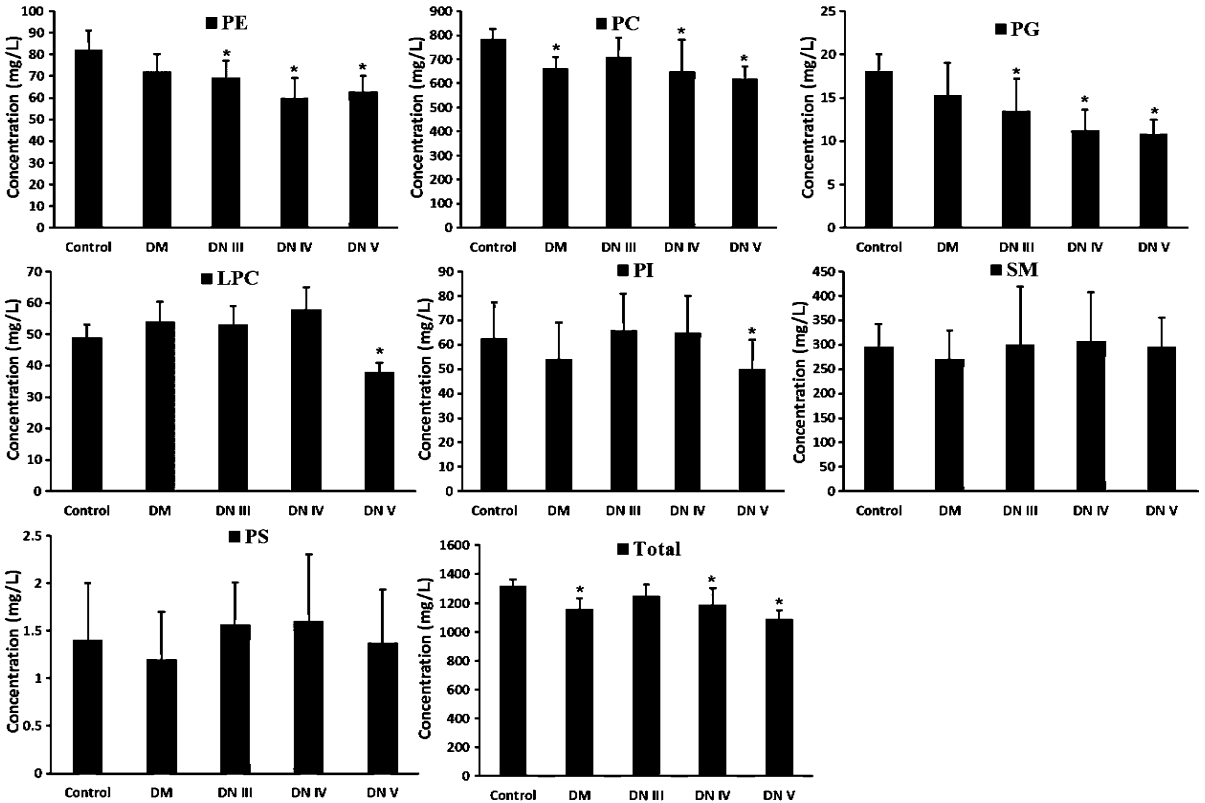

Fig. 9.10 Comparison of seven phospholipids and total phospholipid concentration in each group (*$p < 0.05$ when compared with control group).

kidney damage. Moreover, in DN V, greater reduction in the concentration of PC and decreased hydrolysis of Lyso-PC results in further damage of cell membrane structure and function.

The total amount of phospholipids in DM and DN V patients was much lower, as can be seen from Fig. 9.10, while patients in the other two stages had no significant change.

DN can lead to reduction of intracellular inositol, so that PI synthesis is limited, leading to decreased Na^+, K^+-ATP activity and increased vasodilator prostaglandin production. Eventually, it causes hemodynamic abnormalities, which is a very important symptom of DN.

The content of 20 phospholipids in patients was much lower than normal from Fig. 9.10, particularly in the patients with DN V. It confirmed that the activation of the protein kinase pathway induced increased activity of phospholipase, thereby speeding up the hydrolysis of membrane phospholipids, resulting in decreased phospholipid levels in the patients. At the same time, as the polyol pathway becomes activated, sorbitol breaks the original structure of the cells.

Phospholipids are a major component of cell membranes, and directly affect the physiological function of cells. Decreased concentration of phospholipids will change the composition and permeability of cell membranes and affect the normal function of cells. Therefore, changes in phospholipid content are important biological indexes to diabetes and diabetic nephropathy.

9.3.1.3 Quantitative Study of Fatty Acid Metabolism Quantitative analysis of 15 fatty acids, which are closely related to DN, namely, decanoic acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1n-9), stearic acid (C18:0), oleic acid (C18:1n-9), vaccenic acid (C18:1n-11), linoleic acid (C18:2), arachidonic acid (C20:4), eicosapentaenoic acid (C20:5), eicosatrienoic acid (C20:3), eicosadienoic acid (C20:2), arachidic acid (C20:0), and docosahexaenoic acid (C22:6), were carried out by gas chromatography-mass spectrometry (GC-MS) in the study.[32] The total ion chromatogram is shown in Fig. 9.11.

Based on the results of quantitative analysis, seven pathogenesis-related fatty acids were determined in the study. C20:4, C18:2, C20:2, and C20:3 showed an upward trend with the development of DN. The concentration of four compounds damaged the endothelial cells, resulting in the damage of glomerular, tubular, and small blood vessels in kidney. Among them, the eicosanoid metabolites (C20 family fatty acids, such as C20:4, C20:3, and C20:2) are important in DM and DN. They are mainly produced from the decomposition of membrane phospholipids, and synthesis and food intake of linoleic acid (C18:2). Thus they synthesize inflammatory mediators and signaling substances, such as prostaglandin (PG type), leukotriene (LT type), thromboxane acid (TX class), and overoxidation of arachidonic acid (HETE Class). Arachidonic acid (C20:4) is an important inflammatory mediator. Under normal circumstances, arachidonic acids are mainly stored as fatty acyl groups in the

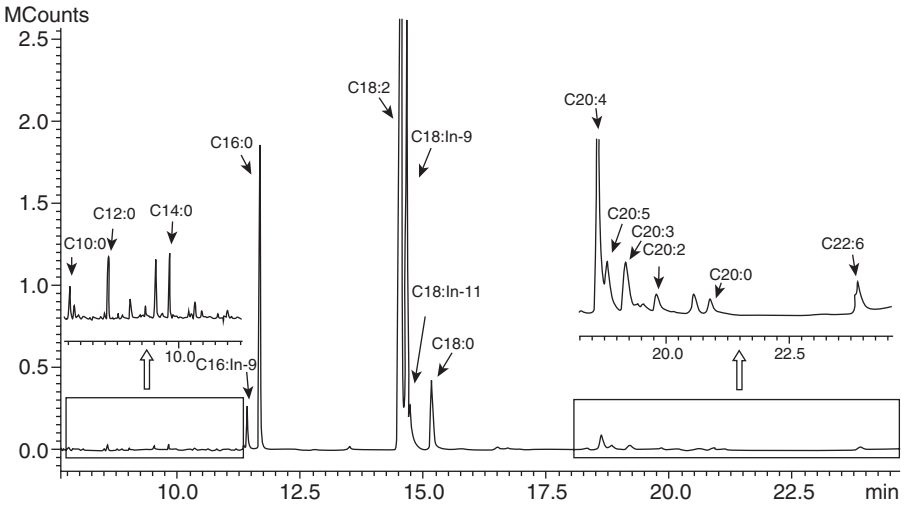

Fig. 9.11 Total ion chromatogram of 15 fatty acids.

membrane phospholipids. However, under the diabetic state, a large number of arachidonic acids dissociate and release nonesterified arachidonic acid (as the form of NEFA), while other increased eicosanoids cause metabolic disorders through inflammatory mediators. Inflammatory mediators disturb the Ca2+ regulation of ion transports, resulting in altered endothelial cell function and accelerated progression of tissue fibrosis.[28] C18:2 is the precursor compound of C20:4, and is the initial material for other inflammatory mediators. The increased concentration of C20:4 and other C20 fatty acids promote the inflammatory response of the body.

In addition, concentration of plasma fatty acid supports the lipotoxicity theory that nonesterified fatty acids are toxic molecules, causing environmental damage within cells, thereby affecting the process of renal fibrosis directly or through cooperation with other factors.

9.3.1.4 Quantitative Study of Purine and Pyrimidine Metabolism A total of 21 purine and pyrimidine metabolites were selected from the pathway, which was closely related to the DN. For the detection of all purine and pyrimidine compounds, two methods were utilized: a UV detector with its wavelength set at 254 nm was used to determine orotic acid, uric acid, cytidine, hypoxanthine, deoxyuridine, thymine, inosine, thymidine, adenosine purine, adenosine, and deoxyadenosine; and a Sciex API 3000 triple-quadruple mass spectrometer was used to quantify β-alanine, sarcosine, cytosine, creatinine, uracil, dihydro-uracil, xanthine, uridine, guanosine, and deoxy-inosine. The positive ion mode was used by the multiple reactions monitoring (MRM) mode.[33] The typical chromatograms of purine and pyrimidine compounds are shown in Fig. 9.12.

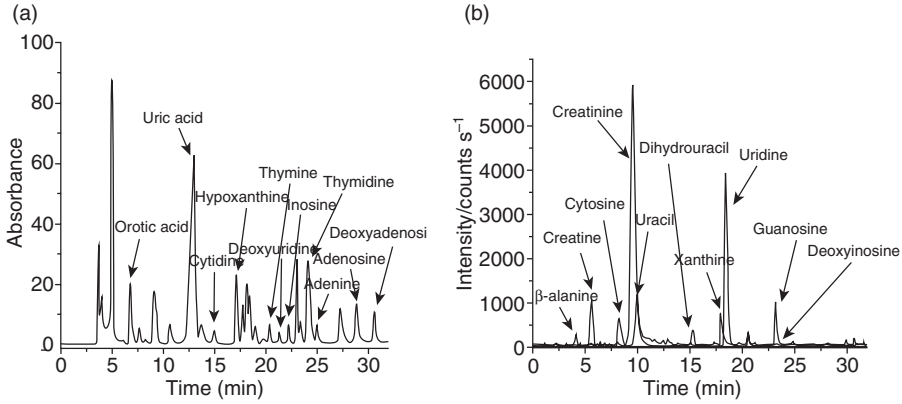

Fig. 9.12 Chromatograms of plasma samples using UV detector (A) and extracted ion chromatogram (B).

Samples of five groups were analyzed, and the concentrations of compounds are shown in Table 9.10.

Uric acid, xanthine, inosine, adenosine, cytosine, cytidine, and thymidine were found to have significant differences among the groups, and thus can be identified as potential markers.

Fig. 9.13 shows the content changes of uric acid and xanthine. With the development of the disease, uric acid levels increased among the groups. The control and the DM group, as well as the DN IV and DN V groups, had significant differences, which can be used to monitor the development of the disease and for early diagnosis. Xanthine level increased among the groups, and the difference between DN IV and DN V was significant, which can be used to monitor the progression.

The content of inosine and adenosine showed significant differences with the development of the disease. Inosine levels increased among the groups, and there were significant differences between DM and DN III, which can aid early diagnosis. With the development of the disease, adenosine levels increased, and there were significant differences among DN III, DN IV, and DN V, which can be used to monitor the course of development. It is beneficial for the early control of the disease and can be used to evaluate the effects of treatments and guide the treatment of diseases.

The content of cytosine and cytidine also increased with the course of development, and showed significant differences between DN IV and DN V, which means they can be used to monitor the progression of the disease. In addition, the cytosine level had a significant difference between DM and DN III and can provide early detection of complications.

With the development of the disease, thymidine showed an increasing trend. There were significant differences among DM, DN III, DN IV, and DN V, which can be used to monitor the development of DN.

TABLE 9.10 Quantitative Analysis of Purine and Pyrimidine Compounds in 5 Groups

Compound	Control	DM	DN III	DN IV	DN V
Creatine	1.226 ± 0.282	0.911 ± 0.207	0.804 ± 0.121	0.571 ± 0.145	0.975 ± 0.213
Orotic acid	0.021 ± 0.003	0.030 ± 0.005	0.031 ± 0.004	0.022 ± 0.011	0.085 ± 0.025
Cytosine	0.115 ± 0.015	0.194 ± 0.038	0.293 ± 0.076	0.305 ± 0.069	0.520 ± 0.167
Creatinine	7.321 ± 0.770	11.282 ± 1.447	19.043 ± 5.201	51.360 ± 14.224	82.101 ± 11.889
Uric acid	46.527 ± 3.060	58.624 ± 5.656*	60.462 ± 8.601*	64.867 ± 7.851*	76.450 ± 5.651*
Cytidine	0.046 ± 0.012	0.051 ± 0.007	0.056 ± 0.014*	0.091 ± 0.021*	0.296 ± 0.069*
Hypoxanthine	0.287 ± 0.036	0.283 ± 0.075	0.247 ± 0.088	0.221 ± 0.064	0.528 ± 0.231
Uridine	1.226 ± 0.096	1.394 ± 0.168	1.031 ± 0.161	1.035 ± 0.204	1.119 ± 0.177
Xanthine	0.477 ± 0.062	0.554 ± 0.159	0.663 ± 0.131*	0.839 ± 0.179*	2.034 ± 0.657*
Thymine	0.035 ± 0.008	0.030 ± 0.007	0.034 ± 0.017	0.028 ± 0.007*	0.077 ± 0.023*
Deoxyuridine	0.194 ± 0.055	0.099 ± 0.027	0.062 ± 0.016	0.124 ± 0.051	0.162 ± 0.056
Inosine	0.077 ± 0.012	0.080 ± 0.015	0.284 ± 0.021*	0.319 ± 0.079*	0.947 ± 0.346*
Adenine	0.165 ± 0.036	0.146 ± 0.055	0.156 ± 0.064	0.172 ± 0.091	0.187 ± 0.042
Thymidine	0.028 ± 0.005	0.039 ± 0.012	0.081 ± 0.030	0.122 ± 0.054	0.339 ± 0.093
Adenosine	0.136 ± 0.030	0.138 ± 0.029	0.295 ± 0.071*	0.540 ± 0.080*	1.870 ± 0.407*

*$p < 0.05$, compared with control group.

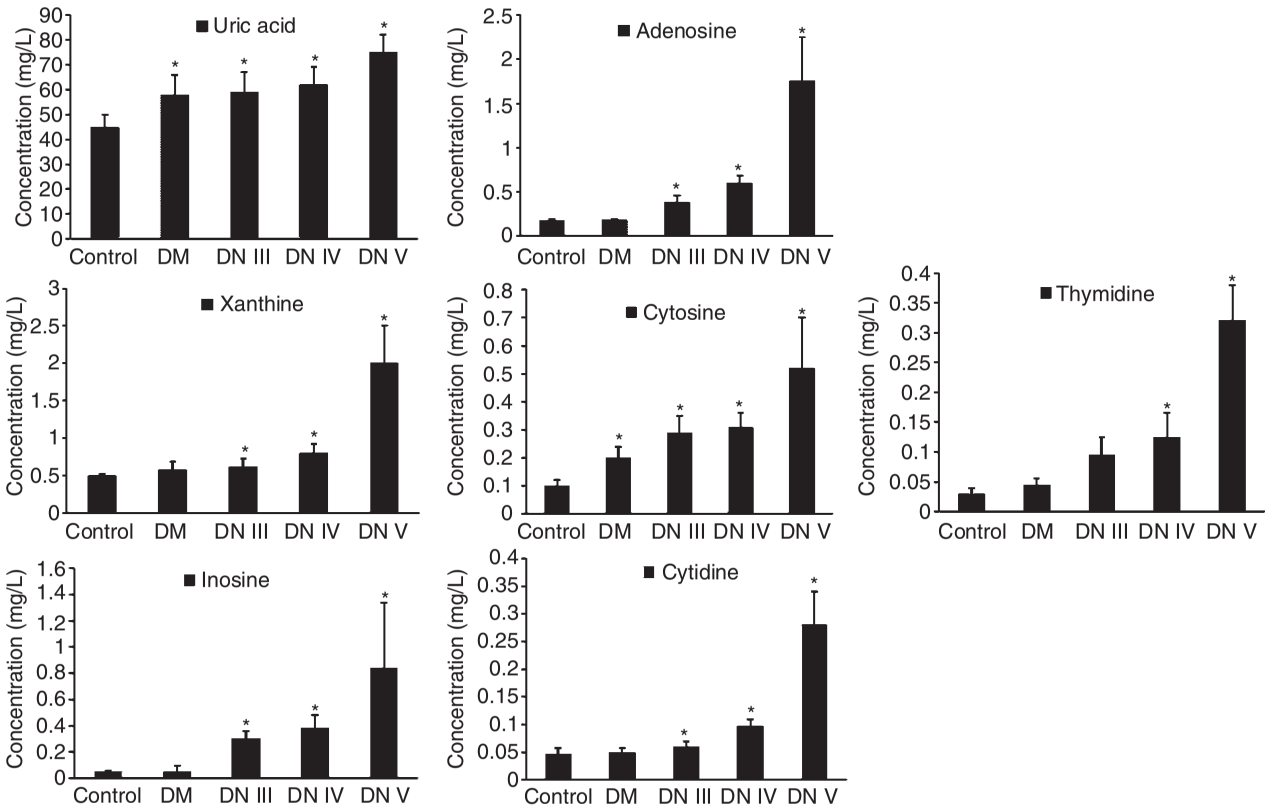

Fig. 9.13 Comparison of purine and pyrimidine metabolites concentration among groups (*$p < 0.05$ when compared with control group).

Adenosine has a very important role in the kidneys, participating in renal electrolysis metabolism, renin release, and renal blood flow. It has a significant relationship with the development of DN.[34, 35] In addition, the treatment of DN blocks the rennin-angiotensin system (RAS).[36] Adenosine is an immunosuppressive agent. In high levels it can cause immune deficiency syndrome. In type 2 diabetes, increased adenosine is likely to facilitate the development of the disease, leading to occurrence of complications. The content of adenosine increases, especially in the DN V stage. The major metabolic pathway that transforms adenosine into inosine is under the action of adenosine deaminase. The reduced activity of adenosine deaminase will increase the adenosine content. Moreover, decreased activity of adenosine deaminase is likely to be an important mechanism of DN.

Uric acid in the body not only has beneficial effects (as a reducing material, involved in redox reaction, antioxidant, and anti-DNA damage), but also has deleterious effects (promote proliferation of vascular smooth muscle, leading to endothelial dysfunction).[37, 38] Uric acid is associated with low renal blood flow, slowing down blood vessels, and activating the RAS. High levels of uric acid can cause proliferation of vascular smooth muscle cell, which can induce irreversible renal damage. Uric acid level will promote the oxidation of low-density lipoprotein cholesterol, which leads to lipid peroxidation.[39–44]

Thymidine increase is also associated with the procession of DN, and it is significantly increased in DN V. It may be caused by increased dTMP. Methotrexate protects the kidney in the human body.[45, 46] The amount of methotrexate is reduced with the course of DN. As dihydrofolate reductase is a competitive inhibitor, reduction in its amount increases folate, thereby causing increased content of dTMP.[47, 48] The conversion of thymidine is carried out by dTMP, and thymine is generated by the conversion of thymidine, resulting in an increase in thymine level. Considering the enzyme activities in the metabolic pathway, the amount of thymidine is determined by the activity of three enzymes. Two such enzymes have been reported. Decreased 5′-nucleotidase activity increases thymidine kinase activity,[49] but these two changes will not increase the thymidine amount. The amount of thymine remains unchanged, and this shows that decreased thymidine phosphorylase activity is necessary for catalyzing thymidine into thymine, which may lead to elevated levels of thymidine. At the same time, it is an important mechanism of nephropathy since elevated thymidine levels can cause endothelial cell DNA damage.

The amount of cytidine and cytosine were gradually increased in this study. Previous studies suggested that phospholipids, PC, and PE gradually decrease with the development of nephropathy. It is reported that the mechanism is due to PKC activation, which leads to the activation of phospholipase. Since cytidine is a substrate of the nucleotide salvage pathway, and PC and PE are closely related to the anabolism, it was suggested that abnormalities in the nucleotide salvage pathway are a possible reason for the phospholipid changes.

9.3.1.5 Quantitative Study of Aminothiols' Metabolism A total of eight aminothiols were chosen for detection in the study, covering nearly all the aminothiols in the body and related to the development of DN, namely cystathionine (Cysta), cysteine (Cys), cysteinylglycine (Cys-gly), homocysteine (Hcy), S-adenosylmethionine (SAM), methionine (Met), glutathione (GSH), and S-adenosyl homocysteine (SAH). These aminothiols were precisely quantified by HP Met 1100 HPLC in tandem with triple-quadruple mass spectrometer (PE SCIEX API 3000, PerkinElmer Sciex, Canada). The chromatogram is shown in Fig. 9.14.

After determination of aminothiols in controls and patients, the content of Hcy, SAM, and SAH were found to be gradually increased with the aggravation of DN. The concentration of Cys increased, while Cys-gly, Met, and GSH decreased in the patients (Table 9.11).

Hcy was significantly higher in patients with renal damage in plasma, because of the decreased clearance rate in the kidneys. When plasma is no longer filtered, renal parenchymal cells are no longer participating in the Hcy metabolism. As shown in the Hcy metabolic cycle, methionine is formed by a folate coenzyme, such as methylenetetrahydrofolate, or through methylation of methyl donor.[50] Therefore, the accumulation of homocysteine that transform into methionine affects the formation of methionine. In addition, SAM is the inhibitor of methylenetetrahydrofolate reductase. When the concentration of SAM increases, synthesis of methyl tetrahydrofolate is suppressed, leading to the accumulation of Hcy, and inhibition of the formation of methionine. In general, the reasons for accumulation of Hcy have been suggested to be as follows: decreased clearance of trans-sulfuration, increased production

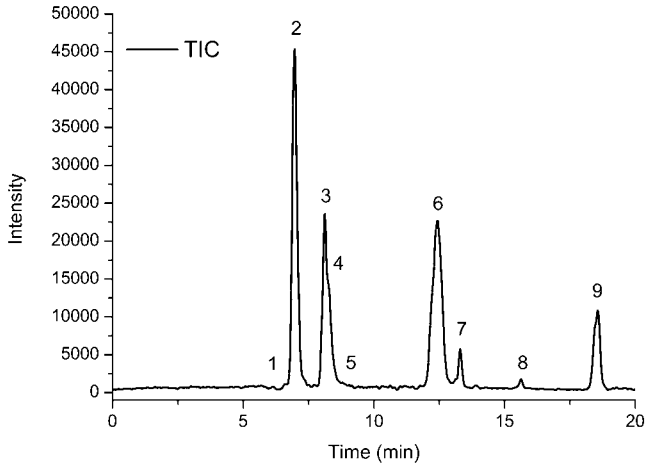

Fig. 9.14 Chromatogram of 8 aminothiols and internal standard. 1: Cysta; 2: Cys; 3: Cys-gly; 4: Hcy; 5: SAM; 6: Met; 7: GSH; 8: SAH; 9: MPG.

TABLE 9.11 Quantitative Results of Aminothiols in Plasma with DM and DN Groups

	Concentration ($Mean \pm t \times s/\sqrt{n}$)				
Compound	Control	DM	DN III	DN IV	DN V
Cys-gly, μmol/L	8.41 ± 0.64	6.98 ± 0.90[a]	6.82 ± 1.30[a]	6.51 ± 1.02[a]	6.25 ± 1.02[a]
SAH, nmol/L	13.89 ± 2.62	16.03 ± 2.89	28.04 ± 6.98[a,b]	53.51 ± 14.42[a,b,c]	304.07 ± 56.13[a,b,c,d]
SAM, nmol/L	32.97 ± 2.57	35.09 ± 4.38	40.04 ± 6.12[a]	54.52 ± 13.32[a,b]	82.35 ± 14.00[a,b,c]
Hcy, μmol/L	5.18 ± 0.53	5.94 ± 1.16	6.78 ± 1.73[a]	8.74 ± 1.77[a,b]	14.28 ± 3.10[a,b,c]
Cysta, nmol/L	23.73 ± 2.88	27.05 ± 4.81	23.55 ± 6.96	28.91 ± 5.74	24.49 ± 4.63
Met, μmol/L	22.17 ± 1.38	18.64 ± 2.64[a]	18.15 ± 3.06[a]	16.76 ± 2.34[a]	15.02 ± 1.30[a,b]
GSH, μmol/L	2.96 ± 0.27	2.39 ± 0.31[a]	2.05 ± 0.31[a]	2.01 ± 0.49[a]	1.96 ± 0.22[a]
Cys, μmol/L	70.33 ± 5.60	88.30 ± 15.31[a]	90.46 ± 23.77[a]	95.26 ± 22.78[a]	97.92 ± 15.05[a]

[a] $p < 0.05$, compared with control group (Student's t-test);
[b] $p < 0.05$, compared with DM;
[c] $p < 0.05$, compared with DN III;
[d] $p < 0.05$, compared with DN IV.

of transmethylation, reduced rate of regeneration, and reduced renal uptake of Hcy and metabolism.

The concentration of SAH in the DN patients was significantly increased. It has been reported that the kidney is the only organ that removes Hcy from the body.[51] In addition, the accumulation of Hcy leads to secondary accumulation of SAH, so that the concentration of SAH is significantly increased.[52, 53] At the same time, increased SAH concentration in plasma can inhibit methyltransferase activity. Methylation is related to many important cellular biosynthesis processes, such as biosynthesis of creatine, epinephrine, carnitine, phospholipids, DNA, and RNA.

GSH opposes oxidative stress and stimulates the exogenous detoxification of chemical substances. GSH was relatively low in the patients with renal injury. The human body maintains low concentrations of Hcy and Cys and stores them as glutathione. GSH and its metabolites, Cys-gly, are formed by γ-glutamyl transpeptidase and dipeptidase. Therefore, enzymes in the glutathione metabolic pathway also affect the concentrations of aminothiols.[54]

9.3.2 The Correlation Analysis Between Metabolites and Clinical Biochemical Indexes

Correlation analysis of quantitative metabonomics and clinical biochemical indices was carried out using SPSS software (Version 14.0; SPSS Inc., USA).

Six compounds in the purine metabolism, uric acid, hypoxanthine, xanthine, inosine, adenine, and adenosine, were selected for correlation analysis. As seen in Table 9.12, uric acid, xanthine, and adenosine were positively correlated with systolic blood pressure and serum creatinine. Moreover, the content of xanthine and adenosine in vivo were positively correlated with urea nitrogen.

Seven compounds in the pyrimidine metabolism, cytosine, cytidine, uridine, thymine, thymidine, deoxyuridine, and creatine, were chosen for correlation analysis with clinical biochemical data. The correlation coefficient was shown in Table 9.13. It can be seen that cytosine, cytidine, and thymidine showed a significant positive correlation with urea nitrogen and serum creatinine. Importantly, the correlation coefficients of cytidine were more than 0.7. Therefore, purine and pyrimidine metabolism were related to renal injury.

In DM patients, PC, SM, and LPC were positively correlated with fasting blood glucose, cholesterol, and triglycerides. PE was mainly correlated with high-density lipoprotein (Table 9.14). In DN patients, PCs were negatively correlated with urea nitrogen, especially PC834 (–0.334, $p < 0.01$) and PC826 (–0.272, $p < 0.01$). Therefore, the PC class is related to kidney injury and can be used as an indicator of diagnosis and drug efficacy evaluation.

The corrections for the aminothiols data and clinical biochemical indicators are listed in Table 9.15. SAH and SAM exhibited significant correlations with serum creatinine, which has been recognized as a clinical index for renal function diagnosis. However, the concentration of serum creatinine does not have

TABLE 9.12 Correlation Between Purine Metabolites and Clinical Parameters

	Uric Acid	Hypoxanthine	Xanthine	Inosine	Adenine	Adenosine
BMI	0.006	0.042	0.15	0.007	0.155	0.256
Time with diabetes	0.12	0.032	0.227	–0.101	0.153	0.092
Age	0.19	–0.03	–0.172	0.006	0.136	0.124
Systolic pressure	0.392**	0.206	0.292*	0.084	0.065	0.485**
Diastolic pressure	0.055	0.065	0.117	–0.127	–0.077	0.24
Urea nitrogen	0.204	0.386**	0.410**	0.207	0.286**	0.658**
Serum creatinine	0.330**	0.394	0.443**	0.112	0.185	0.699**
Glycosylated hemoglobin	0.273	–0.048	–0.24	0.043	–0.172	0.24
Total cholesterol	–0.218	–0.173	0.018	–0.003	–0.196	0.01
Triglyeride	–0.288*	–0.114	–0.008	–0.052	–0.108	–0.127
High density lipoprotein cholesterol	0.027	–0.035	–0.134	0.019	0.05	0.135
Low density lipoprotein cholesterol	–0.167	–0.136	0.081	–0.03	–0.201	–0.049
Fasting blood glucose	–0.262*	–0.118	–0.21	–0.135	–0.117	–0.238

*Significant correlation $p < 0.05$;
**significant correlation $p < 0.01$.

TABLE 9.13 Correlation Between Pyrimidine Metabolites and Clinical Parameters

	Sarcosine	Cytosine	Cytidine	Uridine	Thymine	Deoxyuridine	Thymidine
Age	0.039	–0.105	–0.047	–0.103	0.170	–0.076	–0.048
BMI	0.066	0.021	0.082	–0.168	–0.017	–0.120	–0.130
Fasting blood glucose	–0.152	–0.123	–0.284*	0.040	–0.165	–0.260*	–0.106
Glycosylated hemoglobin	–0.523**	–0.129	–0.073	–0.081	0.016	–0.135	–0.020
Serum creatinine	0.370**	0.590**	0.801**	0.113	0.468**	0.241	0.537**
Urea nitrogen	0.302	0.422**	0.705**	0.017	0.214	0.302*	0.388**
Total cholesterol	–0.209	–0.154	0.015	–0.014	–0.208	0.064	0.181
Triglyceride	–0.147	0.067	–0.054	0.099	–0.119	–0.121	0.015
High density lipoprotein cholesterol	–0.141	–0.076	0.062	0.019	–0.138	0.323**	0.015
Low density lipoprotein cholesterol	–0.157	–0.294*	–0.061	0.023	–0.142	0.001	0.104
Systolic pressure	0.111	0.302*	0.418**	–0.093	0.286*	0.081	0.237
Diastolic pressure	–0.024	0.145	–0.022	–0.041	–0.039	–0.032	–0.132

*Significant correlation $p < 0.05$;
**significant correlation $p < 0.01$.

TABLE 9.14 Correlation Analysis Between Phospholipids Metabolites and Clinical Parameters

	PE-SUM	PG-SUM	PS-SUM	PI-SUM	PC-SUM	SM-SUM	LPC-SUM
Urea nitrogen	–0.038	–0.034	–0.118	–0.141	–0.288*	–0.054	0.041
Serum creatinine	–0.029	–0.064	–0.07	–0.206	–0.185	–0.125	–0.063
Glycosylated hemoglobin	–0.069	–0.013	–0.116	–0.174	–0.005	–0.183	–0.149
Fasting blood glucose	–0.249	–0.188	0.028	–0.067	0.044	0.024	–0.151
Cholesterol	0.083	0.291*	–0.071	0.054	–0.004	0.074	0.141
Triglyceride	0.122	0.215	–0.294*	–0.055	–0.034	–0.22	–0.064
High density lipoprotein cholesterol	0.07	–0.013	0.297*	0.092	0.169	0.287*	0.052
Low density lipoprotein cholesterol	0.06	0.241	–0.044	0.077	0.082	0.124	0.101
Systolic pressure	0.107	–0.003	0.054	–0.168	–0.075	–0.045	0.107
Diastolic pressure	0.092	0.215	0.013	–0.062	0.091	0.016	0.052

*Significant correlation $p < 0.05$;
**significant correlation $p < 0.01$.

TABLE 9.15 Correlations Between Aminothiols Metabolites and Clinical Parameters

	Cys-gly	SAH	SAM	Hcy	Cysta	Met	GSH	Cys	SAM/SAH
Fasting blood glucose	0.132	–0.096	–0.007	0.1	–0.132	–0.102	0.001	–0.116	0.213
Hemoglobin	0.162	–0.073	–0.127	–0.059	–0.181	0.079	–0.053	–0.034	0.119
Glycosylated hemoglobin	–0.042	–0.447**	–0.421**	–0.356**	–0.075	0.216*	0.021	0.06	0.313**
Serum creatinine	–0.09	0.789**	0.574**	0.465**	–0.016	–0.164	–0.07	0.163	–0.667**
Urea nitrogen	–0.01	0.681**	0.604**	0.527**	0.189	–0.163	0.019	0.079	–0.582**
Cholesterol	–0.042	–0.017	0.052	–0.018	–0.025	0.182	0.009	0.096	–0.005
Triglyceride	0.001	0.032	0.088	–0.034	0.023	0.055	0.001	0.258*	0.041
High density lipoprotein cholesterol	–0.117	–0.112	–0.156	–0.108	–0.165	0.04	–0.03	–0.241*	–0.02
Low density lipoprotein cholesterol	0.125	0.03	0.189	–0.095	–0.03	0.254*	–0.004	0.071	0.01
Systolic pressure	0.031	0.376**	0.258*	0.197*	0.039	0.078	0.024	0.202*	–0.355**
Diastolic pressure	0.023	–0.152	–0.195	–0.103	–0.016	0.251*	0.183	–0.047	0.061

*Significant correlation $p < 0.05$;
**significant correlation $p < 0.01$.

a visible increase until the functions of glomerulus decrease to about 50%. In this study, there was no significant difference of serum creatinine levels between the DM and DN III groups (from Table 9.3), while SAH had significant differences between these two groups (DM: 16.03 ± 2.89, DN III: 28.04 ± 6.98, $p < 0.05$). SAH is an important methyl donor, and serum creatinine is an important methyl receptor. Furthermore, the increased concentration of SAH affects the methyl transfer process, leading to an elevated creatinine level. It has been suggested that SAH is more sensitive than serum creatinine, can be considered as a potential biomarker for the development of DN, and was used as an auxiliary diagnosis index of DN.

Considering the limitations of conventional indices used in clinical practices, more sensitive and new indices are desired. The potential biomarkers identified above are feasible for clinical application according to the correlation study between IMPT data and biochemical indices.

9.3.3 Integrated Metabonomics Study Based on TCM Syndrome Differentiation Typing of DN

The two research modes, metabolic fingerprint and quantitative metabolic pathway, were carried out according to TCM syndrome differentiation typing.

9.3.3.1 Metabolic Fingerprint of DN Similar to the Mogensen stages of samples, all the samples were input into SIMCA-P for PLS-DA, and the clinical syndrome differentiation typing of TCM was used as a Y matrix. The score plot is shown in Fig. 9.15.

In the two-dimensional plot of PLS-DA, DM and DN overlap dramatically between the groups according to TCM syndrome differentiation typing. Then all samples were analyzed using three-dimensional score plot, and it was found that the sample was distributed in a pattern according to groups. The more serious the symptoms, the further away the samples were from the control

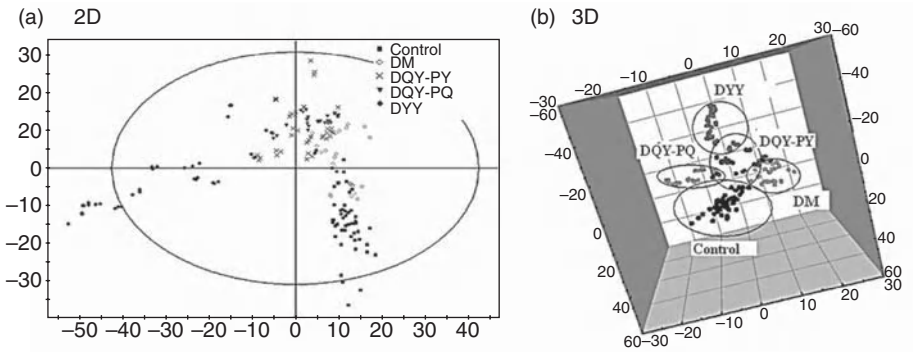

Fig. 9.15 PLS-DA score plot of plasma fingerprints from five groups according to TCM typing.

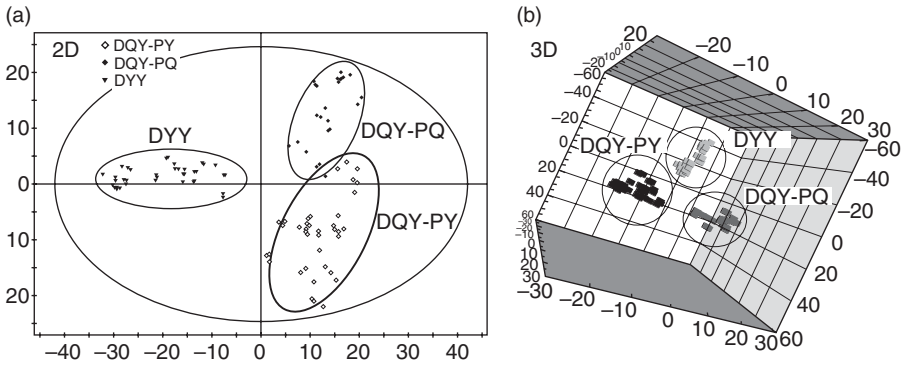

Fig. 9.16 PLS-DA score plot of plasma fingerprints from DQY-PY, DQY-PQ, and DYY groups according to TCM typing.

group. This result showed the different syndromes could be reflected through different metabolites among the groups.

Three TCM syndrome differentiation types were compared and the results are shown in Fig. 9.16. In the figure, it can be seen that the three groups are completely separated.

The results illustrated that TCM syndrome differentiation typing is associated with the metabolic states, and difference of metabolites is the chemical substance basis of TCM syndrome differentiation typing.

9.3.3.2 The Study of Phospholipid Metabolism There were 20 phospholipids in seven classes selected to be quantified according to TCM syndrome differentiation typing, and the results are shown in Table 9.16.

TCM typing showed that the total amount of three PE decreased with the development of the disease, which was consistent with the results of Mogensen staging. It was also demonstrated that the activation of phospholipase caused decreased phospholipid concentration.

The total amount of PG phospholipids was significantly decreased with the course of the development ($p < 0.05$). Levels in patients were lower than levels in healthy subjects, which were similar to the results of Mogensen staging. The low levels of phospholipids were mainly due to the excessive activation of phospholipase, which accelerates hydrolysis of membrane phospholipids. The results suggested that the dramatically decreased concentration of PG phospholipids might be closely related to the early progresses of DM and DN.

The concentration of PI phospholipids in patients of DQY and DYY were lower than in the healthy subjects according to TCM typing. There was no significant difference between the three groups of patients. PC m/z = 854 in patients of DQY and DYY was much lower than in the controls, and decreased gradually with the development of the disease, especially in the DYY patients.

The total concentrations of the selected 20 phospholipids in patients were much lower than in the healthy subjects, especially in the DYY patients. It

TABLE 9.16 Quantitative Results of Phospholipids According to TCM Typing (Conc.: μg/mL)

Class	m/z	Control	DM	DQY-PQ	DQY-PY	DQY	DYY
PE	766	16.20 ± 3.07	14.30 ± 4.26	17.06 ± 4.18	16.88 ± 5.72	16.95 ± 3.74	16.92 ± 4.43
	750	55.18 ± 8.52	45.36 ± 11.82	47.00 ± 17.71	35.84 ± 8.57*	40.06 ± 8.23	31.82 ± 9.88*
	742	11.66 ± 1.55	13.46 ± 2.66	13.77 ± 3.83	12.23 ± 2.74	12.81 ± 2.13	15.01 ± 3.55*
PG	747	13.31 ± 1.80	11.68 ± 2.95	10.47 ± 2.41	9.06 ± 1.51*	9.60 ± 1.25*	6.07 ± 2.20*
	745	4.66 ± 0.60	3.64 ± 0.65*	4.64 ± 0.86	3.75 ± 0.34*	4.09 ± 0.37	4.38 ± 0.76
PS	788	13.06 ± 1.11	11.18 ± 1.62	16.87 ± 9.16	11.88 ± 1.98	13.77 ± 3.48	10.49 ± 1.49
	810	23.99 ± 1.73	21.84 ± 3.33	24.19 ± 3.21	23.02 ± 2.50	23.46 ± 1.88	21.79 ± 3.38
	834	13.56 ± 2.46	10.54 ± 2.14*	11.78 ± 2.38	12.72 ± 2.23	12.36 ± 1.58	12.51 ± 2.48
PI	885	37.32 ± 8.21	31.84 ± 7.67	36.58 ± 11.27	35.98 ± 7.16	36.21 ± 5.86	34.07 ± 10.30
	833	9.08 ± 2.98	8.53 ± 3.28	8.79 ± 4.06	8.99 ± 3.22	8.91 ± 2.43	8.91 ± 3.79
	861	16.38 ± 4.15	13.95 ± 4.10	15.12 ± 5.69	15.47 ± 4.31	15.34 ± 3.29	14.81 ± 5.27
PC	834	11.21 ± 0.75	8.66 ± 1.14*	9.42 ± 1.62*	9.46 ± 1.18*	9.45 ± 0.91*	8.30 ± 1.15*
	802	639.04 ± 34.15	549.49 ± 44.67*	559.47 ± 77.44*	579.32 ± 66.67*	571.81 ± 48.39*	490.58 ± 42.44*
	854	132.68 ± 10.49	114.91 ± 11.87*	111.54 ± 14.65*	117.30 ± 14.33*	115.12 ± 10.06*	106.89 ± 18.31*
SM	775	26.52 ± 1.66	25.63 ± 2.57	28.81 ± 4.45	28.05 ± 2.74	28.34 ± 2.25	25.75 ± 3.41
	747	190.72 ± 10.77	173.92 ± 22.15	197.54 ± 35.71	201.21 ± 22.20	199.82 ± 18.22	192.57 ± 30.78
	845	48.63 ± 3.18	42.34 ± 4.92	46.32 ± 7.85	48.01 ± 5.95	47.37 ± 4.50	47.73 ± 6.84
Lyso-PC	540	30.18 ± 3.08	35.89 ± 7.23	37.15 ± 11.11	32.52 ± 7.19	34.27 ± 5.86	28.53 ± 8.73*
	568	12.57 ± 1.54	13.69 ± 3.51	14.15 ± 4.83	12.67 ± 2.76	13.23 ± 2.39	12.06 ± 5.25
	588	6.89 ± 1.06	7.75 ± 1.93	7.87 ± 2.42	7.29 ± 1.57	7.51 ± 1.28	6.55 ± 2.04

*$p < 0.05$, compared with control group.

confirmed that the activation of the PKC pathway induced the increase of phospholipase activity, thereby speeding up the hydrolysis of membrane phospholipids, resulting in decreased phospholipids. At the same time, as the polyol pathway becomes activated, sorbitol broke the original structure of the cell membrane.

9.3.3.3 The Study of Purine and Pyrimidine Metabolism The related compounds in pyrimidine and purine metabolism were analyzed according to TCM typing, and the results are listed in Table 9.17.

Creatinine, uric acid, xanthine, inosine, adenosine, and cytidine were found to be increased with the development of the disease compared with the controls, DM, DQY-PY, and DQY-PQ. There were significant differences among the groups. Specific analysis of selected metabolites is described as follows.

There was an increase of creatinine levels with the disease development, with significant differences existing among the groups ($p < 0.05$). However, there was no significant difference between DQY-PY and DQY-PQ ($p = 0.22$). Uric acid was found to be increased with the development of the disease, with significant differences between the controls and the patients ($p < 0.05$). The content of xanthine in vivo had an increasing trend with the progression of the disease, and significant differences were found among the control, DQY, and DYY groups ($p < 0.05$).

Creatinine levels gradually increased with the DN development. There were significant differences among the control, DQY, and DYY groups. Meanwhile, DQY-PQ also had significant differences from DQY-PY ($p < 0.05$). The content of adenosine gradually increased with the development of the disease. There were significant differences among the control, DQY, and DYY groups. However, DQY-PQ and DQY-PY had no significant difference.

Cytidine and cytosine levels gradually increased with the development of the disease. There were significant differences among the control, DQY, and DYY groups. However, DQY-PQ and DQY-PY showed no significant difference.

The metabolite concentrations showed obvious regularity among the groups according to TCM syndrome differentiation typing, and showed significant differences among the groups. Inosine, adenosine, and xanthine contents showed significant differences between the DQY and DYY groups.

9.3.3.4 The Study of Aminothiols Metabolism Table 9.18 lists the concentrations of eight aminothiols in the controls and the patients. Cys-gly and GSH gradually decreased with the course of the disease, while SAH, SAM, Hcy, Cysta, and Cys were increased. The contents of Cys-gly, SAH, SAM, Hcy, and GSH showed significant differences between the control and DQY-PY groups, suggesting that the indictors can aid the early diagnosis of DN syndrome differentiation typing. In addition, SAH content showed significant differences between DYY and DQY, indicating that SAH can better reflect the development of the disease.

TABLE 9.17 Quantitative Results of Purine and Pyrimidine According to TCM Typing (Conc.: mg/L)

Compound	Control	DM	DQY-PY	DQY-PQ	DYY
Cytosine	0.115 ± 0.006	0.194 ± 0.095	0.241 ± 0.069*	0.294 ± 0.066*	0.522 ± 0.046*
Creatinine	7.321 ± 0.305	11.282 ± 3.656*	31.410 ± 13.067*	35.448 ± 14.130*	67.003 ± 9.368*
Uric acid	46.527 ± 1.211	58.624 ± 14.295*	60.448 ± 6.995*	69.011 ± 7.953*	69.554 ± 5.353*
Cytidine	0.046 ± 0.005	0.051 ± 0.017	0.111 ± 0.068*	0.125 ± 0.057*	0.223 ± 0.042*
Hypoxanthine	0.287 ± 0.014	0.283 ± 0.189	0.248 ± 0.078	0.322 ± 0.150	0.454 ± 0.087*
Uridine	1.226 ± 0.038	1.394 ± 0.424	1.222 ± 0.215	1.113 ± 0.148	1.153 ± 0.122
Xanthine	0.477 ± 0.024	0.554 ± 0.402	0.608 ± 0.423	0.849 ± 0.563	1.202 ± 0.350*
Thymine	0.035 ± 0.003	0.030 ± 0.018	0.029 ± 0.009	0.044 ± 0.017	0.073 ± 0.010*
Deoxyuridine	0.194 ± 0.022	0.099 ± 0.069	0.107 ± 0.040	0.115 ± 0.037	0.171 ± 0.026
Inosine	0.077 ± 0.005	0.080 ± 0.037	0.107 ± 0.043	0.352 ± 0.104*	0.438 ± 0.165*
Adenine	0.165 ± 0.014	0.146 ± 0.139	0.180 ± 0.093	0.126 ± 0.029	0.205 ± 0.044*
Thymidine	0.028 ± 0.002	0.039 ± 0.031	0.110 ± 0.064*	0.125 ± 0.062*	0.226 ± 0.043*
Adenosine	0.136 ± 0.012	0.138 ± 0.073	0.396 ± 0.255*	0.656 ± 0.416*	1.305 ± 0.256*
Creatine	1.226 ± 0.112	0.911 ± 0.524	0.536 ± 0.136*	0.798 ± 0.207	1.005 ± 0.131

*$p < 0.05$, compared with control group.

TABLE 9.18 Quantitative Results of Eight Aminothiols According to TCM Typing

Compound	Control	DM	DQY-PY	DQY-PQ	DYY
Cys-gly (mg/L)	1.49 ± 0.11	6.98 ± 0.90	1.21 ± 0.21*	1.11 ± 0.14$^{\nabla\nabla}$	1.01 ± 0.17$^{\circ\circ}$
SAH (μg/L)	5.74 ± 1.09	16.03 ± 2.89	22.86 ± 12.55**	34.41 ± 15.58$^{\nabla\nabla}$	100.1 ± 36.9$^{\circ\circ,\star,\triangle\triangle}$
SAM (μg/L)	13.12 ± 1.02	35.09 ± 4.38	18.45 ± 5.31**	22.13 ± 7.19$^{\nabla\nabla}$	35.21 ± 7.84$^{\circ\circ,\triangle\triangle}$
Hcy (μg/L)	699.1 ± 71.9	5.94 ± 1.16	936.8 ± 169.1*	1124 ± 230$^{\nabla\nabla}$	1697 ± 420$^{\circ\circ,\triangle\triangle}$
Cysta (μg/L)	5.26 ± 0.63	27.05 ± 4.81	5.75 ± 1.67	5.92 ± 1.25	7.19 ± 1.26°
Met (mg/L)	2.73 ± 0.20	18.64 ± 2.64	2.75 ± 0.47	2.75 ± 0.32$^{\nabla}$	3.39 ± 0.84
GSH (μg/L)	910.2 ± 83.6	2.39 ± 0.31	667.7 ± 91.6*	637.3 ± 110.3$^{\nabla\nabla}$	625.899 ± 98.1$^{\circ\circ}$
Cys (mg/L)	7.98 ± 0.71	88.30 ± 15.31	9.16 ± 1.91	11.11 ± 2.16	12.91 ± 3.22$^{\circ\circ}$

p value showed the ANOVA results among four groups;
NS: no significant difference;
*$p < 0.05$,
**$p < 0.01$ between DQY-PY and control;
$^{\nabla}p < 0.05$,
$^{\nabla\nabla}p < 0.01$ between DQY-PQ and control;
$^{\circ}p < 0.05$,
$^{\circ\circ}p < 0.01$ between DYY and control;
$^{\star}p < 0.05$ between DYY and DQY-PQ;
$^{\triangle\triangle}p < 0.01$ between DYY and DQY-PY.

Hcy, SAH, SAM, Cys-gly, and GSH had significant differences between the control and DQY-PY groups. Among them, SAH showed significant difference between DQY-PQ and DYY. The elevated Hcy level can be attributed to the phlegm and blood stasis.[55] In addition, blood stasis is generated under the action of multiple pathological states, and is closely related to vascular and endothelial cell injury.[56] The kidney is an important organ for Hcy excretion. Damaged kidney endothelial cells result in decreased clearance of Hcy, so that the content of Hcy would be increased. Increased SAH content in the body causes damage to the kidneys in two possible ways. First, when SAH is converted into Hcy by SAH hydrolase, it will certainly cause the accumulation of Hcy in vivo, so that SAH levels would increase. Second, SAH and Hcy are toxic to the kidney, damaging renal functions. TCM theory proposed that a toxically injured kidney is the core of DN.[60] The revealed SAH and Hcy levels are associated with TCM syndrome differentiation typing.

9.3.4 The Correlation Study of Metabolites and Symptoms

Table 9.19 lists the results of the correlation study of potential biomarkers and clinical symptoms. The results showed that phospholipids had significant negative correlation with blood stasis in DN, and purine, pyrimidine, and aminothiols had significant positive correlation with blood stasis. The main symptoms are JFJC and ZTMM, which shows that the subjective symptom scores of TCM typing are associated with the quantities of the metabolites.

In addition, analysis of different indices based on TCM typing found that the combination of potential biomarkers can achieve a high accuracy rate, indicating that TCM syndromes can be used to determine the classification.

9.4 CONDITION OF METABOLISM AFTER TREATMENT WITH *TANGSHEN* FORMULA (TSF)

TSF is a TCM formula prepared for the treatment of DN. It supplements *qi* and nourishes *Yin*, as well as activating blood circulation to dissipate blood stasis. It contains *Huangqi* (Astragali Radix), *Dihuang* (Rehmanniae Rhizoma), *Sanqi* (Notoginseng Radix et Rhizoma), *Weimao* (Euonymi Ramulus), *Dahuang* (Rhei Radix et Rhizoma), and *Shanzhuyu* (Corni Fructus). Studying the metabolic changes in patients before and after the TSF treatment can be useful for in-depth efficacy evaluation and understanding of the mechanism.

9.4.1 Sample Collecting

In this study, in line with Western medicine diagnosis of DN, according to Mogensen staging criteria, patients with DN in the middle stages were selected (stages III and IV). Patients were then divided into two treatment groups: one group was treated based on Western medicine, and another had the TCM

TABLE 9.19 Results of Correlation Analysis Between Potential Biomarker Concentrations and Main Symptom Scores in DN

	DK*Yin*	DLQ	PD	SB			DK*Yang*		
Compound	YT	QDLY	ZTKZ	JFJC	ZTMM	MSHA	WHZL	FZ	YNP
PE750	–0.052	0.128	–0.181	–0.217	–0.533**	–0.225	–0.068	0.085	0.252
PG747	–0.012	0.1	–0.166	–0.321	–0.416*	–0.269	–0.285	0.088	0.221
PC834	0.200	0.077	–0.237	–0.288	–0.192	–0.285	–0.301	0.207	–0.055
PC802	–0.019	0.29	0.087	–0.362*	–0.353*	–0.155	–0.28	0.17	0.024
PC854	–0.134	0.344*	0.07	–0.100	–0.311	–0.197	–0.182	0.363*	–0.072
Cytosine	–0.258	–0.053	0.117	0.238	–0.019	0.163	0.247	0.18	–0.151
Creatinine	–0.248	–0.249	0.152	0.440**	–0.236	0.402*	0.298	0.206	–0.349*
Uric acid	–0.273	0.07	0.199	0.278	0.015	0.349*	0.105	0.025	–0.312
Cytidine	–0.293	–0.0266	0.17	0.392*	–0.214	0.433**	0.123	0.14	–0.292
Hypoxanthine	0.097	0.028	0.006	0.098	0.009	0.134	0.064	0.157	0.009
Thymine	–0.197	–0.006	0.432**	0.202	–0.08	0.321*	0.1	0.107	–0.09
Inosine	–0.186	–0.201	0.415**	–0.005	–0.225	0.256	0.081	0.366*	0.054
Thymidine	–0.222	–0.124	0.05	0.243	0.009	0.102	–0.088	0.164	–0.311
Adenosine	–0.24	0.152	–0.006	–0.466**	–0.113	0.281	0.124	0.042	–0.492**
Creatine	–0.056	–0.257*	0.266	0.451**	–0.131	0.096	0.533**	0.273	–0.299
Cys-gly	–0.397*	0.091	–0.185	–0.302	0.104	–0.265	–0.079	–0.186	0.386*
SAH	–0.109	–0.052	0.104	0.556**	0.144	0.302	0.332	0.229	–0.388*
SAM	–0.111	–0.350*	0.137	0.227	0.001	–0.05	0.245	0.415*	–0.316
Hcy	0.104	–0.097	0.177	0.255	0.055	0.006	0.312	0.315	–0.2
GSH	0.406*	–0.013	–0.267	0.041	–0.095	–0.109	–0.069	–0.16	–0.019
Cys	0.185	–0.234	0.09	–0.066	0.056	–0.29	0.086	0.081	–0.011

*Significant correlation $p < 0.05$;
**significant correlation $p < 0.01$.

treatment for 6 months. All the patients were further divided into five groups, namely, no treatment group (0 month), 3 months of treatment by Western medicine group, 6 months of treatment by Western medicine group, 3 months of treatment by Chinese medicine group, and 6 months of treatment by Chinese medicine group, according to treatment time and different treatment methods.

9.4.2 The Change of Metabolism after Treatment of TSF

In the study, the metabolic changes of patients with DN after the treatments were studied. Also, the metabolic fingerprint and four metabolic cycles, including purine and pyrimidine, phospholipids, aminothiols, and fatty acid metabolisms, were analyzed.

9.4.2.1 The Metabolic Fingerprint of DN Metabolic fingerprint was used to analyze the samples after drug intervention, and the results were compared among the groups. Comparison methods included Western medicine and Chinese medicine interventions after 3 and 6 months of treatment; and at the same time, the differences between two treatment methods were analyzed, which are shown in Fig. 9.17.

Fig. 9.17 shows the comparison of the metabolic differences between DN III and DN IV, respectively, by the Western and Chinese medicine treatment of the patients. It can be concluded from the figure that the patients with DN improved after the Western and TCM treatments. After treatment with Western drugs and Chinese medicine intervention, the relative quantitative results of potential biomarkers gradually approaches those of the healthy controls, indicating that the diabetic patients were improving.

The potential biomarkers were identified after 6 months of treatment. Further analysis of drug treatment for the metabolic process is as follows:

All the data were input into MarkerLynx software for the cluster analysis of PLS-DA. Loading plot of PLS-DA can find the metabolites, and is

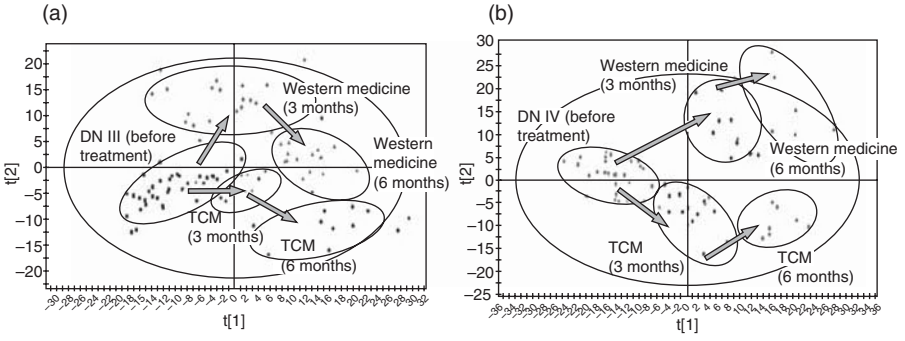

Fig. 9.17 OPLS-DA score plot of patient plasma fingerprints in DN III (A) and DN IV (B) after treatment.

TABLE 9.20 Potential Biomarkers Identified of DN III Treated by TSF

No.	Rt (min)	m/z	Formula	Identification
1	12.0272	782.5714	$C_{44}H_{80}NO_8P$	PC(18:4/18:0) /M+H
2	6.763	524.3716	$C_{26}H_{54}NO_7P$	LysoPC(18:0)/M+H
3	10.599	774.5679	$C_{45}H_{76}NO_7P$	PE(22:6/P-18:1) / M+H
4	10.1859	676.5486	$C_{37}H_{74}NO_7P$	PE(16:0/P-16:0) /M+H
5	1.8992	784.4916	$C_{45}H_{70}NO_8P$	PE(22:6/18:4) /M+H
6	10.2191	703.5683	$C_{36}H_{72}NO_7P$	PE(15:0/P-16:0) /M+ACN+H
7	11.3324	730.5892	$C_{41}H_{80}NO_7P$	PE(20:1(11Z)/P-16:0) /M+H
8	7.514	538.3879	$C_{27}H_{56}NO_7P$	LysoPE(22:0/0:0)/ M+H
9	11.0098	689.5628	$C_{37}H_{73}N_2O_7P$	SM(d18:0/14:1(9Z)(OH)) /M+H
10	10.2257	701.5602	$C_{39}H_{77}N_2O_6P$	SM(d18:0/16:1) /M+H
11	14.1511	732.613	$C_{41}H_{84}N_2O_6P$	SM(d18:1/18:0) / M+H
12	7.6636	323.3046	$C_{20}H_{34}O_3$	15(S)-Hydroxyeicosatrienoic acid/ M+H
13	10.2258	702.5632	$C_{40}H_{80}NO_6P$	CerP(d18:1/22:0) /M+H

TABLE 9.21 Potential Biomarkers Identified of DN IV Treated by TSF

No.	Rt (min)	m/z	Formula	Identification
1	13.8077	818.608	$C_{48}H_{84}NO_7P$	PC(22:6/P-18:0) /M+H [1+]
2	5.7548	523.3601	$C_{23}H_{48}NO_7P$	LysoPC(15:0)/ /M+ACN+H [1+]
3	5.6203	546.356	$C_{28}H_{52}NO_7P$	LysoPC(20:3) /M+H
4	5.9976	1044.711	$C_{67}H_{102}O6$	TG(22:5/20:4/22:5)/M+ACN+H
5	6.7625	525.375	$C_{23}H_{40}N_8O_6$	VPGPR Enterostatin/M+H
6	2.0307	200.0136	$C_4H_{10}NO_6P$	O-Phosphohomoserine/M+H[1+]
7	7.7974	501.377	$C_{21}H_{40}N_8O_6$	Kentsin/Tuftsin/M+H [1+]
8	7.5356	561.4028	$C_{38}H_{56}O_3$	2-Hexaprenyl-3-methyl-6-methoxy-1,4 benzoquinone /M+H [1+]
9	7.5361	297.2881	$C_{16}H_{33}NO$	Palmitic amide/M+ACN+H
10	7.6639	282.2778	$C_{18}H_{35}NO$	Oleamide/M+H

important for classification. According to the VIP values, these metabolites can be considered potential biomarkers related to the TSF treatment. Formulas obtained were searched in the database for further identification, and the results are shown in Tables 9.20 and 9.21.

Table 9.20 lists the results of 13 potential biomarkers of DN III treatment by Western medicine for 6 months and Chinese medicine for 6 months, including PC, Lyso-PC, PE, Lyso-PE, SM, TG, sphingosine, and diacylglycerol.

Table 9.21 lists the results of 10 potential biomarkers of DN IV treatment by Western medicine for 6 months and TCM for 6 months, including PC, PE, SM, PG, MG, and DG.

Summarizing the results of potential biomarkers from patients in DN III and DN IV groups after treatment by TSF indicates that most of them were LPC. LPC is a group of phospholipids containing only one fatty acid. Through

its relative concentration is low, it has important functions. The solubility of phospholipids increases their purifying abilities and accelerates the rate of exchange between the cytoplasm and the membrane. Therefore, LPC is potentially damaging to red blood cells. Lyso-PC is associated with the occurrence of many diseases, as it also can promote inflammation.

Lyso-PC can combine with glucose transfer proteins to inhibit glucose transport. Moreover, through activation of the PKC-α pathway, Lyso-PC can inhibit insulin-stimulated glucose metabolism to promote the formation of peroxide ions within blood and oxidative stress.

It is also reported that Lyso-PC can activate JNK or PKC to inhibit tyrosine phosphorylation, thereby inhibiting insulin signaling. Increased PKC activity causes hyperperfusion and hyperfiltration of glomerulus, and further increased glomerular extracellular matrix synthesis. Recent studies have shown that PKC activation can upregulate cell adhesion molecules in mesangial cells, promote leukocyte adhesion and aggregation in glomeruli, and accelerate the glomerular injury.

Lyso-PC can also inhibit Na^+, K^+-ATPase activity, thereby causing abnormalities in cell structures and functions, and ultimately leading to hemodynamic disorder. Na^+, K^+-ATP enzymes are ubiquitous in cell membranes and are involved in many biochemical processes. Their activity may be associated with the occurrence of many diseases.

9.4.2.2 The Quantitative Study of Phospholipids Nine potential biomarkers identified to be related to diabetic nephropathy were quantified in the study of TSF, including PC m/z = 792, m/z = 826, m/z = 830, m/z = 854, m/z = 802, PE m/z = 750, PI m/z = 885, SM m/z = 747, and Lyso-PC m/z = 540.

Figure 9.18 lists the quantitative results of nine compounds. The figure includes the data after drug administration for both the experimental group and the control group.

After administration, the concentrations of PC m/z = 792, PC m/z = 830, PE m/z = 750, and PI m/z = 885 in the patients significantly improved after 6 months, and were close to the normal level; the concentrations of PC m/z = 826, PC m/z = 854, and PC m/z = 802 had also improved, but there were still significant differences between the patients and the healthy people. In addition, treatments had little impact on LPC 540. Therefore, the treatment had alleviated the disorders of phospholipid metabolism.

The results of nine compounds among the four groups showed that there was a significant difference between the Chinese medicine treatment group and the control group (Western medicine group) (P=0.001), which demonstrated that TSF was better than Western treatment for the disorder of phospholipid metabolism.

9.4.2.3 The Study of Fatty Acid Metabolism The contents of 14 fatty acids after treatment were analyzed in the research, and the results are shown in Fig. 9.19. The fatty acids can be maintained at a relatively stable level in the

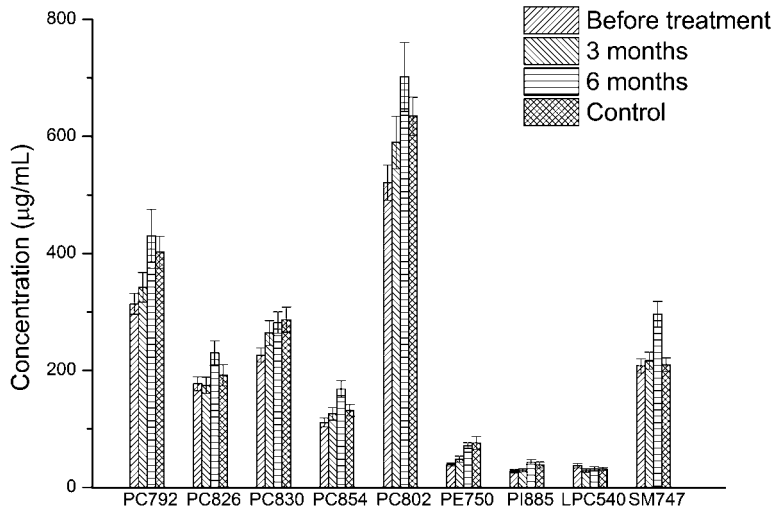

Fig. 9.18 Comparison of nine phospholipid compounds concentration among four groups (before treatment, treatment after 3 months, treatment after 6 months, and control).

body for a long period of time, and ultimately get to a relatively low level after 6 months, which is due to the long-term efficacy of TCM.

The content of certain fatty acids could increase slightly in 3 months, but generally showed a downward trend in 6 months, which may be due to the gentle effects of TCM. In the initial period of the treatment, TCM could not control the progress of the disease, but it eventually restored the balance of fatty acids in the body.

9.4.2.4 The Study of Purine and Pyrimidine Metabolism The quantitative analysis of purine and pyrimidine compounds in plasma samples after treatment was carried out. A total of four compounds among them changed obviously, namely uric acid, xanthine, cytosine, and thymidine.

As shown in Fig. 9.20, thymidine levels increased with the development of DN in the cross-sectional study. Patients before treatment had significantly higher thymidine level than the normal group did. Thymidine levels were significantly decreased in Western and TCM treatment groups after 3 months, and continued to decline during the 6 month period. At the end, the amount of thymidine was closer to normal, indicating that the treatment is effective.

In the cross-sectional study, with the increase in severity, cytosine content of the patients before treatment was significantly higher than that of the normal group. For the Western treatment, cytosine levels decreased significantly in 3 months, and continued to decline in the 6 month period. For TCM treatment, there was significant decrease in 3 months, and the decline was

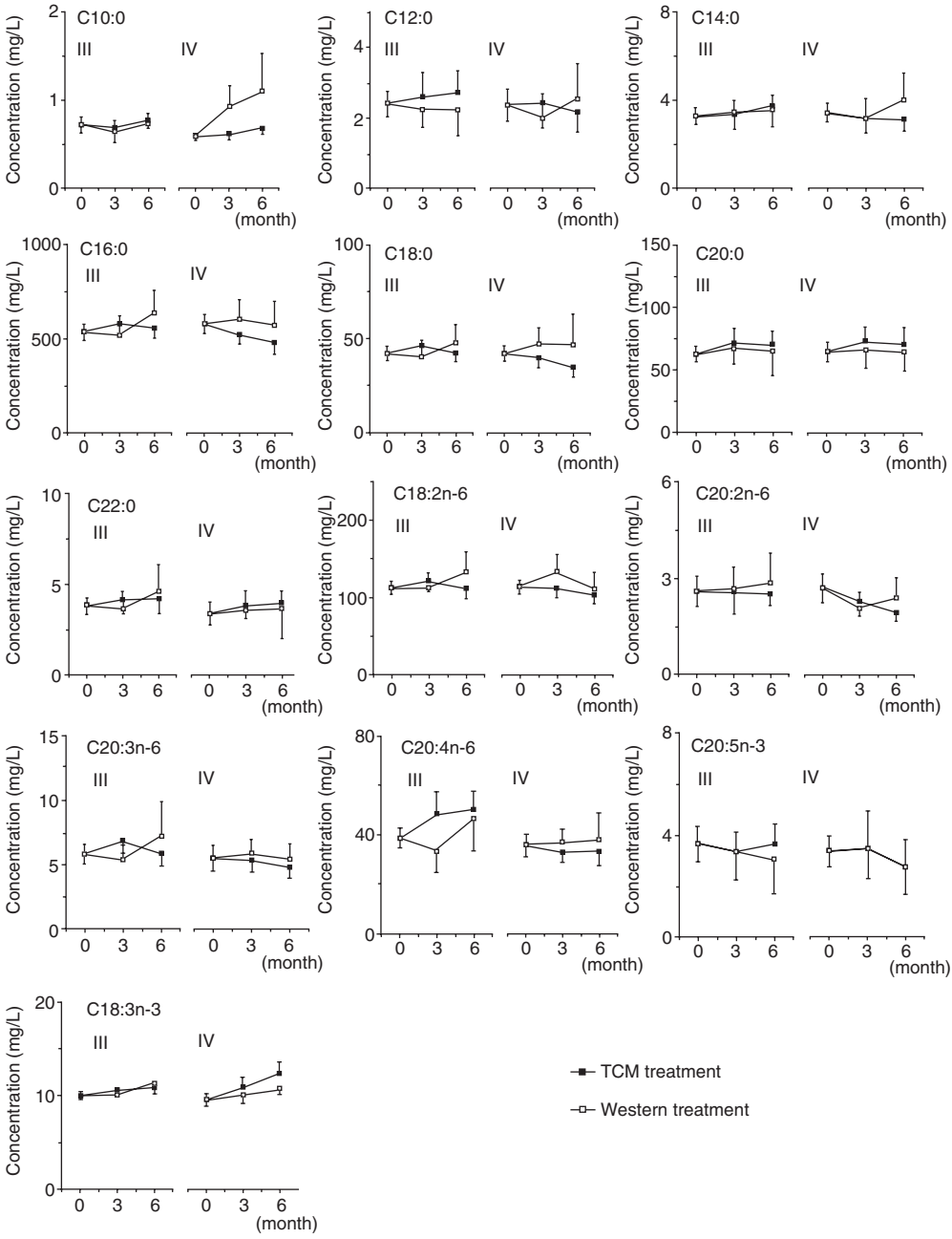

Fig. 9.19 Comparison of fatty acid concentration for different treatment methods.

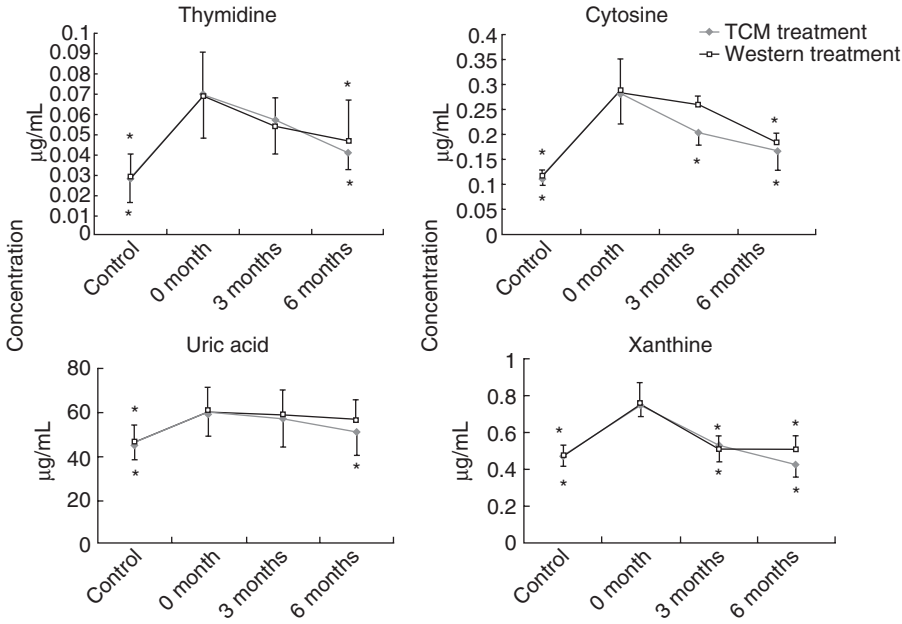

Fig. 9.20 Comparison of purine and pyrimidine compounds concentration for different treatment.

greater than it was in the Western medicine group. The content of cytosine was closer to the normal level, indicating that the treatment is effective.

Uric acid levels increased with the development of DN. For the Western treatment, uric acid levels decreased significantly in 3 months, and continued to decline in 6 months. For TCM treatment, there was significant decrease in 3 months, and the decline was greater than that in the Western medicine group. The content of uric acid was closer to the normal level, indicating that the treatment is effective.

Xanthine levels increased with the development of DN. For the Western treatment, xanthine levels decreased significantly in 3 months, and continued to decline in 6 months. For TCM treatment, there was significant decrease in 3 months, and the decline was greater than that in the Western medicine group. The content of xanthine was closer to the normal level, indicating that the treatment is effective.

In the course of the treatment, uric acid, cytosine, xanthine, and thymidine levels were close to those of the healthy group. Both TCM and Western treatment obtained better results in 3 months. Uric acid, adenosine, xanthine, and thymidine concentrations decreased to near these of the healthy group. After 6 months, the concentration of uric acid and xanthine in TCM treatment decreased, while there was no significant difference in the western treatment. It can be suggested that TCM treatment is more effective than the western treatment.

Considering the composition of TSF in this study, the possible mechanism is described here. The TCM formula used in this study contains many raw herbs that have therapeutic effects on the disease, such as phenols and saponins from *Sanqi*, which clear lipid radicals and superoxide anions. Total flavonoids and total polysaccharides from *Weimao*, and anthraquinones from *Dahuang* can inhibit lipid peroxidation. 8-D-glucopyranose-4,7-dioxyisoflavone from kudzuvine root, polysaccharides PFCC I, PFCA II, and iridoid glycosides from *Shanzhuyu* can inhibit the activity of xanthine oxidase, reducing AGEs.

The amount of uric acid and xanthine in the patients receiving the TCM treatment was greater than that of the patients receiving Western medicine treatment. Therefore, this study speculated that the most important therapeutic mechanism of TCM is from its antioxidant effect, and regulation of the level of oxidative stress.

9.4.2.5 The Study of Aminothiols Metabolism This study analyzed eight aminothiols in different groups. Cys-gly and GSH in glutathione metabolism and Hcy, SAH, and Met in Hcy metabolism had significant changes among the groups. However, Cys and Cysta had no significant change. The results are shown in Fig. 9.21.

The results showed that there were trends in the TCM treatment groups and the Western treatment groups. TCM treatment is relatively palliative, while Western treatment results vary greatly among the patients, because Western medicine relies more on individual sensitivity to drugs. Levels of several compounds returned close to normal, proving that both TCM and Western treatment can effectively improve GSH and Hcy metabolisms of DN III and DN IV patients.

Cysta, GSH, Hcy, Met, and SAH levels in patients after treatment for 3 months showed significant differences compared with their levels before the treatments. GSH and SAH levels after 6 months of treatment were in the healthy range.

9.5 GENOMICS STUDY OF DIABETIC NEUROPATHY

Pathogenesis of DN is based on multiple genes, including cytokines, growth factors, and other factors that participate in glucose metabolism and hemodynamics. Four susceptibility genes, namely CDKN2A, CDKN2B, IGF2BP2, and CDKAL1, were chosen from the research of genome-wide association study. Four sugar metabolism-related genes, namely aldose reductase (AR), advanced glycation end product receptor (AGER), glucose transporter 1 (GLUT1), and insulin-like growth factor 2 (IGF2); four hemodynamic-related genes, namely angiotensinogen (AGT), β3-adrenergic receptor (ADRB3), angiotensin II (AGTR2), and Angiotensin-converting enzyme (ACE) gene; and important genes in basal metabolism, namely PK*C* (PRKCA) and 5,10-

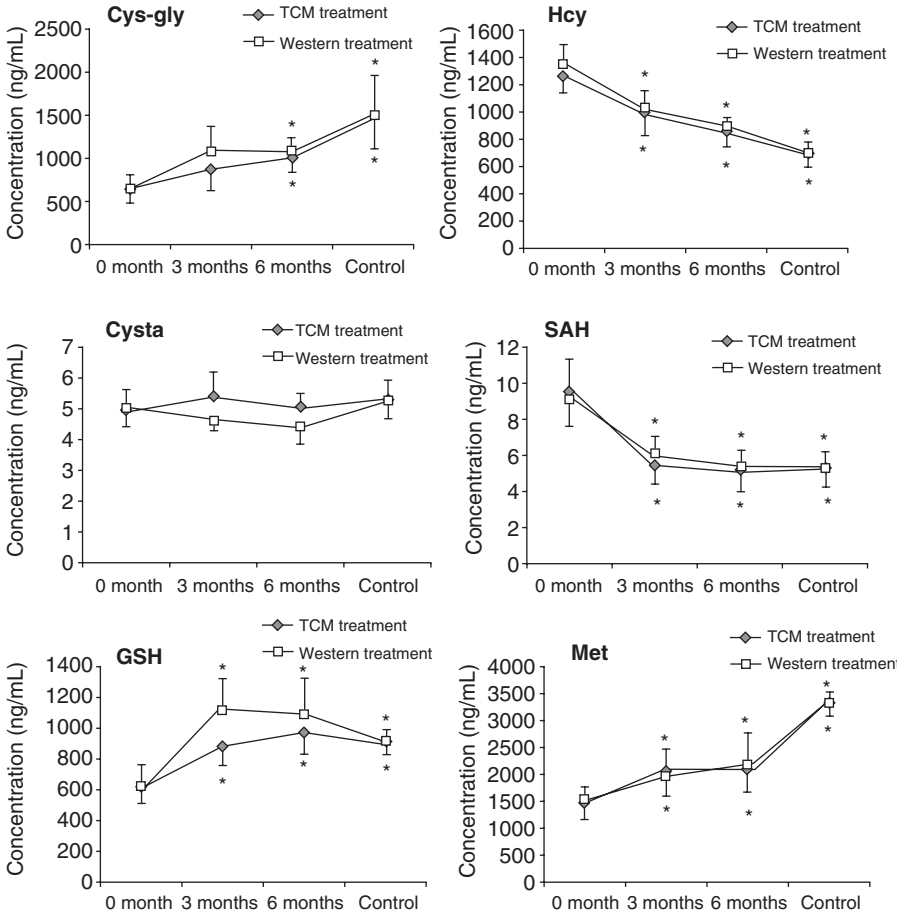

Fig. 9.21 Comparison of aminothiols concentration in TCM treatment group and control group after treatment.

methylenetetrahydrofolate reductase gene (MTHFR), were also chosen for the study.

9.5.1 mRNA Study According to the Mogensen Staging

Based on literature and experimental screening, fourteen diabetic neuropathy-related functional genes were chosen in this study, with GAPDH being the internal standard for the gene expression. The steps for real-time PCR measurement of gene expression included RNA extraction, cDNA preparation, primer design, real-time PCR methodology establishment, sample analysis, and data processing. According to Mogensen's staging, patients were categorized into DM, DN III, DN IV, and DN V groups. The gene expressions within

TABLE 9.22 Quantitative Determination of 14 Genes According to Mongensen Staging

Case/CON	AR	AGER	IGF2BP2	CDKN2A	CDKAL1	CDKN2B	GLUT1
DM	1.45 ± 0.19	0.38 ± 0.29	1.95 ± 2.71	0.88 ± 1.16	0.1 ± 0.10	0.48 ± 0.74	0.32 ± 0.29
DN III	6.56 ± 1.34	0.41 ± 0.49	9.59 ± 4.44	0.41 ± 0.28	0.25 ± 0.57	0.03 ± 0.05	0.71 ± 1.13
DN IV	3.00 ± 2.90	0.69 ± 0.91	12.75 ± 2.92	0.50 ± 0.52	0.04 ± 0.06	0.03 ± 0.06	0.49 ± 0.85
DN V	1.45 ± 1.47	0.29 ± 0.34	1.10 ± 1.72	0.26 ± 0.20	1.26 ± 0.29	1.27 ± 0.48	0.54 ± 0.68
Case/CON	MTHFR	PKC	IGF2	ACE	ADRB3	AGT	AGTR2
DM	0.03 ± 0.01	0.02 ± 0.04	0.21 ± 0.15	0.64 ± 1.17	0.33 ± 0.33	0.36 ± 0.42	0.37 ± 0.31
DN III	0.04 ± 0.07	0.0002 ± 0.0003	1.37 ± 1.12	0.31 ± 0.65	0.38 ± 0.31	0.23 ± 0.15	0.39 ± 0.30
DN IV	0.01 ± 0.01	3.53 ± 2.19	1.88 ± 1.79	0.01 ± 0.02	0.78 ± 1.13	0.09 ± 0.09	0.55 ± 0.73
DN V	0.01 ± 0.01	0.03 ± 0.06	2.31 ± 2.6	0.33 ± 0.55	0.76 ± 1.09	0.16 ± 0.18	0.14 ± 0.11

each of the abovementioned stages were subjected to statistical processing before being compared among different stages. The statistical result was as follows.

9.5.1.1 DN Susceptibility Genes

9.5.1.1.1 CDKAL1 CDKAL1, partially residing in the cytoplasm, has a transmembrane domain without signaling peptides. The expressions of CDKAL1 in brain, muscle, and kidney tissue are suspected to be associated with the function of insulin receptors.[58] The expression of CDKAL1 was significantly different between the controls and type II diabetes patients ($p < 0.05$), so it should be recognized as a susceptibility gene and appreciated in the early diagnosis of diabetes. Its level increased in patients at the DN III stage, indicating that CDKAL1 expression is closely related to the development of the disease. On the other hand, the decreased expression of CDNKAL1 in the patients of DN IV stage along with its increased expression in the patients of DN V stage indicate that the expression of CDKAL1 in DN V stage is significantly different from that of DN III and IV stage patients. It is proposed that CDKAL1 could be an indicator gene for stage diagnosis of DN V. This result could be further used in the study of DM and diabetic neuropathy.

9.5.1.1.2 CDKN2A The difference of CDKN2A expression between the controls and the DM patients was not very obvious, indicating that the susceptibility of the gene was possibly merely reflected in mutation of the gene sequence and specific sites, rather than in the gene expression. Among the three DN stages, CDKN2A levels stayed relatively stable without significant variation. However, its low expression in DN patients compared with healthy subjects suggested the possibility that CDKN2A downregulation could be related to different DN stages.

CDKN2A is a tumor suppressor gene residing in chromosome 9p21, which encodes two different kinds of tumor suppressor proteins. The CDKN2A gene is closely associated with lung cancer, liver cancer, and stomach cancer, and suppresses tumors.[59] It can be inferred that there is a positive relation between downregulation of the CDKN2A gene and development of the disease, as the patients in the DN V stage, the most serious stage, had the minimum expression of CDKN2A. The transition of the disease from the controls to the patients should be recognized as a harmful process for normal tissues and cells. Therefore, downregulation of CDKN2A should be associated with the progression of the disease.

9.5.1.1.3 CDKN2B CDKN2B is an analog of pl6 in the cDNA library that was formed from TGF2β inhibited HaCaT cell line using cDNA of pl6NK4A as the probe, and was cloned by the Beaeh group.[60] CDKN2B is located in the chromosome 9p21 domain, whose abnormal expression (null, mutation, or rearrangement) is observed in multiple tumor and cancer cell lines.[61]

DM patients showed around 50% lower CDKN2B expression compared with the healthy subjects, proving its role as a susceptibility gene of diabetic neuropathy. Meanwhile, the large RSD of CDKN2B expression in both the healthy and the patients with type II diabetes demonstrated the individual variance of its expression. In the patients of DN III and DN IV, expression of CDKN2B was significantly lower than the controls and other patients with type II diabetes. The gene could be used in the prevention and early diagnosis of DN patients. Among DN V patients, the expression of the CDKN2B gene was significantly higher than that in the healthy and patients with other type 2 diabetes, revealing that the CDKN2B gene could be used as an index gene of DN V, in the diagnosis of DN V, and as a potential target of chemotherapy. The high expression of CDKN2B in patients of DN V stage suggested the severity of the disease.

9.5.1.1.4 IGF2BP2 The genetic variation of the IGF2BP2 gene might increase the risk of impaired fasting blood glucose. Susceptible SNP sites are strongly related to fasting glucose, glycosylated hemoglobin, and especially insulin β cell function.[62]

From the results of the quantitative analysis, the expression of IGF2BP2, a diabetes susceptible gene, showed greater variation in patients with diabetes, so it led to unclear separation among the controls and diabetes patients. Also, it was found that the IGF2BP2 gene was strongly correlated with the DN III and DN IV stages, as during these two stages the expression of IGF2BP2 increased significantly. It should be pointed out that the increased gene expression was differentiable among the other three groups, suggesting the possibility of IGF2BP2 being a specific gene in the diagnosis and treatment of patients in the DN III and DN IV stages. In addition, IGF2BP2 expression decreased greatly in DN V, showing the substantial change of the disease. The disease is usually accompanied by some severe physiological and pathological conditions, such as uremia.

It has been widely recognized that the polymorphisms of CDKAL1, CDKN2A, CDKN2B, and IGF2BP2 genes were obvious in type 2 diabetes. However, only CDKAL1 and IGF2BP2 expressions rather than CDKN2A and CDKN2B expressions changed significantly between the controls and diabetic patients. Besides, these genes showed different sensitivity toward the different DN stages. CDKAL1 and CDKN2B were more sensitive to the patients in DN V, while IGF2BP2 was sensitive to the patients in DN III and DN IV. Downregulation of CDKN2B gene was positively related to the severity of DN.

9.5.1.2 Glucose Metabolism-Related Genes

9.5.1.2.1 AR The expression of the AR gene increased from the controls to the DM groups. AR gene expression was significantly increased in patients of DN III, reaching $1.14 \times 10^{-1} \pm 2.32 \times 10^{-2}$. Its expression was 6 to 7 times higher than that of the controls, and 4 to 5 times that of the patients in DM. As

the disease worsened, the expression of the AR gene decreased in the patients of DN IV and DN V. As the AR gene was highly correlated with patients having DN III, it can be used in early prevention and diagnosis of DN.

Patients with type 2 diabetes always have high blood sugar levels, which is an important factor to induce DM to develop into DN.[63] With continued high glucose in the blood, the stress response and adaptability activates the AR gene, causing the expression of AR gene to increase significantly in the early stages of diabetes. The strong expression of the AR gene leads to activation of the polyol pathway, causing increased sorbitol content and accumulation of intracellular osmotic pressure, thus damaging glomerular, tubular, and renal microvascular endothelial cells. Dysfunctional glucose metabolism was found in patients of DN IV and DN V, resulting in decreased AR gene expression.

The expression of the AR gene showed significant difference between the DM and DN groups ($p < 0.05$), showing its effectiveness for the prevention and early diagnosis of DN. AR gene expression is significantly different among the patients in different stages ($p < 0.05$), and can effectively differentiate the patients of DN.

9.5.1.2.2 AGER The expression of the AGER gene in the healthy group was much higher than that in the groups with DM and in different stages of DN, indicating that the reduced expression of AGER was closely related to the occurrence of the disease. The expression of AGER in DM patients was significantly reduced from that of the controls. It may be because the AGER gene is the glycation end product receptor. The patients with type 2 diabetes had disorders in glucose metabolism. There are fewer glycation end products compared with the controls, causing lower expression of the AGER gene in patients. The expression of AGER increased in the patients of DN III, but it still maintained the same level in the type 2 diabetic patients.

Among the three stages of DN, AGER was more sensitive to the patients of DN IV than the other two stages, and its expression was obviously high in DN IV, so it can be used to aid the diagnosis of DN IV.

It was speculated that the AGER gene may be one of the factors that promote the transition of DM into DN. Sustained high levels of blood sugar in patients with type 2 diabetes lead to increased advanced glycation end products. However, the low expression of AGER gene cannot be enough for the metabolism of glycation end products, resulting in the accumulation of advanced glycation end products in the body. In the end, it breaks the glomerular rate and the osmotic balance, resulting in the occurrence and development of DN.

9.5.1.2.3 GLUT1 The changing trend of the GLUT1 gene is consistent with that of the AGER gene during the development of the disease. Its expression in patients with type 2 diabetes was significantly lower than that in the controls. In the three stages of DN, GLUT1 expression did not change significantly,

indicating that the expression of the gene was slightly lower than it was in the controls, but much higher than it was in the patients with diabetes. GLUT1 expression in the DN III patients was higher than in the DN IV and DN V patients, and it significantly increased in the diabetes patients. GLUT1 can also contribute to the early prevention and diagnosis of DN.

It is widely known that high blood glucose is one of the reasons for increasing damage to the glomerular extracellular matrix and kidneys. A growing number of studies have confirmed that the increase of GLUT1 in mesangial cells was more closely related to extracellular matrix functions.[64] It was suggested that GLUT1 and AGER may also lead type 2 diabetes patients to develop DN. From the results of the gene expression, it could be seen that its expression was low in the patients with type 2 diabetes. It is also known that GLUT1 is a glucose transporter. Therefore, low expression of glucose transporter in the blood results in high levels of blood sugar, thereby affecting the metabolic changes and renal functions.

9.5.1.2.4 IGF2 The expression of the IGF2 gene was significantly lower in the patients with type 2 diabetes than the controls, indicating that the IGF2 gene has a high correlation with diabetes. IGF2 expression was significantly increased in the development from DM to DN III, so it could be useful in the early prevention and diagnosis of DN. IGF2 expression levels increase with the progression of the disease, indicating that upregulation of IGF2 is related to the development of the disease.

Low expression of IGF2 in diabetes patients may also lead to high blood sugar in the body and the occurrence of DN. Because low expression of IGF2 is a cause of low insulin levels in patients, the blood glucose levels remain high in the patients.

Kono T et al.[65] found that mRNA of IGF2 could be detected in the fetal liver, kidney, adrenal gland, cerebral cortex, cartilage, skeletal muscle, lung, and placenta using digoxigenin labeled cRNA probe by in situ hybridization and immunocytochemistry, and undifferentiated epithelial cells in renal tubules show the strongest fluorescence. This shows that the IGF2 gene plays an important role in renal tubular function; and it confirms that IGF2 has a close relationship with the occurrence of DN.

Disorders of glucose metabolism can lead to increased intracellular glucose concentrations, induce damages, and promote the development of DN through nonenzymatic glycation and activation of the polyol pathway. Disorders of lipid metabolism can induce the release of inflammatory factors, increase oxidative stress, damage glomerular filtration, and promote glomerulosclerosis. The AR gene is more sensitive to the DN III patients, and can effectively distinguish among the three stages of DN. The AGER gene was more sensitive to the DN IV patients, and the GLUT1 gene was more sensitive to the DN III patients. These two genes can induce the occurrence of DN. The expression of the IGF2 gene showed a positive correlation with the development of the disease.

9.5.1.3 Hemodynamics-Related Genes

9.5.1.3.1 AGT AGT is an angiotensinogen, which is related to the regulation of blood pressure and hemodynamics. AGT expression in type 2 diabetes and different stages of DN patients was significantly lower than its level in the controls. AGT expression was decreased from diabetes to DN III, indicating that the reduction of AGT may cause the occurrence of DN. AGT expression decreased significantly from DN III to DN IV ($p < 0.05$), suggesting obvious hemodynamic change in the DN IV patients. AGT expression was increased in DN V due to the physiological and pathological changes in patients.

The low expression of AGT caused a low level of angiotensinogen in type 2 diabetes and different stages of DN patients, thus affecting their blood pressure and the hemodynamic status of the patients. Therefore, hemodynamic indices in patients were significantly lower than in the control group. This shows that the expression of the AGT gene might be associated with the progression of DN.

9.5.1.3.2 ADRB3 ADRB3 is an adrenergic receptor that regulates blood pressure in vivo. ADRB3 gene expression in type 2 diabetes and DN patients was generally lower than in the controls. In addition, increased expression of the ADRB3 gene had a positive correlation with the development of DN. ADRB3 gene expression may also lower blood pressure and cause some other hemodynamic changes, thus contributing to the occurrence and development of DN.

Different from AGT, the ADRB3 gene is an adrenergic receptor and its expression may be subject to the constraints of adrenaline in the body. Patients' conditions in DN IV and DN V had been more serious, as stress reactions and self-regulations occurred. Moreover, the adrenaline levels were the highest among the stages, which led to relatively strong expression of the ADRB3 gene. ADRB3 gene expression fluctuated in patients with DN IV and DN V, indicating that individual differences of epinephrine levels were obvious in the patients, leading to changes of its receptor ADRB3 gene.

9.5.1.3.3 AGTR2 The AGTR2 gene is the key point of renin-angiotensin regulation, and is important in DN development. The AGTR2 gene is mainly in vascular smooth muscle cells, which mediate the majority of biological effects by AGTR2. AGTR2 is an important candidate gene of the great vessels and microvascular disease in the patients with type 2 diabetes.[65]

AGTR2 gene expression in type 2 diabetes patients and patients in different stages of DN was generally lower compared with the normal amount. AGTR2 is an angiotensin II receptor, and its low expression is closely related to vascular contraction, blood pressure, and other hemodynamic indices, indicating the abnormal hemodynamic status in the patients. AGTR2 gene expression had no significant difference between DM and DN III, suggesting that AGTR2 might have little effect going from type 2 diabetes to DN. The AGTR2 gene was more sensitive to the patients of DN IV, having the highest expression in

the three stages of DN. The expression of AGTR2 was significantly low in DN V patients, indicating that physiological, organizational, and hemodynamic conditions have significantly deteriorated in the DN V patients.

9.5.1.3.4 ACE ACE is a key enzyme in the renin-angiotensin system, can catalyze the conversion of angiotensin I to angiotensin II, and controls vasoconstriction through the angiotensin receptor.[66] It is essential for the blood pressure and hemodynamics.

The ACE gene is genetically similar to the AGT, ADRB3, and AGTR2 genes, and its expression was downregulated in patients with type 2 diabetes and different stages of DN. As an angiotensin-converting enzyme, ACE plays an important role in the regulation of blood pressure and blood flow. ACE expression was significantly reduced from type 2 diabetes to DN III. The ACE gene led to further deterioration of the hemodynamic status in patients with diabetes. Moreover, these patients ultimately developed DN. The expression of ACE was extremely low, at $1.047 \times 10^{-3} \pm 2.608 \times 10^{-3}$ in DN IV patients. After moving into the DN V stage, ACE expression returned to the level of DN III patients, which may be caused by the opposition to the hemodynamic status in the body.

The study found that the expressions of AGT, ADRB3, AGTR2, and ACE genes in patients with type 2 diabetic and different stages of DN were generally lower than the controls. It was indicated that low expressions of these hemodynamic-related genes directly led to abnormal hemodynamic status in the patients, which may be one of the causes for hypertension in the patients with diabetes, and for promoting development of DN in diabetes patients. During the transformation process of DM to DN, AGT and ACE gene expressions were significantly reduced, suggesting that these two genes may be the cause of DN occurrence.

The ADRB3 gene was more sensitive to the patients of DN IV and DN V, and the AGTR2 gene was more sensitive to the DN IV patients. ACE gene expression in DN IV patients is significantly lower than it is in patients in the other groups, and it can be used as a target index gene for the diagnosis of DN IV.

9.5.1.4 Other Related Genes

9.5.1.4.1 PRKCA PKC can activate the plasmatic target enzyme-based regulation process and affect nuclear transcription factors by gene expression regulation. Many of those genes are associated with cell growth and differentiation, and the most important one is the signal transduction protein.

As shown in the table, type 2 diabetes and DN III/V patients had an abnormally low expression of PRKCA compared to the control subjects. However, the PRKCA gene was sensitive to DN IV, as it had much higher expression ($5.243 \times 10^{-2} \pm 3.250 \times 10^{-2}$) in the DN IV patients compared with the control subjects. The abnormality in DN IV revealed distinct changes in PKC-involved

metabolism, suggesting the potential of PKC as a DN stage IV indicator in diagnosis and treatment.

9.5.1.4.2 MTHFR MTHFR is an important component in folic acid metabolism and a key enzyme in methionine metabolism. It functions in the reduction of internal N5, N1-methylenetetrahydrofolate to N5-methylenetetrahydrofolate, and the methylation of Hcy in blood for the maintenance of normal Hcy levels.[67]

It could be concluded that MTHFR is one of the key genes in maintaining normal bioactivity, and its abnormal expression would lead to diseases or other physiological conditions. As seen from the results, type 2 diabetes and DN patients showed decreased MTHFR expression compared with the healthy subjects. MTHFR expression progressively decreased in DN III, IV, and V as the disease worsened, suggesting that it could be a potential index for the DN stage.

PRKCA and MTHFR are both associated with internal signal transduction and metabolism, and show certain relationships between diabetes and DN. PRKCA is highly sensitive toward the patients of DN IV, so that permits its role as an index for DN stage IV. MTHFR showed lower expression in the diabetes and DN patients and was positively related to the severity of the disease, allowing it to be a useful index of the healthiness of the body.

9.5.2 The mRNA Study Based on TCM Syndrome Differentiation Typing

The expressions of 14 genes were corrected by the housekeeping gene, GAPDH. All the DN patients were divided into DQY-PY, DQY-PQ, and DYY groups according to TCM syndrome typing, and the gene expressions were analyzed in each group. The results are listed in Table 9.23.

9.5.2.1 Diabetes Susceptibility Genes

9.5.2.1.1 CDKAL1 Gene The expression of the CDKAL1 gene in DN patients of DQY-PY was much higher, while it was significantly lower than the controls. It showed that the CDKAL1 gene was more sensitive in DQY-PQ. The expression of the CDKAL1 gene decreased from DM to DQY-PY. However, CDKAL1 gene expression fluctuated in patients of DQY-PQ. This may be due to the large individual differences existing according to the syndrome differentiation of TCM types.

9.5.2.1.2 CDKN2A The expression of the CDKN2A gene was gradually reduced with the development from DQY-PY to DQY-PQ to DYY. The results showed that CDKN2A gene expression was positively correlated with the development of DN.

Referring to the theory of TCM, *Yin* was used to emphasize the chemical substance basis of the human body and organs, while *Yang* and *qi* emphasize

TABLE 9.23 Quantitative Determination of 14 Genes According to TCM Syndrome Typing

Case/CON	AR	AGER	IGF2BP2	CDKN2A	CDKAL1	CDKN2B	GLUT1
DM	1.45 ± 0.18	0.38 ± 0.29	1.95 ± 2.71	0.88 ± 1.16	0.09 ± 0.10	0.48 ± 0.74	0.32 ± 0.29
DQY-PY	4.94 ± 0.87	0.43 ± 0.36	8.96 ± 17.13	0.74 ± 1.10	0.05 ± 0.05	1.94 ± 2.37	37.62 ± 9.22
DQY-PQ	2.34 ± 1.78	0.66 ± 0.58	6.67 ± 14.85	0.59 ± 0.58	1.38 ± 2.47	1.08 ± 1.95	0.22 ± 0.29
DYY	1.28 ± 0.08	0.23 ± 0.23	0.18 ± 0.27	0.26 ± 0.21	0.04 ± 0.07	0.01 ± 0.01	0.07 ± 0.11
Case/CON	MTHFR	PKC	IGF2	ACE	ADRB3	AGT	AGTR2
DM	0.03 ± 0.01	0.02 ± 0.04	0.21 ± 0.15	0.64 ± 1.17	0.33 ± 0.33	0.36 ± 0.42	0.37 ± 0.31
DQY-PY	0.01 ± 0.01	0.17 ± 0.4	1.30 ± 1.43	0.53 ± 0.86	0.26 ± 0.36	0.25 ± 0.21	0.35 ± 0.36
DQY-PQ	0.14 ± 0.14	8.16 ± 19.86	2.06 ± 1.92	0.01 ± 0.01	0.33 ± 0.33	0.12 ± 0.07	0.37 ± 0.35
DYY	0.00 ± 0.00	0.004 ± 0.01	1.39 ± 2.14	0.73 ± 1.06	0.95 ± 1.15	0.06 ± 0.05	0.24 ± 0.20

the function of the human body and organ performance. The development of DN patients from DQY-PY to DQY-PQ to DYY reflected change of the disorder from the organ itself, thereby affecting its functions and ultimately leading to deterioration of the organ chemical substance basis and physiological function. As CDKN2A is a tumor suppressor gene, its decreased level also implies the increased risk of cancer in patients.

9.5.2.1.3 CDKN2B The CDKN2B gene is similar to the CDKN2A gene. The expression of CDKN2B significantly decreased with the development from DQY-PY to DQY-PQ. However, CDKN2B gene expression significantly increased from DM to DQY-PY, indicating that there was change of the chemical substance basis and infrastructure in the kidney and other organs of the patients with DN. The gene expression was much lowered in the patients of DYY, having a level of only $1.225 \times 10^{-5} \pm 2.145 \times 10^{-5}$, which is 0.7% that of the control group. It was suggested that change happened on the glomerular structure, thus affecting its function.

CDKN2B gene expression played an important role in the progression of the disease, reflecting the chemical substance basis of the patients' organs that changed with the disease, thereby affecting its function, and ultimately leading to deterioration of the organ structure and function. The low expression level of CDKN2B gene in the patients of DYY provided a reference value for the syndrome differentiation typing of TCM.

9.5.2.1.4 IGF2BP2 The gene expression of IGF2BP2 was found to be similar to the expression of the CDKN2B gene in TCM syndrome differentiation typing. There was positive correlation between the reduction of gene expression and disease progression. At the same time, the expression of IGF2BP2 gene was highly sensitive to the patients of DYY, and its expression in DYY is 2% of that in DQY-PY, which is significantly decreased. It is also an important reference for syndrome differentiation typing of TCM. IGF2BP2 gene expression fluctuates in the patients of DQY-PY and DQY-PQ. This may be due to the large individual differences that exist according to the syndrome differentiation of TCM typing.

The expression of four diabetes susceptibility genes, CDKAL1, CDKN2A, CDKN2B, and IGF2BP2, were more sensitive in the patients of DQY-PQ, and their expressions in DQY-PQ were much higher than the other groups. The gene expression of CDKN2A, CDKN2B, and IGF2BP2 were reduced along with the worsening of the disease, and the changes of the three genes were positively correlated with the development of the disease. In addition, the expressions of these genes in patients of DYY were significantly lower than the other groups, providing reference and confirming the TCM syndrome differentiation typing.

9.5.2.2 Glucose Metabolism-Related Genes

9.5.2.2.1 AR The expression of the AR gene was progressively reduced in different TCM syndrome differentiation types, which was similar to the results

of western staging. The expression levels of the AR gene showed significant differences among the DQY-PY, DQY-PQ, and DYY groups, showing that the AR gene can be used to effectively distinguish the three types of TCM syndrome differentiation typing. In the patients of DQY-PY, the expression of AR gene was 4.9 times higher than that of the healthy subjects, so it can provide authentication information for syndrome differentiation typing.

Deficiency of *qi* and *Yin* showed an abnormal chemical substance basis in the body's organs and tissues. The AR gene is also a type of Aldose reductase. Its high expression indicates that the structural change in the kidneys and other organs may be due to long-term hyperglycemia in vivo.

9.5.2.2.2 AGER Results showed that the expressions of the AGER gene in DM and each differentiation of different syndrome differentiation types in patients were much lower than normal. The expression of AGER gene was more sensitive in DQY-PQ, achieving $2.098 \times 10^{-3} \pm 1.847 \times 10^{-3}$. Its expression was significantly upregulated in the other two syndrome differentiation types.

According to the TCM theory, *qi* refers to the nutrients in the body and one's mental state. The AGER gene is the end product receptor of advanced glycation, which is closely related to transmembrane sugar transportation in vivo. Its function also coincides with the TCM philosophy of *qi*, so that the expression of the AGER gene in DQY-PQ is significantly different from that of the other two syndrome differentiation types.

9.5.2.2.3 GLUT1 The expression of the GLUT1 gene was found to be significantly different in TCM typing and Mogensen staging, reflecting the differences between the two diagnostic methods. The expression of GLUT1 was abnormally high in the DQY-PY patients, reaching $2.296 \times 10^{-1} \pm 5.626 \times 10^{-2}$, and was 37.6 times higher than the control.

GLUT1 in mesangial cells is a major glucose transporter protein, and the abnormal gene expression may lead to glomerular hypertrophy, mesangial expansion, and fibrosis. All of these are related to the structure of the kidney. Moreover, it shows the same philosophy of *Yin* according to TCM. Therefore, the GLUT1 gene shows a high degree of specificity with the DQY-PY patients. It provides a quantitative index for validation and verification of TCM syndrome differentiation typing.

9.5.2.2.4 IGF2 Results showed that IGF2 gene expression was stable in the different TCM syndrome differentiation types of DN, although it was significantly higher than that of the DM patients. This implies that upregulation of the IGF2 gene is closely related to the occurrence of DQY and DYY. At the same time, the IGF2 genes in different syndrome differentiation types of DN patients had significant differences compared to the normal levels. The expression of the IGF2 gene was higher in DQY-PQ patients, as it reached 13.37 ± 12.44. It was useful as a reference for TCM syndrome differentiation typing.

The AR gene was more sensitive to the patients of DQY-PY, and it can effectively distinguish the three types of TCM syndrome differentiation typing. The AGER and IGF2 genes were more sensitive to the patients of DQY-PQ, which also verified that the two genes involved in the transport and metabolism matched with the philosophy of *qi* according to TCM. The GLUT1 gene is related to the physiological changes and structural basis of renal tubules, and corresponds to the chemical substance basis of *Yin* in TCM philosophy, showing the sensitivity and specificity of DQY-PY.

9.5.2.3 Hemodynamics-Related Genes

9.5.2.3.1 AGT AGT gene expression showed a gradual decrease from the control, DM, DQY-PY, and DQY-PQ groups, to DYY. The AGT gene is a angiotensinogen and is involved in the regulation of blood pressure and hemodynamics. Reduced AGT gene expression indicates the abnormality of hemodynamics in patients. The trend of genetic change also reflects the progression of the disease. To some extent, it was proved with the scientific validation of TCM. The expression of AGT gets much lower as the severity of the disease increases. Moreover, it was found that the results of AGT gene expression did not have significant fluctuations in various TCM syndrome differentiation types, indicating that the AGT gene was a better fit for TCM syndrome differentiation typing.

9.5.2.3.2 ADRB3 The expression of the ADRB3 gene was significantly reduced from the healthy level compared to levels in patients with DM, and was near the level of 0.1345 ± 0.1369 with the development of the disease from DQY to DYY. It showed that ADRB3 may not the gene lead to the deficiency of *qi* and *Yin*, so the expression of the gene in DQY and DYY was consisted with its expression in DM. But the ADRB3 gene showed a higher sensitivity in DYY patients, and the expression was significantly higher than in other patients of TCM syndrome differentiation types.

In the DYY patients, the basis of organ function and structure changed, so that ADRB3 expression again increased to approximately the normal level. It may be related to stress and generation of instinctive compensation in the body.

9.5.2.3.3 AGTR2 The changes of the AGTR2 expression gene were consistent with those of the AGT gene, showing lower levels in the patients with DM and different TCM syndrome differentiation types, and was significantly lower than the normal ($p < 0.05$).

It showed hemodynamic abnormalities in the DQY and DYY patients, and gradually deteriorated with the development of the disease. AGTR2 gene expression was the lowest in the DYY patients among various TCM syndrome differentiation types, suggesting the patients of this type with hemodynamic status worsened.

9.5.2.3.4 ACE Gene There was no significant difference of ACE expression in DM and DQY-PY. However, the expression of ACE in the patients with DQY-PQ was only 1.8% of that in the patients with DQY-PY, indicating that the ACE gene was highly sensitive for the deficiency of *qi*. The low expression of the ACE gene may lead to extreme deterioration of hemodynamics and deficiency of *qi* in TCM theory, which is closely related to the TCM syndrome differentiation typing. *Qi*, according to TCM, also reflects the substance of transport and energy status, which may also be the reason for low expression of the ACE gene in the patients. ACE expression in the DYY patients returned to the level of DM and DQY, which may be due to stress and compensatory actions in the body.

Expressions of hemodynamic-related genes AGT and AGTR2 were reduced with the progresses of the disease, corresponding with the TCM syndrome differentiation typing, and reflecting gradual deterioration of the condition. The changes of AGT and AGTR2 genes were positively correlated with the classification of DN according to TCM syndrome differentiation typing. The ADRB3 gene was more sensitive to DYY patients, showing that it could be a better indicator for syndrome differentiation typing of TCM. The ACE gene showed high specificity to the DQY-PQ patients of DN, and its expression was significantly lower than that of the other TCM syndrome differentiation types, so it can provide a quantitative verification and reference index for TCM syndrome differentiation typing diagnosis.

9.5.2.4 Other Related Genes

9.5.2.4.1 PRKCA The PRKCA gene was very sensitive to the DQY-PQ patients. PRKCA expression reached $1.210 \times 10^{-1} \pm 2.94 \times 10^{-1}$ in the DQY-PQ patients, which was significantly higher than that in the controls, DM, and other types of TCM syndrome differentiation typing patients. As a subtype of PKC, PRKCA can activate a variety of enzymes in the cytoplasm, and participate in the regulation of many biochemical reactions. *Qi* in TCM also emphasizes transport and metabolism, and symbolizes the energy required to promote normal functioning of various tissues and organs. Because the PRKCA gene had high specificity and sensitivity in DQY-PQ, it could be a good quantitative index for TCM syndrome differentiation typing. PRKCA gene expression was significantly lower than normal in the patients of other syndrome differentiation types.

9.5.2.4.2 MTHFR The expressions of the MTHFR gene in patients with DM and DN were generally decreased. MTHFR is a key enzyme in folate metabolism, and is a very important enzyme in metabolic processes. Once abnormality occurs, its expression is reduced in the body. MTHFR expression in DN patients was significantly lower than normal. The MTHFR gene was more sensitive to the DQY-PQ patients according to the TCM syndrome differentiation typing, and its expression in the DQY-PQ patients was the

highest among the three TCM syndrome differentiation types. It reached $8.146 \times 10^{-3} \pm 8.146 \times 10^{-3}$, which is 14% of the normal level. Because the condition of DYY had become very serious, the MTHFR expression was at its lowest, $1.961 \times 10^{-5} \pm 4.772 \times 10^{-5}$, less than 1% of the level of the control subjects. Therefore, the severity of the patient can be estimated through the expression of MTHFR.

PRKCA and MTHFR are important genes related to basic signal transduction and metabolism. They represent different characteristics among the TCM groups. The PRKCA gene was highly sensitive to the DQY-PQ patients, so it was a potential target for the treatment of DQY-PQ. MTHFR gene expression was significantly low in DM and different types of TCM syndrome differentiation types. Additionally, MTHFR expression was progressively reduced with the development of the disease. Therefore, the expression of MTHFR gene can be used to monitor changes of human health and the severity of DN.

9.6 THE INTEGRATED BIOMARKER SYSTEM OF DN

The biggest goals in metabonomics are to analyze qualitatively and quantitatively all the metabolites in specific systems simultaneously. However, limited by current technology, these goals are difficult to achieve. Metabolic fingerprints can characterize comprehensive features, called "featured mode," but it is not precise enough in quantification, as most biomarkers found are usually lipids and other biomarkers are not being studied specifically. On the other hand, quantitative determination, known as "microcosmic mode," can provide the exact amount of metabolites and have more specificity. However, the shortcoming of the model is that it is not comprehensive and complete enough, so it ignores a lot of unknown information. Therefore, integration of quantitative metabonomics data and metabolic fingerprint data is more complete and systemic.

For DN research, three aspects of integration, including the integration of quantitative determination and metabolic fingerprints, integration of IMPT and clinical biochemical symptoms, and integration of metabonomics and genetics, were applied in the study. The results of Western medicine staging and TCM typing for sample classification were improved significantly after the integration. The combined potential biomarkers group not only improved disease identification accuracy, but also contributed to tracking the development of the disease and evaluating the treatment efficacy.

Western medicine treats human disease (target organ, target tissue, and target), and oriental medicine treats sick people (syndrome). These two methods were fused to study the overall pattern of the disease. We believe that the characterization of the complex disease needs systematic research, including three levels of indices: pathological indices (biochemical imaging markers) according to western medicine, biomarkers identified from the basic research (genes, proteins, and metabolites), and quantitative indices of TCM theory (overall symptom). IBS was believed to be a new way for translational medicine.

TABLE 9.24 Comparison of Sample Distribution in Two Diagnostic Systems

	Control	DM	DN III	DN IV	DN V	Total
Control	30					
DM		22				22
DQY-PY			10	3	4	17
DQY-PQ			6	7	8	21
DYY			2	4	17	23
Total	30	22	18	14	29	113

9.6.1 The Possibility of Integration of TCM and Western Medicine

Mongensen staging is based on Western medical diagnosis while syndrome typing is based on TCM diagnosis. Although many differences exist between the two medical systems, a high correlation was found in our study. The distribution of all the patients by TCM syndrome typing in the group of Mogensen stages (including DN III, DN IV and DN V) is listed in Table 9.24.

The diagnostic results of illness were similar in both systems. For example, the sickest patients in DN V mostly belonged to DYY, which is the most severe type in TCM syndrome typing. For TCM syndrome typing, the lightest condition was DQY-PY while the most severe one was DYY. Most of the DQY-PY patients belonged to DN III and most DYY patients belonged to DN V. It indicated that there was a certain consistency between Mongensen staging and TCM syndrome typing. As mentioned above, Western diagnosis is pathology-oriented, while TCM syndrome typing pays more attention to holistic symptoms, so there are some differences between TCM syndrome typing and Mongensen staging. For example, two patients (HIII08 and HIII09) were diagnosed as DN III according to Mongensen staging, but were in the most severe group of DYY by TCM syndrome typing. According to clinical records, urine protein of the two patients was 33 mg/24 h and 200 mg/24 h, but these patients had obvious DK*Yang* symptoms of edema (*fu zhong*) and nocturia (*ye niao pin*). In addition, our quantitative determination found that PE750, PC854, SAH, and SAM levels in these two patients were as high as those at the DN V stage. Therefore, urine protein alone used in the diagnosis is one-sided, and the overall state of patients should be considered. IBS is possibly better for comprehensive diagnosis.

Among the three categories of TCM symptoms, DK*Yang* (edema, nocturia) and DK*Yin* (lumbodynia, soreness and weakness of waist and knees) had significant differences between DM and DN, while SB (limbs numbness) had significant differences not only between DM and DN, but also among the three stages of DN, so they can be considered as indices for overall evaluation.

In five significantly differentially expressed genes, the variation tendencies of IGF2BP2, AR, and AGT were completely consistent in the two diagnostic systems, but there were small differences in the expressions of CDKAL1 and

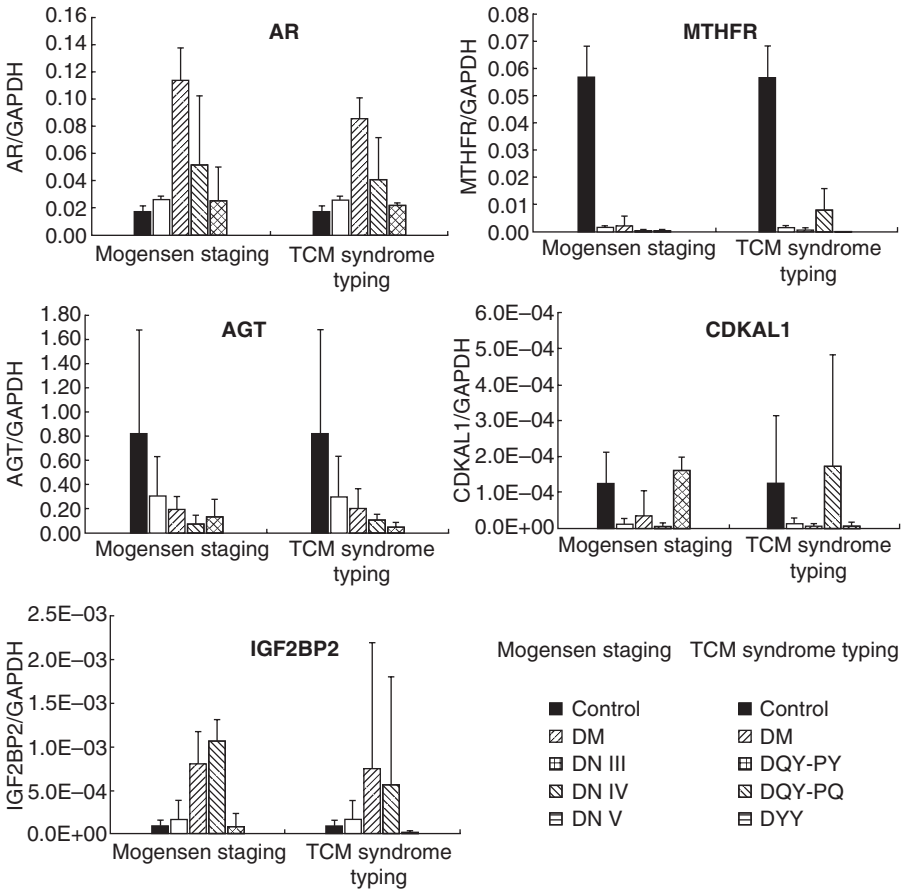

Fig. 9.22 Comparison of gene expression according to Mongensen staging and TCM syndrome typing.

MTHFR (Fig. 9.22). It indicated that the two diagnostic systems had similarity in gene expressions. Some of the genes were also found to have correlations with some specific metabolites identified above. For example, MTHFR is the key enzyme of folate metabolism, the lower expression of which correlated with increased metabolites of SAH and SAM.

According to the results of clinical indices, IMPT, genomics, and TCM symptoms, we suggest that IBS include the pathological and biochemical indexes (urine protein, serum creatinine, and urea nitrogen), IMPT biomarkers (adenosine, uric acid, cytidine, cytosine, inosine, PE750, PC834, PC802, PC854, SAH, SAM, and Hcy), genomics indices (IGF2BP2, AR, AGT, CDKAL1, and MTHFR), and TCM symptoms (DK*Yang*, DK*Yin*, and SB). These indices are potential biomarkers of DN, but further research with large sample sizes is needed to confirm these findings.

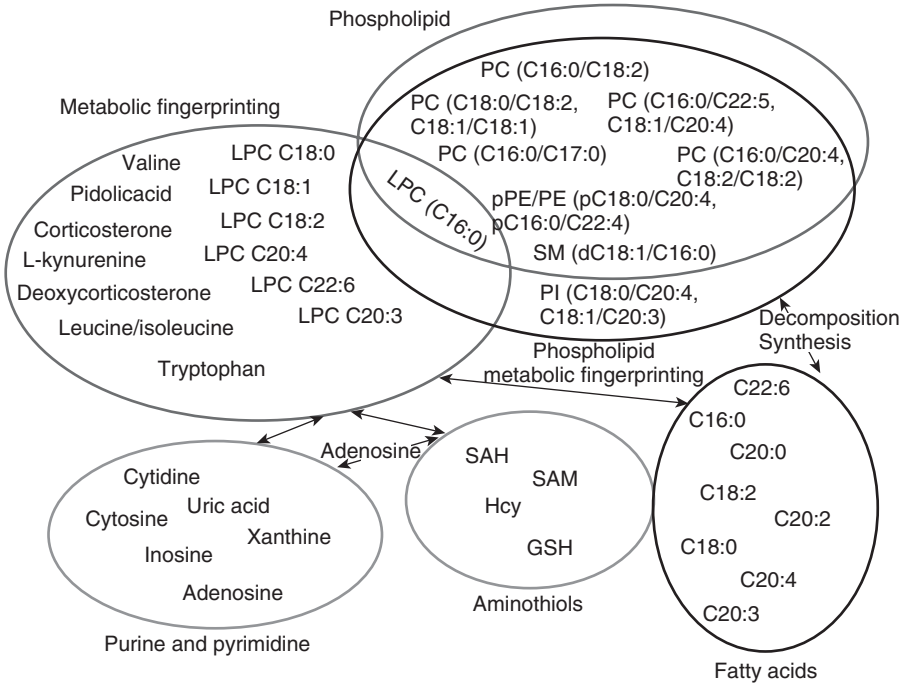

Fig. 9.23 Summary of 41 potential biomarkers and their related metabolic pathways.

9.6.2 Integrated Study of Quantitative Data and Metabonomics Data

After integration of quantitative metabolic data and metabolic fingerprint data, 41 potential biomarkers were identified from the study of DN based on IMPT (Fig. 9.23).

There exists a problem in the comparability between different dimensions and levels of data since quantitative data represent real content of the substance, and fingerprint data represent the peak intensity. A four-step solution for the integration of metabolic fingerprint and quantitative data was proposed (Fig. 9.24). First, the data were processed to change the characters of inversed data. Second, dimensionless processing was carried out, so that the raw data could be converted into dimensionless index values to solve the comparability issue of the data. Third, OSC was used to denoise. Finally, PLS-DA was used for cluster analysis.

Before integration of the data, the PLS-DA results of metabolic fingerprint data are shown in Fig. 9.25A.

The figure showed that there was a little overlap among the control group, DM, DN III, DN IV, and DN IV, but they were basically separated.

Based on the research of metabolic fingerprints, the quantitative data were integrated in the study. Chemotactic and dimensionless processing was carried

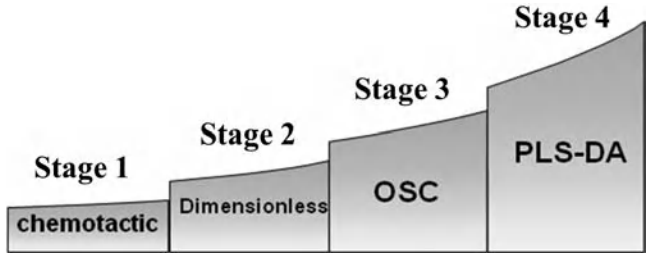

Fig. 9.24 Integrated route of metabolic fingerprint and quantitative determination data.

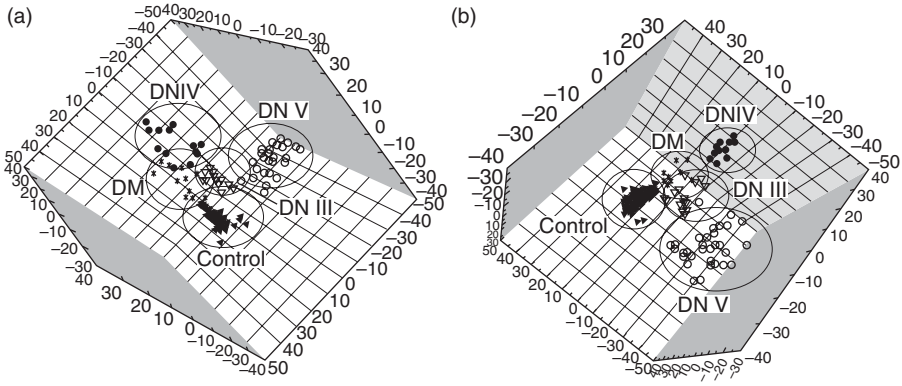

Fig. 9.25 Score plot of PLS-DA results of all the sample fingerprints before integration (A) and after integration (B).

out for integration. Fig. 9.25B illustrates the score plot of PLS-DA results for all the samples after integration. Although there was still slight overlap among the groups after integration, the DN IV group was better separated from the others.

After data analysis using PLS-DA, compounds were screened out based on the VIP list. There was significant difference between the two groups ($p < 0.05$). Qualitative and quantitative metabonomics information of the compounds can be obtained directly from the quantitative data, and the possible formulas of metabolic fingerprint data were found using the Waters MassLynx software. Next, the formulas obtained were input into databases, including http://www.pubchem.ncbi.nlm.nih.gov, http://www.genome.jp/kegg/ligand.htmL, and http://redpoll.pharmacy.ualberta.ca /~aguo/www_hmdb_ca / HMDB/.

Table 9.25 lists all the markers after integration, including the quantitative metabonomics data and metabolic fingerprint data. It shows that there are four categories of potential biomarkers, including Lyso-PC, branched-chain amino acids, fatty acids, and purine metabolism.

TABLE 9.25 Potential Biomarkers Identified after Integration

NO.	Compound	Data Form
1	Lyso-PC(16:0)	Metabolic fingerprint
2	Lyso-PC(18:2)	Metabolic fingerprint
3	Lyso-PC(18:0)	Metabolic fingerprint
4	L-Valine	Metabolic fingerprint
5	Pidolic acid	Metabolic fingerprint
6	Leucine / isoleucine	Metabolic fingerprint
7	L-Tryptophan	Metabolic fingerprint
8	C20:4	Quantitation
9	C18:2	Quantitation
10	Adenosine	Quantitation
11	C20:2	Quantitation
12	C20:3	Quantitation

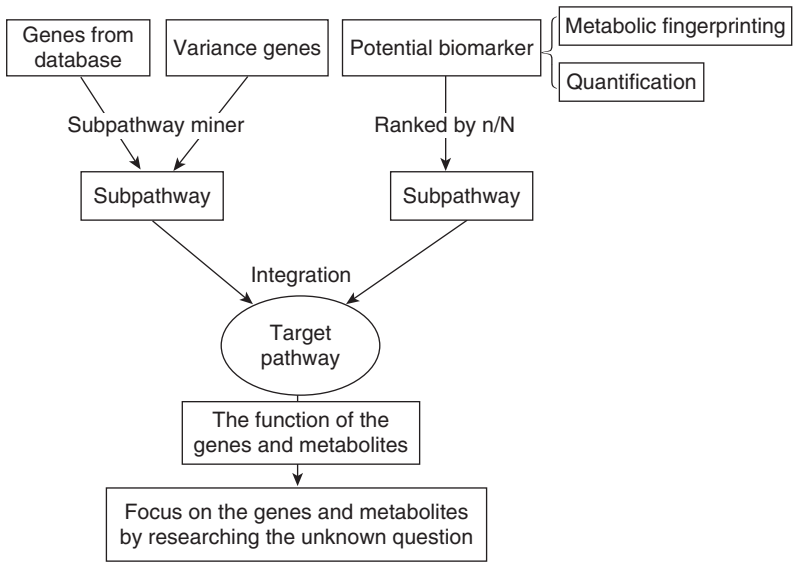

Fig. 9.26 Framework of integrated study of metabolic and genetic research.

These 12 markers were used as integrated indicators to calculate the prediction accuracy of each group (Fig. 9.26). The prediction accuracies were 78.8%, 69.7%, 68.2%, 89.4%, and 86.0%, according to control, DM, DN III, DN IV, and DN V, while the overall prediction accuracy was 78.7%. Compared with the potential biomarkers from metabolic fingerprints, phospholipids fingerprints, and metabolomics study, the prediction accuracy was higher. Although it was lower than the sum of all the potential biomarkers, which was 96.0%, the number of the biomarkers was reduced to 12, which was about one third

TABLE 9.26 Discriminant Accuracy Based on the Use of Different Potential Biomarkers

	Discriminant Accuracy (%)					
Potential Biomarkers	Control	DM	DN III	DN IV	DN V	All
Purine and pyrimidine	86.7	65.2	55.6	94.1	83.3	78.0
Aminothiols	60.0	27.3	43.8	70.0	80.0	57.4
Fatty acid	73.3	47.8	52.9	72.7	53.3	59.5
Phospholipids	56.7	39.1	33.3	31.3	76.0	50.0
Metabolic fingerpriting	46.7	30.4	86.7	66.7	70.0	57.3
Integrative metabolites (12)	78.8	69.7	68.2	89.4	86.0	78.7
Combined metabolites (41)	100.0	86.4	100.0	100.0	96.0	96.0

of the sum of all the biomarkers. The results showed that this integrated method was useful to search for potential biomarkers.

9.6.3 The Relationship Study of Metabolic and Genetic Pathway

In the occurrence and development process of some diseases, such as cardiac disease, diabetes, DN, and cancer, genes, proteins, and metabolites produce some significant changes. New research fields, including genomics, proteomics, and metabolomics, were utilized to screen for important genes, proteins, and metabolites. Particularly, gene chip technology was driven by the development of genomics research, resulting in the discovery of a large number of possible genes associated with certain diseases. In addition, these genes were shared through a number of databases. Bioinformatic techniques were then used to process large amounts of data by annotating the compounds in the pathway, and to reveal the mechanisms of diseases. Methods of annotating genes to KEGG pathways and identifying statistically significant pathways are the most commonly used among them.[69, 70] Many researchers have developed some tools based on annotation to the KEGG pathway, and have been able to find related diseases and physiological pathways as well as overall pathway analysis. Further research on the mechanism and development of new drug targets was also carried out.

To solve the above problems, the method of gene annotation was used to focus the number of channels to improve the accuracy and efficiency of the study by integration of genetic and metabolic data. The method is divided into three steps. First, genes associated with certain diseases were searched in the public databases and our laboratory's self-built database using subpathway software, and subsequently pathway I was formed. Second, metabolites associated with certain diseases were searched in the public databases and our laboratory's self-built database using "R" software, and subsequently pathway II was formed. Third, pathways I and II were compared, and the common pathways were analyzed. Fig. 9.26 shows the roadmap for the annotation.

9.6.3.1 Genes Research for Focus Pathways Genes absorbed in the study were obtained with two methods, one from the public database, and the others from our studies of comparative genomics, which included diabetes susceptibility genes, including CDKAL1, CDKN2A, CDKAN2B, and IGF2BP2; sugar metabolism related genes, including AR, AGER, GLUT1, IGF2; hemodynamic genes, including AGT, ADRB3, AGTR2, ACE; and other functional genes, including PRKCA and MTHFR. All the genes were annotated to the pathways, and the number of genes in each pathway was counted. The number of all the genes that a human has was set as m, and the number of genes that fall into the pathways was set as t. The number of genes submitted was set as n, and the number of the genes annotated in this pathway was r. P-value was obtained using the following formula (9.1):

$$P(X = x > r) = \sum_{x=r}^{t} \frac{\binom{t}{x}\binom{m-t}{n-x}}{\binom{m}{n}} \tag{9.1}$$

The pathway map related to type 1 diabetes, type 2 diabetes, and DN was built, and is shown in Fig. 9.27. In the figure, each pathway associated with the disease had p-values lower than 0.05. There were 14 pathways associated with type 1 diabetes, 63 pathways associated with type 2 diabetes, and 20 pathways associated with diabetic nephropathy. Some common pathways were found for these diseases, such as the interaction of cytokine-cytokine receptor and the VEGF signaling pathway.

9.6.3.2 Metabolites Analysis for Focus Pathways Based on a comprehensive and systematic study of metabolomics of diabetes and diabetic nephropathy, 41 significant metabolites related to the occurrence and development of the diseases were taken in our database. The enrichment analysis was carried out in the KEGG pathway using the "R" program. The number of metabolic markers gathered in a pathway was set as n, the total number of compounds in the pathway was set as N, and then the n/N value was obtained. n/N values greater than 0.1 indicate significantly correlated pathways, n/N values in the range of 0.05–0.1 indicate general correlation, n/N values in the range of 0.02–0.05 indicate weak correlation with the pathway, and n/N values of 0 were not be considered. Table 9.27 shows an ordered list of some related pathways.

9.6.3.3 Combination of Genes and Metabolites in the Focus Pathways There were two pathways overlapping in the gene and metabolite study, namely linoleic acid metabolism and glutathione metabolism. These two pathways had very significant relationships with the occurrence of the disease.

Take the linoleic acid metabolic pathway, for example (Fig. 9.28). There are three metabolites enriched in the pathway, including linoleic acid, arachidonic

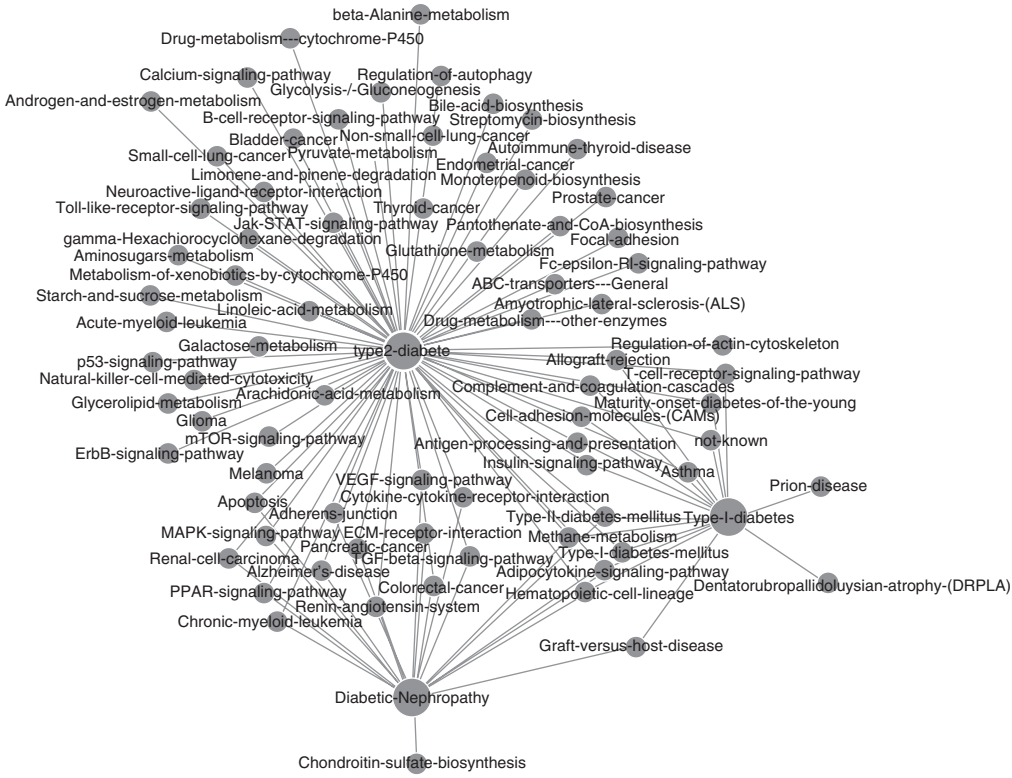

Fig. 9.27 Pathway map of type 1 diabetes, type 2 diabetes, and diabetic nephropathy.

TABLE 9.27 Order List of Related Pathways According to *n*/*N*

Pathway	*N*	*n*	*n*/*N*
Linoleic acid metabolism	26	3	0.115
Cysteine and methionine metabolism	56	4	0.071
Valine, leucine, and isoleucine biosynthesis	28	2	0.071
Valine, leucine, and isoleucine degradation	41	2	0.049
Pyrimidine metabolism	59	3	0.051
Purine metabolism	92	4	0.043
Arachidonic acid metabolism	75	2	0.027
Glutathione metabolism	38	1	0.026
Arginine and proline metabolism	77	2	0.026

acid, and PC. Linoleic acid significantly increased with the development of the disease. From its biological function, its synthesis and intake in vivo is an important source of C20 fatty acids. The raised level of linoleic acid may lead to a higher concentration of C20 fatty acids, resulting in damages to endothelial cells and worsening of the disease.

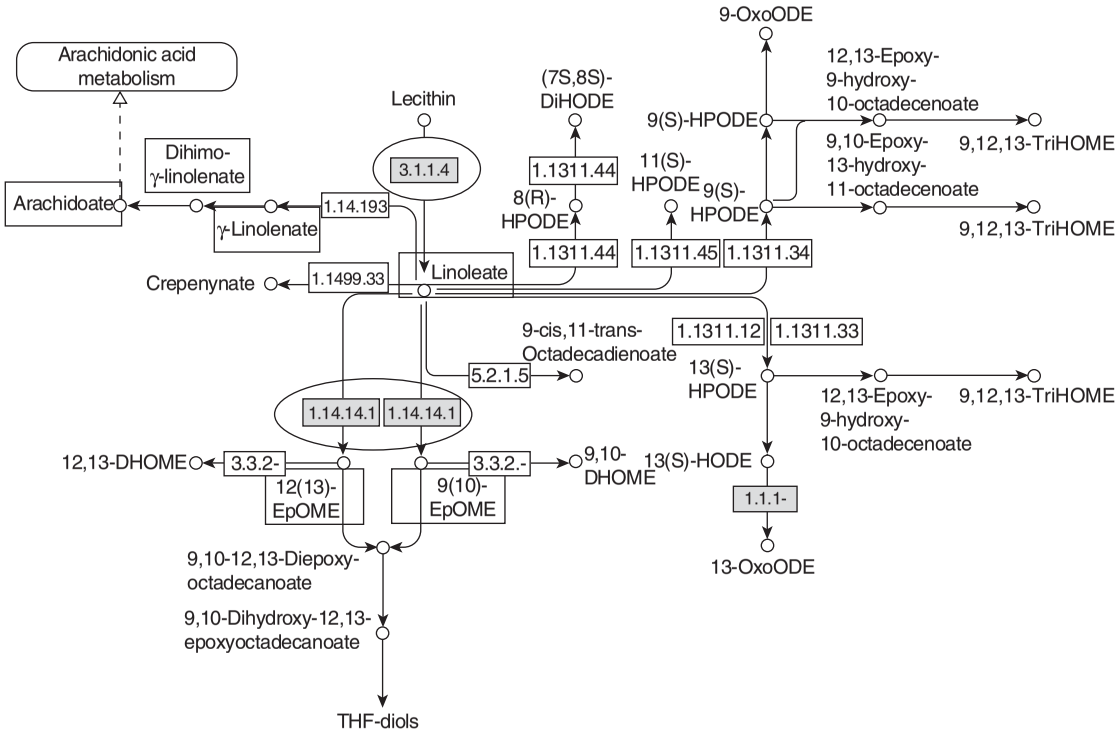

Fig. 9.28 Pathway of linoleic acid metabolism.

Arachidonic acid is an important inflammatory mediator during the development of diseases. Its content change will cause metabolic disorders and disorders related to Ca^{2+} regulation of ion transport, as well as altered endothelial cell function. PC has also been reported to be involved in disorders of obesity and insulin resistance.

The enrichment genes were mainly centered around two enzymes that were circled. The enzyme 3.1.1.4 is phospholipase A2, the major change of its gene set is PLA2. The function of the gene is to control the relevant enzymes to catalyze the hydrolysis of membrane phospholipids and produce nonesterified fatty acids, such as arachidonic acid and LPC in glucose metabolism and insulin secretion. The key genes included PLA2G4A, PLA2G12A, and PLA2G12B.[68] The enzyme 1.14.14.1 is flavinprotein monooxygenase; and its main gene set is the CYP2 gene class, focusing on genes such as CYP2C9, CYP3A4, and CYP3A5.

It is reported that mutations in the CYP2C9 gene can lead to increased metabolic activity of antidiabetic drugs, such as sulfonylureas and columns. It can lower blood concentration in the body.[69] In addition, it is also reported that CYP3A4 and CYP3A5 gene polymorphisms were closed related to multidrug resistance in renal transplant patients.[70]

The disease-related metabolites and genes that were not found in the pathways were also discussed for their physiological functions and significances. As metabolites (boxed) in the pathway, γ-linoleic acid is a precursor of prostaglandins; 8,11,14-eicosatrienoic acid and arachidonic acid compete to combine with cyclooxygenase and lipoxygenase, and convert to prostaglandin E1; but there is little report about 12 (13)-EpOME and 9 (10)-EpOME.

9.7 CONCLUSIONS

Systems biology research of DN had been carried out in this chapter. The approach of metabolic fingerprinting, quantitative analysis of seven classes of phospholipids, 15 fatty acids, eight aminothiols, and 21 purine and pyrimidine compounds, as well as 14 DN-related genes were established in the study. The methods of quantitative metabonomics and the quantitative PCR of target genes were successfully used in the research of DN according to Mogensen stages and TCM types. Our research proposed that complex diseases require more systematic study, including three levels of indices: pathological indexes (biochemical imaging markers) according to Western medicine, biomarkers identified from basic research (genes, proteins, and metabolites), and quantitative indexes from TCM theory (overall symptom).

In addition, a series of methods were used for the integration of metabolomic data, genetic data, and clinical biochemical data. Such methods include the combination of metabolic fingerprinting and the quantitative metabolic cycle, the combination of qualitative and quantitative data, and the combination of genes and metabolites by a variety of analytical instruments,

bioinformatics, and computing technologies. Therefore, the study integrated metabolic, genetic, and clinical studies, making the research of the disease more in-depth and systemic.

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CHAPTER 10

CHEMOMICS RESEARCH ON THE TCM FORMULA OF THE *QINGKAILING* INJECTION

The pharmacological material basis of TCM is the multicomponent mixture (effective component group) with synergistic effects. Its mode of action (mechanism) has been shown to be the complex system regulatory network (at least understood as multicomponent, multitarget, timely ordered integration regulation), so TCM research methodology should also be compatible with this feature. Global systems biology integrated with chemomics is proposed based on the working features of Chinese medicine, which is an innovative methodology. The first few chapters of this book, on related methodologies and techniques, presented these concepts in great detail. They stated that chemomics can be used to study the information characterization and excavation of the chemome of TCM and the contained effective component groups; systems biology can be used to express the biological complex system regulatory network on behalf of the roles of TCM. This chapter takes the TCM formula *Qingkailing* injection as an example, introducing the application of chemomics research in TCM.

Qingkailing injection, a well-known formula of TCM, has been used in clinical practice for nearly 30 years. It comprises eight raw herbs or extracts thereof, including *Jinyinhua* (Lonicerae Japonicae Flos), *Zhizi* (Gardenia Fructus), *Banlangen* (Isatidis Radix), *Shuiniujiao* (Bubali Cornu), *Zhenzhumu* (Margaritifera Usta), baicalin (instead of *Huangqin* [Scutellariae Radix] which is used in the original formula), cholic acid, and hyodesoxycholic acid (instead of *Niuhuang* [Bovis Calculus] in the original formula). It shows good clinical

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efficacy on stroke (cerebral edema), respiratory infections, hepatitis, and so on. However, the pharmacological material basis is not clear, and the mechanism still needs to be confirmed. In addition, the difficulty of the quality control of this preparation and all other problems need to be solved. Quality control of the injection also has serious impacted on the clinical efficacy and safety of the *Qingkailing* injection, and adverse reactions have been reported. With the support of 973 project, our group worked with Prof. Pengtao Li's group from Beijing University of Chinese Medicine, under the guidance of academician Wang Yongyan, choosing the treatment of the "upper-*jiao* fire" (ischemic stroke) as a breakthrough point, putting the chemical research and pharmacology study closely together.

Pharmacological research of *Qingkailing* was led and accomplished by Prof. Pengtao Li in Beijing University of Chinese Medicine. In this research, the doctrines of "toxin-caused injury of cerebral collaterals" of TCM and "inflammatory cascade reaction" of Western medicine about strokes was combined, and the group designed focal complete brain ischemia, the whole brain incomplete cerebral ischemia animal model and brain neurons, glial cell, microvascular endothelial cells, and so on, and totally researched 51 pharmacological related markers,[1–6] which provide an effective guarantee for the pharmacological evaluation of the *Qingkailing* formula and its effective component group.

The pharmacological material basis of *Qingkailing* was studied by a group in Tsinghua University.[7–15] According to the research strategies and methods of chemomics of TCM formula, the work focused on the global chemome and effective chemome of *Qingkailing*, and was carried out on these two levels systematically, screening the effective component group and optimizing the compatibility of *Qingkailing*. The roadmap for chemomics research of *Qingkailing* is shown in Fig. 10.1.

10.1 CHEMOMICS RESEARCH OF *QINGKAILING* INJECTION

10.1.1 TCM Theory of *Qingkailing* Injection

Reconstructed from the ancient classical formula of *Angongniuhuang* pills, the *Qingkailing* injection is a modern Chinese medicine. It has been applied in clinics since 1973, and it has significant effects in reducing cerebral edema, refreshing and inducing resuscitation, and reducing mortality. Now it is widely used for clinical brain embolism emergencies.

Academician Wang Yongyan proposed a hypothesis of the toxin-caused injury of cerebral collaterals, which is based on the study of pathogenesis of TCM, the theory of TCM pathogenesis, clinical characteristics of stoke conditions, and practical information. It was said that the core of the stroke lies in brain collaterals; "deficiency of viscera function, disorder of *qi* and *Xue*, pathogen induced toxin, and the injury of brain collaterals" are the main mechanisms of stroke pathological development and evolution. For the pathogenesis, he pointed out that the key points of the treatment of apoplexy are

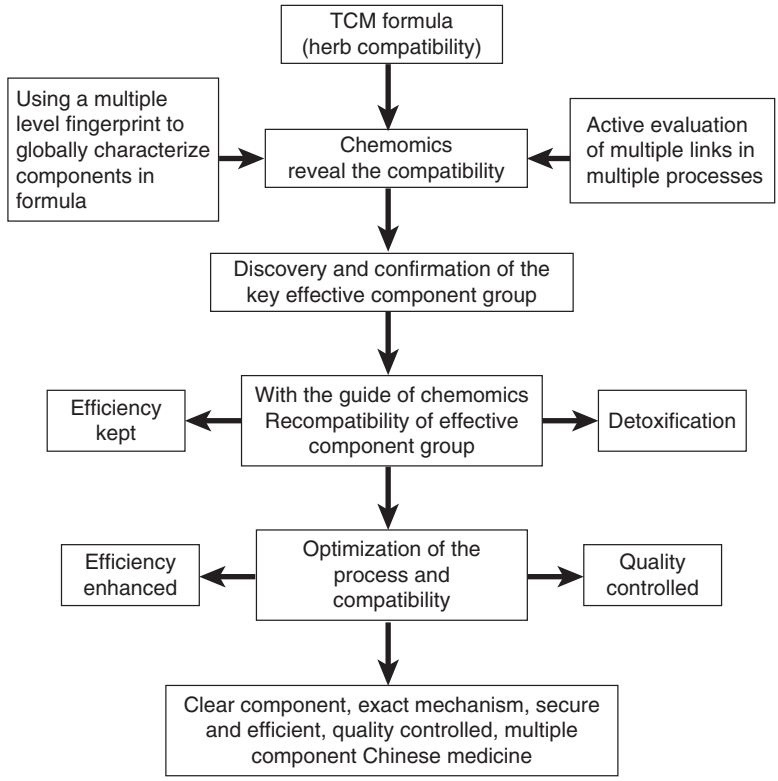

Fig. 10.1 Map for chemomics research of *Qingkailing*.

to dispel the toxins and dredge brain collaterals. Dispelling the toxins is to antagonize the damaging factors and eliminate pathogenic factors; dredging brain collaterals is to navigate the blood and strengthen body resistance.[1] By lowering the permeability of capillaries and reducing cytotoxic edema, the *Qingkailing* injection can reduce the damage range of stroke and reduce neuronal death. By doing these things, it can comprehensively regulate the cascade of ischemic stroke pathological process. This treatment effect is mainly aimed at the toxin-caused injury of cerebral collaterals pathomechanism, and this effect can be achieved by the scientific compatibility of "detoxification and brain collateral dredging" drugs.[2] *Niuhuang* is the main drug in this formula, which has several effects such as clearing heat and relieving convulsion. Its major components are cholic acid and deoxycholic acid. Cholic acid and deoxycholic acid are now used as alternatives to *Niuhuang*, but deoxycholic acid has stronger pharmacological activity than cholic acid. *Shuiniujiao* is the alternative form of rhino horn; they both contain keratin, with similar amino acid species and concentration. Studies have shown that they have similar pharmacological activities, such as cardiac effect, sedation, and detoxification.

Zhenzhumu is the scalloped nacreous layer of cultural *Pteria martensii*, which contains the same amino acid as pearls, and has beneficial effects including heat-clearing and detoxifying, tranquilizing, and improving acuity of vision. *Huangqin* and *Jinyinhua* are both used as effective parts in medicine. The major component of *Huangqin* is baicalin, while the major component of *Jinyinhua* is chlorogenic acid. They both have beneficial effects including heat-clearing and desiccation, anti-inflammation, and detoxification, which can also play a secondary detoxification function with cholic acid and mixed amino acids. *Zhizi* decoction pieces are used in medicine, and these pieces can purge heart fire and triple-*jiao* fire, eliminate heat, detoxify, and disencumber. Geniposides and crocetins have antiphlogistic and cholagogic action. Using *Banlangen* to replace Coptis Chinensis in the *Angongniuhuang* pills ensures the heat-clearing and detoxification functions of formula. The main ingredients of *Banlangen* are nucleosides and amino acids. With the mutual reinforcement between *Huangqin* and *Zhizi*, which have the synergy effect of purging and eliminating heat and playing the common effect on *qi* and *Xue*, the coupled medicine can clear the triple-*jiao* fire, lung heat, and *Xue* heat. *Jinyinhua* and *Banlangen* have a synergistic effect, and cholic acid, *Zhenzhumu*, and *Shuiniujiao* used together can strengthen functions of the whole formula, such as heat-clearing, detoxification, relieving convulsions, subduing wind, and reducing phlegm by resuscitation. In addition, the whole formula has the beneficial effects of heat-clearing, detoxification, reducing phlegm, dredging collaterals, resuscitating, tranquilizing, and allaying excitement.

However, how does the formula compatibility achieve the global comprehensive regulation of the body? What is the pharmacological material basis for the ischemic cascade pathological process? What are the chemical relationships among different biological ingredients'; biological effects? What is the relationship between antagonistic damage and the ability to stop damage from occurring? All these questions have become the core that needs to be answered in successive steps. By clarifying these issues, we will find out the basic pharmacological material basis and biological effect theory of the *Qingkailing* injection and propose compatible theoretical ideas of modern formulas and new drug ideas for treating difficult diseases. (The *Qingkailing* injection can dispel toxins and dredge brain collaterals, resuscitate, eliminate pathogenic factors and strengthen body resistance, and halt the damage caused by cerebral ischemic cascade.)

In order to scientifically explain all the focuses mentioned above, we should first define the effective component group which constitutes the *Qingkailing* injection, aiming for chemical separation and analysis of the effective component group. This is a critical basic component of research. It will lay the theoretical and methodological foundation for in-depth analysis of the focuses mentioned above. Determination of the effective component group in the *Qingkailing* injection is based on the pathological mechanisms of the toxin-caused injury of cerebral collaterals in stroke,[14] and analysis from dispelling of the toxins, relieving obstruction of collateral vessels, and the resuscitation effect.

Cerebral ischemic cascade is the main pathological process of stoke occurrence and development. Cerebral ischemic cascade originates from the microvascular perfusion disorders and leads to free radical generation, lipid peroxidation, damages to vascular endothelial cells, neurogliocytes, and neurons. Glial cell swelling can lead to the vicious cycle of microvascular perfusion disorders at the same time. Therefore, to explore the effective component group of *Qingkailing* injection and the compatibility of the effective component group, the working rule of inhibition of stroke cerebral ischemic cascade in multiple links, we should pay close attention to this reaction in several key aspects.

10.1.2 Global Chemome Research of *Qingkailing* Injection

The global chemome of the *Qingkailing* formula is very complex. In order to characterize such a complex chemome of a TCM formula, we obtain its fingerprint database by multidimensional analysis. We used online combined analysis and plant chemical separation means to identify and analyze chemical compositions as much as possible. By doing this, we can gain the quantitative analysis data with multiple components and establish the *Qingkailing* chemical composition database. Consider that the *Qingkailing* injection contains not only groups of small organic molecules, but also inorganic ions and biological macromolecules. We developed many types of chemical fingerprint methods to characterize them. These types are shown in Fig. 10.2. We established ICP/

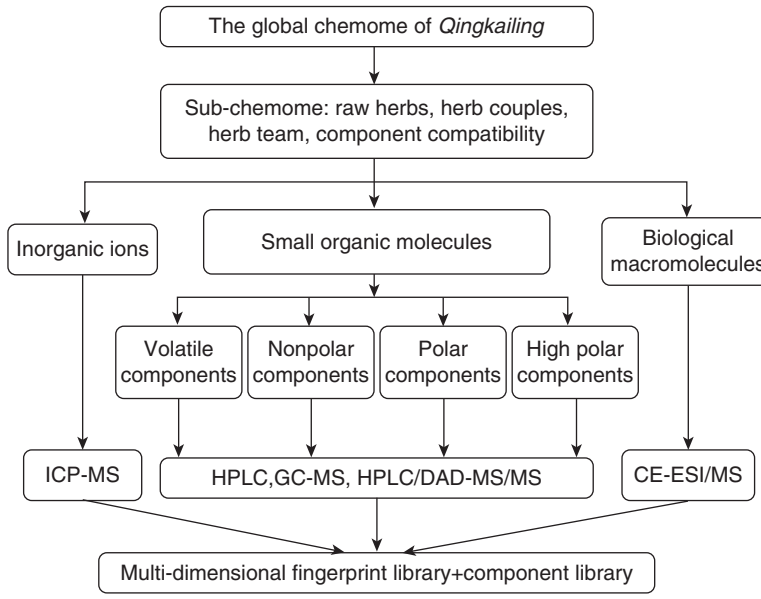

Fig. 10.2 Multidimensional fingerprint used for characterization of *Qingkailing* chemome.

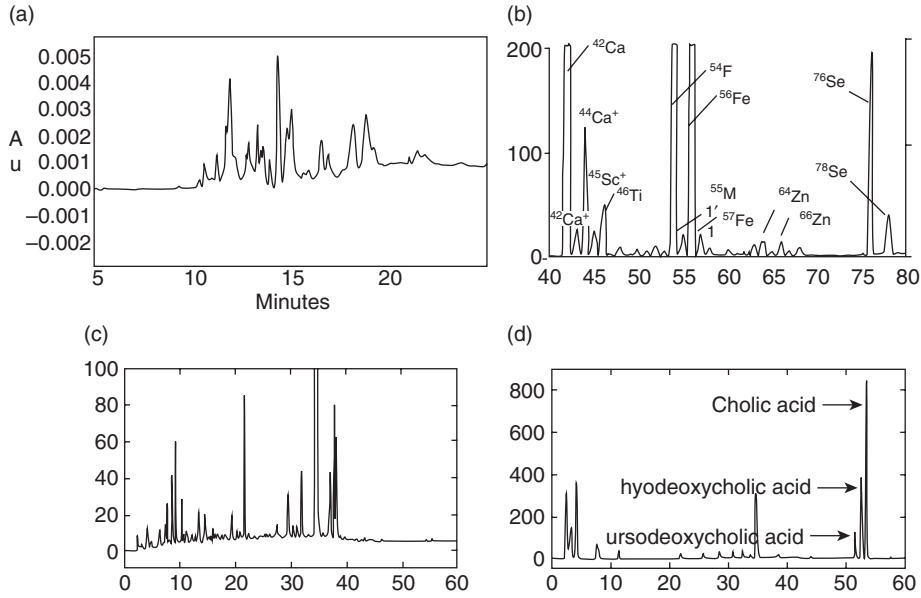

Fig. 10.3 Different type fingerprints of *Qingkailing* formula. A: CE fingerprint of peptide of *Shuiniujiao*; B: ICP-MS fingerprint of inorganic ions of *Zhenzhumu*; C: HPLC-DAD fingerprint of *Qingkailing* (*Zhizi*, *Banlangen*, *Jinyinhua*, baicalin); D: HPLC-ELSD fingerprint of *Qingkailing* (cholic acid, ursodeoxycholic acid, hyodeoxycholic acid).

MS fingerprint for inorganic ions, used a protein fingerprint method for peptide protein and other biological macromolecules, and used a multidimensional fingerprint approach for small organic molecules. The different types of fingerprints of the *Qingkailing* formula are shown in Fig. 10.3.

By a variety of analytical and characterization methods, we identified a total of more than 40 organic components and over 10 inorganic components in the global chemome of the *Qingkailing* injection. By doing these, we can basically determine the main chemical composition of *Qingkailing*. According to the nature of the chemical composition of *Qingkailing*, we can divide these effective parts into nine types of effective component group (that is, the global chemome can be divided into nine subchemomes) and identify their main components:

(1) Cholic acid compositions contain deoxycholic acid, hyodeeoxycholic acid, and taurocholic acid.
(2) Amino acid compositions contain histidine, lysine, aspartic acid, arginine, threonine, serine, glutamic acid, proline, glycine, alanine, cystine, valine, leucine, and so on (they come from *Shuiniujiao*, *Zhenzhumu*, and *Banlangen*).

(3) Inorganic elements contain Ca, Mg, Si, K, Ti, Mn, Fe, Ni, Cu, Zn, Se, Sr, and so on (they mainly come from *Zhenzhumu*). Studies show that the element Ca in *Qingkailing* includes two forms: free inorganic form and organic form.
(4) Flavone compositions contain baicalin, wogonoside, and so on (they mainly come from *Huangqin*).
(5) Organic acids compositions contain chlorogenic acid, isochlorogenic acid, quinic acid, gardenia acid, crocetin, and so on (they mainly come from *Zhizi*, *Jinyinhua*, etc.).
(6) Nucleoside compositions contain adenosine, uridine, guanosine, and so on. (They mainly come from *Banlangen*).
(7) Iridoid glycoside compositions contain geniposide, gardenoside, genipin-gentiobioside, gardoside, and so on (they mainly come from *Zhizi*).
(8) Pigments compositions contain crocetin, gardenia yellow, gardenia blue, gardenia red, and so on (they mainly come from *Zhizi*).
(9) Volatility compositions contain coriandrol, palmitic acid, firpene, and so on.

10.1.3 Effective Chemome Research on *Qingkailing* Injection

On the basis of global chemome research, we established a chemical information matrix on the subchemome and its compatibility mode (chemical information is characterized by the fingerprint method and the qualitative and quantitative results of indicators which are shown in Fig. 10.2.). A related active information matrix is constructed by multiparameter pharmacological indices, and we adopted a component-effect research method to identify the main efficient components and key efficient ingredients. We finally determined the best chemical compatibility of the *Qingkailing* subchemome in treatment of cerebral ischemia injury in accordance with the principle of "guarantee the effects and reduce the toxicity." Four effective component groups, including cholic acids (taurocholic acid, hyocholic acid, hyodeeoxycholic acid, etc.), baicalins (baicalin mainly, as well as deoxy-baicalin, wogonoside, etc.), gardenia iridoid glycosides (geniposide mainly, as well as genipin-gentiobioside, geniposide, etc.), and *Zhenzhumu* hydrolyzes (mainly amino acids), have best chemical compatibility. These four compositions compose a small part of *Qingkailing*, which is the effective chemome of *Qingkailing*. Moreover, we used uniform design to optimize the compatibility amount of the effective component group. The results showed that the small square of *Qingkailing* has the advantage of attenuation and a higher synergy effect than the original formula.

10.1.3.1 Global Characterization of Effective Chemome with Fingerprint When screening for effective chemome from the global chemome (shown as Fig. 10.4), it is necessary to establish an effective method of chemical

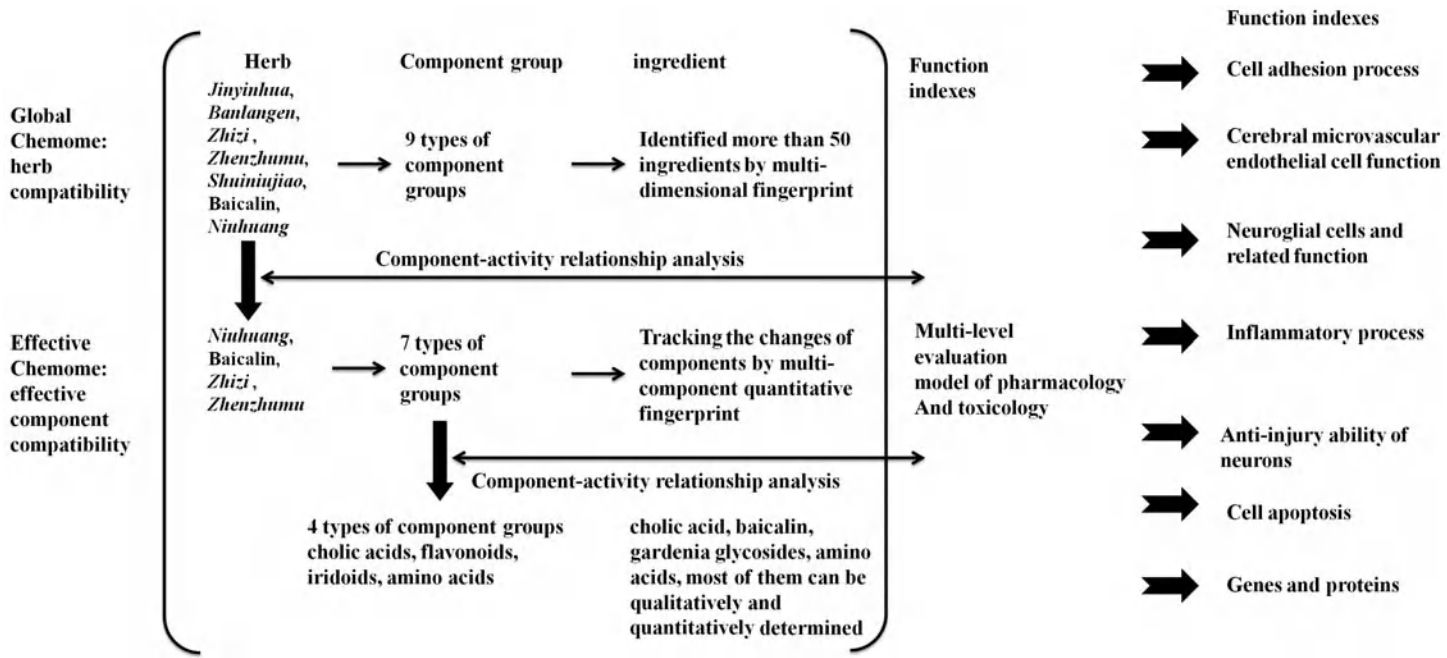

Fig. 10.4 "Top-down" screening process of *Qingkailing* effective chemome.

characterization, which is aimed at achieving comprehensive monitoring and tracking of the chemical information changes in compatibility in the process of changing from the whole formula to the various subchemomes (medicine, the drug, the medicine team and its effective component group, etc.). Only through this method can we achieve the correlation analysis with pharmacodynamics information, and identify and determine the efficient substances group (the compatibility of effective component) in it. In this study, we combined the means of fingerprint and multicomponent quantitative determination method to track and characterize the effective chemome.

Major chemical ingredients of various medicines in *Qingkailing* injection that contribute to the global formula chemical group are as follows:

(1) *Banlangen*: Contributes a certain amount of adenosine compositions and some of the amino acid compositions. However, the common indigo, indirubin pigment, and other pigments ingredients were not detected in the *Qingkailing* injection.
(2) *Jinyinhua*: Contains chlorogenic acid, isochlorogenic acid, quinic acid, Luteolin, caffeic acid and other ingredients.
(3) *Shuiniujiao*: Its hydrolyzate contains amino acids as well as trace elements in the free and bound forms.
(4) *Zhenzhumu*: Contains 95% calcium carbonate; after hydrolysis and precipitation, removing a lot of calcium, a small amount of calcium and other trace elements are left, mainly in the form of chelate, in which the majority of keratin has changed into amino acids through the hydrolysis process, and does not exclude the existence of a small amount of hybrid protein and peptide materials.
(5) *Taurocholic acid*: Contains 93.77%.
(6) *Hyodeoxycholic acid*: Contains 98.8%.
(7) *Baicalin*: Contains 92.27%.
(8) *Zhizi*: Contains geniposide (including geniposide, digoxin geniposide, etc.), organic acids (chlorogenic acid, ursolic acid, etc.), pigments (crocetin, gardenin, gardenia blue, gardenia red, etc.), and polysaccharides.

This study shows that even for the composition in medicine that contributes to the formula, the relative content of these components still matches a regular change to medicine compatibility in formula. The variation rule of chemical information has a significant implication for guiding the selection of the compatibility among effective component groups. With pharmacodynamic validation, we can reveal the compatible relationship of each component in formula.

To better understand the compatible relations of medicines in formulas, we made corresponding studies of the drug team and drug compatibility, including six drug teams: *Huangqin* and *Zhizi*; *Banlangen* and *Jinyinhua*;

Zhenzhumu and *Shuiniujiao*; taurocholic acid and hyocholic acid; a formula containing *Banlangen*, *Zhizi*, *Zhenzhumu*, and *Shuiniujiao*; and another formula containing six medicines, including *Banlangen*, *Zhizi*, *Zhenzhumu*, *Shuiniujiao*, taurocholic acid, and hyocholic acid. In accordance with the same formula of *Qingkailing* and the same analytical conditions, we determined their fingerprints. From the fingerprint determination of the whole formula, single extract, and extracts of drug teams, the fingerprint well demonstrated the relationship between medicinal ingredients and herbs. It can be seen from the results that proper medicine compatibility forms traditional Chinese formulas. The medicine's material composition can be expressed through the fingerprint. The distribution of components in the fingerprint and the changes in quantity and the specific compatible relationship between single medicine and compatible groups composition can provide support for reasonable relationships among material compositions. Moreover, we can also use extract feature information method to analyze the impact of the compatibility mode. Comparing the fingerprint peaks of the *Huangqin* and *Zhizi* drug team with the herb fingerprints, we found that the feature information of compound-related peaks was enhanced, while the other peaks of raw herbs in the compatible process were reduced. Comparing the fingerprint peaks of the *Banlangen* and *Jinyinhua* drug team with the single herb fingerprints, there was a great change in the peak information shown in the fingerprint, a mutation in the interrelationship of compatible ratios, and a reduction in the compound-related peaks from a single medicine to medicine compatible process. Comparing the fingerprint peaks of the *Zhenzhumu* and *Shuiniujiao* drug team with the herb fingerprint, it can be seen that feature information of compound-related peaks had been enhanced, but not significantly. The effect was enhanced among the hydrolyzate, *Banlangen*, and *Jinyinhua*, but its change was little.

10.1.3.2 Specific Characterization of Effective Chemome with Multi-Component Quantification Although a fingerprint can reflect the most comprehensive information of chemical composition, it is a relatively vague way to make qualitative description. It is difficult to obtain accurate expression of specific chemical composition by fingerprint. In order to accurately track and compare the variations of chemical composition information in a compatible process, we should also establish a multicomponent quantitative analysis. The *Qingkailing* injection is made of eight types of raw herbs or extracts, including *Banlangen*, *Zhizi*, *Jinyinhua*, *Huangqin*, *Shuiniujiao*, *Zhenzhumu*, taurocholic acid, and hyocholic acid. For each medicine, we chose representative indicators as quantitative control objectives (the representative indicators are shown in Table 10.1). For all these medicines, except for the animal or mineral material such as *Shuiniujiao* and *Zhenzhumu* that were used with other detection methods, we established a simultaneous determination method for nine representative indicators in other six medicines HPLC/ELSD/DAD.

TABLE 10.1 Multi-Component Quantification of *Qingkailing* Injection

Target Medicine	The Target Component for Quantitative Determination
Banlangen	Uridine, adenosine
Zhizi	Geniposide
Jinyinhua	Chlorogenic acid, caffeic acid
Huangqin	Baicalin
Taurocholic acid	Cholic acid
Hyocholic acid	Hyodeoxycholic acid, bear cholic acid
Shuiniujiao	Total nitrogen
Zhenzhumu	Total calcium, free calcium

According to the chemical structure, the nine target components can be divided into five categories: uridine and adenosine are nucleosides; chlorogenic acid and caffeic acid are organic acids; geniposide and baicalin belong to iridoid glycoside and flavones ingredients respectively; and cholic acid, hyodeoxycholic acid, and bear cholic acid are steroids. There are big differences in the chemical structures and UV absorption characteristics among various components. This feature makes them very difficult to separate and detect simultaneously in conventional chromatographic conditions, as they are often required to be measured one by one in different chromatographic conditions and detection wavelengths. This is especially true of the cholic acids, which only have weak UV end absorption (approximate 192 nm). Conventional UV detection methods have low sensitivity and poor accuracy, so we need to use ELSD and other general-purpose detectors. For many types of components, it is necessary to develop multiple analytical methods and multiple injections, it will take several tries to analyze and cost many reagents. According to the different spectral characteristics of different types of components, we combined a diode array detector and evaporative light scattering detector, establishing an approach for simultaneous determination of five categories (effective component groups) including nine representative indicators in the *Qingkailing* injection by high-performance liquid chromatography coupled with photo diode array detection and evaporative light scattering detection (HPLC-DAD/ELSD). The approach provides a simple, reliable, reasonably new model to simultaneously quantitatively determine multiple components in a traditional Chinese complex compound system.

By HPLC/DAD full wavelength scanning, we found that the UV absorptions of five types of components in *Qingkailing* sample are different. Uridine and adenosine are two nucleoside components, which have maximum absorption at near 254 nm. The maximum absorption of chlorogenic acid and caffeic acid is 330 nm. The maximum absorptions of geniposide and baicalin are 240 nm and 280 nm, respectively. Three cholic acids have similar structures, and all belong to steroid compounds. There is basically no UV response in the

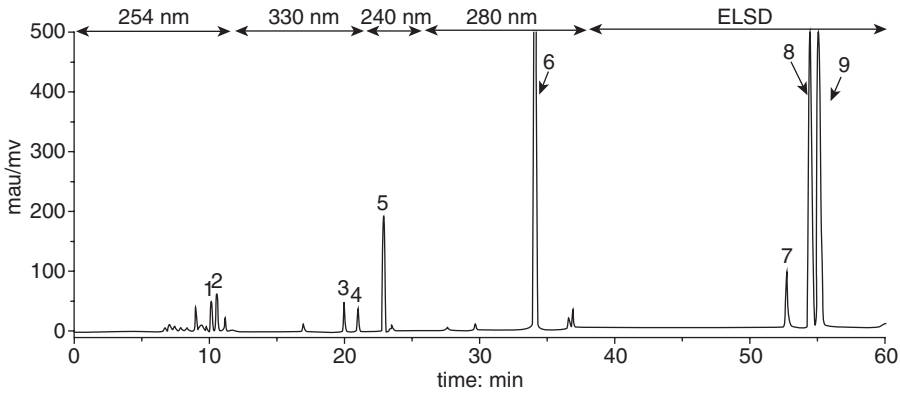

Fig. 10.5 HPLC-DAD/ELSD fingerprint of *Qingkailing* injection. 1: uridine (8.57 min); 2: adenosine (9.10 min); 3: chlorogenic acid (20.47 min); 4: caffeic acid (21.41 min); 5: geniposide (21.68 min); 6: baicalin (34.58 min); 7: ursodesoxycholic acid (51.97 min); 8: cholic acid (53.05 min); 9: hyodeoxycholic acid (54.12 min).

range of 200–590 nm, so it is better to adopt ELSD detection. Therefore, we adopted a single injection and separation by HPLC, coupled with photo diode array detection and evaporative light scattering detection, set four mentioned UV detection wavelengths on DAD to detect six components, (uridine, adenosine, chlorogenic acid, caffeic acid, geniposide, and baicalin), while we adopted ELSD to determine the remaining three components (bear cholic acid, cholic acid, and hyodeoxycholic acid). By these methods, a new approach for determining all five types of bioactive components in the *Qingkailing* injection was developed (typical HPLC-DAD/ELSD chromatograms of the *Qingkailing* injection are shown in Fig. 10.5).

We have also established a separate detection method using ELSD only with the HPLC/ELSD method, but compared with the HPLC-DAD/ELSD method, HPLC/ELSD is not as good as HPLC/DAD not only in sensitivity but also in reproducibility for the other six components besides bile acids in the *Qingkailing* injection. So for taking the simultaneous quantitative determination of all the nine components of *Qingkailing* injection, we recommend the HPLC-DAD/ELSD method. Without increasing analysis time and workload, it can fully combine the advantages of the two detectors and provide good quantitative results.

The developed HPLC-DAD/ELSD analytical method was subsequently applied to simultaneously determine the nine ingredients in 19 batches of the *Qingkailing* injection. The samples were obtained from different compatibilities and producing processes of the *Qingkailing* injection. The results indicate that the concentration of each component in different samples can be accurately and quantitatively reflected. Quantitative information can be more sensitive than fingerprints to reflect differences of chemical information in different samples, especially the subtle changes in active ingredient content of

some features or the drugs'; key ingredients. Therefore, the combination of fingerprint technology and quantitative analysis of multiple components is a necessary mode for the characterization of TCM complex system.

10.1.4 In Vivo Analysis of Effective Components of the *Qingkailing* Injection

In order to study the active ingredients of Chinese herbal formulas that can enter the body and the effects that compound compatibility have on the process of active ingredient compounds in vivo, and to explain the sequential relationship between the process of the active ingredients in vivo and the pharmacological effect, in vivo sample analysis is needed.

After the same amount of all parts of *Qingkailing*, skullcap extract and *Zhizi* extract were administered; cerebrospinal fluid, plasma, and serum samples were collected for analysis after a period of time.

According to the in vivo analysis results, the concentration–time curve of single herbs and the *Qingkailing* injection were obtained after administration and the results are shown in Fig. 10.6. Nos. 403 and 404 were administered baicalin, *Zhizi* extract respectively, and 410 was administered the *Qingkailing* injection.

The measurement results of in vivo samples of *Zhizi* herbs showed several things. The main ingredient is geniposide, which contained 40–50% of the total solids in the extract. Also, geniposide is one of active ingredients with relatively clear pharmacological effects in *Qingkailing* injection. Therefore, geniposide was used as the indicator for *Zhizi* sample analysis in vivo.

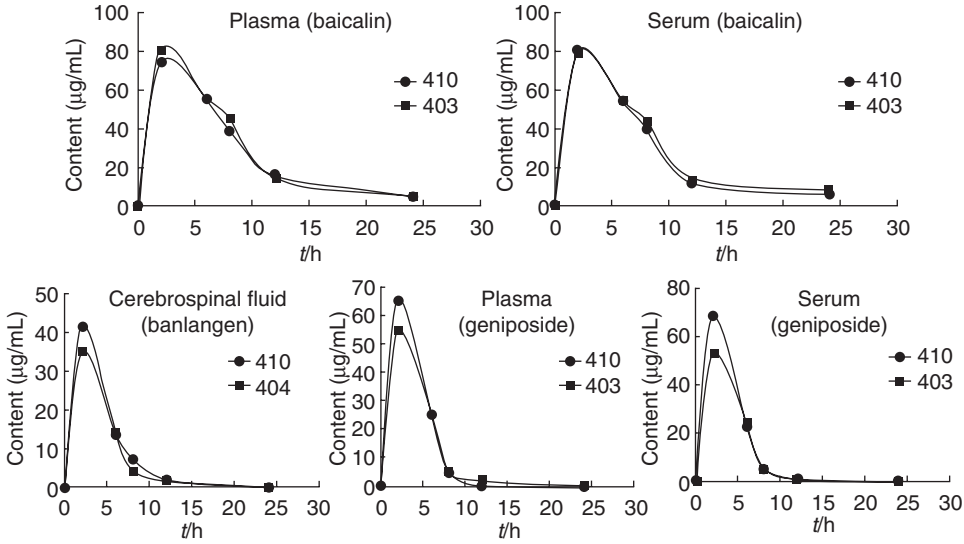

Fig. 10.6 Blood concentration–time curve of ingredients after baicalin, *Zhizi*, and *Qingkailing* injection administration.

Geniposide can be detected in all three types of samples. This illustrated that geniposide can move through the blood–brain barrier and have the same process of accumulation and elimination in three fluid environments. It arrived the peak time at 2–4 h, while concentration in the CSF sample was slightly delayed, but all samples were eliminated within 24 h completely. Geniposide contents in CSF samples attain 50–60% of plasma and serum samples. It showed that the larger part of *Zhizi* component concentrated on the central nervous system. Peak concentration of *Qingkailing* was more than the *Zhizi* extract (the ratio between them was: plasma at 1.18, serum at 1.20, CSF at 1.17). The integral area values of concentration–time curve of *Qingkailing* was also more than that of the *Zhizi* extract, showing that in the same amount of formula, the bioavailability of geniposide in *Qingkailing* with compatibility is better than single *Zhizi* extract. Other components in *Qingkailing* can promote geniposide absorption and delay geniposide elimination time by compatible effect.

The in vivo measurement results of skullcap extracts showed specific characteristics as well. Baicalin content in skullcap extract was not less than 92%. Therefore, we can use baicalin as an indicator to determine *Huangqin* extracts. Baicalin was not detected in the cerebrospinal fluid. It can be speculated that skullcap extract does not pass through the blood–brain barrier. The measurement results of plasma and serum samples showed that baicalin arrived at the peak time at 2–4 h, and most of the baicalin was eliminated after 24 h. Between administrations of skullcap extract and the *Qingkailing* formula, there was no significant difference in the concentration–time curve of baicalin, showing that with the compatible effect of other components in *Qingkailing* formula, there was little effect on the content of baicalin in blood absorption, distribution, and elimination.

Because this study aimed at *Qingkailing* injection treatment of cerebral edema, ingredients of *Qingkailing* that can move through the blood–brain barrier were of concern. Previous in vivo studies have shown that *Zhizi* composition can pass through the blood–brain barrier and maintain a high concentration in the cerebrospinal fluid. But baicalin was not detected in the cerebrospinal fluid. To further verify the results, we performed a study with the above components by blood–brain barrier models in vitro. The results showed that baicalin was not detected at the side of blood–brain barrier, indicating that baicalin may not play a role in the brain through the blood–brain barrier directly, but the effective component group gardenia glycosides and bile acids of *Qingkailing* could pass through the simulated blood–brain barrier, and geniposide permeability was better (Fig. 10.7).

10.2 PHARMACODYNAMIC EVALUATION OF *QINGKAILING* INJECTION

A pharmacodynamic study was completed by Professor Li Pengtao's group in Beijing University of TCM.[1–4] With animal models and cell models, for the

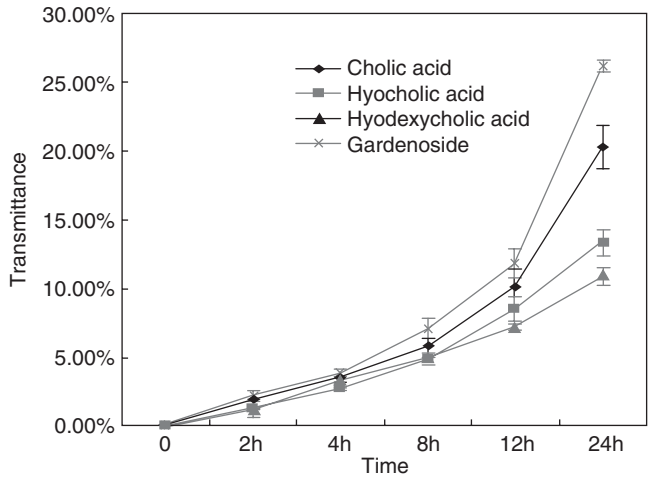

Fig. 10.7 Analysis results of the effective components in *Qingkailing* injection through the simulated blood–brain barrier transmittance.

major pathological aspects, they designed an effective index and evaluated and compared every active component of the whole *Qingkailing* formula. This study established multiple pharmacological evaluation models of anti-ischemic cascade, verified the pharmacological effects of the *Qingkailing* injection, and compared the effect of effective component group of every medicine in the *Qingkailing* formula on the different pathological aspects of cerebral ischemia. This can provide a reference for further study of the compatibility of effective component groups.

10.2.1 Rat Model of Focal Cerebral Ischemia/Reperfusion Injury

The various components of the whole *Qingkailing* formula, including cholic acid, baicalin, *Zhizi* extract, *Jinyinhua* extract, *Banlangen* extract, *Zhenzhumu*, and *Shuiniujiao* hydrolyzate, can significantly improve the animal model's neurological functions; and cholic acid and baicalin can reduce the mortality of the animal model. *Zhizi*, *Jinyinhua*, *Banlangen*, *Zhenzhumu*, *Shuiniujiao*, and other single effective component groups did not have significant or increasing trend effects on the mortality of animal models. With the increase of the effective compatible components, mortality declined, and other effective component groups displayed a significant synergy. The whole *Qingkailing* formula had the most significant effect, and the mortality rate was zero, showing a good synergy for the reduction of toxins and increase of effect.

Jinyinhua, *Banlangen*, *Zhenzhumu*, and *Shuiniujiao* can significantly improve the level of superoxide dismutase (SOD) in brain tissue of animal models. Cholic acid, baicalin, and *Zhizi* do not have a better effect on brain

tissue SOD levels of animal models. *Zhizi* combined with baicalin improved brain tissue SOD levels of animal models significantly, showing that there may be synergy between the two. While *Jinyinhua* and *Banlangen* have no synergy effect, these four herbs have the strongest compatible effect. If there is a weak synergy between the two raw herbs, it would have to be further verified. Cholic acid itself had no effect on the SOD level. Added to *Zhenzhumu* and *Shuiniujiao*, there also was no effect. The effect was not strengthened after adding the effective dose of *Banlangen* and *Zhizi* in *Zhenzhumu* and *Shuiniujiao*.

Banlangen can significantly reduce the lipoperoxide (LPO) content in brain tissue of animal models. The rest of the medicines had no significant effect compared with *Banlangen*. Skullcap and *Zhizi*, which have no effect on it originally, can significantly reduce LPO content in brain tissue of animal model after being combined. Cholic acid, *Zhenzhumu*, and *Shuiniujiao* compatibility also significantly reduced LPO contents in brain tissue. However, its effect is far weaker than the former compatibility. *Zhizi*, *Banlangen*, cholic acid, *Zhenzhumu*, and *Shuiniujiao* compatibility can significantly reduce the LPO content in brain tissue. In summary, skullcap and *Jinyinhua* have no significant impact on the lipid peroxidation process in cerebral ischemia cascade reaction.

Zhizi, *Banlangen*, *Zhenzhumu*, and *Shuiniujiao* can significantly reduce sodium–potassium–ATP enzyme levels in brain tissue of animal models; baicalin has a contrary role, while cholic acid and *Jinyinhua* have no significant effect. Adding cholic acid to *Zhenzhumu* and *Shuiniujiao* and adding baicalin to *Zhizi* can significantly reduce the role of brain tissue sodium–potassium–ATP enzyme level in animal models. It prompted the theory that compatibilities of these two groups have an antagonistic effect on sodium–potassium–ATP enzyme activity. *Jinyinhua* and *Banlangen* compatibility has no significant synergy. *Zhizi*, *Banlangen*, *Zhenzhumu*, and *Shuiniujiao* significantly reduced sodium–potassium–ATP enzyme levels in brain tissue of animal models. After compatibility there is no such effect.

Zhenzhumu and *Shuiniujiao* have a significant improving trend on brain energy charge, while cholic acid, baicalin, and *Zhizi* have no significant effect. *Jinyinhua* and *Banlangen* have an improving tendency. *Zhenzhumu* and *Shuiniujiao* combined with cholic acid have the original effect. Baicalin, *Zhizi*, *Jinyinhua*, and *Banlangen* compatibility still has no significant effect on brain energy charge. Adding skullcap, *Zhizi*, *Jinyinhua*, and *Banlangen* in *Zhenzhumu* and *Shuiniujiao* had a negative impact on the original effects.

Comparing the contents of plasma von Willebrand factor (vWF), we found that *Banlangen* could significantly reduce the plasma vWF level in animal models. Cholic acid, baicalin, *Zhizi*, *Jinyinhua*, *Zhenzhumu*, and *Shuiniujiao* have no significant improving effect on the vWF level of animal models. Cholic acid even has a negative effect. Compatible effects showed that cholic acid, *Zhenzhumu*, and *Shuiniujiao* compatibility, and skullcap and *Zhizi*

compatibility, produced a significant reduction effect on plasma vWF level of the animal model, illustrating that a synergistic effect may exist in these two groups. *Jinyinhua* and *Banlangen* compatibility did not show significant synergy. Each component of *Qingkailing* had no significant effect, but the whole *Qingkailing* formula had a significant effect. Specific synergistic compatibility effects need to be further verified.

10.2.2 Rat Model of Incomplete Cerebral Ischemia/Reperfusion Injury

On the basis of the abovementioned studies, we further analyzed some components and the possible effective compatibility among them. In this study, we took an incomplete rat cerebral ischemia-reperfusion injury model resulting from repeated occlusion of bilateral carotid artery and related biological effects as the evaluation indicator. The role characteristics of each effective component of *Qingkailing* and the effective component compatibility were analyzed.

Through the comparative study of SOD levels in plasma, we found that skullcap, *Zhenzhumu*, and *Shuiniujiao* hydrolyzate could significantly improve the plasma SOD level in the animal model. There was a trend that cholic acid could improve plasma SOD levels in the animal model. *Zhizi*, *Jinyinhua*, and *Banlangen* did not significantly affect plasma SOD levels in the animal model. Compatibility function showed that adding *Banlangen* and *Zhizi* in hydrolyzate can weaken hydrolysis improvement role in plasma SOD. Adding acid also had no significant effect. The whole *Qingkailing* formula had no significant effect on plasma SOD levels.

The interclass comparative study of plasma lactate dehydrogenase (LDH) levels showed that *Banlangen* could significantly reduce the plasma high-density lipoprotein (HDL) levels in the animal model. Cholic acid and baicalin had a tendency of reducing plasma HDL level in animal models. Hydrolysis had no significant effect on plasma HDL level in animal models. Compatible effect study showed that adding hydrolysis and *Zhizi* to *Banlangen* could weaken the reducing plasma SOD level role of *Banlangen*. Further adding acid also had no significant effect. The whole *Qingkailing* formula had no significant effect on the plasma LDL level.

Cholic acid, *Zhizi*, and hydrolysis could significantly reduce plasma vWF levels in animal models. The whole *Qingkailing* formula compatibility weakened the role of cholic acid, *Zhizi*, and hydrolysis, reducing plasma vWF level in animal models.

Cholic acid, *Zhizi*, and hydrolysis had no significant effect on brain water content in animal models. The whole *Qingkailing* formula compatibility can significantly reduce brain water content in animal models, illustrating that there is synergy among all the components.

Comparative analysis of the SOD level in brain tissue showed that cholic acid, baicalin, and *Banlangen* could significantly improve brain tissue SOD

level in the animal model. Hydrolyzate had no significant effect on brain tissue SOD level in animal models. Adding hydrolysis and *Zhizi* to *Banlangen* had no significant effect on the improving brain tissue SOD role of *Banlangen*, indicating that there were no obvious synergies among the three. The compatibility among *Banlangen*, cholic acid, hydrolysis, and *Zhizi* can reduce the improvement of brain tissue SOD caused by *Banlangen* and cholic acid. The whole *Qingkailing* formula compatibility weakened the improving brain tissue SOD role of cholic acid, baicalin, and *Banlangen*.

Cholic acid can significantly reduce brain tissue MDA level in animal models. *Zhizi* and hydrolysis had a tendency which could decrease brain tissue MDA level. Compatible effect tests showed that in the whole *Qingkailing* formula, compatibility could weaken the reduction brain tissue MDA role of cholic acid; hydrolysis, *Banlangen*, and *Zhizi* compatibility had a tendency to improve brain cell energy charge value in the animal model. After the addition of cholic acid, this effect was reduced.

10.2.3 SH-SY5Y Nerve Cell Model

The whole *Qingkailing* formula, the mixed solution of *Zhizi*, *Banlangen*, *Zhenzhumu* hydrolyzate, and the mixed solution of cholic acid, hyocholic acid, *Zhizi*, *Banlangen*, and *Zhenzhumu* hydrolyzate could significantly retard neural cell apoptosis. All components of *Qingkailing* had an obvious antioxidant effect.

10.2.4 Endothelial Cell Model

We also tested the effective component group of *Qingkailing*'s effects on apoptosis and toxicity on ECV304 endothelial cell. The result showed that 5 μL/mL (1 μL *Qingkailing* injection per mL cell culture medium) and 10 μL/mL of the whole *Qingkailing* formula could significantly improve the survival rate of hypoxia-ischemia ECV304 endothelial cells. The survival rate of the 20 μL/mL group was significantly reduced, showing that the protective effect of the whole *Qingkailing* formula on hypoxia-ischemia and reperfusion ECV304 endothelial cells correlates with the dose. The 5 and 10 μL/mL doses had a significant protective effect on the cells, while the 20 μL/mL dose had no protective effect on the cells, and even increased cell damage. This result is also consistent with the cytotoxicity test of the whole *Qingkailing* formula. The 5, 10, and 20 μL/mL doses of cholic acid could significantly reduce the lactate dehydrogenase leakage rate of ECV304 endothelial cells, but had no significant effect on cell survival rate, showing that it had a protective effect on hypoxia-ischemia-reperfused ECV304 endothelial cells. Although the three doses of *Huangqin* could increase the lactate dehydrogenase leakage rate of the cells, the 5 and 10 μL/mL doses could increase the survival rate of hypoxia-ischemia-reperfused ECV304 endothelial cells. The 20 μL/mL dose had a slight decrease in cell survival rate. It was also shown that the 5 and 10 μL/mL doses of skullcap

might have a protective effect on hypoxic-ischemic-reperfused ECV304 endothelial cells, while the 20 μL/mL dose increased the cell damage. Overall, a 10 μL/mL dose of the whole *Qingkailing* formula had the best protective effect on the hypoxic-ischemic-reperfused ECV304 endothelial cells. The expression test of ICAM-1 of the hypoxia-ischemia-reperfused human brain endothelial cells showed that the whole *Qingkailing* formula, cholic acid, hyocholic acid, *Zhizi*, *Banlangen* and *Zhenzhumu* hydrolyzate could inhibit the expression of ICAM-1 of brain endothelial cells.

10.3 RESEARCH ON THE *QINGKAILING* DERIVED FORMULA

10.3.1 Compatibility Screening and Optimization of *Qingkailing* Derived Formula for the Treatment of Cerebral Edema

After establishing the platform of Chinese herbal compound chemome research and pharmacodynamic evaluation, through various compatibility methods which correspond to the changes of chemical composition information and the correlation of pharmacodynamics (research about the components and efficiency of TCM), we needed to screen the effective chemome from the global chemome of TCM formula, that is, to select effective component compatibility with the best therapeutic effect based on the disease being treated. Medicinal chemistry information on TCM formulas is obtained from chemomics research and the pharmacology information matrix is obtained from pharmacology research, which are the basis and premise of component–efficiency-related analysis. Strictly speaking, to obtain an optimal compatibility mode, one should establish every compatibility mode which can cover each subchemome comprehensively, and then do the pharmacodynamic evaluation, using a variety of analysis processes, including regression analysis (linear/nonlinear), association analysis, causal discovery, artificial neural networks, and other mathematical and informatics tools to establish component–efficiency relationship prediction model. However, due to the large number of TCM and Chinese herbal components, and their complex chemical compositions, it is not feasible to be in full accordance with theoretical combinations of the design approach to compatibility mode because of the workload. However, there are two advantages of TCM formula selection that must be fully used. First, the selected initial formula should have definite clinical effects, so you can take the efficiency as a reference for the following selection of compatibility. Second, the compatibility theory of TCM, such as the coupled medicines/medicine team experience compatibility, can be used to reduce the components'; compatibility. We also need to take advantage of a variety of information processing tools, such as the contribution method of informational characteristics on fingerprint analysis. These tools will be described later here.

In this study, using the *Qingkailing* injection (original formula) as a reference, we made the pharmacodynamic evaluation (see Section 10.2) on the

single herb extracts of herbs, the four couplet medicines (*Huangqin* and *Zhizi*, *Banlangen* and *Jinyinhua*, *Zhenzhumu* and *Shuiniujiao*, and cholic acid and hyocholic acid), four drug teams (hydrolysate of *Zhenzhumu* and *Shuiniujiao* and extracts of *Banlangen* and *Zhizi*; cholic acid, hyocholic acid, hydrolyzate of *Zhenzhumu*, and *Shuiniujiao*; hydrolysate of *Zhenzhumu* and *Shuiniujiao*, extracts of *Banlangen* and *Zhizi*-cholic acid, hyocholic acid, and baicalin extracts of *Zhizi*; and *Banlangen*, *Jinyinhua*, and *Zhenzhumu*) in accordance with *Qingkailing* injection formula and the preparation process for the samples, and according to chemome information (including fingerprints and determination with multiple indicators).

10.3.1.1 Compatibility Screening and Optimization Guided by Fingerprint Characteristic Information Analysis Fingerprint characteristic information analysis has been described in detail in Chapter 3. By the fingerprint feature information extraction, classification, and comparative analysis, one can use the chemical point of view to judge the selection from taste, the couplet medicines, medicine team, and its effective component group for the entire contribution and impact of characteristics information to guide formula compatibility and development of new drugs.

The basic principle uses the original *Qingkailing* injection fingerprint as a reference, and assumes that the global fingerprint of the original formula and compatibility are reasonable (because of its efficiency has been proved by clinical practice). Then selective analysis of the fingerprint peaks or peak groups (on behalf of the appropriate herbs or component groups) affecting and contributing on the global fingerprint helps to judge the effect of the medicine or component on the whole formula. Regarding the three fingerprints of *Qingkailing* injection, only the most representative HPLC/DAD fingerprint is discussed here (Fig. 10.8). In the figure, peak 26 is geniposide, peak 27 is geniposide isomer, peak 30 is chlorogenic acid, peak 32 is baicalin, peak 35 is scutellarin, and peak 37 is crocin and other mixed peaks. Around

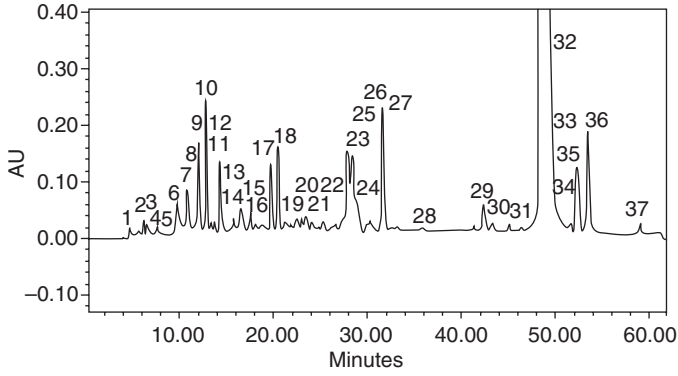

Fig. 10.8 HPLC-UV fingerprint of *Qingkailing* injection.

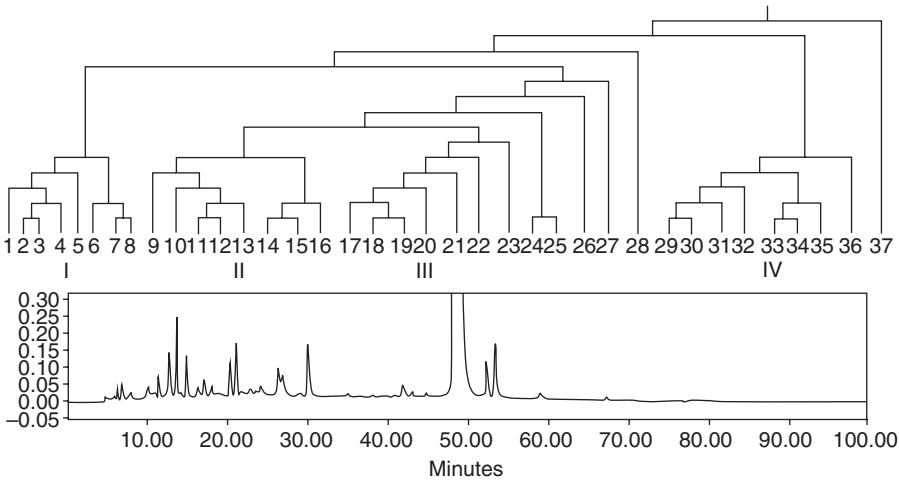

Fig. 10.9 Peak clustering of the *Qingkailing* injection according to retention time.

70 min is the cholic acid region, which includes three components, hyodesoxycholic acid, deoxycholic acid, and cholic acid. Due to the low response of cholic acid in the HPLC-UV fingerprints, an additional test of HPLC/ELSD was needed, which is not stated or shown in the figure.

Fingerprints of *Qingkailing* injection was clustered by retention time. Fig. 10.9 shows the clustering of *Qingkailing* injection fingerprint according to peak retention time. The clustering of retention times was repeated on 10 batches of *Qingkailing* injection and the results could be repeated.

From Fig. 10.9, the *Qingkailing* injection's 37 fingerprint peaks can be divided into five categories, I = {1–8}, II = {9–16}, III = {17–23}, IV = {29–36}, and the remaining isolated peaks would be in the V class; in *Qingkailing* injection fingerprint data, baicalin (peak 32) accounted for 80% of the total response, while the rest of the peak response was below 1%. So while evaluating, the baicalin peak was generally excluded, and the remaining 36 peaks were evaluated. The percentage of the respective 5 categories are: I: 2.55%; II: 4.81%; III: 4.68%; IV: 84.52% (after removing baicalin it is 5.05%); and V: 3.42%. The compared similarities are (including baicalin peak basis): I: 0.9999; II: 0.9993; III: 0.9993; IV: 0.119; and V: 0.9992, but the characteristics are difficult to identify. However, calculating after deducting the baicalin peak, the results are: I: 0.9456; II: 0.6630; III: 0.6903; IV: 0.4335; and V: 0.6471. These show clear differences; and the results can be divided into three parts {I}, {II, III, V}, {IV}. {II, III, V} as a class compared to the whole formula (excluding baicalin) had similarity of 0.251. As a result, the category {II, III, V} and {IV} can be considered the characteristic peaks.

From the classification of fingerprints, because of the heavy effects of baicalin, the baicalin peak needs to be hidden in the analysis. After removal of baicalin, the main features of the response focused on the {II, III, V} and

{IV}. First analyzing the IV, we could find that the main peaks {33–36} from *Huangqin* by matching with the medicine fingerprint. Keeping this main peaks and removing the other fingerprint peaks, and then comparing these results with the whole formula fingerprint resulted in a similarity of 0.9501. In the previous analysis, it can be seen that after the removal of IV, the similarity was 0.4335, indicating that the main feature in this type of information is from *Huangqin*, so the characteristic information of IV are peaks 33–36.

Analyzing {II, III, V} the same way, the main peaks from *Zhizi* and some new material peaks in V. Retaining the major peaks, removing all other peaks and compared with the whole formula, the similarity was 0.992, so that feature information obtained in class V are peaks 24 and 27. Peak 27 is Geniposide. To analyze the rest of {II, III}, one should combine the relationship between the whole formula fingerprint and medicine fingerprint.

Analyzing the similarity between the whole formula fingerprint and fingerprints of intermediates of the *Qingkailing* injection, such as hydrolysate of *Shuiniujiao* and extracts of *Jinyinhua* and *Banlangen*, we found that they were incomparable. But after comparison of the fingerprints, the main response of *Zhenzhumu*, *Shuiniujiao*, and *Banlangen* could all fall into class I and II region of the whole formula. Comparing the fingerprints of *Jinyinhua* and the whole formula, it showed that in the 30–40 min range were *Jinyinhua* herb peaks that were not in the whole formula, and the major fingerprint peaks of *Jinyinhua* all fall in the region of II and III. Given this result, we can divide *Qingkailing* injection fingerprint into three main areas: before 22 min include hydrolysate of *Zhenzhumu* and *Shuiniujiao*, *Banlangen*, and *Jinyinhua* herbs mixed area; 22–35 min included *Zhizi* herbs and the main new produced substances region; and 35–65 min included mainly the *Huangqin* area. However, chlorogenic acid and analogues existed in the 35–40 min period but only in small amounts. Thus, the peaks in the fingerprint analysis were not considered. Combined with the grouping of herbs, their classification should be reidentified. According to attribution, the original class III peaks 17–19 should be incorporated in the original class I and II; class III peak 23 should be incorporated into class V, while peak 24 as the peak of the new material was considered at the same time. So merging the discussions of the above characteristic information of class IV and V, we divided the *Qingkailing* injection fingerprint characteristic information into two categories: I′ = {1–19 numbers of peaks in the characteristic information}, II′ = {23, 24, 27, 33–36}. After removing peaks 1–19, the similarity was 0.2516 when compared with whole formula, while removing peak II′ resulted in a similarity of 0.1508. When only retaining II′, after the removal of the remaining peaks, the similarity was 0.5548. Just retaining I′ and II′, the similarity was 0.9785, showing that it was reasonable to consider only the feature information of II′. The above calculations did not include the main peak of baicalin.

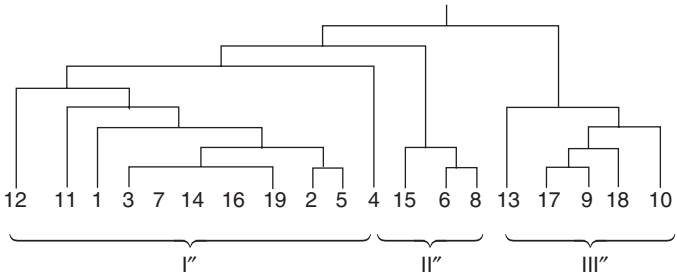

Fig. 10.10 Classification for I′ according to the intensity of the signal.

I′ is the extracts of three herbs mixed fingerprint region. In order to extract characteristic information, it must be reclassified. The classification was according to the size of the signal, and the results are shown in Fig. 10.10.

The fingerprints are divided into three areas, I″ = peaks {1–5, 7, 11, 12, 14, 16, 19}, II″ = peaks {6, 8, 15}, and III″ = peaks {9, 10, 13, 17, 18}. After removing peaks 1, 2, 3, and comparing with the entire class, I" category accounted for 23.5% of the response class; after the removal, the similarity was 0.9735. II″ response category accounted for 15.1% of class; after the removal, the similarity was 0.8615. III″ category accounted for 61.4% class response; after the removal, the similarity was 0.2365. Therefore, the characteristic information of I′ is peaks {6, 8–10, 13, 15, 17, 18}; the main featured information is peaks {9, 10, 13, 17, 18}. Compared with the herbs, peaks 6, 8, 15 did not have any medicine attribution. Peak 9 was from *Banlangen* and *Jinyinhua*; peak 10 was from *Zhenzhumu* and *Shuiniujiao*; peaks 13, 17, and 18 were from *Banlangen*. Comparing the three little categories of I″, II″, and III″ again among them, after the removal of peaks {9, 10}, the similarity was 0.0547; while the removal of peaks {13, 17, 18} led to a similarity of 0.3409. The conclusion is that the former plays a more important role than the latter.

From the above, we found that I′ = peaks {6, 8–10, 13, 15, 17, 18}. To verify its characteristic, after the removal of peaks 1 through 19, compared with all formula, the similarity was 0.2516; in the peaks 1–19, after the removal of I′, compared with the original 1–19 data, the similarity was 0.441; After removing I′, the similarity compared to the whole formula was 0.4655; while removing the fingerprint peaks range from 1 to 19 which are not included in I′, the similarity was 0.9744 compared with the whole formula.

By this analysis, the characteristic information of the *Qingkailing* injection's fingerprint can be obtained: I′ + II′ = peaks {6, 8–10, 13, 15, 17, 18, 23, 24, 27, 33–36}; peaks 6, 8, 15, 23, and 24 did not have medicine attribution; the similarity between feature information and the whole formula was 0.9464, and had a high correlation, while the similarity of other general fingerprint information was 0.1181.

The above characteristic information can be divided into A, B, C, D, E, and F—6 small classes in accordance with the attribution of the herbs, of which class A = peaks {6, 8, 15, 23, 24} were unattributive peaks. After its removal, compared with the whole class, the similarity was 0.850. Class B is peak {9}, and belongs to *Jinyinhua* herbs. After its removal, compared with the whole class, the similarity was 0.8142. Class C is peak {10}, and belongs to *Zhenzhumu* and *Shuiniujiao*. After its removal, compared with the whole class, the similarity was 0.6470. Class D is peak {27}, and belongs to *Zhizi*. After its removal, compared with the whole class, the similarity was 0.4439. Class E is peaks {33–36}, and belongs to *Huangqin*. After its removal, compared with the whole class, the similarity was 0.2131. Class F is peaks {9, 13, 17, 18}, and belongs to *Banlangen*. After its removal, compared with the whole class, the similarity was 0.5450. For the peak 9 attributed to *Jinyinhua* and *Banlangen*, by comparison with *Banlangen*, retaining peak 9 and replacing peaks 13, 17, and 18 with 0, and then compared with F, the similarity was 1.0. On the contrary, retaining peaks 13, 17, and 18 and replacing peak 9 with 0, and then comparing the results with F, the similarity was 0.094. This shows that peak 9 belongs to the *Banlangen* class, and has effects that are weaker than the other three. Peak 9 also differs more than nine times in similarity with other three peaks, so it is considered as the characteristic information of *Jinyinhua* in the comparisons. The importance of various types can be seen from Fig. 10.11.

Fig. 10.11 shows the compatibility of *Qingkailing* injection material characteristics. *Huangqin* and *Zhizi* have the most important roles; *Zhenzhumu*, *Shuiniujiao*, and *Banlangen* second; and *Jinyinhua* and nonattributed materials last. This conclusion seems to go against with the original theory. Originally, the *Zhenzhumu* and *Shuiniujiao* are the *Chen*, but their roles shown here are not great. So it could be noted that UV detection for the determination of the

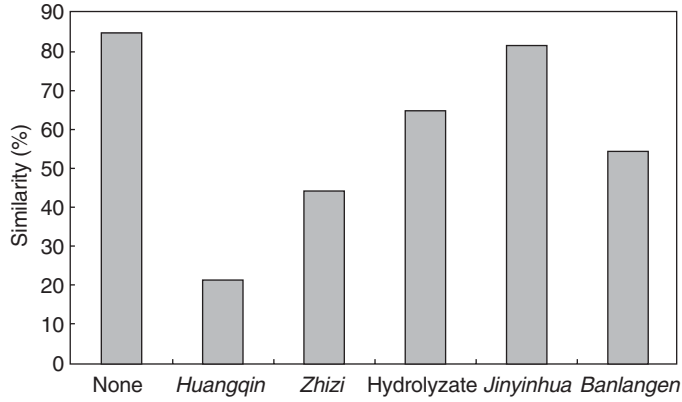

Fig. 10.11 Similarity analysis after removing indicated herb (hydrolyzate: hydrolyzed solution of *Zhenzhumu* and *Shuiniujiao*). The smaller value of similarity is, the greater contribution to the activity of the whole formula.

display was under the HPLC-DAD fingerprint, but the main bioactive materials of *Zhenzhumu* and *Shuiniujiao* are some inorganic ions and some biological macromolecules, and the information in HPLC-DAD fingerprint was insufficient. Therefore the above conclusions do not have great conflict with the original theory.

In the above studies, after knowing the attribution of every feature peak in the fingerprint of the whole formula, the contribution of different compatibilities to the feature of fingerprint of the whole formula was further compared.

Considering the similarity values, after the removal of feature information and comparison with the whole formula, the smaller the value is, the greater the importance of the information. The specific exhibition is the actual similarity value. The RSDs ($n = 5$) are all less than 10%.

The comparison of the *Huangqin* and *Zhizi* couplet medicine is shown in Fig. 10.12. The comparative similarity values are as follows: *Huangqin* was

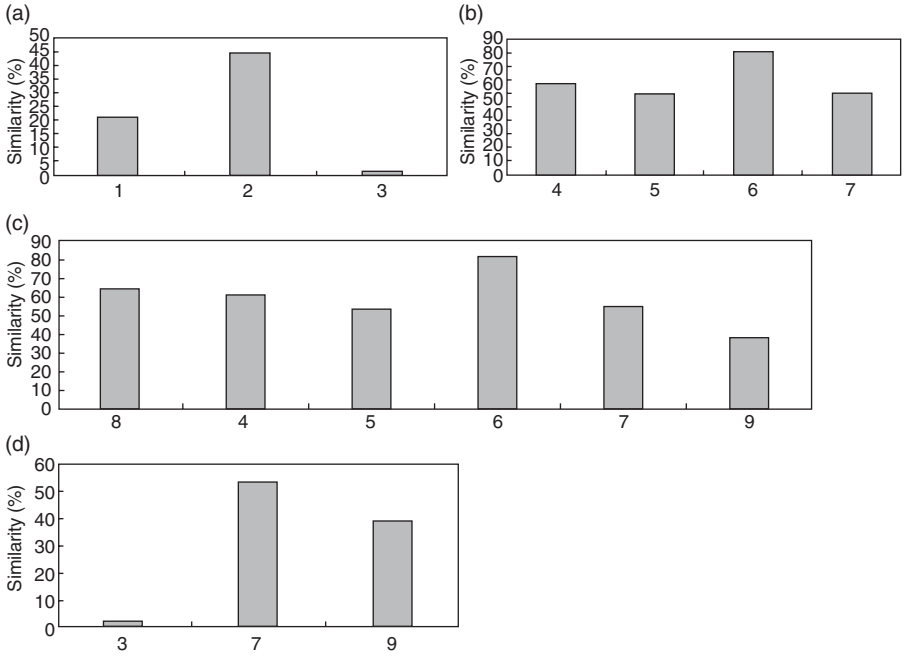

Fig. 10.12 Comparison of the changes of similarity among removing indicated herb couples. 1: *Huangqin*; 2: *Zhizi*; 3: *Huangqin* and *Zhizi*; 4: *Banlangen* without *Jinyinhua*; 8: hydrolyzed solution of *Zhenzhumu* and *Shuiniujiao*; 9: *Banlangen*, *Jinyinhua*, and hydrolyzed solution of *Zhenzhumu* and *Shuiniujiao*. A: removing herb couples of *Huangqin* and *Zhizi*; B: removing herb couples of *Banlangen* and *Jinyinhua*; C: removing herb team of *Banlangen*, *Jinyinhua*, and hydrolyzed solution of *Zhenzhumu* and *Shuiniujiao*; D: removing herb couples of A or B and herb team of C.

0.2131; *Zhizi* was 0.4439; and the *Huangqin* and *Zhizi* drug team was 0.0147. The results indicate that *Huangqin* and *Zhizi* in *Qingkailing* injection are important, so when compatibility of the two medicines is made, the importance of the feature information is strengthened.

The comparison of the *Banlangen–Jinyinhua* couplet medicine is shown in Fig. 10.12. The comparative similarity values are as follows: *Banlangen* was 0.5450; *Jinyinhua* was 0.8142; the *Banlangen–Jinyinhua* drug team was 0.5450. The characteristic information of *Banlangen* and *Jinyinhua* are overlapped. In other words, the information of *Banlangen* may include the information of *Jinyinhua*. If excluding all the features of *Jinyinhua*, the similarity of *Banlangen* changed to 0.6107. The importance of *Banlangen* and *Jinyinhua* characteristics information is obviously inferior to that of *Huangqin* and *Zhizi*. When making the compatibility of *Banlangen* and *Jinyinhua*, the information is strengthened slightly, but still insignificant.

The comparison between hydrolyzate of *Zhenzhumu* and *Shuiniujiao*, and extracts of *Banlangen* and *Zhizi* can be seen in Fig. 10.12. Compared similarity values are as follows. The similarity of hydrolyzate of *Zhenzhumu* and *Shuiniujiao* was 0.647, indicating that its importance is not obvious, which may be related to the poor reflection of these two herbs'; characteristic information in the fingerprints. When comparing *Zhenzhumu* and *Shuiniujiao* hydrolyzate, and *Banlangen* and *Jinyinhua*, the characteristic information became strengthened, but the change was still insignificant.

Comparison of these drug teams shows that the compatibility of *Huangqin* and *Zhizi* had the biggest impact on whole formula, while other drug combinations can have an insignificant impact on whole formula.

10.3.1.2 Pharmacodynamic Verification for the Compatibility of Effective Component Groups Fingerprint analysis of the characteristics only lets us understand compatibility relationship on chemical characteristics. There is some reference value, but cannot replace the analysis of pharmacodynamics. Efficacy of the study results also must be verified, then rationale of the compatibility needs to be confirmed. Therefore, the pharmacodynamic evaluation is conducted simultaneously with chemical analysis of the compatibility of the formula, the couplet medicine, and drug team.

Qingkailing injection consists of several components, including *Huangqin*, *Zhizi*, *Banlangen*, *Jinyinhua*, *Zhenzhumu*, *Shuiniujiao*, cholic acid, and hyocholic acid (instead of the original calculus bovis). In accordance with the basic process of preparing the *Qingkailing* injection, all the samples from every sector in the circuit process are reserved, and a series of preparations is formed. The effective component group of single herb preparation and preparation process are included. The effective material content and pH value of single herb in each step of the process of preparation are the same as in the *Qingkailing* injection. Blank control (water for injection) preparation is set up, and 11 total preparations are formed and numbered. In

addition, the compatibility of the effective component group in accordance with formula of *Qingkailing* injection was made. Calculus bovis is for *Niuhuang* detoxification resuscitation, *Zhenzhumu* and *Shuiniujiao* are for resuscitation refreshing, baicalin and *Zhizi* are for purging fire and detoxification, and *Jinyinhua* and *Banlangen* are for dissipating heat and detoxification. These four drugs teams are attributed. The next steps are assuring the compatibility of these drug teams, setting up the whole formula, and numbering the groups. The efficacy of bioactive components was conducted by single-blind method.

10.3.1.2.1 Analysis of the Effective Component Groups of Qingkailing *Based on Cerebral Ischemia Animal Model* Using the focal cerebral ischemia-reperfusion model made by ligation of the middle cerebral artery of rat and the incompletion cerebral ischemia animal model made by repeated occlusion of both sides of carotid arteries of rat, taking the level of neurologic impairment and mortality as evaluation indicators, combining with the biochemical analysis of series indicators of metabolic injury, we screened the effective component groups from *Qingkailing* injection.

Including cholic acid, baicalin, *Zhizi* extracts, *Jinyinhua* extracts, *Banlangen* extracts, *Zhenzhumu*, *Shuiniujiao* hydrolyzate, and other effective parts in *Qingkailing* injection can significantly improve the model animal neurological deficit symptoms. These results showed that there is significant synergy among the various parts of the formula, and the *Qingkailing* injection was the most significant one.

Cholic acid and baicalin can reduce mortality in animal models, though the impact of the remaining individual parts on mortality of the animal models was not significant or even negative. The mortality rates of every compatible group declined significantly. As the compatibility of the efficacy of some substances increased, mortality further declined. Mortality with the *Qingkailing* injection was zero, showing ability of the ingredients to reduce poison and increase efficiency.

For the antiperoxidation of lipids, *Jinyinhua*, *Banlangen*, *Zhenzhumu*, and *Shuiniujiao* can significantly improve the level of SOD in brain tissue of the animal model. Cholic acid, baicalin, and *Zhizi* had no effect on SOD levels in brain tissue. Compatibility of baicalin and *Zhizi* had significantly improved the SOD levels in brain tissue of models; other compatible ingredients did not display synergy. *Banlangen* could significantly reduce LPO content in brain tissue of the animal model; the rest of the drugs had no significant impact. But after compatibility of baicalin and *Zhizi*, contents of LPO could be significantly reduced in the brain; after compatibility of cholic acid, *Zhenzhumu*, and *Shuiniujiao*, LPO content in brain tissue was effectively reduced. *Zhizi*, *Banlangen*, cholic acid with *Zhenzhumu*, and *Shuiniujiao* compatibility could significantly reduce LPO in the brain tissue. Baicalin and *Jinyinhua* had no significant effect on the lipid peroxidation process.

Zhizi, *Banlangen*, *Zhenzhumu*, and *Shuiniujiao* can significantly reduce sodium-potassium-ATP enzyme activity in brain tissue in the animal model. Baicalin had the opposite effect, and cholic acids and *Jinyinhua* had no significant effect. The compatibility of cholic acid, *Zhenzhumu*, and *Shuiniujiao* and the compatibility of baicalin and *Zhizi* could weaken the brain tissue of the reducing effect of Na-K^+-ATP activity, suggesting that between the two sets of compatibility likely have enzyme antagonism activity. The compatibility of *Banlangen* and *Jinyinhua* did not show significant synergy.

Zhenzhumu and *Shuiniujiao* could have a significant trend toward improvement in the brain. Cholic acid, baicalin and *Zhizi* had no significant effect. *Jinyinhua* and *Banlangen* brought about a certain improvement trend. After the compatibility of *Zhenzhumu*, *Shuiniujiao*, and cholic acids, the original effect still remained, while the compatibility of baicalin and *Zhizi* and *Jinyinhua* and *Banlangen* still had no significant effect on cerebral energy charge. *Zhenzhumu*, *Shuiniujiao*, baicalin, *Zhizi*, *Jinyinhua*, and *Banlangen* brought about a negative impact on their original effect.

Banlangen could significantly reduce the vWF level in plasma of the animal models. Cholic acid, baicalin, *Zhizi*, *Jinyinhua*, *Zhenzhumu*, and *Shuiniujiao* did not significantly improve the vWF level in the animal model; cholic acid even had a negative impact. However, the compatibility of cholic acid, *Zhenzhumu*, and *Shuiniujiao*, and the compatibility of baicalin and *Zhizi*, resulted in a significant reduction in plasma vWF in the animal models. The compatibility of these two groups showed synergies, but did not show significant synergy with the compatibility of *Jinyinhua* and *Banlangen*. The *Qingkailing* injection had significant effects. Cholic acid and baicalin could significantly reduce the level of NOS in brain tissue of the animal models, but *Zhizi*, *Jinyinhua*, *Banlangen*, *Zhenzhumu*, and *Shuiniujiao* made no improvement on the brain tissue NOS levels of the animal model. The compatibility effect did not show significant synergy.

10.3.1.2.2 Selection of the Qingkailing *Effective Component Groups with Cell Model* We selected effective materials by cell model with the use of cultured neurons, neurogliocytes, and endothelial cells, in nontoxic to normal cultured cell doses.

10.3.1.2.2.1 EFFECT ON NEURONS The whole *Qingkailing* formula had an obvious protective effect on neurons in certain doses. In this formula, the compatible mixed solution of *Zhizi*, *Banlangen*, and the hydrolyzate of *Zhenzhumu* with *Shuiniujiao* can protect neurons greatly. It has the ability to promote neuronal (SH-SY5Y) proliferation, inhibit neuronal apoptosis and lipid peroxide production, and increase SOD and NOS activity of the antilipid peroxide system. Also, it increases the concentration of NO. The compatible mixed solution of cholic acid, hyodeoxycholic acid, *Zhizi*, *Banlangen*, and the hydrolyzate of *Zhenzhumu* with *Shuiniujiao* has significant toxic effects on neurons. It inhibits neuronal apoptosis and lipid peroxide production and

increases SOD and NOS activity and the concentration of NO. However, compared with the compatible mixed solution of *Zhizi*, *Banlangen*, and the hydrolyzate of *Zhenzhumu* with *Shuiniujiao*, the effect is a little reduced. Single-drug studies have shown that for certain doses, cholic acid and *Zhizi* have significant protective effects, while hyocholic acid and baicalin show a high level of toxic effects. High doses of baicalin has lower inhibitory effects on SH-SY5Y neurons than low doses. Low-dose *Jinyinhua* and the hydrolyzate of *Zhenzhumu* with *Shuiniujiao*, as well as high-dose *Banlangen*, can protect neurons greatly. Therefore, with compatibility of different doses or different constituents, *Qingkailing* effective materials can reduce the deficiency of a single herb, leading to the best efficacy of *Qingkailing*.

10.3.1.2.2.2 EFFECT ON ENDOTHELIAL CELLS The whole *Qingkailing* formula has significant protective effects on endothelial cells in a certain dose. In this formula, the compatible mixed solution of *Zhizi*, *Banlangen*, and the hydrolyzate of *Zhenzhumu* with *Shuiniujiao* has significant protective effects on endothelial cells, playing a role in promoting endothelial cell (ECV304) proliferation. Another compatible mixed solution, which consists of cholic acid, hyocholic acid, *Zhizi*, *Banlangen*, and the hydrolyzate of *Zhenzhumu* with *Shuiniujiao*, shows a weaker promotion effect on endothelial cell proliferation than the solution of *Zhizi*, *Banlangen*, and the hydrolyzate of *Zhenzhumu* with *Shuiniujiao*, whereas its toxicity increased. The studies on single components have shown that cholic acid, baicalin, and the hydrolyzate of *Zhenzhumu* with *Shuiniujiao* promote endothelial cell proliferation. High doses of *Zhizi* and hyocholic acid also have comparatively strong effects on promoting endothelial cell proliferation. On the contrary, *Jinyinhua* and *Banlangen* inhibit the growth of endothelial cells significantly.

10.3.1.2.2.3 EFFECT ON NEUROGLIOCYTES The whole *Qingkailing* formula can inhibit neurogliocytes. This inhibition of low-dose solution is significantly lower than the high-dose one. In this formula, the compatible mixed solution of *Zhizi*, *Banlangen*, and the hydrolyzate of *Zhenzhumu* with *Shuiniujiao* protects glia cells, while another mixed solution, which consists of cholic acid, hyocholic acid, *Zhizi*, *Banlangen*, and the hydrolyzate of *Zhenzhumu* with *Shuiniujiao*, inhibits them. Cholic acid, hyocholic acid, and baicalin each protect neurogliocytes significantly in low doses, but inhibit the cells comparatively strongly with high doses. *Zhizi*, *Jinyinhua*, *Banlangen*, and the hydrolyzate of *Zhenzhumu* with *Shuiniujiao* all inhibit neurogliocytes slightly.

Comprehensively, the effect of each component of *Qingkailing* injection on different pathological aspects of cerebral ischemia has a relative specificity. These components have various effect relationships (at the same time) such as effectiveness and ineffectiveness, and compatible synergy and attenuation. The couplet medicines, such as baicalin and *Zhizi*, play a key role in many aspects and have a strong synergy. *Zhenzhumu* is irreplaceable in decreasing the Na-K-ATPase level in brain tissue, improving brain energy charge, and

other aspects. The compatibility of cholic acid, *Zhenzhumu*, and baicalin–*Zhizi* makes synergy. *Banlangen* and *Jinyinhua* have effects on some sections, but the efficacy synergy of the two is not obvious after being processed with the *Qingkailing* injection procedure and the same proportion compatibility. Combined with fingerprint analysis, *Banlangen–Jinyinhua* compatibility changes a lot compared with extracts of the single herbs in the fingerprints, and their component peaks differ significantly. These prove that compositions change before and after the compatibility. This compatibility shows little characteristic information in the *Qingkailing* formula fingerprint. In other words, it makes little contribution to the *Qingkailing* formula. With the principle of ensuring efficiency and reducing toxicity in mind, the *Qingkailing* effective component group for cerebral ischemia were cholic acids, baicalins, *Zhizi*, and *Zhenzhumu* (called "*Qingkailing* derived formula" in this book).

10.3.2 Optimization of Preparation Process for the Effective Component Groups in Qingkailing-Derived Formula

In the studies above, when used to select compatibility, test samples were prepared completely according to the process of the original *Qingkailing* injection formula at the beginning. According to the determined composition of *Qingkailing*-derived formulas, herbal medicines in the original *Qingkailing* injection can be replaced by cholic acids, baicalins, and *Zhenzhumu* hydrolyzate correspondingly, except for *Zhizi* in its raw form. In order to make the feeding of effective component groups feasible, a further study in the extraction process of *Zhizi*'s effective components has been made after determining the compatibility of *Qingkailing*-derived formula. With the indicator as geniposide, a type of iridoid glycosides that takes a great proportion in the original formula, a comprehensive survey was made to optimize the extraction process of *Zhizi*'s effective components from the extraction solvent and methods. Eventually, the optimized extraction process of *Zhizi* is as follows. The *Zhizi* was extracted by water and precipitated by ethanol, and then eluted by macroporous resin column (specific parameters not described here). The effective component for feeding should meet the standard fingerprints of *Zhizi*'s effective components and the content standard of effective components (geniposide ≥ 70%, crocins ≥ 16%).

Original formula injections were used as controls; a *Qingkailing*-derived formula sample (A) was prepared with the original process, another *Qingkailing*-derived formula (B) was prepared with the optimized *Zhizi* extraction process, and with the same compatible amount as the original formula. Wistar rats were made into (middle cerebral artery thrombosis [MCAT]) cerebral infarction models to compare the formula efficacies with different preparation processes using SOD, MDA, and other indicators in brain tissue (Table 10.2).

These results show that the *Qingkailing*-derived formula prepared with processes A and B can significantly reduce MDA levels and recover the

TABLE 10.2 Determination Results of *Qingkailing* Derived Formula Active Indicators

Group	Amount	SOD	MDA
Normal	10	136.4 ± 23.11	0.94 ± 0.365
Model	10	110.2 ± 28.74*	1.51 ± 0.532**
Original	10	113.8 ± 23.55*	1.32 ± 0.553*
A	10	123.8 ± 15.81	1.14 ± 0.368$^{\triangle}$
B	10	131.5 ± 28.04	0.95 ± 0.286$^{\triangle\triangle}$

Compared with normal group: *$p < 0.05$, **$p < 0.01$; compared with model group: $^{\triangle}p < 0.05$, $^{\triangle\triangle}p < 0.01$.

elevated SOD level in the brain tissue of the model group. Between the two processes, B is more prominent. It could be considered that group B is more meaningful for ischemic injury, especially for the lipid peroxidation process, which is an important aspect. More results of the following optimization and efficacy experiments show that, in terms of treatment of cerebral edema, *Qingkailing*-derived formula is close to the original formula in some evaluations but is significantly better than the latter in many pharmacological evaluations after optimization. In addition, group B has the lightest color in its appearance, showing that it has less pigment impurity.

10.3.3 Optimization of the Ratio of the Effective Component Groups for Qingkailing Derived Formula

10.3.3.1 Experimental Design for Dose-Effect Correlation Dose–effect correlation is an important part of drug research and development. A *Qingkailing*-derived formula dose–effect correlation experiment was designed from the "quantity" and "efficiency" aspects.

From the "quantity" perspective, the amounts of herbal medicines in *Qingkailing*-derived formulas were arranged by uniform design. Uniform design is one of the statistical experimental design methods, and it complements many other experimental design methods, such as orthogonal design, optimal design, rotating design, robust design, and Bayesian design. Experimental design is a method to choose the most effective test points within the test domain, obtain the respective observations by testing, and analyze data to obtain the test conditions with the optimal response. Therefore, the goal of experimental design is to obtain system information as sufficiently as possible with the least amount of trials. Uniform design can achieve this goal better. The mathematical principle of uniform design is the uniform distribution theory in number theory. This approach draws on the research of the number theory method in approximate analysis and combines the number theory

with multivariate statistics, belonging to the pseudo-Monte Carlo method category. Uniform design only deals with even distribution of test points within the test range. The starting point of selection of trial representative points is "dispersed," regardless of whether the selection is "neat and comparable." It ensures test points with uniform distribution statistical properties, making each level of each factor with only one trial. The test points of any two elements are pointed at the grid points of the plane, and each row and each column has only one test point. It focuses on even distribution of test points within the test domain and getting the most information through the least amount of tests. Therefore, the number of tests is much lower than that of the orthogonal design, making uniform design particularly suitable for multiple factor, multilevel testing and the conditions that system models are completely unknown. For example, when the test has m factors, and each factor has n levels, there will be a total of n^m combinations for a full trial. Orthogonal design selects n^2 points from these combinations, while uniform design selects n points with the help of uniform distribution in the number theory. The number theory method makes test points evenly spread within the range of integration and sufficiently close to various values of integrated function. It is convenient for computers to make statistical models. For *Qingkailing*-derived formula composition design, although the number of factors involved (herbal medicine) and levels are not very large, the regression analysis of uniform design plays a very important role in determining the optimal choice. In addition, because TCM usually consists of a number of herbs and its composition scope is even broader, uniform design was selected to arrange composition trials instead of normal orthogonal design. This research is to study the possibility of popularizing this method for commonly used formula research.

From the "efficiency" point of view, *Qingkailing*-derived formulas generate overall comprehensive regulatory effects on ischemic cascade of pathological processes in stroke treatment. According to this principle, six relevant key indicators were studied. Ischemic cascade is a major pathological process in a stroke's occurrence and development. Ischemic cascade begins with microvascular perfusion disorders, which result in a generation of many free radicals and stimulate lipid peroxidation to damage vascular endothelial cells, neurogliocytes, and neurons. Then, glial cell swelling leads to the vicious cycle of microvascular perfusion disorders. With the "poison damaging brain network" micropathogenesis of stroke, in the pathological process above, damaging the brain is a function of the toxin. Free radical and toxic substances of metabolism such as NO are generally reflected by LPO of free radical-stimulated lipid peroxidation products and NOS expression level. As for dispelling exogenous pathogens, reductions of the damage above are evaluated by indicators. Strengthening body resistance capability is the enhancement of the resistance to damage. The influence factor can be internal cytokines that act against damage or removal, such as free radical scavenger SOD, or materials that protect their own cell activities, such as Na-K-ATPase.

TABLE 10.3 U9 (3 × 3 × 3 × 3) Uniform Design Table

No.	X1	X2	X3	X4
1	1(1)	1(2)	2(4)	3(8)
2	1(2)	2(4)	3(8)	3(7)
3	1(3)	2(6)	1(3)	2(6)
4	2(4)	3(8)	3(7)	2(5)
5	2(5)	1(1)	1(2)	2(4)
6	2(6)	1(3)	2(6)	1(3)
7	3(7)	2(5)	1(1)	1(2)
8	3(8)	3(7)	2(5)	1(1)
9	3(9)	3(9)	3(9)	3(9)

X*n* (*n* = 1–4) are four herbs.

We used the whole animal model for the major pathological aspect to design the efficacy indexes and optimize the *Qingkailing*-derived formula composition ratio.

10.3.3.1.1 Uniform Design for Formula Based on the findings above, the four ingredients are determined as cholic acids (including hyocholic acid and taurocholic acid; purity > 90%), *Zhizi* (extract), *Zhenzhumu* (hydrolyzate), and baicalin (purity > 95%). There are three levels of magnitude. A mixed uniform design with four factors and three levels is adopted, taking U9 (3 × 3 × 3 × 3) (Table 10.3).

Nine formulas have been designed according to the uniform design table. The formulas are created by uniform design. Group 10 has the same proportion as the *Qingkailing* formula (four herbs are prepared in accordance with the original *Qingkailing* formula), group 11 is the original *Qingkailing* formula.

10.3.3.1.2 Pharmacological Experimental Design *Experimental animals and grouping*: Healthy male SD rats with clean grade, weight 330 ± 20 grams, were used. There were 156 rats randomly divided into 24 h ischemic model groups: drug 1, drug 2, drug 3, drug 4, drug 5, drug 6, drug 7, drug 8, drug 9, drug 10, and drug 11 group (each group *n* = 12). The normal group (*n* = 12), drug 1, drug 2, drug 3, drug 4, drug 5, drug 6, drug 7, drug 8, and drug 9 groups were separated according to the nine component drugs in Table 10.3. Drug 10 corresponds to the same proportion in the *Qingkailing* formula; drug 11 corresponds to the original *Qingkailing* injection.

Preparation of animal models: Rats were anesthetized with peritoneal injection of 10% chloral hydrate (0.35mL/100 g). An incision was made on the middle of the neck and the left external carotid artery was exposed under a surgical microscope. Along the external carotid artery, its branches were separated into the occipital artery and superior thyroid artery. Then they were occluded with an electric knife. The distal end of external carotid artery was

ligated, letting the end of external carotid artery free. A wedge incision was cut in the beginning part of the stump, and a fishing line was inserted (diameter 0.25 mm, heated the head end to 0.3 mm in diameter, spherical) from here. The line goes from the external carotid artery into the carotid artery via the bifurcation and reaches the start point of the middle cerebral artery, blocking the blood supply of the middle cerebral artery. The external carotid artery stump is tied closed with sutures. Excess fishing line was cut and the incision in the neck was sutured. No fishing line was inserted in the normal group while the other steps are the same as the surgical group. The successful animal model was selected (features of successful models: awaked rats shows ipsilateral Horner syndrome and contralateral forelimb-oriented paralysis), sampling was conducted according to different ischemic times.

Methods of administration: administration was done by peritoneal injection. Apart from the normal group and model group, other groups took a prophylactic injection 1 h before being modeled. Different groups were administered 0.3 mL/100 g. The injections above were diluted with saline to 0.9 mL/100 g in order to reduce intraperitoneal mucous membrane irritation. This was done at an interval of 4 h. The treatment groups were administered once again with a 12 h interval. The model group was injected with saline 0.9 mL/100 g intraperitoneally.

Sample handling: All groups were anesthetized at different ischemic time points. Carotid artery blood was sampled by intubation. A small amount of heparin was used for anticoagulation. After going through 3500 rpm/min in a centrifuge for 15 min, plasma was stored in the –20°C refrigerator in preparation for testing vWF. An abdominal incision was made to expose the inferior vena cava. Venous blood (3 mL) was pumped slowly with a syringe and injected into test tubes. The test tubes were then set aside at room temperature for 10 min, then centrifuged at 3500 rpm/min for 10 min. Serum aliquots were stored in a –20°C refrigerator prepared for neuron-specific enolase test. In decapitated animals, the ischemic side (left side) of the brain striatum and cortex were quickly removed to the ice tray. They were weighed and added with iced saline (0.25 mL/100 mg). These tissues were homogenized in an ice bath at 4°C, and centrifuged at 3500 rpm/min for 15 min. Supernatant aliquots were placed in a –20°C refrigerator for testing tumor necrosis factor-α (TNF-α), inerleukin-1β (IL-1β), malondialdehyde (MDA), and inducible nitric oxide synthase (iNOS).

Indices: On the basis of determined herbs of the simplified formula, 6 indices were selected for efficacy evaluation, including TNF-α protein, IL-1β protein, vWF protein, neuron-specific enolase protein content, iNOS activity, and MDA content. They are closely related to multilink inhibition in the ischemic cascade.

10.3.3.2 Pharmacodynamic Evaluation of Different Qingkailing *Derived Formulas*

After 24 h of cerebral ischemia, brain tissue of the model group had an increasing level of TNF-α protein (compared with the normal group

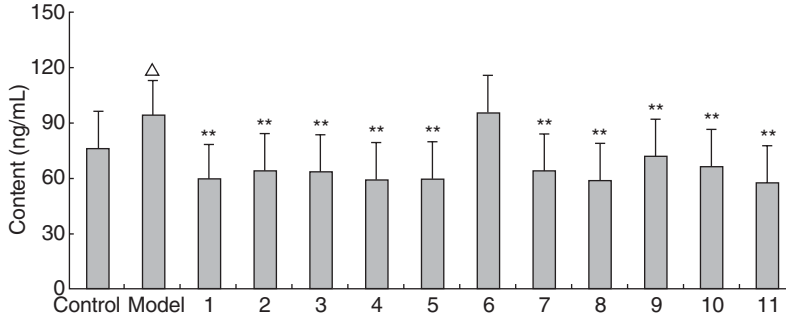

Fig. 10.13 Effect of different formulas on the levels of TNF-α in ischemic brain tissue. Compared with normal group: $^{\triangle}p < 0.05$; compared with model group: $^{*}p < 0.05$, $^{**}p < 0.01$.

$p < 0.05$). After 24 h of ischemia, each of the six drug-treated groups had significantly lower amounts of TNF-α protein level in the brain tissues compared with the model group (model group $p < 0.01$) (Fig. 10.13).

Studies have shown that the expression and levels of the tumor necrosis factor-α (TNF-α) in plasma and brain tissue of cerebral ischemia/reperfusion injury had increased. The damaging factors of cerebral ischemia in the early stage are mainly from TNF-α expressed by ischemic neurons and microglia: TNF-α can induce cells to go through necrosis, which is characterized by cell swelling and cell dissolution, and apoptosis which is characterized by the disassembly of DNA into DNA fragments and the shrinkage of organelles, at different stages and different areas. The results showed that with the suture method in the duplicated rat MCAO model, after 24 h of ischemia, the level of TNF-α protein in the brain tissue of the model group was significantly higher than in the normal group. After analysis of the drugs that have effects on levels of TNF-α protein, we found that, with the exception of formula group-6, each drug-treated group can significantly inhibit the increased levels of TNF-α protein.

After 24 hours of After 24 h of cerebral ischemia, the brain tissue had an increased level of IL-1β protein (compared with the normal group, $p < 0.05$). After 24 h of ischemia, the drug-treated group 4 had a level of IL-1β protein that was lower than that of the model group ($p < 0.05$). Drug-treated groups 2, 6–9, and 11 had levels of IL-1β protein that were higher than that of the model group ($p < 0.05$ or $p < 0.01$) (Fig. 10.14).

Cerebral ischemia can induce IL-1β mRNA synthesis and increased expression of the protein in the brain, leading to significantly increased levels of IL-1β. In the state of cerebral ischemia, the sustained production of IL-1β could result in a sharp pathological increase in its concentration by proinflammatory response, promoting the release of free radicals and enhancing the role of excitatory amino acid toxicity mechanisms which are involved in nerve

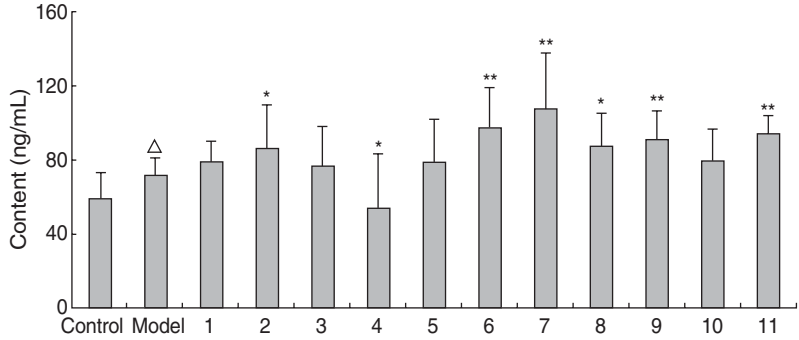

Fig. 10.14 Effect of different formulas on the levels of IL-1β in ischemic brain tissue. Compared with normal group: $^{\triangle}p < 0.05$; compared with model group: $^{*}p < 0.05$, $^{**}p < 0.01$.

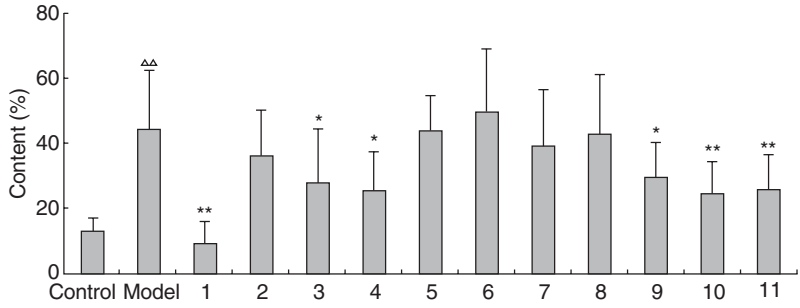

Fig. 10.15 Effect of different formulas on the levels of vWF in ischemic brain tissue. Compared with normal group: $^{\triangle\triangle}p < 0.01$; compared with model group: $^{*}p < 0.05$, $^{**}p < 0.01$.

tissue damage. This study showed that after 24 h of cerebral ischemia, levels of IL-1β protein in brain tissue were significantly increased. After 24 h of ischemia, drug-treated group 4 could inhibit the levels of IL-1β protein increased in ischemic brain tissue, but in addition to the drug-treated group 4, the rest of each of the drug-treated groups did not show inhibition of the increased levels of IL-1β protein; and drug-treated groups 2, 6–9, and 11 showed that they promoted the trend of the expression of IL-1β.

Compared with the normal group, after 24 h of cerebral ischemia, levels of vWF in plasma were significantly increased ($p < 0.01$). Compared with the model group, drug-treated groups 1, 3, 4, and 9–11 showed that levels of vWF in plasma were decreased ($p < 0.05$ or $p < 0.01$) (Fig. 10.15).

Endothelial cell coagulation factor VIII-related antigen vWF is synthesized mainly by vascular endothelial cells. When vascular endothelial cells are damaged, such as though cerebral ischemia and atherosclerosis, it can lead to increased levels of vWF in plasma. Levels of vWF in plasma can be used as

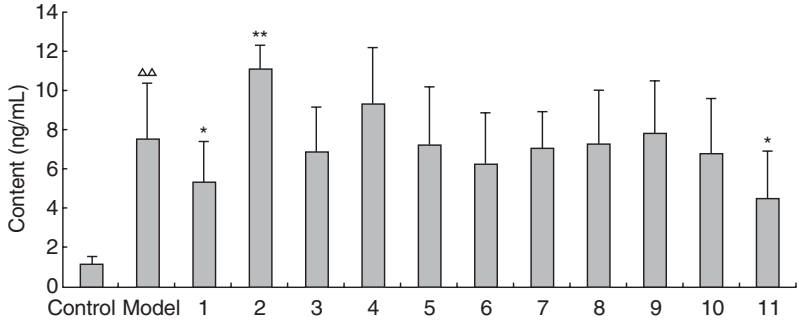

Fig. 10.16 Effect of different formulas on the levels of neuron-specific enolase in rat serum. Compared with normal group: $^{\triangle\triangle}p < 0.01$; compared with model group: $^{*}p < 0.05$, $^{**}p < 0.01$.

an important index for the severity of cerebral vascular disease and can be used as an index for judgment of recovery. Compared with the normal group, after 24 h of cerebral ischemia, levels of vWF in plasma were significantly increased. The drug-treated groups 1, 3, 4, and 9–11 could inhibit the increasing of levels of vWF in plasma, and had good protection of vascular endothelial cells.

Compared with the normal group, after 24 h of cerebral ischemia, levels of serum neuron-specific enolase protein were significantly increased ($p < 0.01$). Compared with the model group, drug-treated groups 1 and 11 had decreased levels of serum neuron-specific enolase protein ($p < 0.05$ or $p < 0.01$). Drug-treated group 2 had a significantly higher level of neuron-specific enolase compared with the model group ($p < 0.01$) (Fig. 10.16).

After focal cerebral ischemia, levels of neuron-specific enolase were increased in the blood and cerebrospinal fluid for 2–5 days, having a linear relationship between the concentration level and the size of infarct volume. The study showed that after cerebral ischemia for 24 h, levels of serum neuron-specific enolase protein were significantly increased. Drug-treated groups 1 and 11 inhibited the increased levels of serum neuron-specific enolase protein, indicating some neuroprotective effects; and drug-treated group 2 showed a trend for elevated levels of neuron-specific enolase, potentially having some side effects.

Compared with the normal group, 24 h after cerebral ischemia, levels of activity of iNOS significantly decreased in the brain tissue ($p < 0.01$). Ischemic injury may be related to excessive consumption of the iNOS. There was no significant difference between each drug-treated group and model group. Drug-treated group 7 even had a tendency that further reduced the level of iNOS compared with the model group ($p < 0.01$), showing that *Qingkailing* might not have an obvious role in this pharmacological index (Fig. 10.17).

After 24 h of cerebral ischemia, levels of MDA were increased in the brain tissue (compared with the normal group $p < 0.05$). Around 24 h after ischemia,

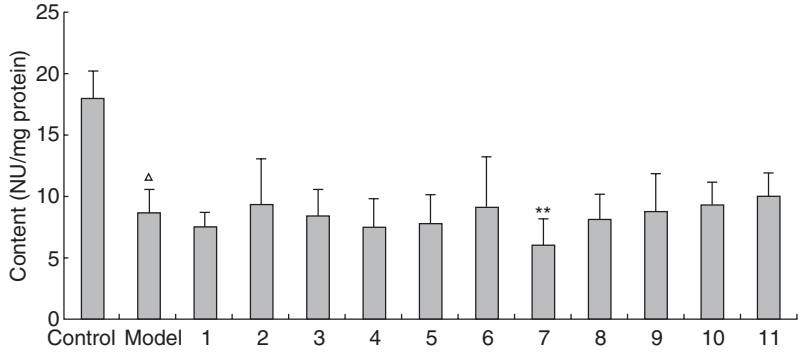

Fig. 10.17 Effect of different formulas on the levels of activity of iNOS in cerebral ischemia tissue. Compared with normal group: $^{\triangle}p < 0.05$; compared with model group: $^{*}p < 0.05$, $^{**}p < 0.01$.

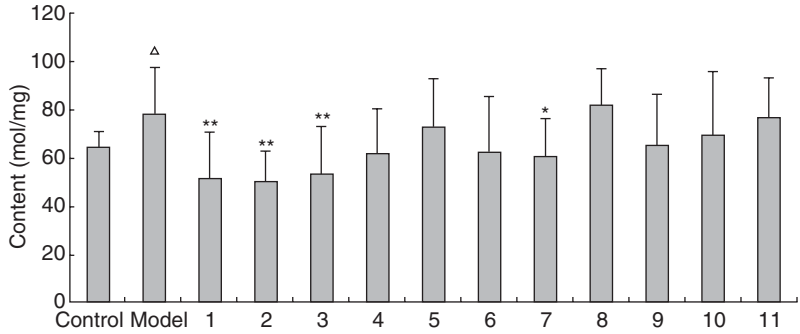

Fig. 10.18 Effect of different formulas on the levels of MDA in cerebral ischemia tissue. Compared with normal group: $^{\triangle}p < 0.05$; $^{\triangle\triangle}p < 0.01$; compared with model group: $^{*}p < 0.05$, $^{**}p < 0.01$.

compared with the model group, drug-treated groups 1–3 and 7 had decreased levels of MDA in brain tissues ($p < 0.01$ or $p < 0.05$) (Fig. 10.18).

MDA is the metabolite of the peroxidation of biofilms unsaturated fatty acid caused by oxygen-derived free radicals. The free radical chain reaction mediated by the oxygen-derived free radicals is an important factor in acute cerebral ischemia neuronal damage. Anti-free radical treatment can reduce the damage of cerebral ischemia and improve neurological function. The results indicated that after 24 h of cerebral ischemia, levels of MDA were increased in the brain tissue. The drug-treated groups 1–3 and 7 could inhibit the increased levels of MDA in ischemic brain tissue.

Considering the previous discussions, drug-treated groups 1, 3, and 4 showed good neuroprotective effects, and the remaining drug-treated groups showed little effect, or played an improvement role in some indices while in the other indices showed obviously aggravated damage.

10.3.3.3 Regression Analysis Under the same composite group, we took the average values of the pharmacodynamic index, and then summed the whole average value of the pharmacodynamic index in the same composite group. The result was the total value of pharmacological effects of this formula; the total values of the pharmacological effects in different combinations of formulas were sorted from low to high. Since the various kinds of pharmacological effect indices were inhibitory, the smaller the total value of the pharmacological effects, the better it is. The results are shown in Table 10.4.

Groups 3, 5 and 6 are excellent formulas; group 5 is the best formula, followed by group 6, then 3. Most of the simplified formulas of *Qingkailing* are better than the whole formula of *Qingkailing*, indicating that the simplified formula of *Qingkailing* groups has an attenuation effect compared with the whole formula of *Qingkailing* groups, while some synergies are obvious as well. A formula of *Qingkailing* comprises four raw herbs which have the same composite proportion as the original formula of *Qingkailing*, and had similar pharmacological effects as the original *Qingkailing* formula, indicating that the discarded components have no obvious effects on pharmacological indices above.

After obtaining the experimental results of uniform design, we conducted regression analysis (the whole formula of *Qingkailing* is not included in the regression duing to the different variables).

First, linear regression analysis was conducted and the results of correlation coefficient and residual analysis are shown in Tables 10.5 and 10.6.

According to the above analysis, the result is not ideal. For example, the correlation coefficient is small and the results of analysis of variance are not statistically significant. Because the total variance is certain, the residuals had a larger proportion, while the variance explained by regression analysis had a smaller proportion, indicating that the results of the regression analysis were not statistically significant. Therefore, the relationship between the original data and the efficacy may not be linear, so we applied nonlinear regression analysis.

In uniform design, the representation of the various factors at different levels of the trial sites is very strong. There is a nonlinear relationship between the pharmacodynamic index and various drugs, but during regression analysis one often does not consider the third item of each factor and interaction among them. Here we only considered the first item and second item of the various factors. The matrix is as follows:

$$\mathbf{Y}=\begin{bmatrix} y_1 \\ y_2 \\ \vdots \\ y_n \end{bmatrix}=\begin{bmatrix} 1 & x_{11} & x_{12} & x_{13} & x_{14} & x_{11}^2 & x_{12}^2 & x_{13}^2 & x_{14}^2 \\ 1 & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots \\ 1 & x_{n1} & x_{n2} & x_{n3} & x_{n4} & x_{n1}^2 & x_{n2}^2 & x_{n3}^2 & x_{n4}^2 \end{bmatrix}\cdot\begin{bmatrix} b_1 \\ b_2 \\ \vdots \\ b_n \end{bmatrix}=\mathbf{X}\cdot\mathbf{B},$$

where **Y** represents the matrix of the total efficacy and **X** represents the data matrix of the dosage of each raw herbs. **B** is the corresponding regression coefficients.

TABLE 10.4 Rank List for Pharmacodynamic Index in Different Combinations of Formulas

Group	1	2	3	4	5	6	7	8	9	10	11
NSE	–2.148	–3.491	–3.906	8.278	–8.003	–10.965	–1.060	–1.823	0.687	–0.756	–1.853
Il-1β	1.567	–4.552	–8.463	–15.870	–12.119	–5.603	3.857	2.505	2.188	1.391	2.607
TNF-α	–1.050	–0.866	–0.971	–1.082	–1.069	–0.919	–1.178	–1.009	–0.981	–0.853	–0.792
iNOS	–0.517	0.856	–0.777	0.366	–0.475	0.672	–0.270	–0.667	0.355	0.325	0.779
MDA	–0.751	–1.684	–0.941	–1.350	–1.010	–0.932	–1.043	0.854	–1.067	–0.865	–0.303
vWF	–1.989	–1.050	–0.344	–0.074	–1.260	2.216	–0.770	–0.872	–0.356	–0.213	–0.368
SUM	–4.888	–10.787	–15.402	–10.732	–22.936	–15.531	–0.464	–1.012	0.826	–0.971	0.07
SORT	6	4	3	5	1	2	9	7	11	8	10

NSE, neuron-specific enolase; SUM, the total value of pharmacological effects in the same formula; SORT, the serial number of according to the total value of pharmacological effects in order from low to high.

TABLE 10.5 Correlation Evaluation Form

Correlation Coefficient R	R^2	Adjusted R^2	Standard Deviation
0.578	0.334	–0.199	8.87989

Independent variables: *Zhenzhumu*, baicalin, cholic acid, *Zhizi*. Dependent variable: The value of the total effect.

TABLE 10.6 Residual Analysis

	Sum of Squares	Df	Mean Square	F	Significance Probability
Regression	198.812	4	49.453	0.627	0.664(a)
Residual	394.262	5	78.852		
Total	592.074	9			

Independent variables: *Zhenzhumu*, baicalin, cholic acid, *Zhizi*. Dependent variable: The value of the total effect.

TABLE 10.7 Regression Results of Different Combinations of Variables

Variables (the Amount of Herbs) Combinations										
A	B	C	D	A^2	B^2	C^2	D^2	R	F-test	P
0	1	1	0	1	1	1	1	0.9301	3.2049	0.18347
0	1	1	1	1	1	1	0	0.9384	3.6876	0.15574
0	1	1	1	1	1	1	1	0.9454	2.4039	0.32502
1	0	1	1	1	1	1	1	0.9454	2.4039	0.32502
1	1	0	1	1	1	1	1	0.9454	2.4039	0.32502
1	1	1	0	0	1	1	1	0.9402	3.8122	0.14970
1	1	1	0	1	1	1	1	0.9454	2.4039	0.32502
1	1	1	1	0	1	1	0	0.9453	4.1968	0.13328
1	1	1	1	0	1	1	1	0.9454	2.4039	0.32502
1	1	1	1	1	1	1	0	0.9454	2.4039	0.32502
1	1	1	1	1	1	1	1	0.9454	2.4039	0.32502

A, cholic acid; B, baicalin; C, *Zhizi*; D, *Zhenzhumu*.

In order to get optimal results, we considered all possible combinations of various factors. According to the binomial theorem, the eight variables could be combined as $2^8 - 1$ times (here, minus 1 means that all variables are not selected, leaving only the constant term). Using least squares regression, the results that the coefficients exceeded 0.9 were shown in Table 10.7. As shown in the table, the regression result of the row combination with shading is superior, because its correlation coefficient is comparatively large, and the probability had a significantly low level.

TABLE 10.8 Correlationship Evaluation Form

Correlation Coefficient r	r^2	Adjusted r^2	Standard Deviation
0.945	0.894	–0.681	4.5831

Independent variables: *Zhenzhumu*, baicalin, cholic acid, *Zhizi*. Dependent variable: The value of the total effect.

TABLE 10.9 Residual Analysis

	Sum of Squares	Df	Mean Square	F	Significance Probability
Regression	529.059	6	88.177	4.198	0.133(a)
Residual	63.015	3	21.005		
Total	592.074	9			

Independent variables: *Zhenzhumu*, baicalin, cholic acid, *Zhizi*. Dependent variable: The value of the total effect.

We used Statistical Product and Service Solutions (SPSS) to deal with the row combination with shading, the regression results in Tables 10.8, 10.9, and 10.10.

From the results of correlation coefficients and analysis of variance, we can see that the regression results are more satisfactory. The results of the analysis of variance did not reach a significantly higher level, but were also close to 10%, belonging to fairly significant levels.

According to the results of the correlation coefficient table, the regression equation is as follows:

$$\begin{aligned}\text{The total effect value} = {} & 94.854 + 1.688 \times \text{Cholic acid} + 11.090 \times \text{baicalin} + \\ & 2.468 \times \textit{Zhizi} + 0.198 \times \textit{Zhenzhumu} - 0.810 \times \text{baicalin} \\ & \text{squared} - 0.040 \times \textit{Zhizi}\ \text{squared}\end{aligned}$$

However, when comparing the efficacy index contribution of each medicine, due to the different units of various factors, standardized coefficients should be used. The results of standardization are shown in the "standardized coefficient" column of Table 10.10. The results show that the contributions of baicalin and *Zhizi* were larger, while the contributions of cholic acid and *Zhenzhumu* were relatively smaller. From the signs of coefficients in regression equations, cholic acid and *Zhenzhumu* were all positive, indicating that the smaller their dosage, the smaller the total effective index, and the better efficacy. Note that the signs of coefficients of the first item and second item of baicalin and *Zhizi* are opposite. The direct analysis results from the data are as follows: in the simplified formula of *Qingkailing* group, when the dosages of baicalin and *Zhizi* are within a certain range (or less), the indices of efficacy had a positive contribution; when they reached or exceeded a certain amount,

TABLE 10.10 Correlation Coefficients of Different Combinations of Medicines

	Nonstandardized Coefficient		Standardized Coefficient			B Value Range (95% Confidence Interval)	
	B	Standard deviation	Beta	T	Importance	Lower	Limit
Constant	–94.854	19.434		–4.881	0.016	–156.702	–33.006
Cholic acid	1.688	0.577	0.900	2.924	0.061	–0.149	3.526
Baicalin	11.090	3.384	4.221	3.277	0.047	0.321	21.860
Zhizi	2.468	0.677	4.696	3.646	0.036	0.314	4.622
Zhenzhumu	0.198	0.081	0.754	2.451	0.092	–0.059	0.455
Baicalin squared	–0.810	0.282	–3.950	–2.870	0.064	–1.708	0.088
Zhizi squared	–0.040	0.011	–4.877	–3.544	0.038	–0.076	–0.004

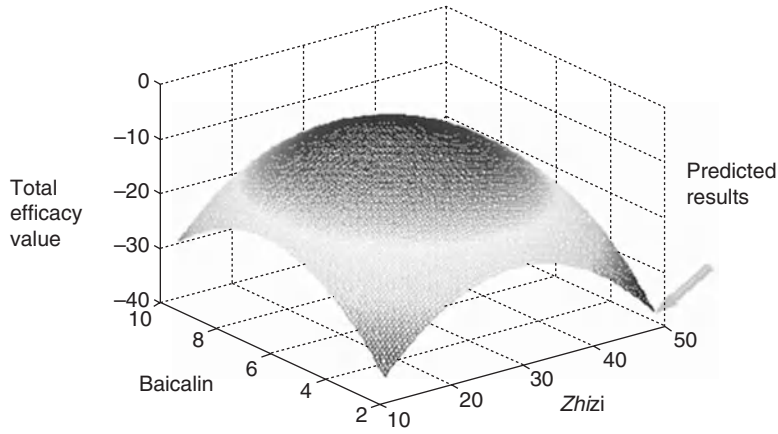

Fig. 10.19 Optimization of prediction map of the *Qingkailing*-derived formula. (See color insert.)

they would produce a negative contribution to the efficacy index, because the premise is that the dosage of each raw herbs is not less than 1.

Adopting the relatively simple method to find the best formula, the four factors (the dosage of four raw herbs) in their respective levels between highest and lowest can generate n points; according to the regression equation, we calculated each level of the total effect values, choosing the corresponding formula of the best pharmacodynamic index. We selected two strongly influential factors, baicalin and *Zhizi*, and the total efficacy value in accordance with this method was mapped in three-dimensional space (Fig. 10.19). Obviously the larger the n value is, the more precise the obtained results are (where $n = 100$). Prediction of the best combination: cholic acid = 3.5, baicalin = 2.5, *Zhizi* = 50, *Zhenzhumu* = 25; the predicted results of the total effect values are –38.981.

With comprehensive analysis of the advantages of the efficacy index values between the two groups, the formulas of *Qingkailing* groups 1, 3, and 4 showed good neuroprotective effects. Score line evaluation showed that the optimal formulas of *Qingkailing* were groups 3, 5, and 6. Considering the results of effect analysis and queuing analysis of the total score, we determined that the optimal compatibility is the formula of *Qingkailing* group 3 among the 11 groups. The results of score line evaluation and nonlinear regression analysis predicted differently from the optimal compatibility. Possible reasons are that the former is essentially a linear method for choosing from the existing 11 groups which has the most negative value of the total effect, considering which is optimal, but not necessarily the best solution; the latter is the result of optimal prediction based on a nonlinear regression optimization, which is the theoretical optimal value, but also requires further experimental validation.

10.3.4 Interpretation of the Pharmacological Mechanism of Effective Component Groups

Based on the previous study, in order to reveal the integrated regulation effect played by the effective component group and explain the meaning of the theory of formula compatibility on multiple aspects and multiple target effects, compatibility principle research was carried out comprehensively on the optimized *Qingkailing*-derived formula.

10.3.4.1 Effect on the Injury and Reaction Process of Ischemic Vascular Endothelial Cells

10.3.4.1.1 Effect on the Expression of Triggering Cell Adhesion-Related Cytokine The cell adhesions that occur in the blood vessel include primary adhesion and firm adhesion; and the main cell adhesion factors that mediate the primary adhesion and firm adhesion of leukocyte and endothelial cells include E-selectin, P-selectin, and ICAM-1. This adhesion is the main initiative step that causes further inflammation, while P-selectin is a sign of platelet activation. Changes in plasma P-selectin levels, to a certain extent, may reflect the destruction of cerebral ischemia levels. For high expression of ischemic primary adhesion E-selectin, except *Zhenzhumu*, the effective component group of baicalin, cholic acid, and *Zhizi* showed significant inhibition on ischemia after 12 and 24 h; for the high expression of ischemic primary adhesion P-selectin, the active components of cholic acid and *Zhizi* showed significant inhibition after 12 h of ischemia, and baicalin and *Zhenzhumu* showed significant inhibition after 24 h of ischemia, inhibiting leukocyte and endothelial cell adhesion in the primary adhesion, which intervenes further strong adhesion between the two to achieve the purpose of anti-inflammation. As to the high expression of ICAM-1 of firm adhesion, baicalin and *Zhenzhumu* mainly showed significant inhibition after 12 h of ischemia, while the active components of cholic acid and *Zhizi* needed 24 h. The compatibility among the four components showed significant inhibition on the high expression of three adhesion factors at two time points. So baicalin has a strong inhibitory effect on the expression of ICAM-1 and E-selectin of brain ischemia after 12 h, while the effective component group of *Zhizi* and cholic acid showed high inhibition at 24 h. *Zhenzhumu* could inhibit the expression of ICAM-1 after ischemia but not on E-selectin.

10.3.4.1.2 Effect on Excretion of Tension Regulator Factor in Brain Microvascular Endothelial Cells Endothelin (ET-1), mainly from vascular endothelial cells, is known as the most powerful endogenous vasoconstrictor. It can cause spastic contraction of vascular smooth muscle. Plasma ET-1 levels will be increased significantly when cerebral ischemia occurs, leading to low perfusion after ischemia, even causing the no-reflow phenomenon and increasing tissue damage. Every component of *Qingkailing*-derived formulas did not

have significant inhibitory effect on the increase of plasma ET-1 levels after 12 h of ischemia. The active components of *Zhizi* and the compatible formula displayed a certain inhibitory effect on the increase of plasma ET-1 levels after 24 h of ischemia, and the latter inhibitory effect was better than the former one. There was synergy among the multiple active components inhibiting the expression of plasma ET-1.

TXA_2, which is mainly produced by the platelets, is a strong vasoconstrictor, prompting platelet aggregation, inducing thrombotic formation, activating neutrophils and mononuclear macrophages, and increasing cerebral ischemia and the inflammation that occurs after. PGI_2 is produced mainly by endothelial cells. It can relax vessels, expand cell gaps, and inhibit adhesion of neutrophils and endothelial cells. Microvascular endothelial cells are damaged when cerebral ischemia occurs, leading to increased synthesis and release of TXA_2. With a relative lack of or reduced PGI_2 synthesis, the TXA_2/PGI_2 ratio is increased, causing spastic contraction of cerebral vessels and platelet adhesion and aggregation, blocking blood flow in microcirculatory blood and triggering and increasing ischemic hypoperfusion, leading to further damage to brain tissue. The metabolites of TXA_2 and PGI_2 are TXB_2 and 6-keto-$PGF_1\alpha$, respectively. In addition to *Zhenzhumu*, cholic acid, and baicalin, effective component groups of *Zhizi* and compatible formulas showed different degrees of inhibition of expression of plasma TXB_2 after cerebral ischemia for 12 h, reducing the TXB_2/6-keto-$PGF_1\alpha$ ratio significantly, playing ideal role in microvascular relaxation and endothelial cells protection. After ischemia for 24 h, baicalin and compatible formulas still could inhibit the high expression of plasma TXB_2 to decrease the TXB_2/6-keto-$PGF_1\alpha$ ratio. *Qingkailing*-derived formulas mainly inhibited the high expression of plasma TXB_2 to reduce the TXB_2/6-keto-$PGF_1\alpha$ ratio, improving microvascular spasms.

10.3.4.1.3 Protective Effect on Brain Microvascular Endothelial Cells

Nuclear factor κB (NF-κB) is considered to be one of the initiating mechanisms of vascular endothelial cell damage, while the increase of plasma vWF levels is considered as a sign of endothelial cell damage. The effective component group of *Qingkailing*-derived formulas could inhibit the expression of NF-κB of ischemic brain tissue in different degrees. Only *Zhenzhumu* reduced the release of vWF after cerebral ischemia for 12 h; and for the effective component group of *Zhizi*, cholic acids, and their compatible formula, the time required to reduce the release of vWF was 24 h. It showed that the effective component group which could inhibit the expression of plasma ET-1 and/or reduce the ratio of TXB_2/6-keto-$PGF_1\alpha$ and the effective component group that could inhibit vWF release into blood were different; there was no obvious correlation between the effects of them. These results suggest that the effect of microvascular spasm and relief and the protective effect of vascular endothelial cells are two different aspects. The effective component group can protect vascular endothelia by spasmolysis as well as play the spasmolysis role or protect vascular endothelia singly. Of course, there maybe other ways

of letting the medicine ultimately play a protective role of microvascular endothelium.

10.3.4.1.4 Promotion of Proliferation and Repair of Endothelial Cells Vascular endothelial growth factor (VEGF) is the specific mitogen of endothelial cells. It has the biological characteristic of promoting proliferation of endothelial cells. Vascular endothelial growth factor receptor (VEGFR) is specifically present in vascular endothelial cells. When cerebral ischemia occurs, hypoxia is a signal to activate the VEGF/VEGFR system, promoting the high expression of ischemic penumbra VEGF. The latter can recover blood flow rapidly, prevent tissue ischemia and necrosis, reduce the infarct area, and ultimately reduce the injury of ischemia in the brain by accelerating vascular endothelial proliferation to induce a great deal of angiogenesis in the penumbra and establish collateral circulation. When cerebral ischemia happens, endothelial cells, neurons and neurogliocytes, macrophages, and so on show overexpression of VEGF, VEGF mRNA, and the expression around the ischemic area. Each component of *Qingkailing*-derived formulas could significantly promote the generation of VEGF after ischemia for 12 and 24 h, especially cholic acid. As for effects on VEGFR1 targets, only active components of *Zhizi* and compatible formulas demonstrated significant effects, and the effects of *Zhenzhumu*, baicalin, and cholic acid were not obvious.

10.3.4.2 Effect on Inflammatory Response Damage After Cerebral Ischemia

10.3.4.2.1 The Inhibitory Effect on Inflammatory Cytokine Response When cerebral ischemia happens, IL-1β, TNF-α and other inflammatory cytokines will be produced by nerve cells and neurogliocytes. IL-1β and TNF-α were synthesized by endothelial cells after ischemia combined with IL-1R; TNF-αR expressed by neurons and vascular endothelial cells in ischemic vulnerable areas causes inflammation. This plays an important role in the process of cerebral ischemia cascade reactions. Interference with the synthesis and secretion of these factors is expected to reduce ischemic inflammatory injury and size of the infarct area. The effective component group of the *Qingkailing*-derived formula and its compatible formulas could inhibit the expression of IL-1β after ischemia for 12 and 24 h in brain tissues. There was no significant effect on the expression of TNF-α after ischemia for 12 h in brain tissues; while *Zhenzhumu*, the effective component group of *Zhizi*, cholic acids, and the compatible formula showed effective inhibition on the TNF-α expression in brain tissues.

10.3.4.2.2 Promoting Effect on Protective Factor Reaction It is considered that IL-6, TGF-β, and HSP70 play a role in the protection of damaged brain tissues after the cerebral ischemia. IL-6 is synthesized and secreted by the stimulation of neurons microneurogliocytes and endothelial cells from ischemic damage; TGF-β, which is produced by activated T cells and monocytes/

macrophages, can function as an antioxidant, prevents apoptosis, adjusts inflammation, adjusts microglia and astrocyte response in ischemic brain injury, and links multiple aspects of ischemic brain injury. TGF-β receptor is expressed in the central nervous system, mainly in the ischemic area of local neurons, activated astrocytes, endothelial cells, and macrophages. Ischemia- and hypoxia-induced expression of HSP70 can enhance the protective effect on neurons. The effective component group of *Zhizi*, baicalin, cholic acid, and compatible formulas could protect neurons by promoting the secretion of IL-6 in the early stage of ischemia (12 h after ischemia); compatible formulas did not show significant synergy. For the responsive expression of TGF-β, every treatment group did not show an enhanced expression effect 12 h after ischemia; only the active components of *Zhizi* and compatible formulas showed a promoting effect on the synthesis of TGF-β 24 h after ischemia. For the TGF receptor (TGF-βR_1), each treatment group did not show any effect after ischemia for 12 h, but after ischemia for 24 h, compatible formulas of *Zhizi*, *Zhenzhumu*, baicalin, and cholic acid showed significantly enhanced expression. Each treatment group did not affect the HSP70 content significantly 12 h after ischemia in brain tissues; only baicalin and the compatible formula increased HSP70 content significantly 24 h after ischemia in ischemic brain tissues.

10.3.4.3 Effects on the Reactivity of Neurogliocytes and Neurotrophic Factors Expression

10.3.4.3.1 Effects on Activation of Neurogliocytes After cerebral ischemia, the response of stimulated astrocytes is far beyond the nutritional support of neurons and degeneration of necrotic tissue scavenging effect, and is also associated with complex molecular biological changes. During the early stages of cerebral ischemia, it can enhance tolerance to low sugar and hypoxia, and has significant protective effect on ischemic neurons by regulating the concentration of extracellular K^+, uptaking glutamate, expressing neurotrophic factors, and so forth. Glial fibrillary acidic protein (GFAP) is the structural protein of microastrocytes. Its content significantly increases in reactive astrocytes; S-100 is the cytoplasm of astrocyte calcium-binding protein, which can adjust the morphology and metabolism of astrocytes. GFAP and S-100 protein in acute cerebral ischemia are in significantly high expressions. In the ischemic 12 h period, apart from *Zhenzhumu*, the expression of GFAP was much more enhanced in each component of *Qingkailing*-derived formulas and compatibility groups than in the model group, promoting the activation of astrocytes. Baicalin and bioactive components of *Zhizi* have the most significant effects. Extended to 24 h after ischemia, astrocytes of the model group were further activated, but the treatment group did not appear to facilitate the expression of GFAP. In the ischemic 12 h period, cholic acid, *Zhenzhumu*, and the compatibility group can significantly promote the expression of S-100, but in the

ischemia 24 h period, this effect disappears. It is presented as promotion for gliocyte activation in the earlier stages.

10.3.4.3.2 Effects on Neurotrophic Factors Neurotrophic factors (NTF) are a group of protein or peptide molecules that have special nutritional effect on nerve tissues. NGF, bFGF, and BDNF are the most studied neurotrophic factors. NGF mainly comes from neurons in the brain, and also from neurogliocytes. In the early period of brain injury, NGF responsively plays a protective role for neurons. bFGF within brain tissue is mainly produced in astrocytes, and it can also be secreted by neurons and vascular endothelial cells. It promotes survival and repair or regeneration of damaged neurons. BDNF is mainly produced in the neurons and astrocytes of the central nervous system. In the case of ischemia, the expression of BDNF and its receptor trkB are increased to protect neurons from damage by stabilizing the concentration of Ca^{2+} in cell, reducing NMDA receptor function, acting against endogenous toxic substances damage, and so forth. *Qingkailing*-derived formulas have protective effects on cerebral ischemia neurons, mainly through promoting the production of bFGF to protect nerve cells, promoting neuronal ischemic tissue to produce NGF; the BDNF neuroprotective effect may not be the effective mechanism. In the former effects, cholic acid plays a dominant role in the biological effects, but in stabilizing the damaged neurons, reducing the level of metabolic synthesis, and enhancing the ability of neurons to withstand hypoxia, geniposide and *Zhenzhumu* play a major role.

10.3.4.4 Effects on Apoptosis-Related Proteins in the Early Period of Ischemia In the process of ischemic penumbra degradation, cell death and its resulting expansion of irreversible damage lesions may be achieved through the apoptosis mechanism. Apoptosis is initiated by a variety of types of stimulation of apoptosis signals, and regulated by a "waterfall" activation process involving endogenous genes, enzymes, and signaling pathways. The caspase system plays an important role in the process of apoptosis, as the final process of apoptosis is achieved through the activation of caspase. Caspase-3 is the key protease of apoptosis in mammalian cells. P53 is a protein that is expressed in the severely damaged parts in damaged neurons of the ischemic brain, and can induce apoptosis of neurons. Its mechanism may be that P53 protein is a direct transcription factor, which directly and rapidly improves the expression of Bax, and reduces the expression of Bcl-2 protein. Therefore, inhibiting the expression of caspase-3 and P53 can protect ischemic tissues. This study showed that cerebral ischemia activated the expression of caspase-3 and P53. Bioactive components of *Zhizi*, cholic acid, and compatibility formula had significant inhibition on the expression of caspase-3 in the cerebral ischemia 12 h period, but this effect disappeared 24 h after ischemia, showing that effective component group of *Qingkailing*-derived formulas cannot inhibit the expression of caspase-3 and inhibit neuronal apoptosis, which further shows the effect of

timeliness. During these two periods, each component of the *Qingkailing*-derived formula and its compatibility formula did not show effective inhibition on the expression of P53.

10.3.4.5 Cell Model Analysis of Multiple Aspect Effect Mechanisms

Using SH-SY5Y neural cells and ischemia and hypoxia/reperfusion cells of mouse brain microvascular endothelial cells as a cell model, we determined the contents of mitochondrial activity, mitochondrial membrane potential, cytochrome-C levels, Caspase-3 protein expression, MAP2 protein expression, apoptosis rate, SOD activity, and MDA of nerve cells, as well as the cells'; cytotoxicity to brain microvascular endothelial cells. We observed the effective features of each component of the *Qingkailing*-derived formula and its compatibility formula. Taking the original *Qingkailing* injection as comparison, the results showed that each drug group showed varying degrees of positive effects.

10.3.4.5.1 The Protective Effects on Neuronal Mitochondrial Function Mitochondria are important organelles and the energy source for cell metabolism. The activity of mitochondrial and membrane potential can reflect the integrity of mitochondrial function. Low oxygen and glucose deprivation can significantly reduce the activity of mitochondrial and membrane potential. Cholic acid, baicalin, the *Zhizi* effective component group, *Zhenzhumu*, *Qingkailing*-derived formula, and the original *Qingkailing* formula can effectively protect the activity of mitochondria against oxygen–glucose deprivation/reperfusion damage on the nerve cells, increase membrane potential, and stabilize cell membrane potential level. Cholic acid, baicalin, and the *Zhizi* effective component group had an obvious effect and had a trend of increasing efficiency after compatibility, and the *Qingkailing*-derived formula had a better effect than the original *Qingkailing* formula.

10.3.4.5.2 Effects on Cell Apoptosis Mechanism Mitochondrial cytochrome C has dual functions: regulating cell energy metabolism and apoptosis; and not only regulating methods of cell death through the regulation of cellular energy metabolism, but also direct-mediated apoptosis by causing transduction and amplification of apoptosis signal. The release of mitochondrial cytochrome C plays an important role in the process of apoptosis. After cytochrome C is released into the cytoplasm, it triggers the activation of the caspase cascade, and results in cell death. After oxygen-glucose deprivation and reperfusion, mitochondria releases cytochrome C to significantly increase its content within the cells, and expression of caspase protein increases. Cholic acid, baicalin, bioactive components of *Zhizi*, *Zhenzhumu*, *Qingkailing*-derived formula, and the original *Qingkailing* formula can all reduce the release of cytochrome C, reducing the expression of caspase protein significantly and inhibiting cell apoptosis rate. This effect did not show differences among each drug group.

Microtubule-associated protein combines with tubulin, and is involved in the composition of microtubule. Microtubule is a component of the cytoskeleton system, and plays important roles in the maintenance of neuronal morphology, material transport, and neurotransmitter function. Microtubule-associated protein binds with tubulin and is involved in the composition of microtubule. The occurrence of abnormal microtubule is a sign of serious dysfunction of neurons. After oxygen-glucose deprivation and reperfusion, the contents of neuronal microtubule-associated protein are significantly reduced. Cholic acid and the *Qingkailing*-derived formula showed a significant inhibitory effect.

10.3.4.5.3 Function of Anti-Free Radical Damage Free radical damage is an important part of ischemic cascade reactions. It mainly causes lipid peroxidation leading to cell membrane damage. MDA is a product of lipid peroxidation; its content can reflect the degree of lipid peroxidation. Superoxide dismutase (SOD) plays a vital role in the balance of body's oxidation and antioxidants, which can remove superoxide anions and hydrogen peroxide to protect the integrity of cell membrane structure and function. After oxygen–glucose deprivation and reperfusion, there is a significant increase the content of MDA in nerve cells, and significant inhibition of the activity of SOD. Cholic acid, baicalin, bioactive components of *Zhizi*, *Zhenzhumu*, the *Qingkailing*-derived formula, and the original *Qingkailing* formula can all dramatically reduce MDA content and increase SOD activity in nerve cells. Moreover, the *Qingkailing*-derived formula is the strongest in improving SOD activity of nerve cells, and is significantly better than cholic acid, baicalin, bioactive components of *Zhizi*, *Zhenzhumu*, and the original *Qingkailing* formula, showing the synergies after compatibility.

10.3.4.6 Comprehensive Analysis From the combination of the display level of main effects and effective components in cerebrospinal fluid, the effects of baicalin are focused on the intervention of cell adhesion in pathological aspects, the role of cholic acid is about injury and damage resistance of inflammatory cell factors in the process of inflammation, *Zhenzhumu* shows multiple linked cell protective effects, and bioactive components of *Zhizi* play a protective role of clearing damage and improving antidamage abilities in pathological links. The compatibility effect has significant theoretical compatibility characteristics of TCM formulas, and is closely related to the pathogenesis of toxic brain damage. The bioactive components of *Zhizi* are the *Jun* of the formula, and have the effects of detoxification, dredging collaterals, refreshing, and resuscitation by working on the main targets of multiple aspects in the cascade of cerebral ischemia and the pathological process. Baicalin is mainly aimed at adhesion-leading inflammation and microvascular poor areas, has clearing heat and detoxification effects, and can enhance the effects of bioactive components of *Zhizi* in clearing heat and detoxification, and dredging collaterals. Cholic acid has the effects of cooling blood detoxification,

inflammation, and nerve cell protection in the inflammatory damage links, and can also enhance the effects of bioactive components of *Zhizi* in detoxification and dredging collaterals, and dissipating phlegm for resuscitation and a refreshing feeling. The sedation and tranquilization effect of *Zhenzhumu* shows multiple links to protection of the brain, strengthening the resuscitating and refreshing effects of the whole formula. These three drugs are the *Chen*, and is the system of one *Jun* to three *Chen*.

The *Qingkailing*-derived formula has multiple links to inhibitory effects for cerebral ischemic injury cascade; and every aspect of these effects has factors of inhibiting injury (dispelling exogenous pathogen) and the improvement of anti-injury protection (strengthening body resistance), but generally shows advantages for the effect of dispelling exogenous pathogens. Different components have relatively focused targets, and the feature of changing by time. The *Qingkailing*-derived formula is better than the original *Qingkailing* formula in many aspects, which is inextricably linked with the elimination of some insignificant components of ischemic stroke and the reoptimization process of the compatibility.

10.4 CONCLUSIONS

TCM is complex, and its functional indications are very extensive. Using integrated chemomics, we can characterize its functional indications and their relevant material bases. For some specific indications, selecting scientific and rational methods and indices of active evaluation to establish the levels of chemome characterization methods and utilizing chemome screening systematically for traditional Chinese formulas can lead to the development of modern components of TCM with basically known effective component groups and pharmacological effects.

In this study, chemomics was used to screen the effective component group of the *Qingkailing* injection from top to bottom, and eliminate some unnecessary and potential side effective component groups, retaining four compatible types of effective component groups. Considering the efficacy evaluation, the compatibility of these four effective component groups well maintained the original treatment of stroke (cerebral edema) of the *Qingkailing* injection. Hence, we believe that these four compatible components can represent the effective compounds of *Qingkailing* injection in the treatment of stroke. In a comparison of the fingerprint of the simplified formula and the original formula of *Qingkailing*, we could see that the *Qingkailing*-derived formula has a more simple, effective component group and could be quantitatively controlled. And the relationships of compounds are clearer on the fingerprints. Looking at the comparisons of efficacy evaluation, the efficacy of the *Qingkailing*-derived formula is no worse than the original formula in general and even has significant advantages in some indices.

Chemomics, as one of the "omics" research methods, provides a viable method platform to develop new drugs from TCM (including traditional formulas of TCM, components of TCM, or herbal medicines), which shows broad application prospects in the research and development of new drugs from formulas of TCM.

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CHAPTER 11

INTEGRATED GLOBAL SYSTEMS BIOLOGY FOR THE RESEARCH AND DEVELOPMENT OF CHINESE MEDICINE *SHUANGLONG* FORMULA

11.1 BRIEF INTRODUCTION TO THE *SHUANGLONG* FORMULA

Shuanglong formula (SLF), a combination of ginseng and *Danshen* at a ratio of 7:3, was designed by Professor Lianda Li, one of the most famous specialists at Xiyuan Hospital of China Academy of Chinese Medical Sciences, and has been used for the clinical treatment of cardiovascular diseases (CVDs) such as myocardial infarction and angina pectoris for several years. When designing a formula in traditional Chinese medicine (TCM), ginseng with the effectiveness of *qi* is the principal element, whereas *Danshen* with the effectiveness of activating circulation and dispersing stasis of blood are adjuvant components that contribute to the effects of ginseng. Previous studies based on myocardial infarction (MI) models of rats, pigs, and dogs showed that SLF alone or combined with mesenchymal cell transplantation could reduce myocardial infarct area and the degree of myocardial injuries, improve cardiovascular function, and increase myocardial blood and myocardial capillary density.[1–5] Moreover, the Chinese miniature pig MI model from interventional cardiac catheterization blocking coronary blood flow was shown in Fig. 11.1. Compared with the model group (Fig. 11.1A), the myocardial infarct area was obviously decreased and the neovessels was observed in the infarct area in the SLF combined with the mesenchymal cell transplantation group (Fig. 11.1B).

Ginseng of SLF is the dried root of Panax Ginseng C.A. Meyer, which belongs to the family of Araliaceae, and ginsenosides and panaxan are the

Systems Biology for Traditional Chinese Medicine, First Edition. Guoan Luo, Yiming Wang, Qionglin Liang, and Qingfei Liu.

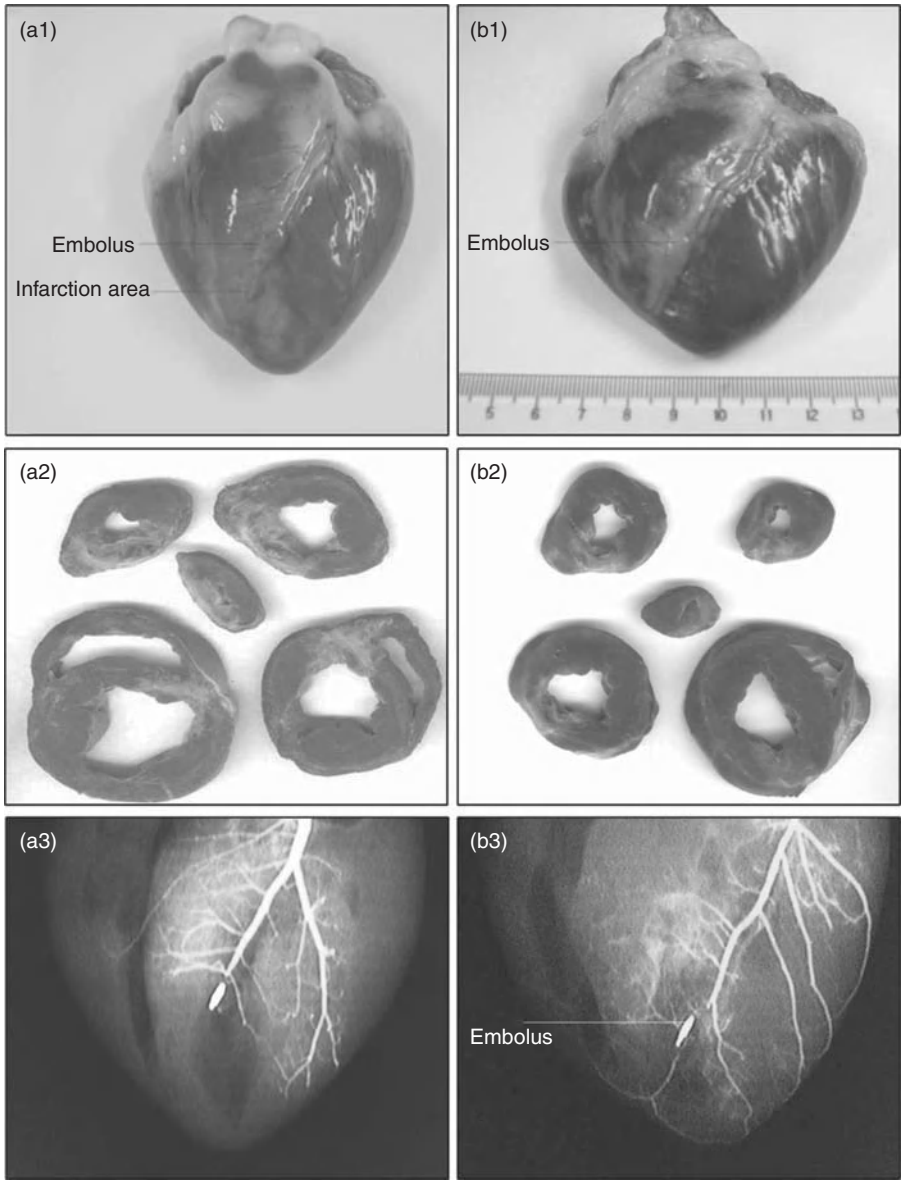

Fig. 11.1 Myocardial infarction of Chinese miniature pigs. A: model group; B: SLF + MSCs group. (See color insert.)

principle ingredients. Currently, over eighty different ginsenosides have been identified from different parts of ginseng.[6] They are classified into three subclasses: Protopanxadiol, such as Rb_1, Rb_2, Rc, Rd, Rh_2, M - Rb_1, and M-Rd; Protopanxatriol, such as Re, Rf, Rg_1, Rg_2, and Rh_1; and oleanolicacid, such as Ro and Ri. The panaxan mainly includes monosaccharide, oligosaccharide, and polysaccharose. Among these, polysaccharose is the bioactive ingredient and some of them, such as SA, SB, PA, and Panax Notoginseng, can be separated from ginseng pectin.[7] Numerous reports have shown that ginsenosides could protect against myocardial damage by increasing 6-keto-$PGF_{1\alpha}$, decreasing lipid peroxidation,[8] having antioxidant effects,[9, 10] and regulating enzyme activity.[11, 12] Both Rb_1 and Re are said to reduce myocardial apoptosis and myocardial ischemia reperfusion injury.[13] Under the condition of MI, ginsenoside Rg_l could reduce myocardial enzyme release, promote vascular (VEGF) and hypoxia inducible factor-1α (HIF-lα) expression, reduce myocardial infarct area, promote angiogenesis, and increase Bcl-2/Bax ratio.[14]

Danshen of SLF is the dried root of salvia militorrhiza that belongs to the family of Labiatae, including alcohol and water-soluble extracts.[15–19] The water-soluble constituent, 3,4-hydroxybenzyl lactic acid, has the basic chemical structure of salvianolic acids.[20] A number of water-soluble components, such as salvianolic acid A, B, C, D, E, F, G, H, and I; tetramehtyl salvinoalic acid A; rosmarinic acid; methyl rosmarinate; isosalvianolic acid C; baicalin; caffeic acid; β-sitosterol; ursolic acid; protocatechualdehyde; isoimperatorin; daucosterol; hesperitic acid; and alkannic acid were found. Most of them were obtained by binding the protocatechualdehyde with other organic acids.[21, 22] One study demonstrated that magnesium tanshinoate B, a bioactive compound isolated from salvia miltiorrhiza, could prevent apoptosis in cardiomyocytes in the ischemic heart, probably through the inhibition of SAP kinase activity.[23] Other studies found that protocatechualdehyde, at a concentration of 4 $\mu mol \cdot L^{-1}$, could restore the changed transmembrane potential and reduce the damage of ectogenesis O^{2-}.[24]

Both SLF and the single herb (ginseng or *Danshen*) can exert therapeutic efficacy in the treatment of CVDs. Nevertheless, SLF was designed based on clinical experience. Several concerns are raised: Can the rationality and science behind SLF be verified and clearly explained? What are the effective substances? Can the efficacy and mechanism of SLF be dissected from the perspective of modern medicine and the life sciences? Here we present a system-to-system (S2S) model by integrating chemomics and systems biology, which is called integrative systems biology, to study the interaction of the drug system and the biological system. Based on the integrative system biology model, a new formula, the *Shuanglong* derived formula (NSLF6), was developed for the traditional Chinese medicine SLF, a clinical effective medicine, with comparatively clear phytochemical composition and mechanism, and quality that can be controlled. The roadmap of the study is shown in Fig. 11.2.

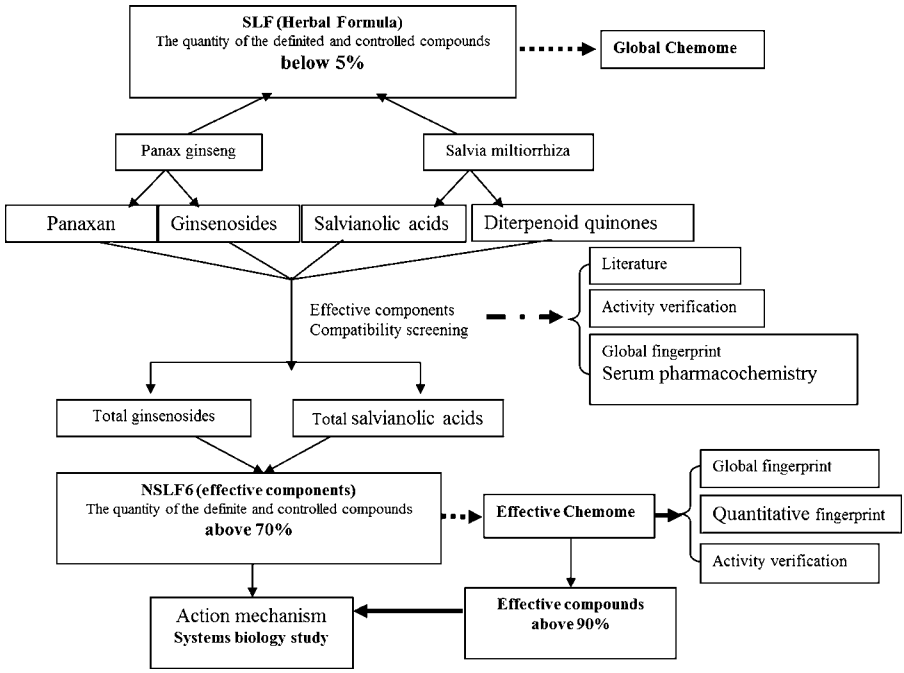

Fig. 11.2 Screening and discovery of NSLF6 based on the system-to-system mode.

11.2 CHEMOMICS STUDY OF THE *SHUANGLONG* FORMULA

11.2.1 Global Chemomics and Serum-Chemical Research of the *Shuanglong* Formula

The global chromatographic fingerprint of the decoction of SLF was established by UPLC/TOF-MS. A total of 73 compounds in SLF were separated and identified on the basis of retention time, m/z, and available conference standards (shown in Table 11.1), among which 48 compounds were from ginseng, 25 compounds were from *Danshen*, and 8 compounds were unidentified. The comparative study of components in plasma of rats before and after administration of NSLF6 was conducted by UPLC-TOF/MS (shown in Fig. 11.3). As shown in Table 11.1, the main hydrophilic components from SLF were gensenosides and salvianolic acids, suggesting that gensenosides and salvianolic acids are likely to be the effective materials for MI.

As shown in Table 11.1, only 25 compounds were detected at 45 min after administration SLF and other compounds were not detected. This may be due to the facts that: those compounds were absorbed into the blood but were rapidly metabolized and eliminated in the body; they were decomposed by intestinal flora and absorbed mainly in the form of metabolites; and their contents or absorption rates were too low, which resulted in concentrations in serum below the detection point.

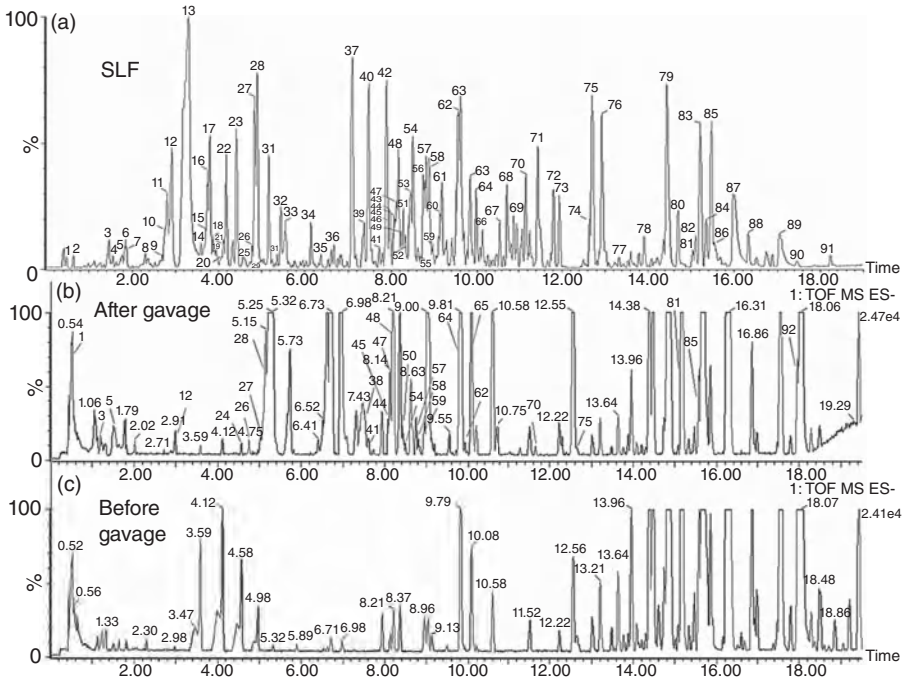

Fig. 11.3 Total ion chromatograms of SLF extracts (A) and serum before (C) and after (B) rats by gavage with SLF based on UPLC-TOF/MS.

11.2.2 Preparation and Characterization of Subchemome of SLF

We pulverized ginseng and *Danshen* to particles with diameters about 0.5–2 cm, and then the particles were isolated and extracted. We collected the eluents, vacuum concentrated them to be strong liquid, and finally dried them to dry paste in hot air cycle at 60°C. We washed the resin column with 95% ethanol.

Based on the screening above, we obtained a simplified prescription, combining total ginsenosides (TGS) and total salvianolic acids (TSA) in equal ratio as present in the original formula SLF, named "NSLF6." The quantity of identified and controllable compounds in SLF was below 5%, whereas that in NSLF6 was above 70% (Fig. 11.4). The quantitative fingerprints of total ginsenosides and total salvianolic acids were established by HPLC-DAD. As shown in Table 11.2, the total contents of quantitative compounds such as ginsenosides Rb_1, Rg_1, and Ro; salvianolic acid B; and lithospermic acid accounted for 74.4% of total solids. Thus, most of the constituents in NSLF6 have been identified and quantified so that we have established a stringent quality control, which provided the chemical basis for unveiling its pharmacological mechanism.

TABLE 11.1 List of Global Chemome for SLF

No.	R.T. (min)	Selected Ion	m/z	Elemental Composition	In Vivo	In Vitro
1	0.54	[M-H]	197.0272	$C_9H_{10}O_5$	3-(3,4-Dihydroxyhenyl) lactic acid	Propanoid acid
2	0.64	[M-H]	313.0923	$C_{17}H_{14}O_6$	Salvianolic acid F	
3	1.26	[M-H]	137.0224	$C_7H_6O_3$	Protocatechuic aldehyde	Protocatechualdehyde
4	1.55	[M-H]	383.0956	$C_{21}H_{20}O_7$	Salvianolic acid K	
5	1.62	[M+COOH]	315.1078	$C_{27}H_{22}O_{12}$	Salvianolic acid H	Isoimperatrin
6	1.83	[M-H]	417.1034	$C_{20}H_{18}O_{10}$	Salvianolic acid I	
7	1.87	[M-H]	371.0975	$C_{19}H_{16}O_8$	Salvianolic acid D	
8	2.31	[M-H]	537.1421	$C_{27}H_{22}O_{12}$	Salvianolic acid M	
9	2.70	[M-H]	555.1463	$C_{27}H_{24}O_{13}$	Salvianolic acid G	
10	2.80	[M-H]	359.1027	$C_{18}H_{16}O_8$	Salvianolic acid L	
11	2.80	[M-H]	717.2053	$C_{36}H_{30}O_{16}$	Salvianolic acid F	
12	2.91	[M-H]	537.1498	$C_{27}H_{22}O_{12}$	Rosmarinic acid	Salvianolic acid I
13	3.30	[M-H]	717.2090	$C_{36}H_{30}O_{16}$	Salvianolic acid J	
14	3.43	[M-H]	537.1498	$C_{27}H_{22}O_{12}$	Ginsenoside Rs_2	
15	3.69	[M-H]	445.0963	$C_{21}H_{18}O_{11}$	Lithospermic acid	
16	3.71	[M-H]	491.1032	$C_{26}H_{22}O_{10}$	Salvianolic acid B	Salvianolic acid C
17	3.80	[M-H]	717.2072	$C_{36}H_{30}O_{16}$	Ginsenoside F_4	
18	3.87	[M-H]	455.3541	$C_{30}H_{48}O_3$	20-Gluco-ginsenoside Rf	
19	3.87	[M+COOH]	831.4751	$C_{41}H_{70}O_{14}$	Salvianolic acid E	
20	4.06	[M-H]	419.0966	$C_{19}H_{18}O_8$	Notoginsenoside-R_1	
21	4.12	[M+COOH]	731.2222	$C_{31}H_{50}O_3$	9′-Methyl lithospermate B	
22	4.17	[M+COOH]	1007.6335	$C_{48}H_{82}O_{19}$	Methyl rosmarinate	
23	4.41	[M+COOH]	977.5273	$C_{47}H_{80}O_{18}$	9′ Methyl salvianolic acid B	Notoginsenoside-R_1
24	4.51	[M+COOH]	519.3562	$C_{30}H_{50}O_4$	Ginsenoside Re	Protopanaxatriol
25	4.59	[M+COOH]	537.1498	$C_{27}H_{22}0_{12}$	Lithospermic acid/isomer	
26	4.77	[M-H]	493.1253	$C_{26}H_{22}O_{10}$	Salvianolic acid A	Salvianolic acid A
27	4.83	[M+COOH]	991.5528	$C_{48}H_{82}O_{18}$	Ginsenoside Re	
28	4.89	[M+COOH]	845.5647	$C_{42}H_{72}O_{14}$	Ginsenoside Rf	Ginsenoside Rf

29	4.90	[M+COOH]	459.3927	$C_{29}H_{50}O$	β-Sitosterol	
30	5.16	[M-H]	885.5041	$C_{45}H_{74}O_{17}$	Ginsenoside malonyl-Rg_1	Ginsenoside malonyl-Rg_1
31	5.25	[M-H]	491.0979	$C_{19}H_{16}O_8$	Isosalvianolic acid C	Isosalvianolic acid C
32	5.44	[M-H]	1031.5356	$C_{51}H_{84}O_{21}$	N.I.	
33	5.55	[M-H]	885.5643	$C_{45}H_{74}O_{17}$	Malonyl-Rg_1 isomer	
34	6.15	[M+COOH]	815.4805	$C_{41}H_{70}O_{13}$	Ginsenoside F_3	
35	6.38	[M-H]	745.2044	$C_{27}H_{24}O_{13}$	Dimethyl salvianolate B	
36	6.67		1033.6500		N.I.	
37	7.09	[M+COOH]	845.5662	$C_{42}H_{72}O_{14}$	Ginsenoside Rg_1	Ginsenoside Rg_1
38	7.41	[M-H]	377.1961	$C_{21}H_{29}O_6$	N.I.	Propanoid acid-gluA
39	7.33	[M+COOH]	1285.6573	$C_{59}H_{100}O_{27}$	Notoginsenoside-R_4	
40	7.47	[M+COOH]	815.5520	$C_{41}H_{70}O_{13}$	Notoginsenoside-R_2	
41	7.56	[M-H]	329.2471	$C_{20}H_{26}O_4$	Salviol	Salviol
42	7.88	[M+COOH]	829.5691	$C_{42}H_{72}O_{13}$	Ginsenoside Rg_2	
43	7.90	[M+COOH]	815.4859	$C_{41}H_{70}O_{13}$	Ginsenoside F_5	
49	8.18	[M+COOH]	1075.6365	$C_{55}H_{92}O_{23}$	Ginsenoside Rs_2	
50	8.19	[M+COOH]	931.6228	$C_{54}H_{94}O_9$	N.I.	Compound K oleate
51	8.27	[M+COOH]	1001.5022	$C_{49}H_{80}O_{18}$	Ginsenoside R_0	
52	8.28	[M+COOH]	683.4992	$C_{36}H_{62}O_9$	Ginsenoside F_1	Ginsenoside F_1
53	8.42	[M+COOH]	1255.6363	$C_{58}H_{98}0_{26}$	Ginsenoside Ra_2	
54	8.47	[M+COOH]	1123.5933	$C_{53}H_{90}O_{22}$	Ginsenoside Rb_2	Ginsenoside Rb_2
55	8.50	[M-H]	1165.5982	$C_{56}H_{92}O_{25}$	Ginsenoside malonyl-Rb_2	Ginsenoside malonyl-Rb_2
56	8.77	[M+COOH]	1123.5909	$C_{53}H_{90}O_{22}$	Ginsenoside malonyl-Rb_3	
57	8.80	[M+COOH]	1123.5868	$C_{53}H_{90}O_{22}$	Ginsenoside Rc	Ginsenoside Rc
58	8.88	[M-H]	1165.5994	$C_{56}H_{92}O_{25}$	Ginsenoside malonyl-Rc	Ginsenoside malonyl-Rc
59	8.96	[M-H]	339.0806	$C_{18}H_{12}O_7$	Salvianolic acid G	Salvianolic acid
60	9.16	[M+COOH]	1195.6102	$C_{56}H_{94}O_{24}$	Panas quinquefolium R_1	
61	9.19	[M-H]	1093.5857	$C_{53}H_{90}O_{23}$	N.I.	
62	9.50	[M+COOH]	1165.7053	$C_{55}H_{92}O_{23}$	Ginsenoside Rs_1	
63	9.56	[M+COOH]	991.5594	$C_{48}H_{82}O_{18}$	Ginsenoside Rd	Ginsenoside Rd
64	9.82	[M+COOH]	503.2664	$C_{30}H_{50}O_3$	Protopanoxadiol	Protopanoxadiol

(*Continued*)

TABLE 11.1 (*Continued*)

No.	R.T. (min)	Selected Ion	m/z	Elemental Composition	In Vivo	In Vitro
65	9.84	[M+COOH]	521.3710	$C_{30}H_{52}O_4$	N.I.	Protopanaxatriol
66	10.10	[M+COOH]	991.5609	$C_{48}H_{82}O_{18}$	Ginsenoside Rd isomer	
67	10.56	[M-H]	961.5531	$C_{48}H_{82}O_{19}$	220-Gluco-ginsenoside Rf isomer	
68	10.72		1033.5769		N.I.	
69	10.91		961.6228		N.I.	
70	11.16	[M+COOH]	811.5568	$C_{42}H_{70}O_{12}$	Ginsenoside Rg_4	
71	11.43	[M+COOH]	811.5567	$C_{42}H_{70}O_{12}$	Ginsenoside Rg_5	
72	11.81		665.4831		N.I.	
73	11.96	[M+COOH]	829.4880	$C_{42}H_{72}O_{13}$	Ginsenoside Rg_2 isomer	
74	12.62	[M+COOH]	505.3546	$C_{30}H_{52}O_3$	Protopanoxadiol	Protopanoxadiol
75	12.70	[M+COOH]	829.5677	$C_{42}H_{72}O_{13}$	Ginsenoside F_2	
76	12.92	[M+COOH]	829.5697	$C_{42}H_{72}O_{13}$	Ginsenoside Rg_3	
77	13.32	[M+COOH]	799.4894	$C_{41}H_{70}O_{12}$	Ginsenoside MC	
78	13.91		595.2895		N.I.	
79	14.43		1191.5946		N.I.	
80	14.69		723.4442		N.I.	
81	15.03	[M+COOH]	667.4415	$C_{36}H_{62}O_8$	Ginsenoside Rh_2	Ginsenoside Rh_2
82	15.11	[M+COOH]	851.4934	$C_{51}H_{98}O_6$	Tripalmitin	
83	15.21	[M+COOH]	811.4869	$C_{42}H_{70}O_{12}$	Ginsenoside Rg_6	
84	15.43	[M+COOH]	649.3914	$C_{36}H_{60}O_7$	Ginsenoside Rh_3	
85	15.48	[M+COOH]	811.4849	$C_{42}H_{70}O_{12}$	Ginsenoside Rk_1	
86	15.58	[M+COOH]	667.4438	$C_{36}H_{62}O_8$	Compound K	Compound K
87	15.97		433.2725		N.I.	
88	16.33	[M+COOH]	625.2377	$C_{28}H_{36}O_{13}$	Eleutheroside B	
89	17.08	[M-H]	455.2816	$C_{30}H_{48}O_3$	Oleanolic acid	
90	17.46	[M+COOH]	461.26677	$C_{27}H_{44}O_3$	Tgogenin	
91	18.22	[M+COOH]	649.4343	$C_{36}H_{60}O_7$	Ginsenoside Rk_2	

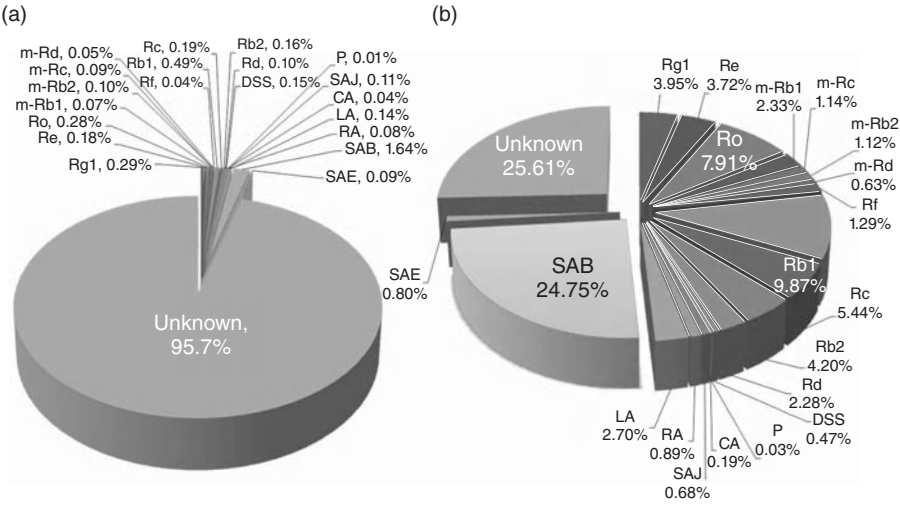

Fig. 11.4 Percentage of definite components in SLF (A) and NSLF6 (B).

TABLE 11.2 Contents of Major Constituents in SLF and NSLF6

Components	SLF (%)	NSLF6 (%)
Ginsenoside Rg_1	0.29	3.95
Ginsenoside Re	0.18	3.72
Ginsenoside Ro	0.28	7.91
Ginsenoside m-Rb_1	0.07	2.33
Ginsenoside m-Rc	0.09	1.14
Ginsenoside m-Rb_2	0.10	1.12
Ginsenoside m-Rd	0.05	0.63
Ginsenoside Rf	0.04	1.29
Ginsenoside Rb_1	0.49	9.87
Ginsenoside Rc	0.19	5.44
Ginsenoside Rb_2	0.16	4.20
Ginsenoside Rd	0.10	2.28
Propanoid acid	0.15	0.47
Protocatechualdehyde	0.01	0.03
Caffeic acid	0.04	0.19
Salvianolic acid J/Isomer	0.11	0.68
Rosmarinic acid	0.08	0.89
Lithospermic acid	0.14	2.70
Salvianolic acid B	1.64	24.7
Salvianolic acid E	0.09	0.80
Total	4.30	74.4

11.2.3 Distribution of NSLF6

The comparative study of components in intestines, liver, and plasma of rats before and after administration of NSLF6, total amount of ginsenosides, and total amount of salvianolic acids were conducted by UPLC-TOF/MS. Plasma (from portal vein and systemic circulation) and tissue (duodenum, jejunum, ileum, colon, and liver) samples were collected at 1 and 3 h after administrations. The MS spectra were processed using MarkerLynx, and the structures of the components were identified based on accurate elemental compositions, available free databases, and reference standards (shown in Table 11.3).

After introduction of a complex drug system (such as NSLF6) into the enteron, some constituents were metabolized by the digestive enzymes or bacteria of the gastrointestinal tract. The metabolites and the rest of the constituents were absorbed by intestinal tract and imported into the hepatic portal vein through mesenteric capillaries, underwent the first-pass metabolism in the liver, and finally exerted efficacies on the target organ through systemic circulation after entering the inferior vena cava.

11.2.3.1 Components That Can Be Absorbed into the Blood and the Main Absorption Sites As shown in Table 11.3, among the 68 components identified in NSLF6, 12 of them, including propanoid acid, 9′-methyl lithospermate B, salvianolic acid D, rosmarinic acid, salvianolic acid B, salvianolic acid C, isosalvianolic acid C, and ginsenosides Rb_1, Rb_2, M-Rb_1, Re, and Rd isomer could be detected in the small intestine and absorbed into the blood as prototypes. Although some components with high content, such as propanoid acid, salvianolic acid B, and ginsenosides Rb_1 and Re, were partly metabolized in the gastrointestinal tract and liver, they could still be detected in the small intestine and absorbed into blood as prototypes, while other components with low content were stable in vivo and could also be detected in the small intestine and absorbed into blood as prototypes. Additionally, some components, such as propanoid acid, salvianolic acid D, salvianolic acid B, and ginsenosides Re, Rf, and Ng-R_1, could be detected in the duodenum but not in other sections of the small intestine, suggesting the duodenum was the main absorption site of these components. Meanwhile, salvianolic acid E, carnosol, M-Rb_2, and Rb_3 could not be detected in the small intestine but were absorbed into the blood as prototypes, which suggested they were completely absorbed in the stomach.

11.2.3.2 Ginsenosides Could Promote Absorption of Salvianolic Acid Results in Table 11.3 show that absorptions of salvianolic acids are different between rats' administration of NSLF6 and total salvianolic acids: salvianolic acids with high content, such as propanoid acid, salvianolic acid B, salvianolic acid C, and isosalvianolic acid C, could be detected in the duodenum after administration of total salvianolic acids while they were not detectable after administration of NSLF6, which may be caused by ginsenosides Rb_1

TABLE 11.3 NSLF6 In Vitro and Its Effective Components In Vivo Distribution

NSLF6 In Vitro	Duodenum	Jejunum	Ileum	Colon	Portal Vein	Liver	Systemic Circulation
3-(3,4-Dihydroxyhenyl) lactic acid	D1	D1,S1	D1,D3,S1,S3	–	S1,S3,D1,D3	–	S1,S3,D1,D3
9′-Methyl lithospermate B	D1,D3,S1	D1,D3,S1,S3	D1,D3,S1,S3	D1,D3,S1,S3	S1,D1,S3	S1	S1,D1
Salvianolic acid D	–	D1, S1	D,D3,S3	–	S1,D1,D3	–	S1,D1,D3
Rosmarinic acid	D1,D3,S1	D1,D3,S1,S3	D1,D3,S1,S3	D1,S3	S1,S3,D1,D3	–	S1,S3,D1,D3
Salvianolic acid B	D1	S1	D1,D3,S1,S3	–	S1,S3,D1,D3	–	S1,S3,D1,D3
Salvianolic acid C	D1	D1,S1	D1,D3,S3	–	S1,S3,D1,D3	–	S1,S3,D1,D3
Isosalvianolic acid C	D1	D1,D3	D1,D3	–	S1,S3,D1,D3	–	S1,S3,D1,D3
Ginsenoside Rb_1	R1,R3,S1	R1,R3,S1,S3	R1,R3,S1,S3	R,R3,S1,S3	S1,S3,D1,D3	–	S1,S3,D1,D3
Ginsenoside Rb_2	R1,R3,S1	R1,R3,S1,S3	R1,R3,S1,S3	R1,R3,S1,S3	S1,S3,R1,R3	–	S1,S3,R1,R3
Ginsenoside malonyl-Rb_1	R1,S1	R1,S1	R1,R3,S1,S3	–	S1,S3,R1,R3	–	S1,S3,R1,R3
Ginsenoside Re	R1,S1	S1,S3	R1,R3,S1,S3	–	S1,R1,R3	–	S1,R1
Ginsenoside Rf	–	R1	–	–	S1,R1,R3	–	S1
Notoginsenoside R_1	–	R1,S1	R1,S1	–	R1,R3	–	R1
Rd isomer	R1,R3,S1	R1,R3,S1,S3	R1,R3,S1,S3	R1,R3,S1,S3	S1	S1,S3	S1
Salvianolic acid E	–	–	–	–	S1	–	–
Lithospermic acid	–	–	–	–	D1,D3	–	D1,D3
Ginsenoside malonyl-Rb_2	–	–	–	–	S1,S3,R1,R3	–	S1,S3,R1,R3
Carnosol	–	–	–	–	S1,S3,D1,D3	–	S1,S3,D1,D3
Ginsenoside Rb_3	–	–	–	–	S1,S3,R1,R3	–	S1,S3,R1,R3
Propanoid acid-B-Gluconic acid (metabolite)	D3,S1,S3	D1,D3,S1,S3	D1,D3,S1,S3	D1,D3,S1,S3	S1,S3,D1,D3	–	S1,S3,D1,D3
Protopanoxadiol (metabolite)	–	–	–	–	S1,S3,R1,R3	–	S1,S3,R1,R3

(*Continued*)

TABLE 11.3 (*Continued*)

NSLF6 In Vitro	Duodenum	Jejunum	Ileum	Colon	Portal Vein	Liver	Systemic Circulation
Salvianolic acid H	–	D1,S1	D,S,S3	–	–	–	–
Salvianolic acid I	D1	D1,S1	D1,D3,S1,S3	–	–	–	–
Salvianolic acid L	D1,D3,S1	D1,D3,S1,S3	D1,D3,S1,S3	D3,S3	–	–	–
Salvianolic acid A	D1,S1	D1,S1	D1,D3,S3	–	–	–	–
Ginsenoside Rd	R1,R3,S1	R1,R3,S1,S3	R1,R3,S1,S3	R1,R3,S1,S3	–	S1,S3	–
Methyl rosmarinate	–	D1,S1	D1, D3	–	–	–	–
Ginsenoside malonyl-Rg_1	–	R1,S1	R1,R3,S1,S3	–	–	–	–
Salvianolic acid J	D1	S1	S1	–	–	–	–
Dimethyl salvianolate B	D1,D3,S1	D3,S1	S3	–	–	–	–
Ginsenoside Ra_3	R3	R3	–	–	–	–	–
Ginsenoside Mc	–	R1	–	–	–	–	–
Notoginsenoside R_4	–	R1,S1	R1,R3,S1,S3	–	–	–	–
Ginsenoside F_3	R1,R3	R1,R3,S1,S3	R1,R3,S1,S3	–	–	–	–
Ginsenoside Ra_1	R1,S1	R1,S1	R1,R3,S1,S3	–	–	–	–
Ginsenoside Ra_2	R1,S1	R1,S1	R1,R3,S1,S3	–	–	–	–
Ginsenoside malonyl-Rc	R1,S1	R1,S1	R1,R3,S1,S3	–	–	–	–
Notoginsenoside R_2	R1,R3	R1,R3,S1	R1,R3,S1	R1,R3,S1	–	–	–
Panas quinquefolium R_1	–	R1	R1,R3,S3	–	–	–	–
Ginsenoside malonyl-Rd	–	R1,S1	R3,S3	–	–	–	–
Ginsenoside Rs_1	R1	R1,S1	R3,S3	–	–	–	–
Ginsenoside R_0	R1,R3,S1	R1,R3,S1,S3	R1,R3,S1,S3	–	–	S	–
Ginsenoside Rg_2	R1,S1	R1,S1	R1,R3,S1,S3	R1,R3	–	–	–
Ginsenoside Rh_1	R1,R3,S1	R1,S1	R1,R3,S1,S3	–	–	–	–
Ginsenoside Rg_6	R1,R3,S1,S3	R1,R3,S1,S3	R1,R3,S1,S3	R1,R3,S1,S3	–	S1	–
Ginsenoside Rk_2	–	R1,S1	–	–	–	–	–

Ginsenoside Rc	–	S1	R3,S3	–	–	–	–
21-Gluco-ginsenoside Rf	–	R1,S1	–	–	–	–	–
20-Gluco-ginsenoside Rf	–	R1,S1	R1,R3,S1,S3	–	–	–	–
Rg_2 isomer	–	R1,R3,S1	R1,R3,S1,S3	R1,R3,S1,S3	–	–	–
Ginsenoside Rg_4	R1,S1	R1,S1	R1,R3,S1,S3	–	–	–	–
Ginsenoside Rg_1	–	R1,S1	–	–	–	–	–
Ginsenoside Rg_5	R1,S1	R1,R3,S1,S3	R1,R3,S1,S4	–	–	–	–
Ginsenoside F_2	–	R1,S1	R1,R3,S3	–	–	–	–
Ginsenoside Rs_2	–	R1,S1	R1,R3	–	–	–	–
Ginsenoside Rg_3	R1,R3,S1	R1,R3,S1,S3	R1,R3,S1,S3	–	–	–	–
Ginsenoside Rf	–	R1,S1	–	–	–	–	–
Ginsenoside Rk_1	–	–	S1	–	–	–	–
Tripalmitin	–	R1,S1	R1,R3,S1,S3	–	–	–	–
Salvianolic acid K	–	–	–	–	–	–	–
Salvianolic acid M	–	–	–	–	–	–	–
Salvianolic acid G	–	–	–	–	–	–	–
Salvianolic acid F	–	–	–	–	–	–	–
Compound K	–	–	–	–	–	–	–
Ginsenoside Rh_2	–	–	–	–	–	–	–
Ginsenoside Rh_3	–	–	–	–	–	–	–
Ginsenoside F_4	–	–	–	–	–	–	–

Note: S, D, and R represent NSLF6, salvianolic acid, and ginsenoside; "1" means that component can be detected at 1 h after administration and "3" means that component can be detected at 3 h after administration.

and Rd from NLSF6. Ginsenosides Rb_1 and Rd may promote the absorption of the four components in the duodenum by inhibiting the cholinergic nervous system, improving the small bowel excitement, and extending drug residence time in the duodenum.[25]

11.2.3.3 Changes of the Components That are Not Absorbed into the Blood in the Small Intestine As shown in Table 11.3, salvianolic acids K, M, G, and F, and ginsenosides c-k, Rh_2, Rh_3, and F_4 were not detected in the small intestine and plasma, which suggested that they were metabolized by the digestive enzymes in the stomach. Additionally, 38 components, including salvianolic acids H, I, L, and A, and ginsenosides Ra_1, Ra_2, Ra_3, Rg_2, and Rg_3, were detectable in different sections of the small intestine, but not in the blood, which suggested that they were metabolized by the enterobacteria in the intestinal tract.

Among the 68 components identified in NSLF6, 16 were absorbed into the portal vein and only 15 were imported into the systemic circulation, except that salvianolic acid E was metabolized by the liver. The other components were metabolized in the gastrointestinal tract.

11.3 PHARMACODYNAMIC EVALUATION OF SLF EFFECTIVE INGREDIENTS

Both herbs, ginseng and *Danshen*, could exert certain efficacies on CVDs. From the Chinese medicine perspective, the combination of the two herbs could benefit *qi*, activate blood circulation, exert synergy efficacies, and produce effects on multiple targets. In order to verify the synergies of the two herbs and screen the best proportion of the combination, the isoproterenol hydrochloride (ISO)-induced and coronary artery ligation-induced MI rat models were used to develop a new formula, NSLF6, from the original formula of SLF and pharmacodynamic evaluation was conducted.

11.3.1 Activity Verification of the SLF

11.3.1.1 Method Dried and pulverized materials of ginseng (500 g), *Danshen* (500 g), and different combinations of ginseng : *Danshen* (6:4, 7:3, 8:2, each of 500 g) were ground up and then refluxed with 4000 mL of water for 60 min twice. After cooling, the extracted solutions were filtered through a glass filter covered with filter paper. The solutions were condensed under decompression to roughly 150 mL and finally were freeze dried. The positive control drug was the *Danshen* agent.

Male Sprague-Dawley rats were randomly divided into eight groups as follows: ginseng group, *Danshen* group, ginseng : *Danshen* (6:4) group, ginseng : *Danshen* (7:3) group, and ginseng : *Danshen* (8:2) group. They received an IP injection with the test solutions of ginseng, *Danshen*, ginseng : *Danshen* (6:4), ginseng : *Danshen* (7:3), and ginseng : *Danshen* (8:2) at a dose of 5 g/kg·w·d

(equal to 7.5 mL/kg·w·d) each. Additionally, the sham surgery group and model group received an IP injection with the same volume of vehicle. The positive drug control group received an IP injection of *Danshen* agent solution at a dose of 2 g/kg·w·d. The eight animal groups were administrated as above for 7 consecutive days. On days 6 and 7, all animals received an IP-injected ISO at a dose of 30 mg/kg except for the control group.

All the rats were fixed in position and two-lead electrocardiograms (ECGs) were recorded by MPA 2000 biosignal analytical system. Before the injections, normal ECG recordings were made for at least 5 min until they were well balanced. The ECGs in all groups after 1 h of the final injection at 0.5, 1, 2, 5, 10, 15, and 20 min were recorded and compared. After ECG measurements, the blood was collected from the vena cava and then centrifuged at 3500 rpm for 10 min at 4°C. The supernatant obtained was frozen immediately, stored at –80°C, and thawed before analysis. After that, the rats' hearts were taken out immediately and fixed in 10% formalin. Serum concentrations of lactate dehydrogenase (LDH), nonesterified fatty acid (NEFA), and creatine kinases (CK) were measured using an enzymatic UV test following the instructions of commercial assay kits. The heart samples were fixed and embedded in paraffin wax. Histologic sections (4–5 μm) of the paraffin-embedded tissues were stained with hematoxylin-eosin. The sections were examined under light microscope, and photomicrographs were taken. The areas of MI of the left ventricle were detected with a Leica image analysis system (10×), and infarct size (%) was expressed as myocardial infarct size/left ventricular area × 100%.

11.3.1.2 Results and Discussion

11.3.1.2.1 Electrocardiogram Analysis The effect of ISO on the J point of rats' ECG is shown in Table 11.4. The ECG alterations, especially the degrees of J point alterations, are generally considered as the main index to evaluate animals' MI. In this study, we found that the J point of ECG in MI model rats elevated slowly for 1 min after ISO injection, and then continuously decreased within 20 min. The J point at each time point within 20 min was significantly different from those in control rats ($p < 0.05$). The therapeutic effects were different among the treatment groups and the combination-based treatment groups were better than the single drug (ginseng or *Danshen*) groups against ECG changes. Furthermore, the combination of ginseng and *Danshen* at a ratio of 7:3 proved to have the best therapeutic efficacy on MI rats.[26]

11.3.1.2.2 Serum Biochemical Measurement Compared with control rats, the concentrations of CK ($p < 0.01$), LDH ($p < 0.01$), and malondialdehyde (MDA) ($p < 0.001$) in the model rats were significantly increased, while the levels of superoxide dismutase (SOD) ($p < 0.05$) were remarkably decreased (Table 11.5). Among treatment groups, a statistically significant restoration in CK, LDH, SOD, and MDA levels was observed in rats treated with the bitherapy groups (6:4, 7:3, 8:2) compared with those treated with monotherapy

TABLE 11.4 Effects of SLF on ΔJ of Electrocardiogram in MI Rats Induced by Isoproterenol at Different Time Points ($x \pm s$, $n = 9$)

Group	Dose (g/kg·d)	5 min	10 min	15 min	20 min
Model	–	–0.167 ± 0.091	–0.176 ± 0.101	–0.179 ± 0.098	–0.194 ± 0.086
Control	–	0.009 ± 0.021***	0.004 ± 0.025***	0.003 ± 0.029***	0.003 ± 0.031***
Positive control	2	–0.133 ± 0.158	–0.123 ± 0.147	–0.118 ± 0.149	–0.108 ± 0.136
Ginseng:*Danshen* (6:4)	5	–0.095 ± 0.093	–0.077 ± 0.099*	–0.089 ± 0.099*	–0.070 ± 0.093***
Ginseng:*Danshen* (7:3)	5	–0.060 ± 0.070**	–0.061 ± 0.062**	–0.064 ± 0.057**	–0.054 ± 0.061***
Ginseng:*Danshen* (8:2)	5	–0.081 ± 0.101	–0.054 ± 0.138**	–0.052 ± 0.130**	–0.051 ± 0.142***
Danshen	5	–0.155 ± 0.103	–0.151 ± 0.126	–0.140 ± 0.114	–0.118 ± 0.112
Ginseng	5	–0.093 ± 0.086	–0.100 ± 0.076	–0.108 ± 0.110	–0.087 ± 0.080

*$p < 0.05$.
**$p < 0.01$.
***$p < 0.001$ versus model group.

TABLE 11.5 Effects of SLF on the Activities of LDH, CK, SOD, and MDA in Serum of MI Rats Induced by Isoproterenol ($x \pm s$, $n = 9$)

Group	Dose (g/kg·d)	LDH ($\mu mol \cdot mL^{-1}$)	CK ($\mu mol \cdot mL^{-1}$)	SOD ($\mu mol \cdot mL^{-1}$)	MDA ($\mu mol \cdot L^{-1}$)
Model	–	0.27 ± 0.12	4.24 ± 1.79	44.55 ± 6.09	5.51 ± 1.30
Control	–	0.12 ± 0.06**	2.12 ± 0.72**	50.90 ± 3.96*	2.18 ± 0.80***
Positive control	2	0.22 ± 0.08	3.01 ± 0.77	47.34 ± 5.87	4.10 ± 1.84
Ginseng:*Danshen* (6:4)	5	0.13 ± 0.07*	2.26 ± 1.07*	49.65 ± 5.00*	3.85 ± 1.38**
Ginseng:*Danshen* (7:3)	5	0.15 ± 0.07*	2.64 ± 1.08*	50.41 ± 4.79*	3.91 ± 1.73*
Ginseng:*Danshen* (8:2)	5	0.15 ± 0.08*	2.72 ± 1.15*	49.65 ± 3.93*	3.79 ± 1.59*
Danshen	5	0.18 ± 0.09	3.81 ± 1.43	47.55 ± 6.36	4.31 ± 1.63
Ginseng	5	0.15 ± 0.10*	2.89 ± 1.40	47.05 ± 7.61	4.30 ± 2.00

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$ vs model group.

TABLE 11.6 Effects of SLF on Cardiac Infarction Area in MI Rats Induced by Isoproterenol ($x \pm s$, $n = 9$)

Group	Dose (g/kg·d)	Cardiac Infarction Area %
Model	–	9.94 ± 4.19
Control	–	1.01 ± 0.87**
Positive control	2	7.72 ± 2.36
Ginseng:*Danshen* (6:4)	5	6.91 ± 2.03*
Ginseng:*Danshen* (7:3)	5	6.87 ± 2.93*
Ginseng:*Danshen* (8:2)	5	6.70 ± 2.12*
Danshen	5	7.65 ± 3.11
Ginseng	5	8.10 ± 3.86

*$p < 0.05$.
**$p < 0.001$ versus model group.

(ginseng or *Danshen*). Although there was a recovery trend toward normal levels in the monotherapy groups, it was not statistically significant ($p > 0.05$) with the exception of LDH in ginseng group ($p < 0.05$). Furthermore, the combination of gisneng and *Danshen* at a ratio of 7:3 was shown to exert the best therapeutic efficacy on MI rats.

11.3.1.2.3 Determination of Myocardial Infarct Size As shown in Table 11.6, compared with the model group, the infarct areas of different combinations of ginseng:*Danshen* (6:4, 7:3, 8:2) showed a significant decrease ($p < 0.05$), but those from the *Danshen* and ginseng groups were not significantly changed. Each monotherapy reduced infarct size in MI rats to a certain degree, but neither of them were equal to the bitherapy groups. The result also supported the existence of synergic effects of bitherapy.

The rat model of ISO-induced MI has been widely used to evaluate several cardiac dysfunctions. Previous studies have shown that the pathophysiological changes following ISO administration are comparable to those taking place in human MI.[27] However, their mechanisms are still unclear and are considered to be related to lipid peroxidation, membrane permeability, oxygen consumption, and calcium overload.[28, 29]

The results showed that the changes of the J point of ECG and the degree of the myocardial enzyme were significantly increased in the model group, which suggested myocardial ischemia and cell damage had occurred. In experimental myocardial ischemia, the degrees of myocardial enzyme release and ECG's J point change were considered important indicators of the degree of myocardial damage. Compared to the model group, the different combinations of ginseng and *Danshen* could obviously inhibit the change of J point, decrease the serum LDH, CK, and MDA levels, increase SOD activity, and decrease infarct size. There was no significant difference among 6:4, 7:3, and 8:2 groups ($p > 0.05$). The combination of ginseng and *Danshen* at the ratio of 7:3 proved to be most effective on MI by comprehensive evaluation of all the data.

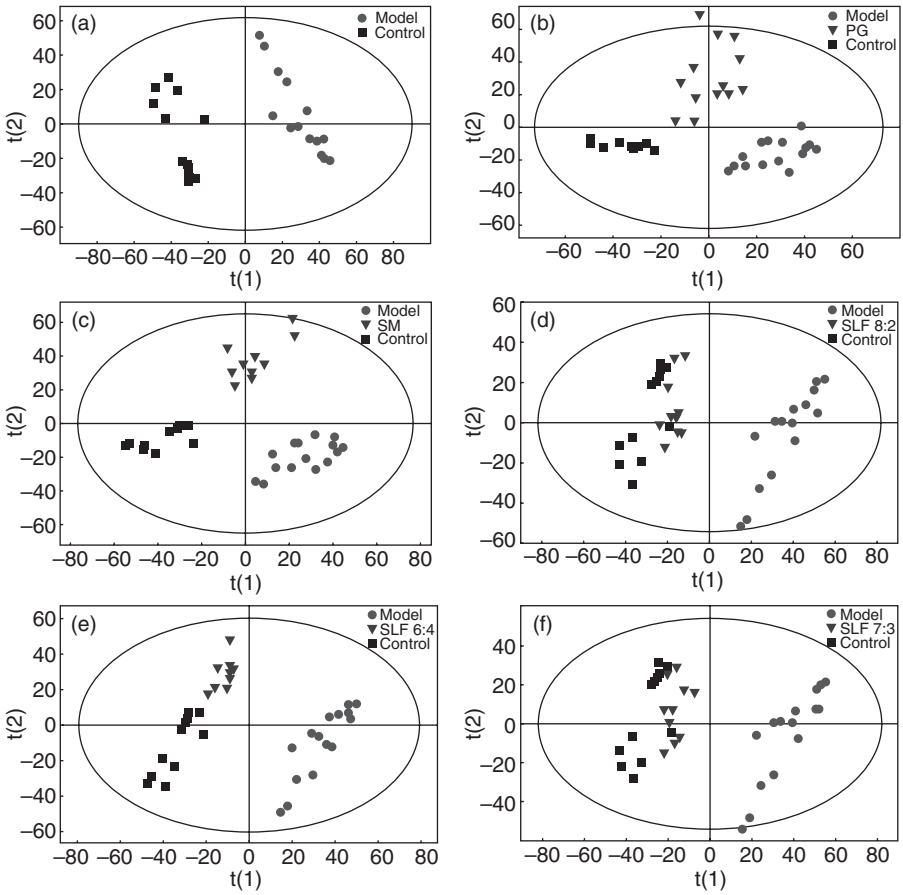

Fig. 11.5 PCA score plots of ISO-induced MI rat serum fingerprints.

11.3.1.3 Metabonomic Results and Discussion

11.3.1.3.1 PCA Analysis Metabonomic results of isoproterenol-induced MI rats are shown in Fig. 11.5. The metabolic state of model group was far away from the normal control group (Fig. 11.5A), indicating the success of ischemia model. Both ginseng group (Fig. 11.5B) and *Danshen* group (Fig. 11.5C) were mixed with the model group and away from the control group. Among the groups of control, model, and different combinations of ginseng and *Danshen* (8:2, 7:3, and 6:4), the group of 7:3 (as original ratio as SLF) was most similar to the control group but different from the model group, as shown in Fig. 11.5D–F. These results showed that the therapeutic effect was different among the treatment groups and the combination-based treatment groups were better than the single drug (ginseng or *Danshen*) groups. Furthermore, the combination of ginseng and *Danshen* at a ratio of 7:3 proved to have the best therapeutic efficacy on MI rats.

11.3.1.3.2 Potential Biomarkers and Pathways Thirteen lipid species, including Lyso-PCs and fatty acids as potential biomarkers of the control and ISO-induced rats were identified, as shown in Table 11.7. The biochemical mechanisms of those relevant metabolites as well as the formation of MI were then investigated.

The metabonomic analysis conducted in the present study showed ISO-induced changes in the profiles of certain endogenous metabolites. In Table 11.7, most Lyso-PCs were downregulated by ISO intervention, while the levels of fatty acids were changed variously. These results suggested that an impaired lipid metabolism plays a deleterious role in the formation of MI.

Lyso-PCs, as endogenous phospholipids, are very important in the pathophysiological change of myocardial tissue. Fatty acids are important constituents of all cell membranes, including endothelial and myocardial cells. As shown in Table 11.7, a decrease in Lyso-PCs and various alterations in fatty acids in ISO-treated rats might have been due to the breakdown of membrane phospholipids. Accelerated phospholipids degradation has been previously reported to cause myocardial injury in ISO-treated rats. These alterations may ascribe to the activation of phospholipase A_2, which mediates the release of specific fatty acids from Lyso-PCs. It could be presumed that several biological factors, such as the activation of protein kinase C (PKC) pathway, enhance the activity of phospholipase A_2 and reduce the Lyso-PCs generation. Several studies have also shown that the phospholopase A_2 in the heart was selective for arachidonic acid containing phospholipids. The elevations of arachidonic acid in serum may contribute to lipotoxicity in myocardium. In any event, the various disorders of fatty acids as well as Lyso-PCs degradation indicate an underlying lipid disturbance is present in ISO-induced rat model that is well associated with the formation of MI.

11.3.2 Screening Effective Ingredients in SLF

The serum-chemical and chemomics research results indicated that ginsenosides and salvianolic acids might be the effective components of SLF for MI. To further verify the above deduction, the two herbs (ginseng and *Danshen*) in SLF were divided into four parts: TGS, the remainder of ginseng (RPG, mainly containing panaxan, TGS removed), TSA, and the remainder of *Danshen* (RSM, mainly containing diterpenoid quinones, TSA removed). After cross combinations (equal to the ratio of 7:3 of ginseng to *Danshen*), their efficacies were evaluated using an ISO-induced MI rat model.

11.3.2.1 Method Male Sprague-Dawley rats (200 ± 20) were obtained from the center of laboratory animals at Tsinghua University. The animals were randomly divided as shown in Table 11.8. The rats were anesthetized with urethane (1.3 g/kg, IP) 1 h after 7 days. Before starting the injection of ISO (10 mg/kg), 5 min of normal ECG recordings were made. The ECGs of all animals after ISO injection at 20 min were recorded and compared. After

TABLE 11.7 Identification of Significantly Differential Endogenous Metabolites in the Serum of ISO-Induced MI Rat Treated with SLF

No.	R. T. (min)	Selected Ion	Mass (m/z)	Elemental Composition	Model	SLF	Control	Identification Results
1	6.4	$[M+COOH]^-$	564.3301	$C_{27}H_{51}NO_9P$	4464.96(↓)	6072.78	5226.69	Lyso-PC (C18:2)
2	6.6	$[M+COOH]^-$	612.3301	$C_{31}H_{51}NO_9P$	5409.74(↓)	6267.72	5227.34	Lyso-PC (C22:6)
3	6.7	$[M+COOH]^-$	588.3301	$C_{29}H_{51}NO_9P$	14244.8(↓)	11226.9	12514.7	Lyso-PC (C20:4)
4	7.5	$[M+COOH]^-$	540.3301	$C_{25}H_{51}NO_9P$	59455.1(↓)	46986.5	52586.8	Lyso-PC (C16:0)
5	8.0	$[M+COOH]^-$	566.3458	$C_{27}H_{53}NO_9P$	8658.46(↓)	9983.50	10279.8	Lyso-PC (C18:1)
6	9.1	$[M+COOH]^-$	640.3128	$C_{13}H_{20}N_5O_{16}P_3$	3276.38(↑)	2960.55	3331.33	Unknown
7	9.9	$[M-H]^-$	480.3090	$C_{23}H_{47}NO_7P$	1128.58(↓)	4019.89	3923.95	Lyso-PC (C15:1)
8	10.0	$[M+COOH]^-$	568.3614	$C_{27}H_{55}NO_9P$	68080.1(↑)	41194.1	53028.9	Lyso-PC (C18:0)
9	11.8	$[M-H]^-$	327.2329	$C_{22}H_{31}O_2$	6215.17(↓)	6743.73	7392.36	Docosahexaenoic acid
10	12.1	$[M-H]^-$	303.2329	$C_{20}H_{31}O_2$	5406.36(↑)	4245.12	4505.60	Arachidonic acid
11	12.4	$[M-H]^-$	279.2329	$C_{18}H_{31}O_2$	3433.98(↓)	4481.87	4743.54	Linoleic acid
12	13.8	$[M-H]^-$	255.2339	$C_{16}H_{31}O_2$	3294.71(↑)	1765.8	1848.60	Palmitic acid
13	14.3	$[M-H]^-$	281.2486	$C_{18}H_{33}O_2$	2458.71(↓)	3055.35	3587.81	Oleic acid
14	16.4	$[M-H]^-$	283.2637	$C_{18}H_{35}O_2$	4841.94(↑)	3570.76	3449.11	Stearic acid

"↑" represents a higher level of metabolites; "↓" represents a lower level of metabolites compared to the model group.

TABLE 11.8 Dosage and Compatibility

No.	Group	Animals	Dose (mg/kg)
1	Model	10	–
2	Control	10	–
3	TGS + TSA	10	99:107
4	TGS + TSA + RPG	10	99:107:743
5	TGS + TSA + RSM	10	99:107:224
6	TSA + RPG + RSM	10	99:743:224
7	TGS + RPG + RSM	10	107:743:224
8	RPG + RSM	10	743:224
9	TGS + TSA + RPG + RSM	10	99:107:743:224
10	Positive control drug	10	50

TABLE 11.9 Effects of SLF Extracts on 2 min ΔJ of ECG in MI Rats Induced by Isoproterenol ($x \pm s$, $n = 10$)

No.	Group	ΔJ (mv)	T test
1	Model group	–0.1347	
2	Control group	0.0090	$p < 0.001$
3	TGS + TSA	–0.0421	$p < 0.01$
4	TGS + TSA + RPG	–0.0404	$p < 0.01$
5	TGS + TSA + RSM	–0.0625	$p < 0.05$
6	TSA + RPG + RSM	–0.0986	$p > 0.05$
7	TGS + RPG + RSM	–0.1480	$p > 0.05$
8	RPG + RSM	–0.1613	$p > 0.05$
9	TGS + TSA + RPG + RSM	–0.0194	$p < 0.01$
10	Positive control	–0.0881	$p > 0.05$

TGS, total ginsenosides; RPG, the remainder of ginseng (mainly containing panaxan, TGS removed); TSA, total salvianolic acids; RSM, the remainder of *Danshen* (mainly containing diterpenoid quinones, TSA removed). Positive control drug: Diltiazem. $p < 0.05$, $p < 0.01$, $p < 0.001$ versus model group.

ECG measurements, blood was collected from the inferior caval vein and then centrifuged. Supernatant were obtained for CK, LDH, and NEFA measurement.

11.3.2.2 Pharmacodynamic Results and Discussion

11.3.2.2.1 Effect on the ECG of MI Rats The ΔJ point of the rats' ECG was shown in Table 11.9. After ISO injection, the J point of ECG was significantly changed. Compared with the model group, the ΔJ of the control, TGS + TSA, TGS + TSA + RPG, TGS + TSA + RSM, and TGS + TSA + RPG + RSM groups were significantly decreased ($p < 0.05$), whereas there were no significant differences ($p > 0.05$) in the TSA + RPG + RSM, TGS +

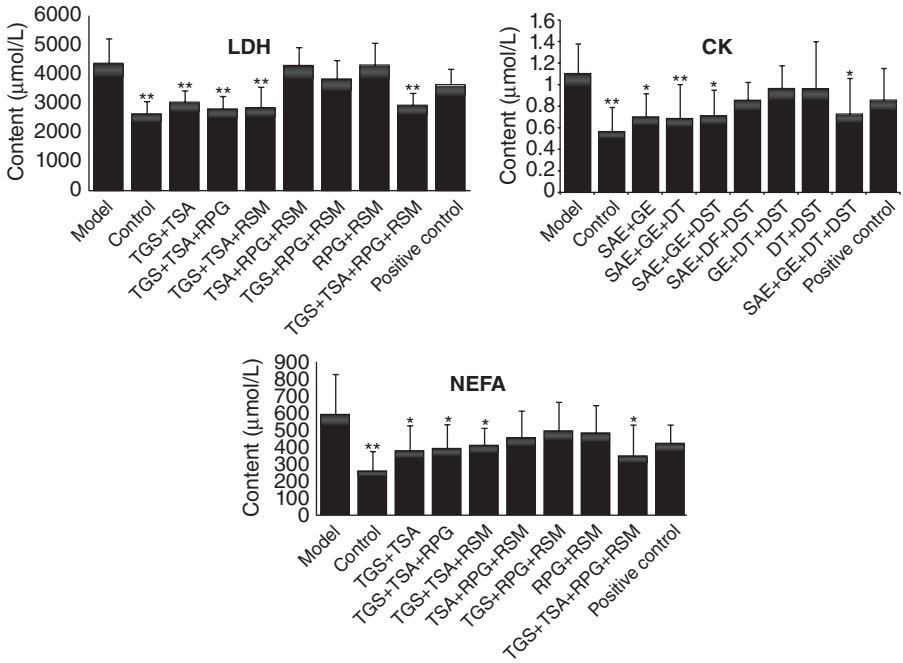

Fig. 11.6 Comparison of LDH, CK, and NEFA activity in serum of ISO-induced MI rats administered different combinations. TGS, total ginsenosides; RPG, the remainder of PG (mainly containing panaxan, TGS removed); TSA, total salvianolic acids; RSM, the remainder of SM (mainly containing diterpenoid quinones, TSA removed). *$p < 0.05$, **$p < 0.01$ versus model group.

RPG + RSM, RPG + RSM, and positive control group. Among the treatment groups, the combination groups including TGS + TSA performed the best in the inhibition of the J point change of MI rats.

11.3.2.2.2 Serum Biochemical Measurement As shown in Fig. 11.6, a significant reduction ($p < 0.05$) in CK, LDH, and NEFA levels was observed in rats treated with TGS + TSA, TGS + TSA + RPG, TGS + TSA + RSM, and TGS + TSA + RPG + RSM compared with the model group. There were no statistically significant differences ($p > 0.05$) in the TSA + RPG + RSM, TGS + RPG + RSM, and RPG + RSM groups compared with the model group. The activities of TGS + TSA were almost the same as those of the TGS + TSA + RPG + RSM group. Besides, the results were confirmed by the ECG and metabonomic studies.

The above results demonstrated that TGS + TSA had the same pharmacological activities as SLF and the removal of the remaining parts (RPG, RSM) had no significant effect on activities. Furthermore, the combination-based treatment groups in the absence of either or both ginsenosides and salvianolic

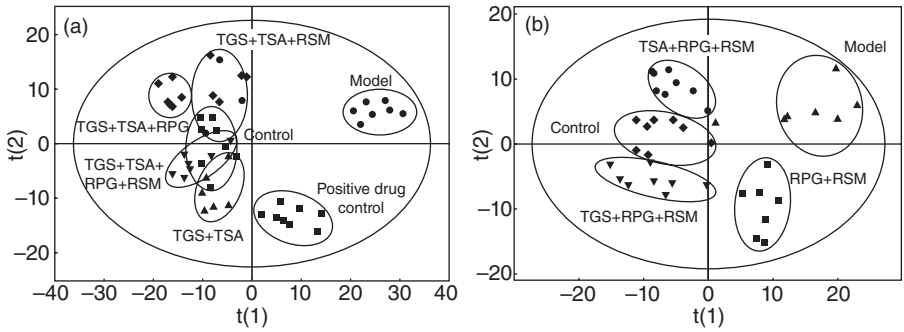

Fig. 11.7 PLS-DA score plots of rat serum after administration. A: PLS-DA score plot of rat serum from control, model, positive control, and groups containing TGS and TSA; B: PLS-DA score plot of rat serum data from control, model, and TGS and/or TSA groups.

acids had little anti-ischemic efficacy. All the results proved that ginsenosides and salvianolic acids were the effective components of SLF for MI.

11.3.2.3 Results of Metabonomic Study

11.3.2.3.1 PLS-DA Analysis The serum samples of the 10 group rats were analyzed by UPLC/TOF-MS and the data were processed by PLS-DA in the software SIMCA-P package. Fig. 11.7A shows that clear separation of the control and model rats could be observed, indicating that there was significant difference in serum samples collected from the two groups; the TGS + TSA + RSM, TGS + TSA + RPG, TGS + TSA + RSM + RPG, and TGS + TSA groups were mixed with the normal control but away from the model group, indicating recovery of the disturbed metabolism state; the positive control drug group was obviously away from the model group and close to the normal control group, indicating the recovery trend of the disturbed metabolism state. Fig. 11.7B indicates that the TGS + RSM + RPG and TSA + RSM + RPG groups moved to the normal control group and the RSM + RPG was far away from the normal control group, indicating little or no anti-ischemic efficacy. The results suggested that the activities of TGS + TSA were almost the same as those of the TGS + TSA + RSM + RPG group and the ginsenosides and salvianolic acids were the effective components of SLF for MI.

11.3.2.3.2 Identification of Potential Biomarker Variables that significantly contributed to the clustering and discrimination were identified according to a threshold of VIP in the projection values (VIP > 1). As shown in Table 11.10, among the 14 variables, 12 were identified on the basis of accurate elemental compositions, available databases, and available reference standards. These identified significantly differential endogenous metabolites were lysophosphatidylcholines (Lyso-PC) and fatty acids, indicating that an impaired lipid

TABLE 11.10 Identification of Potential Biomarkers

No.	RT (min)	Selected Ion	Exact Mass(Da)	Composition	Identification
1	2.41		723.50		unknown
2	7.49	$[M+HCOO]^-$	540.3316	$C_{25}H_{51}NO_9P$	Lyso-PC (C16:0) (↓)
3	9.92	$[M+HCOO]^-$	568.3623	$C_{27}H_{53}NO_9P$	Lyso-PC (C18:0) (↓)
4	6.68	$[M+HCOO]^-$	564.3318	$C_{27}H_{51}NO_9P$	Lyso-PC (C18:2) (↓)
5	2.58		949.671		Unknown
7	18.59	$[M+HCOO]^-$	465.3023	$C_{27}H_{48}O_3$	3α,7α,26-trihydroxy-5β-cholstane(↑)
8	6.69	$[M+HCOO]^-$	588.3317	$C_{29}H_{51}NO_9P$	Lyso-PC (C20:4) (↓)
9	8.03	$[M+HCOO]^-$	566.3473	$C_{27}H_{53}NO_9P$	Lyso-PC (C18:1) (↓)
10	6.62	$[M+HCOO]^-$	612.3319	$C_{31}H_{51}NO_9P$	Lyso-PC (C22:6) (↓)
11	12.29	$[M-H]^-$	279.2331	$C_{18}H_{31}O_2$	Linoleic acid (↑)
12	14.19	$[M-H]^-$	281.2470	$C_{18}H_{33}O_2$	Oleinic acid (↑)
13	9.83	$[M+HCOO]^-$	480.3079	$C_{23}H_{47}NO_9P$	Lyso-PC (C15:1) (↓)
14	12.03	$[M-H]^-$	339.2315	$C_{22}H_{44}O_2$	Docosanoic acid (↓)

"↑" represents a higher level of metabolites; "↓" represents a lower level of metabolites compared to the model group.

metabolism (including phospholipid and fatty acid metabolisms) plays a deleterious role in the formation of MI.

11.3.3 Pharmacodynamic Validation of NSLF6

Based on the screening above, we obtained a simplified prescription, combination of TGS and TSA in equal ratio as present in the original formula SLF, named "NSLF6." In this section, the myocardial infarct area, serum enzyme activities, and the vascular surface density of the infarct area of the MI rats were studied to further evaluate and validate the efficacy of NSLF6 on experimental myocardial infarction.

11.3.3.1 Method Diltiazem was used as the positive control drug. The myocardial infarction of rats was performed by ligating the left ventricular coronary artery, as described previously.[30] MI rats were randomly divided into three groups: NSLF6 group, the positive control drug group, and the model group; being force fed 0.5% CMC-Na solution of NSLF6 (5 g/kg·w·d), diltiazem (50 mg/kg·w·d), and the same volume of vehicle (equal to 10 mL/kg·w·d), respectively. Additionally, there were the sham surgery group and the control group, force fed the same volume of 0.5% CMC-Na solution as in the model group. The five animal groups were administered to as above for 14 consecutive days.

Samples of urine at 14:00 to 20:00 were collected on days 1, 7, and 14. The fresh urine samples were immediately centrifuged at 3500 rpm for 10 min at room temperature, to remove particle contaminants, and the supernatants

were stored at –80°C until UPLC/TOF-MS analysis. After that, the rats were anesthetized and subjected to necropsy. Blood samples were collected from the inferior caval vein after 1 h of the final administration and then centrifuged at 3500 rpm for 10 min at 4°C. The supernatant obtained was frozen immediately, stored at –80°C, and thawed before analysis. The rats' hearts were taken out immediately and fixed in 10% formalin. Serum concentrations of LDH and CK were measured using an enzymatic UV test following the instructions of commercial assay kits.

11.3.3.2 Results of Pharmacodynamic Study

11.3.3.2.1 Effects of NSLF6 on Myocardial Infarct Rats' Enzymes On day 14, biochemical measurements showed a significant elevation in the levels of CK and LDH in the model group as compared to controls and the sham surgery group (Table 11.11), indicating the success of ischemia model after coronary artery ligation and lower levels of self-recovery. Among treatment groups, a statistically significant restoration in CK and LDH levels was observed in rats treated with NSLF6 compared with those treated with the positive control drug. These results demonstrated that NSLF6 performed well in recovering the clinical biochemistry in the MI model.[31]

11.3.3.2.2 Effects of NSLF6 on Myocardial Infarction The heart samples were fixed and embedded in paraffin wax, and 4–5 μm of histological sections of the paraffin-embedded tissues were stained with hematoxylin-eosin. The sections were examined under light microscope, and photomicrographs were taken. The areas of MI of the left ventricle were detected with a Leica image analysis system (10×), and infarct size (%) was expressed as myocardial infarct size/left ventricular area × 100%.

As shown in Table 11.12, compared with the model group, the infarct areas of the NSLF6 group ($p < 0.01$) were significantly reduced. The positive control drug reduced infarct size in MI rats to a certain degree, but there were no

TABLE 11.11 Effects of NSLF6 on Activities of LDH and CK in Serum on MI Rats Induced by Coronary Artery Ligation ($x \pm sd$)

Group	Dose (g/kg·d)	Sample	CK (μmol·ml^{-1})	LDH (μmol·ml^{-1})
Model	–	9	2.15 ± 0.43$^{\#}$	15.60 ± 2.75$^{\#}$
Control	–	10	0.81 ± 0.53	8.28 ± 3.20
Sham surgery	–	10	1.36 ± 0.35**$^{\#}$	8.82 ± 4.07**
NSLF6	5	11	0.86 ± 0.32**	9.02 ± 2.20**
Positive control	0.05	7	1.37 ± 0.39**	12.43 ± 1.69*$^{\#}$

Positive control drug: diltiazem.
*$p < 0.05$.
**$p < 0.01$ versus model group.
$^{\#}p < 0.05$ versus control.

TABLE 11.12 Effects of NSLF6 on Cardiac Infarction Area in MI Rats Induced by Coronary Artery Ligation ($x \pm sd$)

Group	Dose (g/kg·d)	Samples	Cardiac Infarction Area (%)
Model	–	10	22.04 ± 3.84
NSLF6	5	10	9.16 ± 1.37*#
Positive control	0.05	10	16.05 ± 3.71

Positive control drug: diltiazem.
*$p < 0.01$ versus model group.
#$p < 0.05$ versus control.

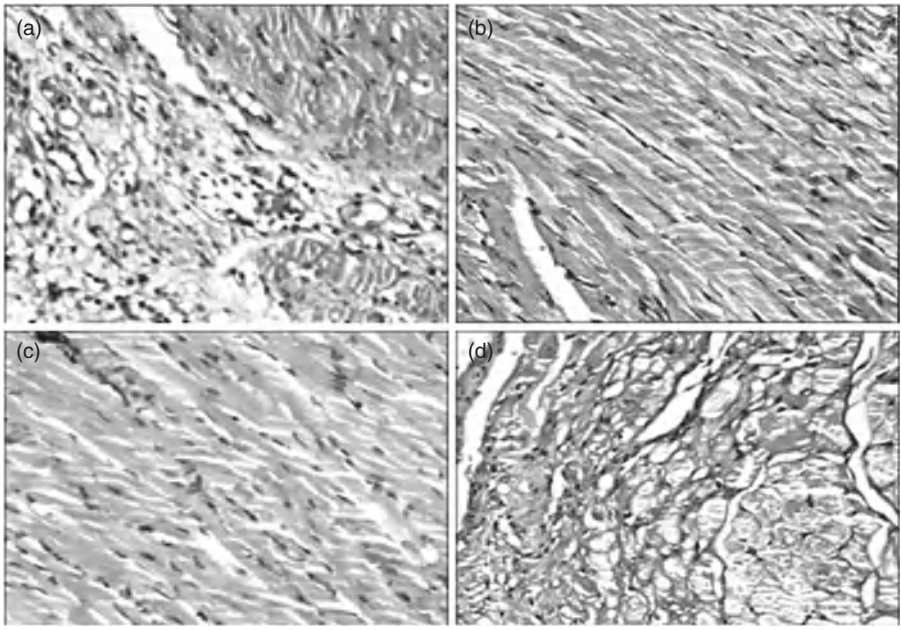

Fig. 11.8 Effect of NSLF6 on coronary artery ligation-induced myocardial injury. Hematoxylin and eosin staining was used to visualize the formalin-fixed sections of cardiac muscle tissue of rats (magnification 100 diameters). A: model group; B: sham surgery group; C: NSLF6 group; D: positive control drug group. (See color insert.)

significant differences ($p > 0.05$). There were no infarct areas in the sham surgery group. The above observations suggested that NSLF6 did have some therapeutic efficacy on MI.

As shown in Fig. 11.8A, histopathological examination of myocardial tissue of the model group showed clear myonecrotic areas. There was complete myonecrosis with fibroblastic proliferation and presence of chronic inflammatory cells. Moreover, marked edema and vacuolar changes along with subendocardial myonecrotic patches were clearly visible in the model group. However, in the sham surgery group (Fig. 11.8B), integrity of myocardial cell

membrane was observed with the exception of some striated muscle rupture. In the positive control drug group (Fig. 11.8D), focal myonecrosis with myophagocytosis and lymphocytic infiltration (myocarditis) were observed, whereas in the NSLF6 group (Fig. 11.8C), no confluent area of multiple subendocardial damage was seen and a reduction in inflammatory cells was observed. The above observations suggested that the NSLF6 and the positive control drug groups could decrease myocardial damage, but the NSLF6 group exerted better effects on MI compared with the positive control drug group.

11.3.3.2.3 NSLF6 Promotes Angiogenesis in MI Rats After routine dehydration, the heart samples fixed in 10% formalin were embedded in paraffin wax and then sliced. The histological sections were stained with factor VIII immunochemistry and then examined under Anymicro DSS image analysis system. The number of vessels of the left ventricle was measured by calculating the positive stained factor VIII, and the number of vessels per square millimeter of the surface density was measured by randomly observing five horizons per slice.

As shown in Table 11.13, compared with the sham surgery group, the vascular density of the model group was not statistically significant, indicating that the spontaneous compensatory angiogenesis was not developed after myocardial infarction. Compared to the model group, there were significant differences ($p < 0.05$) in the vascular density of treatment groups, suggesting both NSLF6 and the positive control drug could promote angiogenesis in infarcted areas.

This study demonstrated that NSLF6 could promote the formation of coronary collateral vessels, protect ischemic myocardium, and reduce infarct area. Ginsenoside Rg_1 and Re were reported to promote the growth of vascular endothelial factor,[32, 33] indicating that the effects of angiogenesis of NSLF6 was from ginsenoside Rg_1 and Re.

NSLF6 could significantly reduce the CK and LDH activities of MI rats induced by coronary artery ligation, the degree of myocardial injury, and myocardial infarct size, as well as promote angiogenesis and increase myocar-

TABLE 11.13 Effect of NSLF6 on Angiogenesis in MI Rats ($x \pm sd$)

Group	Dose ($g \cdot kg^{-1}$)	Samples	Area Density of Vessels (one/mm^2)
Model	–	5	51.1 ± 18.3
Sham surgery	–	5	50.2 ± 12.9
NSLF6	5	5	111.5 ± 13.3**#
Diltiazem hydrochloride	0.05	5	77.5 ± 9.7*

Compared with the model group, *$p < 0.05$, **$p < 0.01$; compared with the diltiazem hydrochloride group, #$p < 0.05$.

dial blood supply. The data showed that NSLF6 has obvious therapeutic efficacy on rat MI induced by coronary artery ligation.

11.3.3.3 Metabonomic Analysis

11.3.3.3.1 Partial Squares-Discriminant Analysis The activities of NSLF6 were evaluated by metabonomics using MI rats induced by coronary artery ligation. Fig. 11.9A shows that the endogenous metabolites in the model group were obviously different from those in the normal control group, indicating the success of ischemia model. After two weeks' survival without medication (Fig. 11.9B), the trajectory of the model group was far away from the position of the normal control, which excluded the possibility of self-cure of the MI rats during the experiment. As shown in Fig. 11.9C, the metabolic state of the NSLF6 group was far away from the initial position on the first day after coronary artery ligation while the trajectory direction gradually moved to the initial space during two weeks' medication, indicating the recovery of the disturbed metabolism state by NSLF6. In Fig. 11.9D, both the NSLF6 and positive control groups moved to the normal control group, but the NSLF6

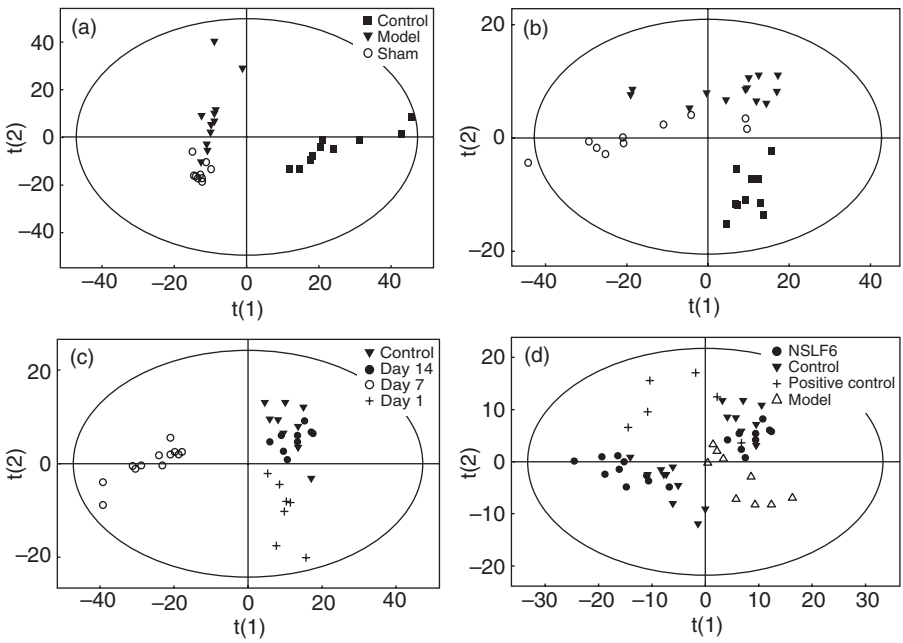

Fig. 11.9 PLS-DA scores plots of rat urine data. A: dynamic mean-centered PLS-DA score plot of rat urine data of model, sham surgery, and control groups on day 1; B: dynamic mean-centered PLS-DA score plot of rat urine data of model, sham surgery, and control groups on day 14; C: dynamic mean-centered PLS-DA score plot of rat urine data of NSLF6 group on days 0, 1, 7, and 14; D: dynamic mean-centered PLS-DA score plot of rat urine data of NSLF6 and positive control drug groups on day 14.

group exhibited better performance in the recovery of metabolism than the positive control group. These results suggested that NSLF6 had a significant efficacy in coronary artery ligation induced MI rats.

11.3.3.3.2 Potential Biomarkers and Pathways Related to MI Variables (metabolites) that significantly contributed to the clustering and discrimination were identified according to a threshold of variable importance in the projection values (VIP > 1), which could be generated after partial squares-discriminant analysis (PLS-DA) processing. As shown in Table 11.14, 17 metabolites were identified on the basis of accurate elemental compositions and context of retention time with available databases.

In addition, most of the interested metabolites were involved in metabolic processes related to myocardial energy metabolism, including the tricarboxylic acid (TCA) cycle (citrate and oxalosuccinate), pentose phosphate pathway (6-phosphogluconic acid and D-glucuronic acid 1-phosphate), and amino acid metabolism (*N*-acetyl-DL-tryptophan and *N*-acetylglutamine). Under the condition of MI, myocardial blood flow became inadequate to meet oxygen demand and myocardial ischemia developed, resulting in reduced formation of adenosine triphosphate (ATP) via aerobic mechanisms and accelerated anaerobic ATP production by glycolysis. Meanwhile, the levels of citrate and oxalosuccinate in TCA cycle were decreased, whereas the lactate[34, 35] via glycolysis was increased. The lactate represents the end product of anaerobic or nonoxidative glycolysis and has been used as a marker of ischemia in patients and in experimental animal studies.[36, 37] Increased 6-phosphogluconic acid and D-glucuronic acid 1-phosphate suggested a pentose phosphate pathway transferred to the energy supply pathway from the main reducing power (NADPH) productive pathway when the TCA cycle was blocked. Furthermore, the TCA cycle was not only the pathway of sugar decomposition but also the pathway of oxidation of fuel molecules, such as fatty acids and amino acids. Therefore, the reduction of TCA cycle intermediates also led to the reduction of fatty acid and amino acid metabolites, such as *N*-acetyl-DL-tryptophan and *N*-acetylglutamine.

In conclusion, myocardial metabolism was dramatically altered by myocardial ischemia. The rates of oxygen consumption and ATP production were reduced, leading to a reduction in ATP content, high rates of glycolysis, lactate accumulation, and decreased intracellar pH.

11.3.4 Dose-Dependent Efficacy of NSLF6

To compare the metabolic differences between the NSLF6 in low dose and high dose, the MS data were processed by PCA. Fig. 11.10 indicates the low dose group showed a trend away from the model group and toward the control group. The group receiving NSLF6 in high doses trended away from the model group. The above results showed that NSLF6 in high doses exerted better performance in protecting the ISO-induced myocardial ischemia and was

TABLE 11.14 Identification of Significantly Differential Endogenous Metabolites in the Urine of Coronary Artery Ligation Induced MI Rats Treated with NSLF6

ID	RT (min)	Selected Ion	Mass(m/z)	Elemental Composition	Identification Results	Control	Day 1	Day 7	Day 14
1	9.08	$[M-H]^-$	595.2056	$C_{36}H_{40}N_2O_6$	Urobilinogen	333.26	49.00↓	160.01	243.93
2	4.30	$[M-H]^-$	192.0601	$C_{10}H_{11}NO_3$	2-Methylhippuric acid	145.98	82.20↓	105.23	123.35
3	8.77	$[M-H]^-$	201.0161	$C_7H_7O_5P$	Benzoylphosphate	145.34	31.57↓	94.71	100.68
4	2.73	$[M-H]^-$	211.9975	$C_8H_7NO_4S$	L-Aspartyl-4-phosphate	321.96	67.04↓	193.13	329.32
5	3.79	$[M-H]^-$	242.9937	$C_9H_{12}N_2O_6$	Unknown	13.49	80.64↑	60.24	48.76
6	3.34	$[M-H]^-$	245.0096	$C_{13}H_{14}NO_3$	*N*-Acetyl-D-tryptophan	355.00	36.98↓	267.41	260.32
7	0.66	$[M+COOH]^-$	254.9791	$C_6H_{10}O_8$	Glucaric acid	121.55	184.73↑	111.77	102.43
8	3.21	$[M-H]^-$	273.0051	$C_6H_{11}O_{10}$ P	D-Glucuronic acid 1-phosphate	114.43	349.74↑	104.20	102.71
9	5.98	$[M-H]^-$	283.0793	$C_{10}H_{12}N_4O_6$	Xanthosine	235.83	45.36↓	137.40	182.92
10	4.29	$[M+COOH]^-$	385.1398		Unknown	255.59	176.35↓	169.79	220.05
11	5.96	$[M-H]^-$	567.1706	$C_{30}H_{48}O_{10}$	Deoxycholic acid 3-glucuronide	239.91	51.74↓	155.13	220.57
12	4.46	$[M-H]^-$	648.9041	$C_{10}H_{17}N_5O_{17}P_4$	*N*-Ligoceroylsphingosine	145.93	57.72↓	76.32	103.78
13	9.09	$[M-H]^-$	297.0969	$C_{19}H_{38}O_2$	Nonadecanoic acid	298.85	59.01↓	211.02	286.32
14	9.12	$[M+COOH]^-$	417.1176		Unknown	38.34	90.25↑	77.65	53.16
15	1.62	$[M+COOH]^-$	246.9890	$C_8H_{14}N_2O_4$	Sebacic acid	9.43	77.68↑	14.16	8.97
16	2.68	$[M-H]^-$	330.0281	$C_{10}H_{14}N_5O_6P$	Deoxyadensine monophosphate	3.01	58.59↑	10.64	1.16
17	3.21	$[M-H]^-$	277.0214	$C_{12}H_{15}N_4O_2S$	Isovaleryglucuronide	64.31	28.19↓	32.51	44.63
18	3.21	$[M-H]^-$	357.1075	$C_{11}H_{23}N_2O_7PS$	2-Phenylaminoadenosine	193.63	41.04↓	118.15	158.43
19	3.24	$[M-H]^-$	275.0216	$C_6H_{13}O_{10}P$	6-Phosphogluconic acid	8.81	156.90↑	17.23	35.80
20	4.45	$[M-H]^-$	646.9079	$C_{42}H_{81}NO_3$	Ceramide	94.32	204.52↑	175.93	124.40
21	4.89	$[M+COOH]^-$	338.0870	$C_{10}H_{13}N_5O_5$	Guanosine	89.29	33.58↓	54.00	77.29
22	1.07	$[M-H]^-$	227.9934	$C_5H_{12}NO_7P$	5-phosphoribosylamine	45.35	20.41↓	30.31	47.05
23	1.87	$[M-H]^-$	188.9813	$C_6H_6O_7$	Oxalosuccinate	45.03	15.88↓	41.40	38.15
24	3.21	$[M-H]^-$	178.0415	$C_9H_9NO_3$	Hippuric acid	188.68	60.85↓	167.21	140.67
25	4.91	$[M-H]^-$	187.0008	$C_7H_8O_4S$	*N*-Acetylglutamine	84.50	20.92↓	29.80	74.60
26	3.70	$[M-H]^-$	336.070	$C_{11}H_{19}N_3O_7S$	S-(Hydroxymethyl)glutathione	69.98	27.69↓	57.63	41.43

Day 1, 7, and 14 refer to the time of treatment by NSLF6. "↑" represents a higher level of metabolites; "↓" represents a lower level of metabolites compared to the model group.

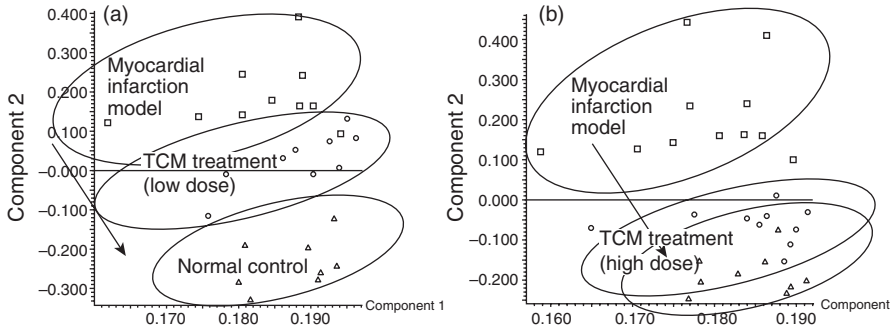

Fig. 11.10 PCA score plots of normal control, model, NSLF6 in low dose (A) and high dose (B) groups.

closer to the normal group, which suggested that NSLF6 treated the MI in a dose-dependent way.

11.4 SYSTEMS BIOLOGY STUDY OF THE MECHANISM OF DIRECTED DIFFERENTIATION OF STEM CELLS INDUCED BY NSLF6

Former pharmacodynamic studies showed that SLF alone or combined with stem cell transplantation could treat myocardial infarction. To further elucidate the mechanism, genomic, proteomic, and metabolic studies using the systems biology method were carried out to understand the role and mechanisms of SLF in stem cell directional differentiation.

11.4.1 Genomics Study of the Mechanism of Directed Differentiation of Stem Cells Induced by NSLF6

In this section, the action mechanism of rat bone marrow mesenchymal stem cells (BMMSCs) directly differentiated into the myocardial cells affected by NSLF6 was studied based on genomics by technologies of immunofluorescence focus imaging and gene chip and gene expression analysis of quantitative detection.

11.4.1.1 The Immunofluorescence Highlights Autoradiography Results Showed That SLF Induced the Differentiation of Stem Cells into Myocardial Cells A complete set of technology platforms of rat BMMSC separation, purification, culture, and drug-induced differentiations of stem cells into myocardial cells were established. And the specific methods of the operation below were reported in our previous work.[38–42] The rat BMMSCs were identified by flow cytometry, immunofluorescence, and cell morphology analysis, which showed that the cells used in experiments were the rat BMMSCs and maintained their characteristics in the proliferation.

BMMSCs were chosen as experimental materials and 5-aza as a positive control drug. Immunofluorescence highlight autoradiography technology was used to detect the cardiomyocyte-specific proteins expressions of Troponin I (cTnI) and myosin heavy chain (MHC). In this study, the BMMSCs were cultured with serum-containing drugs. Serum-containing drugs were divided into: "5-aza," "components" (total salvianolic acid + ginsenoside), "ingredients" (salvianolic acid B + ginsenoside Rb_1), "5-aza + components," and "5-aza + ingredients" group. Additionally, a "blank" (without any drugs) group was a negative control. In these groups, 5-aza was designed as a positive control, as it was reported that 5-aza could induce BMMSCs to differentiate into cardiomyocytes; components and ingredients were designed for verifying whether the TCM SLF could induce BMMSCs to differentiate into cardiomyocytes, or these two groups had the same effect in quantity; 5-aza + components and 5-aza + ingredients were designed for testing whether component or ingredient can enhance the inductive effect of 5-aza and which group was better. Meanwhile, to verify the specific reaction of cardiomyocyte-specific proteins, neonatal rat cardiomyocytes were chosen for the positive control experiment.

A number of papers reported that when induced by 5-aza after 20 days, BMMSCs began to differentiate into cardiomyocytes. Therefore, four time points, days 10, 20, 30, and 40, were chosen for the immunofluorescence highlights autoradiography technology test.

Fig. 11.11 shows that the cTnI and MHC proteins were expressed in purified rat cardiomyocytes and not expressed in the four time points in the blank group, consistent with previous reports. On day 10, in groups 5-aza, components, ingredients, and 5-aza + components, the expressions of cTnI and MHC were not observed. On day 20, weak expression was observed. On day 30, cTnI and MHC were obviously expressed in all groups but with different fluorescence intensity, the strength order of which was 5-aza, components, 5-aza + components, and 5-aza + ingredients. The results showed that the drugs in the groups components, ingredients, 5-aza + components, and 5-aza + ingredients could induce BMMSCs to differentiate into cardiomyocytes on day 20, which suggested that the SLF had the function of inducing BMMSCs to differentiate into cardiomyocytes. Moreover, a gene chip analysis was conducted in the following study to elucidate the mechanism of differentiation induced by SLF at the molecular level.

11.4.1.2 Cluster Analysis of Gene Chips and Gene Screening The immunofluorescence imaging can only answer the question of whether the BMMSCs differentiated into the cardiomyocyte-like cells. It cannot explain the mechanism of its transformation. Therefore, gene chip assay was further conducted. Although gene chip is a high-throughput microarray technology, its accuracy is limited. To explain the changes of differentially expressed genes screened by gene chip under the SLF, quantitative real-time PCR was used to quantify the key gene obtained by gene chips.

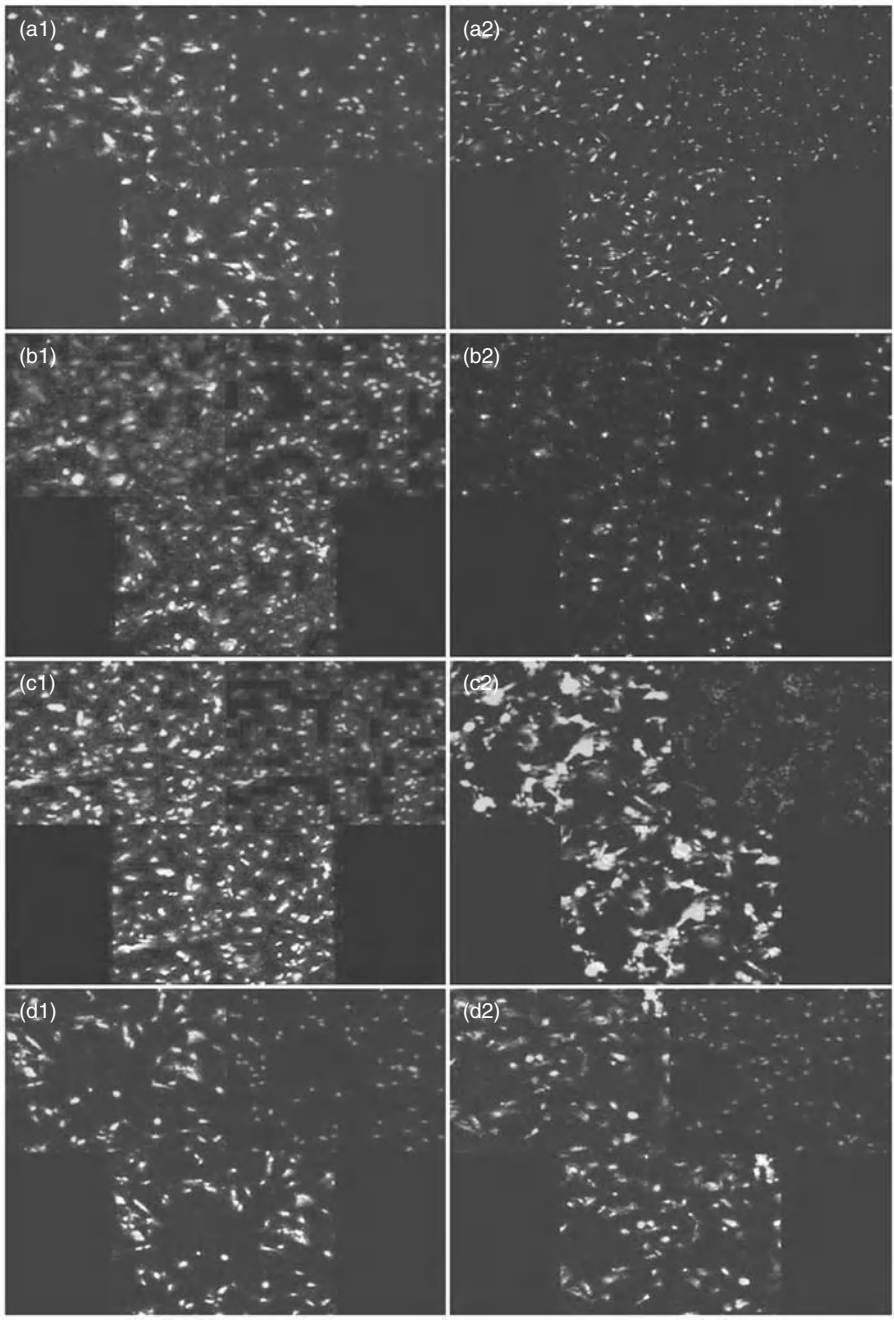
(a1)
(a2)
(b1)
(b2)
(c1)
(c2)
(d1)
(d2)

Fig. 11.11 Immunofluorescences of the MHC and cTnI of BMMSCs induced by different combinations of TCM on day 30. Each picture consists of three photos. The left superior one is a photo of fluorescent reaction antibody staining; the right superior one is a photo of PI nuclear staining; the lower one is a photo including both fluorescent antibody reaction staining and PI nuclear staining. The pictures marked 1 are results of MHC expression; the pictures marked 2 are results of cTnI expression. A, B, C, and D are, respectively, "ingredient" group, "ingredient + 5-aza" group, "component" group, and "component + 5-aza" group. (See color insert.)

11.4.1.2.1 Cluster Analysis of Gene Chips Meanwhile, the gene chip was used to screen differentially expressed genes from the six groups (blank, 5-aza, components, ingredients, 5-aza + components, and 5-aza + ingredients) at the four time points. The gene chip was rat genome Oligo-chip (5705) from Capitalbio Company. OmniGridTM microarray (Genomic Instrumentation Services, Inc., San Carlos, CA, USA) was used for Oligo-storeroom.

In this study, 12 rats' housekeeping genes were used as a positive control, 12 artificial synthetic genes with no homologues to rat genes of 70 mer oligo DNA as a negative control, and 8 yeast genes as external standard. The lattice was divided into 48 submatrices, each of which had 20 rows and 21 columns. The space between two points was 205 μm, and the diameter of each point was about 150 μm. Each gene duplicated three points. Meanwhile, in order to eliminate the differences between luciferase cy3 and cy5 labeled cDNA and the differences in the process of scanning, the exchange of fluorescence experiments in compound treatment and in rt-PCR were conducted. The cy3 labeled RNA of treated cells and cy5 labeled RNA of control cells were in the same group; the cy3 labeled RNA of control cells and cy5 labeled RNA of treated cells were in an another fluorescent exchanging group. The value of genes' change is equal to the average of these two groups. For each compound, two sets of independent experiments were conducted. Thus, two DNA chips were necessary for each compound to obtain its gene expression profile data.

The chips were scanned by LuxScan 10KA dual-channel laser scanner (CapitalBio Corporation) after washing, and the fluorescence image signal was processed by GenePix Pro 4.0 software (Axon Instruments Corporation) for data analysis, and it transformed the image signal to a digital signal; the digital signal was output by ratio manner. The data were processed by the cluster 3.0 software which is the most commonly used method of hierarchical clustering algorithm in the analysis of DNA microarray chips.

11.4.1.2.2 Results of Gene Screening Cluster analysis (Fig. 11.12) showed that the samples of days 10 and 20 congregated a category and days 30 and 40 constituted a category, indicating that gene chip results were consistent with the previous conclusion that the differentiation of BMMSC into cardiomyocytes induced by drugs took place when cultured for 20 days. Meanwhile, at time points of days 10 and 20, the components, ingredients, and 5-aza groups

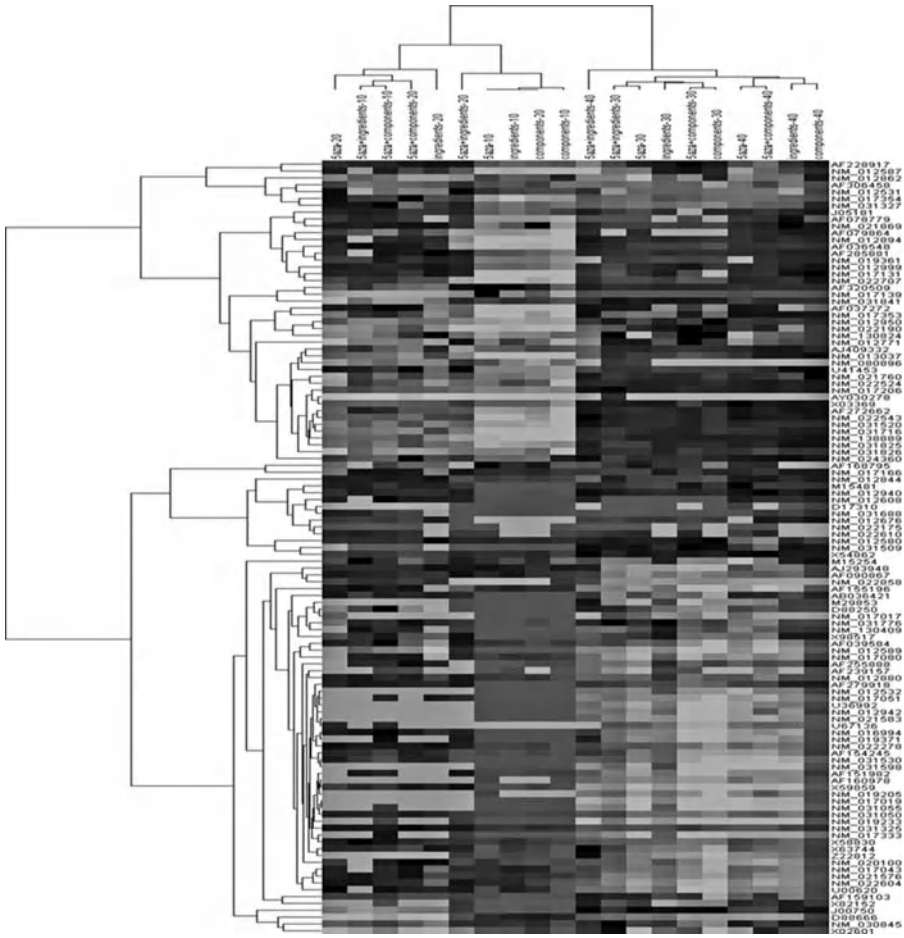

Fig. 11.12 Gene cluster analysis of differentiation of BMMSC into cardiomyocytes induced by drugs.

were closer to each other than to the other two groups, whereas at time points of days 30 and 40, the components and ingredients groups were similar while the other three groups had no cluster regularity.

In order to select the genes related to cell differentiation in a drug-induced process, the gene chips data were normalized using Lowess method, and finally the T-test approach was used to determine the differentially expressed genes at the standard of 95% significant difference and double expression. First, the ratio ≥ 2 or ratio ≤ 0.5 analysis were done for 12 sets of data from four time points and the three groups of 5-aza, components, and 5-aza + components, and 129 differentially expressed genes were selected. Meanwhile, 180 differentially expressed genes were selected in the groups of 5-aza, ingredients, and

TABLE 11.15 Four Differentially Expressed Genes in the "Ingredients" Group

GB.accession	Gene.name	Description
D17310	3αHSD	3-Alpha-hydroxysteroid dehydrogenase
NM_022175	PEM	Placentae and embryos oncofetal gene
NM_031688	SNCG	Synuclein, gamma
Z18877	2′5′-AS	2′5′ oligoadenylate synthetase

TABLE 11.16 Nine Differentially Expressed Genes in the "Components" Group

GB Accession	Gene Name	Description
NM_012589	Il6	Interleukin 6
NM_017019	Il1a	Interleukin 1 alpha
NM_019233	Ccl20	Chemokine (C-C motif) ligand 20
NM_022175	PEM	Placentae and embryos oncofetal gene
NM_031055	Mmp9	Matrix metallopeptidase 9
NM_031530	Ccl2	Chemokine (C-C motif) ligand 2
NM_031598	Pla2g2a	Phospholipase A2, group IIA
NM_031688	Sncg	Synuclein, gamma
Z18877	2′5′-AS	25 Oligoadenylate synthetase

5-aza + ingredients. According to international practices of 12 categories of gene function classification criteria (www.geneontology.org), the 129 and 180 selected genes were divided into 10 categories with the following functions: transport, transcription regulation, signaling, stress reaction, metabolism, development, cell organization and biogenesis, differentiation, cell cycle, cell adhesion, and apoptosis. On this basis, we proceeded analysis with the ratio ≥ 8 or ratio ≤ 0.125. Four significantly differentially expressed genes in component groups (as shown in Table 11.15) and nine genes in ingredient groups (as shown in Table 11.16) were further selected, of which three genes are the same in the two groups. Therefore, a total of 10 significantly differentially expressed genes were found as key genes. The functions of the 10 key genes were also attributable to the aforementioned 10 gene functions.

To understand the molecular mechanism of confocal immunofluorescence assay and gene chip cluster results, these 10 highly differentially expressed genes were quantified by real-time PCR.

11.4.1.3 Quantitative Detection of 10 Highly Differentially Expressed Genes

11.4.1.3.1 Quantitative Results of the Functional Gene The primers (Table 11.18) were designed for 10 selected genes, using the rat GAPDH as housekeeping gene. The quantitative detection of gene expression was conducted using RT-crystal-Cycler636 real-time PCR (Capitalbio). After the optimum reaction condition and composition for each gene was established, the PCR

products after amplification were purified. The concentration of purified PCR products was measured by ultraviolet spectrophotometer, and then the standard curve of each gene for quantitative detection was established. Based on this standard curve of known concentration, the absolute concentration of samples' gene expression was detected. To eliminate the operational errors between two samples, the absolute concentration of each gene was divided by concentration of housekeeping gene GAPDH. Thus, the rate of each gene was processed at a same benchmark, and the relative concentration was used in our study.

The results showed that the gene expression of the blank group changes with time, especially for Il6. The expression of Il6 significantly increased on day 30, which was considered to be the change of cells' own growth and reproduction. Therefore, this physiological change in gene expression was considered background noise. When we analyzed the data, the blank value was subtracted from the concentration of each sample first.

Based on the above calculation, the relative concentrations of the 10 selected genes were listed in Table 11.17. The concentrations of Il1a, Pla2g2a, Ccl20, and Ccl2 changed in the similar trend. The highest expression appeared on day 10 except in the components group, whereas the lowest expression showed on day 20, and the expression increased after 20 days. In the components group, the lowest expression appeared on day 30, and the genes showed rising trend on day 40. The functions of these four genes were reported as: Il1a was mainly involved in the process of regulation, cell cycle, fever, apoptosis, anti-apoptosis, chemotaxis, cellular signal transduction, cell proliferation, and negative regulation of cell proliferation[27, 43–45]; Ccl2 was involved in phosphorylation of protein amino acids, calcium steady-state, anti-apoptosis, chemotaxis, inflammation response, humoral immune response, cell adhesion, the G protein signal transduction, coupling cyclic nucleotide second messenger, JAK–STAT cascade, organ morphogenesis, and stimulation response[46–49]; Pla2g2a was related to lipid catabolism and the fat growth of embryos[50–55]; and Ccl20 was involved in chemotaxis, signal transduction, cellular signal transduction, and stimulation responses.[56] Meanwhile, the expression of the 5-aza group decreased on day 20, consistent with the result of confocal immunofluorescence assay, indicating that 20 days was the time point at which differentiation took place. Thus, we conclude that once these four genes fully unregulated in 10 days, it would affect the proteins and promote the differentiation on day 20 in spite of the genes' downregulation on day 20. Only the components group showed the lowest expression on day 30, and the confocal immunofluorescence assay results also showed that the components was better than the 5-aza on day 30. Based on the physiological function of four genes and the result of drug induction, the efficacy of TCM was considered to last longer.

The result of Mmp9 gene expression indicated that only the components group lasted upregulating in all four time points, and highest expression appeared on day 20, while other groups showed lowest expression on day 20.

TABLE 11.17 Quantitative Results of the 10 Highly Differentially Expressed Genes

Samples	Il1a	Il6	Pla2g2a	Mmp9	Sncg	Ccl20	Ccl2	PEM	2′5′-AS	3α-HSD
0 day-blank	1.72E-03	3.82E-02	8.64E-04	2.08E-04	1.78E-02	1.75E-02	1.87E-02	1.77E-01	2.69E-04	1.84E-02
10 day-blank	1.20E-03	3.85E-02	4.03E-04	5.71E-05	7.23E-04	−5.23E-02	7.34E-03	7.64E-03	−9.96E-07	8.13E-04
20 day-blank	3.46E-02	3.08E-01	1.56E-01	6.26E-03	1.73E-03	−2.49E-02	2.62E-01	3.61E-03	2.21E-06	9.70E-05
30 day-blank	2.32E-02	1.42E+00	6.93E-02	1.33E-03	7.96E-02	−1.50E-02	7.85E-02	5.37E-02	1.55E-05	1.89E-03
40 day-blank	7.94E-03	1.61E-02	1.71E-02	5.49E-04	1.24E-03	1.71E-02	1.16E-01	1.36E-01	1.11E-04	1.56E-03
10 day-5aza	1.47E-01	6.36E-02	2.87E-01	2.01E-02	8.31E-03	−7.88E-03	1.40E+00	7.50E-03	3.77E-05	2.26E-02
20 day-5aza	5.28E-04	7.07E-03	8.84E-04	1.75E-03	8.78E-04	−2.53E-02	1.41E-02	9.40E-03	1.02E-04	1.73E-04
30 day-5aza	3.95E-03	1.31E-01	6.14E-03	1.96E-03	1.55E-02	−1.43E-02	7.63E-02	1.69E-02	3.53E-05	4.95E-03
40 day-5aza	6.01E-04	5.49E-03	3.19E-03	7.42E-04	5.16E-03	3.37E-05	6.53E-05	2.05E-02	2.80E-06	1.40E-04
10 day-component	1.41E-01	1.40E+01	2.08E-01	6.82E-03	5.83E-03	−5.23E-02	5.87E-01	1.87E-02	−2.63E-07	9.60E-03
20 day-component	6.41E-02	1.69E-03	2.35E-01	1.66E-02	1.73E-03	−2.31E-02	8.06E-01	1.70E-02	6.40E-05	2.02E-03
30 day-component	1.10E-03	1.87E-02	2.18E-03	3.81E-03	9.05E-04	−1.49E-02	3.99E-02	8.22E-04	5.50E-05	3.59E-03
40 day-component	3.98E-03	5.38E-02	1.54E-02	1.78E-03	1.45E-04	9.30E-05	1.74E-01	2.48E-03	5.15E-06	1.59E-03
10 day-ingredient	5.12E-04	1.07E-02	3.40E-04	9.26E-04	3.60E-03	−5.23E-02	2.93E-02	2.06E-02	−1.20E-06	6.75E-04
20 day-ingredient	5.98E-04	2.65E-02	5.53E-04	1.59E-03	7.23E-03	−2.53E-02	9.56E-03	5.48E-02	3.91E-05	1.05E-04
30 day-ingredient	6.56E-03	8.79E-03	3.29E-02	3.19E-03	1.12E-02	−1.50E-02	1.84E-01	3.04E-02	1.08E-05	3.18E-03
40 day-ingredient	1.49E-03	4.18E-02	3.17E-03	9.30E-04	4.92E-03	9.16E-05	5.57E-02	1.27E-02	−2.43E-07	2.61E-03
10 day-component+5	9.72E-04	7.03E-03	2.85E-03	1.59E-03	1.64E-03	−5.22E-02	3.11E-01	2.94E-02	1.20E-05	6.59E-04
20 day-component+5	8.57E-04	2.76E-02	2.60E-04	8.82E-04	1.10E-03	−2.33E-02	1.56E-02	2.15E-02	1.10E-05	6.03E-05
30 day-component+5	1.42E-03	1.55E-01	4.87E-03	1.13E-03	1.52E-02	−1.47E-02	6.63E-02	2.10E-02	9.09E-05	2.79E-03
40 day-component+5	1.55E-03	6.16E-02	1.49E-03	4.41E-03	4.22E-03	1.75E-02	2.68E-02	1.77E-01	2.69E-04	7.08E-04
10 day-ingredient+5	1.18E-03	8.43E-02	2.04E-03	6.34E-04	1.02E-03	−5.23E-02	3.37E-02	7.64E-03	−9.96E-07	2.51E-04
20 day-ingredient+5	1.33E-03	2.83E-02	2.27E-03	4.29E-04	6.73E-03	−2.49E-02	1.57E-01	3.61E-03	2.21E-06	6.52E-04
30 day-ingredient+5	7.39E-03	6.49E-02	4.32E-03	2.09E-03	4.73E-03	−1.50E-02	1.86E-01	5.37E-02	1.55E-05	1.53E-03
40 day-ingredient+5	2.07E-03	4.43E-02	5.21E-03	1.92E-03	6.97E-03	1.71E-02	1.09E-01	1.36E-01	1.11E-04	8.11E-04

TABLE 11.18 Primer Sequence Information of Significantly Differentially Expressed Genes When BMMSCs Was Induced

Gene Name	Primer Type	Primer Sequence	Length of PCR Product
GAPDH	Upper	GCATCCTGGGCTACACTGAG	163 bp
	Lower	TCCACCACCCTGTTGCTGTA	
IL6	Upper	CAAGAAAGACAAAGCCAGAG	156 bp
	Lower	CTTAGCCACTCCTTCTGTGA	
IL1a	Upper	GGGAGGAGACGACTCTAAA	147 bp
	Lower	ATGAGGTCGGTCTCACTAC	
Ccl2	Upper	CTCAGCCAGATGCAGTTAA	151 bp
	Lower	TCTCTCTTGAGCTTGGTGA	
Ccl20	Upper	TGGGTTTCACAACACAGATG	143 bp
	Lower	CTTGGTTCTTAGGCTGAGGA	
Pem	Upper	ATCAGTGTGTCCAGAGTGCA	147 bp
	Lower	CATCTTACTCCCCATCTTGC	
Mmp9	Upper	TTGAAGTCTCAGAAGGTGGA	141 bp
	Lower	ACTCACACGCCAGAAGTATT	
Pla2g2a	Upper	TGGTGCTGTGTGACTCATG	159 bp
	Lower	AGCTTTATCGCACTGGCAC	
Sncg	Upper	GTCAGCAGCGTCAACACAGT	154 bp
	Lower	CTCCACTCTTGGCCTCTTC	
3αHSD	Upper	AAGCGGATCAAAGAGCTAA	137 bp
	Lower	GTAAATGGATGATTGGGATG	
22′5′-AS	Upper	GGTGGGAACCAAGAAGGCT	148 bp
	Lower	GGGTTCACAGCAGGATGCA	

This is because that Mmp9 is a proteolytic enzyme involved in peptidoglycan metabolism, protein hydrolysis, catabolism of collagen, and the matrix reconstruction in the process of atherosclerosis development. When tissue is ischemic, Mmp9 significantly increases and dissolves collagen, causing vascular damage and bleeding.[57–61] Galis and his colleagues studied the expression of MMPs, and found all MMPs could be detected in atherosclerosis, proving that MMPs participated the matrix reconstruction in the development of atherosclerosis. [62–63] Previous studies confirmed that when MMP9 is over expressed in human atherosclerotic plaque with increased activity,[64–67] decomposition of collagen fragments in atherosclerotic plaque was found. Combined with the induction effect of the components group, and considering the long-term reparation effect of TCM SLF for myocardial infarction or myocardial necrosis, we found that TCM is more conducive for repairing myocardial necrosis organizations. According to gene function and the drug effect results, we defined the induction trend of MMP9 as the full-time upregulation of gene expression and the promotion matrix reconstruction mode.

For the IL6 gene, except for the result of the components group on the day 10 time point, the changing trends of all groups were very similar; these groups

showed downtrend from day 10 to day 30, and reached the lowest expression on day 30. Compared with the blank group in which the Il6 gene showed the highest expression on day 30 and the lowest expression on day 40, cells treated with drugs got apoptosis from day 30 to day 40. The Il6 gene expression was significantly changed on day 10. Considering this special effect mode of the components group, and according to the function of Il6 which was involved in cytokines, neutrophil apoptosis, acute phase response, humoral immune response, cell surface receptors, signal transduction, positive regulation of cell proliferation, negative regulation of cell proliferation, and negative regulation of chemokine biosynthesis,[68–71] we suggested that TCM was more conducive than 5-aza for the healthy metabolism and development of cells. Therefore, the induction trend of the Il6 gene was defined as the BMMSCs' basic metabolism promotion mode.

Regarding the Sncg gene, each group showed a similar trend which obviously downregulated on day 30, in which the components groups were slightly high and 5-aza group downregulated lowest. Sncg variation was a key to tumor progression, and involved in growth, invasion, metastasis, and prognosis[72] of cancer cells. With drug induction effects, the component group of SLF was most effective for cancer inhibition while the effect of 5-aza group was the weakest. Therefore, the induction trend of Sncg gene was defined as BMMSCs' cancer inhibition mode. Compared to 5-aza, TCM was more effective for cancer inhibition, suggesting that SLF may reduce the possibility of canceration.

The expression result of 2′5′-AS, Pem, and 3αHSD genes showed that the components group was higher than other groups but lower than the 5-aza group in 10 days, and they were high or low with no regularity from day 20 to day 40. There was little difference from day 10 to day 40 in the blank group, and the result calculated with the blank group as control had no significant change. Meanwhile, the functions of these three genes are related to the cells' basic life indications: the Pem (polymorphic epithelial mucin) gene is an intracellular signal transduction molecule, which is not expressed in adult cells, but in germ cells and related to differentiation of embryonic cells and development of gonadal cells[73–74]; the 2′5′-AS (Specific 'oligo adenylate synthase) gene is a medium for validating interferon, with biological functions of antivirus, inhibiting DNA synthesis and cell growth, and regulating immune response[75]; and 3αHSD (3-α-hydroxybutyrate-dehydrogenase) is one of the initial enzymes in the steroids metabolic pathway. The total bile acid (TBA) in serum is a derivative of 3-α-hydroxy steroids. When catalyzed by 3αHSD, TBA generates corresponding 3-deriva-ketones steroids through dehydrogenation in which NAD^+ is reduced to $NADH^+H^+$, which further regulates the activity of 6-phosphogluconate dehydrogenase and participates in the metabolism of glucose.[76–78] BMMSCs can be induced to differentiate into cardiomyocytes, and the basic life indications of each group are enhanced, however, the degrees of drug-induced reaction are different, varying with cell aging and apoptosis. Thus, the induction mode of these three genes was defined as the basic life indication enhancement mode.

11.4.1.3.2 Investigation of Gene Function and Mechanism Comprehensive analysis of the five groups of drug induction results showed that the change of gene expression in the ingredients group, the 5-aza + ingredients group, and the 5-aza + components group were significantly lower than that in the 5-aza group and the components group. Therefore, we mainly conducted comparative analysis between the 5-aza group and the components group. According to the regulation of gene expression, the starting time point, and the period of drug efficacy, the drug induction was divided into five modes: TCM long-time induce mode; full-time upregulation of gene expression and promotion of matrix reconstruction mode; the BMMSCs' metabolism promotion mode; the BMMSCs' canceration inhibition mode and the basic life indication enhancement mode.

The known functions of these genes were analyzed. It was found that 10 major categories of basic gene functions were related to life maintenance (Fig. 11.13). In the process of differentiation from BMMSCs into cardiomyocytes, the TCM SLF not only stimulated the genes related

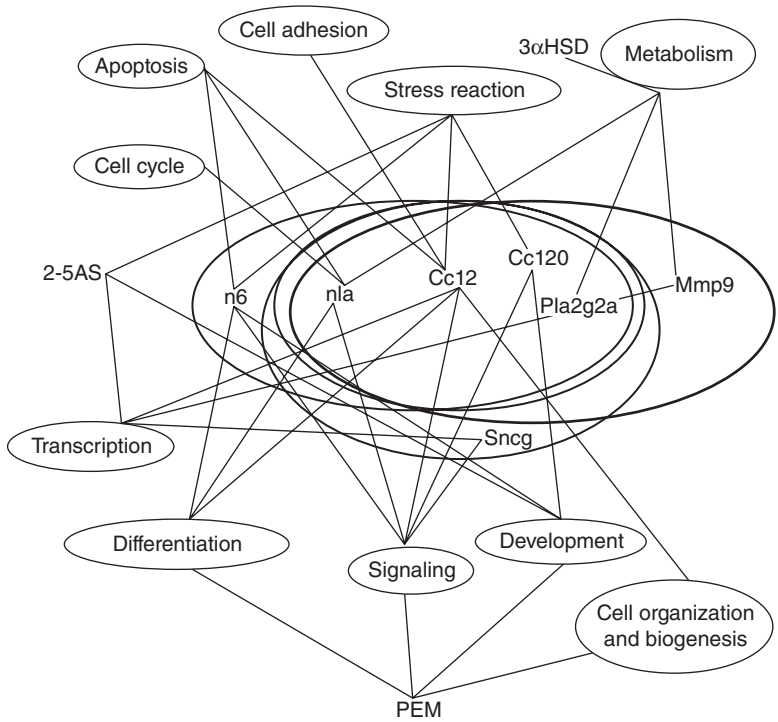

Fig. 11.13 Functional relationship of the 10 differentially expressed genes. These genes related to the differentiation from BMMSCs into cardiomyocyte-like cells induced by NSLF6.

to the basis of life for a long time, but also affected functional genes related to cell matrix reconstruction, the promotion of metabolism, and cancer inhibition, and finally induced development and differentiation of BMMSCs.

Ten genes were selected and verified in this study; however, not only these genes were effective during the BMMSCs' differentiation into cardiomyocytes, which should be regulated by a network of genes. Our analysis showed that Il1a, Pla2g2a, Ccl20, and Ccl2 genes could play an important role in differentiating BMMSCs to cardiomyocytes, while other genes involved in matrix reconstruction of hardening vasculature, promotion of BMMSCs' metabolism, inhibition of BMMSCs' canceration, enhancement of basic life indication, and other auxiliary functions. Therefore, differentiation of BMMSCs into cardiomyocytes by drugs is manipulated through interactions of different genes, networks, and functions.

In addition, genes that were significantly effective in the 5-aza group and components group did not show a more effective inducement and differentiation trend, but a weaker response in 5-aza + components group. This indicated that TCM cannot play a complementary role for 5-aza. Moreover, the effect of TCM's components was much stronger than that of TCM's ingredients, which is likely because that the TCM has multiple target sites and each ingredient works independently. Therefore, all ingredients exist together and affect each other, increasing the effect and reducing the side effects, which finally conducts a better therapeutic effect.

Second, the immunofluorescence assay and quantitative gene detection showed that the induction effect of the 5-aza group appeared earlier than that of TCM inducing groups, but component group was more effective than the 5-aza group in the time point whose apparent value change most; and results of the quantitative detection of gene showed that the expression changes of key genes in the components group lasted longer than those of the 5-aza group. Thus, 5-aza became effective in a short time, while it was the opposite for TCM. TCM has significant effects on genes which are fundamental to inheritance, and TCM takes a longer time from quantity to quality, and ultimately achieves better results. Therefore, the Western medicine has an immediate effect after administration, but it takes long time for TCM. We found that the effect of the TCM SLF on differentiation of BMMSCs to cardiomyocytes is not as good as 5-aza, however, the improving effect of TCM is better than that of 5-aza.

In conclusion, SLF based on the TCM theory of "supplementing *qi* and nourishing blood, promoting blood circulation to remove blood stasis" can cure myocardial infarction, while in molecular biology analysis we believe that the mechanism has a direct effect on functional genes related to inducement and differentiation of BMMSCs into cardiomyocytes, consequently repairs the myocardial necrosis caused by myocardial infarction and ultimately achieves the changes in epigenetics.

11.4.2 Proteomic Study of the Directional Differentiation Mechanism of Stem Cells Interfered with by NSLF6

11.4.2.1 Differential Expression Analysis of the Directional Differentiation Mechanism of Stem Cells Interfered with by NSLF6 In this section, the effect of the Chinese medicine SLF and its main active ingredients on the directional differentiation of rat in vitro mesenchymal stem cells (MSCs) into cardiomyocytes was studied using the established MSCs model. Meanwhile, the proteins obtained in the process of MSC differentiation induced by SLF were characterized. Eventually, we attempted to understand the mechanism of MSC differentiation from the protein perspective by comparative proteomics analysis.

11.4.2.1.1 Cell Isolation and Differentiation MSCs were isolated from Sprague-Dawley rat bone marrows. The details of isolation, identification of MSCs phenotype, and cardiomyocyte-like cells derived from MSCs induced by 5-aza have been reported previously.[42] In this study, 5-aza was used as a positive inducer. MSCs at passage 3–5 were used for this experiment. The medium of the 5-aza group was replaced by complete medium (CM, 10% FBS in DMEN-F12, Gibco) containing 10 μM 5-aza (Sigma) for 24 h, and then cells were cultured with CM without 5-aza. The medium of SLF treatment group was replaced by CM containing 10^{-6} g/mL SLF, and the SLF ingredients group was replaced by CM containing 10^{-6} g/mL SLF ingredients. In the SLF + 5-aza group and the SLF ingredients + 5-aza group, MSCs were induced by 10 μM 5-aza for 24 h, then the mediums were respectively replaced with CM containing 10^{-6} g/mL SLF and the SLF ingredients.

About 10^6 cells were collected by gentle trypsinization after 30 days of incubation. Triplicate undifferentiated MSCs ($n = 3$) and induced-differentiated cells ($n = 3$) were collected for future experiments. As a positive control for immunofluorescence, cardiomyocytes were isolated from the neonatal SD rats (3 days) following the reference method. The cells were cultured in 6-well plates with CM.

11.4.2.1.2 Quantitative Analysis of SLF The main ingredients of SLF were identified using HPLC and shown in Tables 11.19 and 11.20. More than 80% of the ingredients of ginsenosides and salvianolic acid can be qualitative and quantitative.

11.4.2.1.3 Immunochemical Staining To ensure the differentiation of rat MSCs induced by SLF and SLF ingredients, the cardiac-specific proteins, including cardiac troponin I (cTnI) and cardiac myosin heavy chain (cMHC), were identified by immunofluorescence. Fig. 11.14 shows the staining results. Cell nucleus was stained with PI. Actin was chosen as a reference protein, and neonatal rat cardiomyocytes used as a positive reference. Noninduced MSCs stained negatively for the two protein markers.

TABLE 11.19 Main Components in Ginsenosides

No.	Components	Relative Content (%)	No.	Components	Relative Content (%)
1	Rb_1	14.15	7	Rd	4.52
2	Rb_2	11.57	8	m-Rb_1	3.94
3	Ro	10.05	9	m-Rb_2	3.63
4	Rg_1	9.90	10	Rf	2.64
5	Rc	9.33	11	m-Rc	1.98
6	Re	7.68	12	m-Rd	1.28
Total			80.67		

TABLE 11.20 Main Components in Salvianolic Acid

No.	Components	Relative Content (%)
1	Salvianolic acid B	78.96
2	Rosmarinic acid	3.38
3	Salvianolic acid A	1.12
Total	83.46	

Fluorescent signals were detected in all induced groups except the negative control group at the 30th day of differentiation. As shown in Fig. 11.14, both SLF and its main ingredients can in vitro induce rat bone marrow mesenchymal stem cells into cardiomyocyte-like cell. Our previous research has found that SLF bad better effects during the process of MSC differentiation compared to the other groups.[79] Data from HPLC reveal that Rb_1 and Sal B were the main ingredients of ginsenosides and salvianolic acid, respectively, but they seem to be not the sole ingredient responsible for the functions of SLF in the cardiomyocyte differentiation from rat MSCs. As an organic whole, the synergy of each substance of SLF remains to be further clarified. When comparing SLF group and SLF + 5-aza group, the fluorescent signal of SLF + 5-aza group was less than that of the SLF group, probably due to toxicity resulting from simultaneous action of SLF and 5-aza.

11.4.2.1.4 Proteomic Analysis After PDQuest 7.1 software analysis, more than 800 protein spots were detected in each gel, as shown in Fig. 11.15. Cardiomyocyte-like cells, as the transition state cells, have significant differences in their morphology, function, and protein expressions for MSCs and cardiomyocytes. 5-Aza is widely recognized as an inducer for MSCs differentiating into cardiomyocyte-like cells. First of all, the change trends of differentially expressed proteins was investigated in the 5-aza group and the groups treated by TCM. Then the differences and similarities of the differentially expressed proteins were analyzed. Due to the good separation and certain richness, 36 differentially expressed protein spots were identified between the 5-aza group and control group. Among them, the expressions of 14 proteins

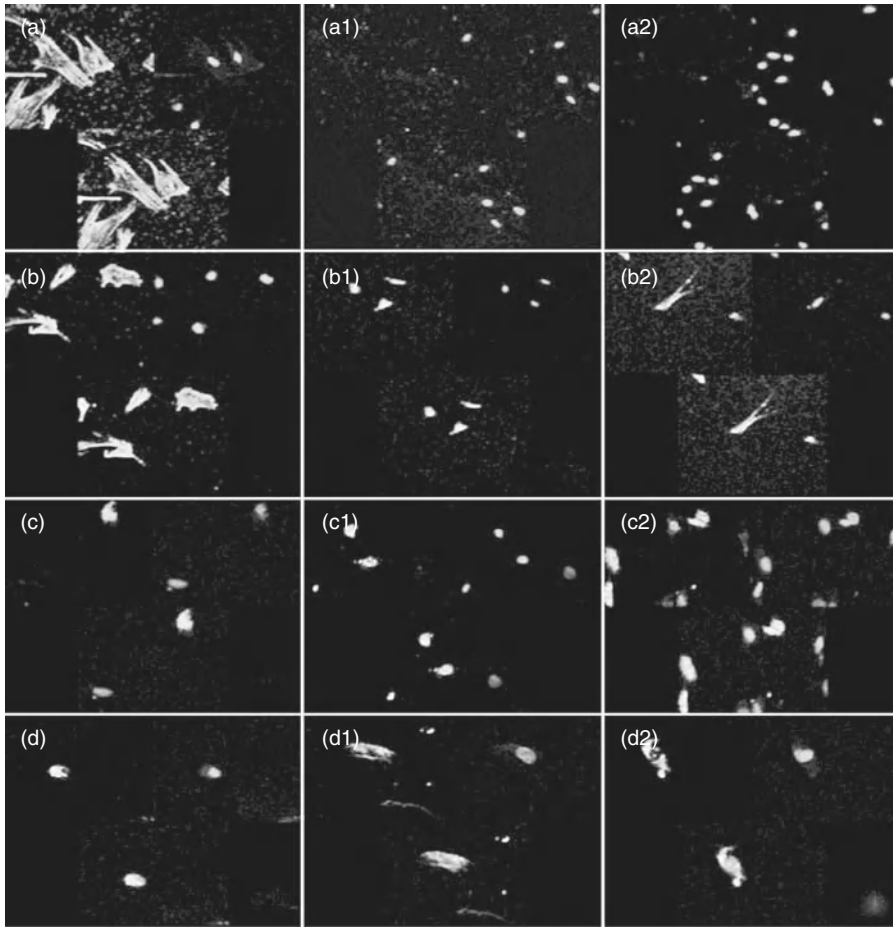

Fig. 11.14 Immunofluorescence results of MSCs induced by TCM. Undifferentiated MSCs were immunostained positively for actin (A), but negatively for cMHC (A1) and cTnl (A2), while rat cardiomyocytes were positive for all three examined antibodies (B: actin; B1: cMHC; B2: cTnl); differentiated MSCs induced by 5-aza (C1: cMHC; C2: cTnl), SLF (D1: cMHC; D2: cTnl), or SLF ingredients (E1: cMHC; E2: cTnl) were positive for both cardiac-specific protein markers, cMHC and cTnl. (See color insert.)

were significantly decreased after 5-aza induction, while 21 proteins were significantly increased. Interestingly, there was only one protein expressed in differentiated MSCs. The 14 downregulated proteins were all greatly reduced for the SLF group as well as the 5-aza group. However, there were partially reduced proteins in the remaining groups (nine for the SLF + 5-aza group, seven for SLF ingredients group, and six for SLF ingredients + 5-aza group). In the 36 differentially expressed protein spots, the protein change of the SLF-induced group was quite similar to the 5-aza-induced group, then were the

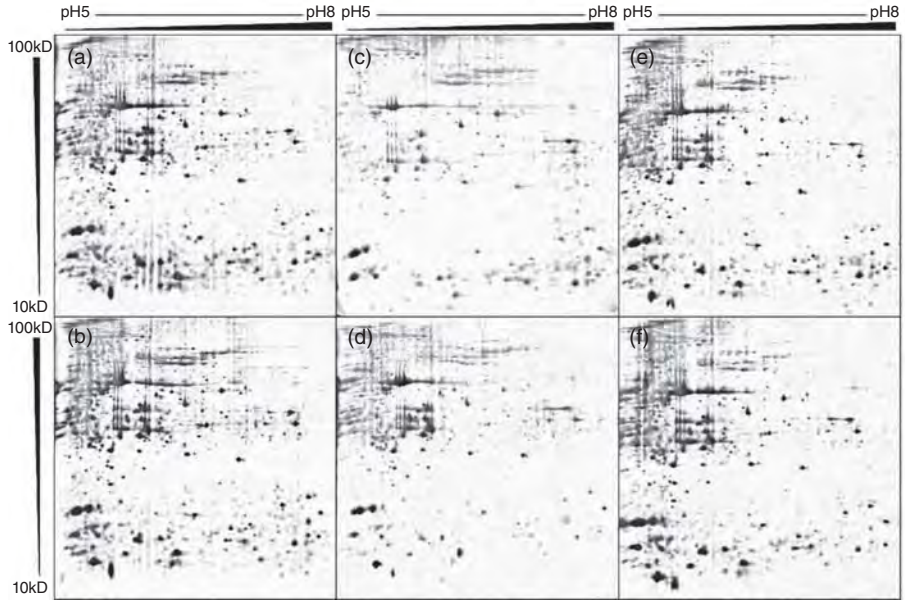

Fig. 11.15 Two-dimensional gel map of protein expression in each group. A: MSCs; B: 5-aza induced group; C: SLF ingredients group; D: SLF ingredients + 5-aza group; E: SLF induced group; F: SLF + 5-aza group.

SLF + 5-aza group, SLF ingredients group, and the SLF ingredients + 5-aza group.

11.4.2.1.5 Identification of the Differentially Expressed Protein Based on the difference of protein profiles, the protein spots of interest in the differentially expressed proteins were cut off from the gels and subjected to trypsin digestion, followed by peptide extraction for MS analysis and peptide fingerprinting analysis. The peptide mass fingerprinting (PMF) method was used to identify 13 proteins via the relevant database, and related data are shown in Figs. 11.16 and 11.17 and Table 11.21.

In this study, the protein expressional profiling of cardiomyocyte-like cells differentiated from rat MSCs was reported. There were 36 proteins modulated by 5-aza and SLF during the process of cardiomyocyte differentiation. Among them, 13 proteins with the most change were successfully identified.

11.4.2.2 Development of the Relational Protein Interaction Network Moreover, protein functional analysis found that the differentially expressed proteins were mainly related to the pathways such as MAPK,[80] Rho,[81] Ca^{2+},[82] and Wnt.[83] By further analysis of the differentially expressed proteins, the relational protein interaction network was preliminarily obtained (Fig. 11.18). These results suggested that SLF might mainly regulate the internal ion

Fig. 11.16 Differentially expressed proteins identified by MS.

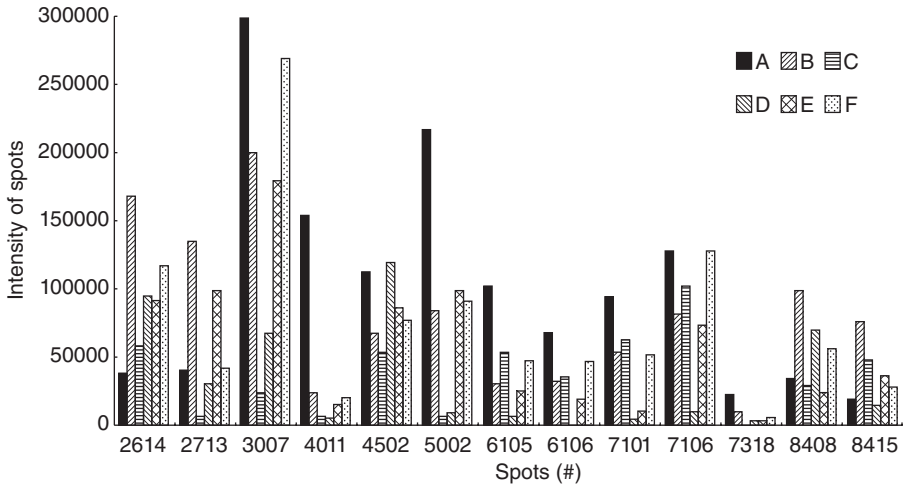

Fig. 11.17 Expression changes of differentially expressed proteins in each group. A: the control group (undifferentiated MSCs); B: the 5-aza group; C: the SLF group; D: the SLF + 5-aza group; E: the SLF ingredients group; F: the SLF ingredients + 5-aza group.

TABLE 11.21 Identification Results of Differentially Proteins During the Differentiation of MSCs Induced by NSLF6

No.	SSP No.	Protein Name (Gene Name)
1	2614	Vimentin (Vim)
2	2713	Prolyl 4-hydroxylase, beta polypeptide (P4hb)
3	3007	Polymerase (DNA directed), delta 1, catalytic subunit (Pold1)
4	4011	Hsc70-ps1 (Hspa8)
5	4502	Pkm2 protein (Pkm2)
6	5002	S100 calcium binding protein A11 (S100a11)
7	6105	Nucleoside diphosphate kinase B (Nme2)
8	6106	Similar to ribosomal protein S12 (Rps12)
9	7101	Actin, cytoplasmic 1 (Actb)
10	7106	Eukaryotic translation elongation factor 2 (Ef-2)
11	7318	Peroxiredoxin 4 (Prdx4)
12	8408	Gapd protein (Gapd)
13	8415	Fructose-bisphosphate aldolase A (Aldoa)

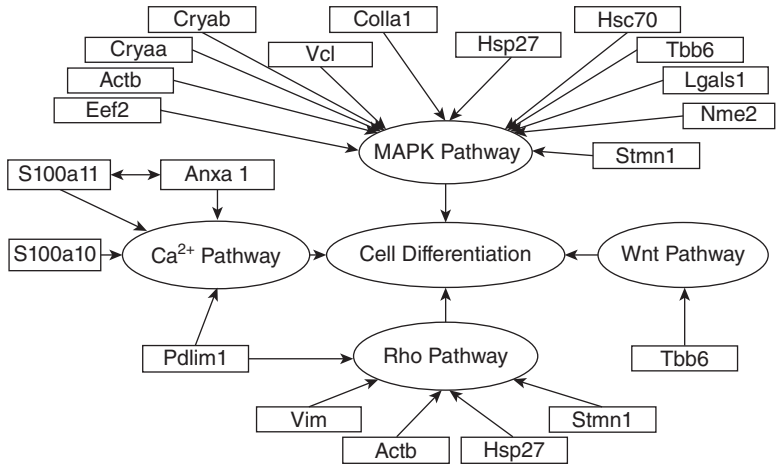

Fig. 11.18 Relational signaling pathway network of differentiation of BMMSC into cardiomyocytes.

concentration and energy metabolism to display the efficacy. Previous studies showed these proteins play important roles in cell proliferation, differentiation, and apoptosis, which may explain the functions of SLF and its ingredients in MSC differentiation to cardiomyocyte-like cells.

5-Aza, a cytidine analogue, can induce differentiation of bone marrow stem cells into myocardial cells. However, it can also lead to changes in DNA structure which have teratogenic and carcinogenic effects. Therefore, MSC differentiation induced by TCM instead of 5-aza is more beneficial to follow-up research and its ultimate clinical application. Fluorescent staining results

showed that SLF and SLF ingredients could induce MSC differentiation into cardiomyocyte-like cells as well as 5-aza, although previous research found that SLF had better effects during the process of MSC differentiation between all the groups. Due to the different batches in the experiment, the inducing ability of SLF and SLF ingredients was reidentified in this study. Moreover, the protein expression changes were investigated.

From the results of protein analysis, the changes of differential expression proteins had a high similarity between SLF and 5-aza treatment groups. However, there were still many differences. Both SLF and 5-aza had the ability to induce MSC differentiation into cardiomyocyte-like cells though each had its unique regulatory mechanism. According to the biological function, a part of the 13 differentially expressed proteins might be involved in the cell proliferation or differentiation through different signaling pathways, such as eukaryotic translation elongation factor 2. Moreover, the introduction of TCM also raised some novel expression changes for proteins during the proliferation or differentiation, similar to ribosomal protein S1. The detection, identification, and further functional analysis of those differentially expressed proteins would facilitate understanding of the process of MSC directed differentiation, thus guiding further study of the efficacy, mechanism, and protein targets of TCM. The experimental results showed that the effects of SLF were better than SLF ingredients for MSC directed differentiation. It was illustrated that the efficacy of TCM formula originated from the compatibility of the substances in the formula, and the individual one or two active ingredients could not represent the complex whole. Therefore, modern systems biology technologies were used to elucidate TCM mechanisms.

11.4.3 Metabonomic Study on the Directional Differentiation of Stem Cells Interfered with by NSLF6

Studies have shown that the Chinese medicine SLF has exact effects on MI and have demonstrated that SLF could promote the differentiation of MSCs into cardiomyocyte-like cells (type myocardial cells).[38–39, 84] The genomics and proteomics studies have been introduced above. In this section, we will introduce the metabonomic study on the on the directional differentiation of stem cells interfered with by NSLF6.

11.4.3.1 Establishment of the Quantitative Metabonomics

11.4.3.1.1 Preassessment and Goal Setting of the Metabolite Group in the Process of Stem Cell Directional Differentiation The results of drug intervention in stem cells will eventually lead to intracellular changes in metabolite levels, which are the cells metabonomics studies. Taking the detection limit of methods into account, metabolisms or pathways, commonly including glycolysis, tricarboxylic acid cycle, pentose phosphate pathway, nucleotide metabolism, and amino acid metabolism, were preassessed. According to the basis

of previous work, the expression of the proteins in the process of MSC differentiation was closely related to energy metabolism.[38–39, 84] Thus, we focused on energy metabolism of the following compounds (Table 11.22) when the methods were established and optimized.

Standard reversed-phase chromatography yields little retention for amino acids and nucleotides because of their hydrophilic and ionic character. In this study, ion pair chromatography was chosen for the separation of those compounds. Thus an ion pair chromatography–mass spectrometry analytical method for simultaneous measurement of more than 50 compounds by Agilent 1100 LC/MSD Trap was developed. ESI negative ion mode was chosen for the analyzers being acids and the mass range was m/z 50–1200.

TABLE 11.22 Metabolites of Cellular Energy Metabolism

Metabolite	Formula	MW	Metabolism
A(adenosine)	$C_{10}H_{13}N_5O_4$	267.24	Nucleotide
dA(deoxyadenosine)	$C_{10}H_{13}N_5O_3$	251.24	Nucleotide
AMP	$C_{10}H_{14}N_5PO_7$	347.22	Nucleotide
ADP	$C_{10}H_{15}N_5P_2O_{10}$	427.21	Nucleotide
ATP	$C_{10}H_{16}N_5P_3O_{13}$	507.18	Nucleotide
G(guanosine)	$C_{10}H_{13}N_5O_5$	283.24	Nucleotide
dG(deoxyguanosine)	$C_{10}H_{13}N_5O_4$	267.24	Nucleotide
GMP	$C_{10}H_{14}N_5PO_8$	363.22	Nucleotide
GDP	$C_{10}H_{15}N_5P_2O_{11}$	443.21	Nucleotide
GTP	$C_{10}H_{16}N_5P_3O_{14}$	523.18	Nucleotide
C(cytidine)	$C_9H_{13}N_3O_5$	243.22	Nucleotide
dC(deoxycytidine)	$C_9H_{13}N_3O_4$	227.22	Nucleotide
CMP	$C_9H_{14}N_3PO_8$	323.20	Nucleotide
CDP	$C_9H_{15}N_3P_2O_{11}$	403.19	Nucleotide
CTP	$C_9H_{16}N_3P_3O_{14}$	483.16	Nucleotide
dT(thymidine)	$C_{10}H_{14}N_2O_5$	242.23	Nucleotide
dTMP	$C_{10}H_{15}N_2O_8P$	322.21	Nucleotide
dTDP	$C_9H_{15}N_3P_2O_{11}$	402.19	Nucleotide
dTTP	$C_9H_{16}N_3P_3O_{14}$	482.17	Nucleotide
U(uridine)	$C_9H_{12}N_2O_6$	244.20	Nucleotide
dU(deoxyuridine)	$C_9H_{12}N_2O_5$	228.20	Nucleotide
UMP	$C_9H_{13}N_2PO_9$	324.18	Nucleotide
UDP	$C_9H_{14}N_2P_2O_{12}$	404.16	Nucleotide
UTP	$C_9H_{15}N_2P_3O_{15}$	484.14	Nucleotide
Lys	$C_6H_{14}N_2O_2$	146.19	Amino acid
Asn	$C_4H_8N_2O_3$	132.12	Amino acid
Ser	$C_3H_7NO_3$	105.09	Amino acid
Orn	$C_5H_{12}N_2O_2$	132.16	Amino acid
Gly	$C_2H_5NO_2$	75.07	Amino acid
Ala	$C_3H_7NO_2$	89.09	Amino acid

(*Continued*)

TABLE 11.22 (*Continued*)

Metabolite	Formula	MW	Metabolism
Arg	$C_6H_{14}N_4O_2$	174.20	Amino acid
Thr	$C_4H_9NO_3$	119.12	Amino acid
Pro	$C_5H_9NO_2$	115.13	Amino acid
His	$C_6H_9N_3O_2$	155.16	Amino acid
Cys	$C_3H_7NO_2S$	121.16	Amino acid
Val	$C_5H_{11}NO_2$	117.15	Amino acid
Cre	$C_4H_7N_3O$	113.12	Amino acid
Asp	$C_4H_7NO_4$	133.10	Amino acid
Glu	$C_5H_9NO_4$	147.13	Amino acid
Met	$C_5H_{11}NO_2S$	149.21	Amino acid
Ile	$C_6H_{13}NO_2$	131.17	Amino acid
Leu	$C_6H_{13}NO_2$	131.17	Amino acid
Phe	$C_9H_{11}NO_2$	165.19	Amino acid
Trp	$C_{11}H_{12}N_2O_2$	204.23	Amino acid
Tau	$NH_2CH_2CH_2SO_3H$	125.15	Amino acid
G6P	$C_6H_{13}O_9P$	260.14	Glucolysis
F6P	$C_6H_{13}O_9P$	260.14	Glucolysis
FBP	$C_6H_{14}O_{12}P_2$	340.12	Glucolysis
DHAP	$C_3H_7O_6P$	170.06	Glucolysis
GAP	$C_3H_7O_6P$	170.06	Glucolysis
GBP	$C_3H_8O_{10}P_2$	266.04	Glucolysis
3PG	$C_3H_7O_7P$	186.06	Glucolysis
2PG	$C_3H_7O_7P$	186.06	Glucolysis
PEP	$C_3H_5O_6P$	168.04	Glucolysis
Pyr	$C_3H_4O_3$	88.06	Glucolysis
Lac	$C_3H_6O_3$	90.08	Glucolysis
CoA	$C_{21}H_{36}N_7O_{16}P_3S$	767.53	Tricarboxylic acid cycle
AcCoA	$C_{23}H_{38}N_7O_{17}P_3S$	809.57	Tricarboxylic acid cycle
Cit	$C_6H_8O_7$	192.12	Tricarboxylic acid cycle
Isocit	$C_6H_8O_7$	192.12	Tricarboxylic acid cycle
Akg	$C_5H_6O_5$	146.10	Tricarboxylic acid cycle
SucCoA	$C_{25}H_{40}N_7O_{19}P_3S$	867.61	Tricarboxylic acid cycle
Suc	$C_4H_6O_4$	118.09	Tricarboxylic acid cycle
Fum	$C_4H_4O_4$	116.07	Tricarboxylic acid cycle
Mal	$C_4H_6O_5$	134.09	Tricarboxylic acid cycle
Oxa	$C_4H_4O_5$	132.07	Tricarboxylic acid cycle
FAD	$C_{27}H_{33}O_{15}N_9P_2$	785.55	Tricarboxylic acid cycle
$FADH_2$	$C_{27}H_{35}O_{15}N_9P_2$	787.57	Tricarboxylic acid cycle
NAD^+	$C_{21}H_{27}N_7O_{14}P_2$	663.43	Glucolysis, tricarboxylic acid cycle
NADH	$C_{21}H_{29}O_{14}N_7P_2$	665.44	Glucolysis, tricarboxylic acid cycle
UDP-Glc	$C_{15}H_{24}N_2O_{17}P_2$	566.31	Pentose phosphate pathway
UDP-GlcNAc	$C_{17}H_{27}N_3O_{17}P_2$	607.36	Pentose phosphate pathway
$NADP^+$	$C_{21}H_{28}N_7O_{17}P_3$	743.41	Pentose phosphate pathway

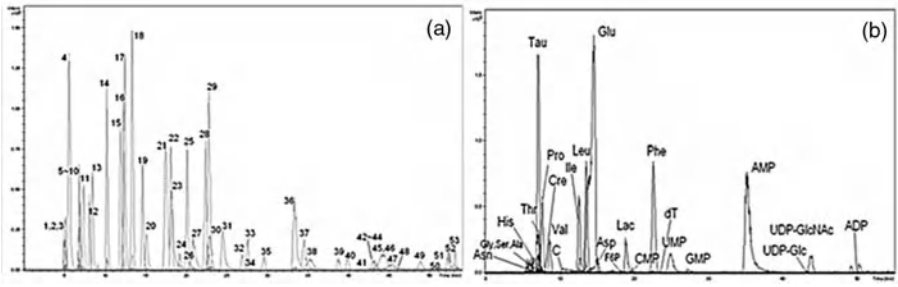

Fig. 11.19 Multiple extracted ion chromatograms of mixed standard solution (A) and metabolites of cells (B). The compounds corresponding to the peak numbers were shown in Table 11.23.

11.4.3.1.2 Preparation of Calibration Standards Fifty-three compounds were selected as analyzers and the mixed standard solution was prepared at the concentration of 16.6μg/mL for each standard. After optimization of chromatography and mass spectrometry conditions, the chromatograms of the 53 standards were established as shown in Fig. 11.19 and Table 11.23.

As shown in Table 11.23, broken behaviors of those compounds under ESI include dehydration, decarbonylation, dipolymerization, dephosphorylation, conjunction with acetate ions, conjunction with water molecules, and addition of purine base or pyrimidine base.

11.4.3.1.3 Preparation of Samples Preparation of cell samples refers to the thesis. As shown in Fig. 11.24B, 30 compounds in stem cells could be separated and detected simultaneously using the established method.

11.4.3.1.4 Method Validation The methods were validated by determining the linearity, sensitivity, and intra- and interday precision. The values for the method validation showed that the method fulfilled the criteria for bioanalytical analysis.

11.4.3.2 Metabonomic Study

11.4.3.2.1 Experimental Design and Grouping Drug intervention started in the third generation of MSCs. To illustrate the changes resulting from the intervention, four groups were designed as follows: the control group, the induced group (add 100 μL of 5-aza solution on the day intervention to a solution at the concentration of 10^{-6} mol/L, after 24 h retrieve the complete medium), the component group (TSA + TGS at the concentration of 10^{-6} g/mL), and the ingredient group (salvianolic acid B + Rb_1 at the concentration of 10^{-6} g/mL). The samples were collected on days 0 (before induction), 10, 20, and 30, as shown in Fig. 11.20.

TABLE 11.23 Retention Times and Fragments of Analytes

No.	Metabolite	R.T.	MW	$[M-H]^-$	Fragments
1	Lys	5.1	146.19	144.8	–
2	Orn	5.1	132.16	131.2	–
3	Arg	5.2	174.20	173.0	–
4	His	5.6	155.16	153.9	–
5	Gly	6.7	75.07	74.4	–
6	Tau	6.8	125.15	124.0	248.8$[2M-H]^-$
7	Asn	6.8	132.12	131.0	113.2$[M-H_2O-H]^-$
8	Ser	6.9	105.09	104.3	74.4$[Gly-H]^-$
9	Ala	6.9	89.09	88.3	–
10	Thr	7.0	119.12	118.1	–
11	Pro	7.4	115.13	114.1	–
12	Cre	8.1	113.12	112.1	–
13	Val	8.5	117.15	116.1	–
14	C	10.2	243.22	242.0	277.9$[M+2H_2O-H]^-$; 110.2$[C'-H]^-$
15	dC	11.9	227.22	225.9	285.9$[M+Ac]^-$; 261.9$[M+2H_2O-H]^-$
16	Ile	12.2	131.17	130.1	–
17	U	12.5	244.20	242.9	278.9$[M+2H_2O-H]^-$; 110.1$[U'-H]^-$
18	Leu	13.4	131.17	130.1	–
19	Glu	14.6	147.13	146.0	128.1$[M-H_2O-H]^-$; 102.2$[M-CO_2-H]^-$
20	Asp	15.1	133.10	132.0	88.3$[Ala-H]^-$
21	G6P	17.5	260.14	258.8	97.1$[H_2PO_4]^-$; 518.8$[2M-H]^-$
22	G	18.1	283.24	281.9	150.0$[G'-H]^-$
23	F6P	18.2	260.14	258.9	97.1$[H_2PO_4]^-$; 518.9$[2M-H]^-$
24	Lac	19.2	90.08	89.2	–
25	dG	20.1	267.24	265.9	332.9$[2M-H]^-$
26	DHAP	20.3	170.06	168.9	338.8$[2M-H]^-$; 97.1$[H_2PO_4]^-$;
27	CMP	20.8	323.20	321.9	97.1$[H_2PO_4]^-$
28	Phe	22.3	165.19	164.0	–
29	dT	22.8	242.23	241.0	276.9$[M+2H_2O-H]^-$; 125.2$[dT'-H]^-$
30	Pyr	22.9	88.06	87.2	–
31	UMP	24.5	324.18	322.9	646.8$[2M-H]^-$
32	GMP	26.8	363.22	361.9	725.0$[2M-H]^-$
33	A	27.6	267.24	266.0	134.1$[A'-H]^-$; 301.9$[M+2H_2O-H]^-$; 325.9$[M+Ac]^-$; 533.0$[2M-H]^-$
34	NAD^+	27.9	663.43	661.9	134.1$[A'-H]^-$; 540.0[M-Nicotinamide]$^-$
35	dA	29.5	251.24	250.0	309.9$[M+Ac]^-$; 286.0$[M+2H_2O-H]^-$; 134.1$[A'-H]^-$; 500.9$[2M-H]^-$
36	dTMP	33.3	322.21	320.9	642.9$[2M-H]^-$;
37	AMP	34.4	347.22	345.9	–
38	Mal	35.1	134.09	132.7	115.1$[M-H_2O-H]^-$
39	Akg	38.5	146.10	145.0	101.2$[M-H_2O-H]^-$
40	Fum	39.8	116.07	115.1	71.4$[M-CO_2-H]^-$
41	CDP	42.1	403.19	401.9	97.1$[H_2PO_4]^-$

TABLE 11.23 (*Continued*)

No.	Metabolite	R.T.	MW	$[M-H]^-$	Fragments
42	UDP-Glc	42.9	566.31	565.1	–
43	FBP	43.2	340.12	338.9	97.1$[H_2PO_4]^-$
44	UDP-GlcNAc	43.3	607.36	606.1	565.1[UDPGlc-H]$^-$; 97.1$[H_2PO_4]^-$
45	Cit	44.2	192.12	190.9	–
46	Isocit	44.2	192.12	191.0	–
47	UDP	45.3	404.16	402.8	806.8[2M-H]$^-$
48	GDP	46.2	443.21	441.9	–
49	ADP	48.8	427.21	425.9	–
50	CTP	50.4	483.16	481.9	97.1$[H_2PO_4]^-$
51	FAD	52.0	785.55	784.1	392.0$[M-2H]^{2-}$
52	CoA	52.5	767.53	766.0	382.4$[M-2H]^{2-}$;
53	AcCoA	53.0	809.57	808.1	403.4$[M-2H]^{2-}$;

A, adensine; G, guanine; C, cytosine; dT, thymine; U, uracil; Ac, acetyl.

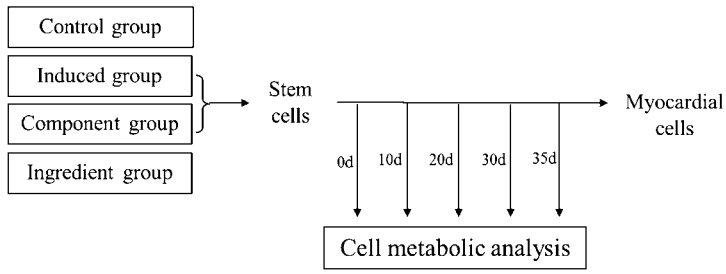

Fig. 11.20 Experimental design of directed differentiation of stem cells.

11.4.3.2.2 Metabolic Trajectory Analysis To comfirm that the stem cells converted into cardiomyocyte-like cells after a certain time induction, the MHC and cTnI as recognized cardiac-specific proteins were chosen. They were identified by immunofluorescence analysis (see Section 11.4). The results showed that 5-aza, components from SLF, and ingredients from SLF could promote the stem cells converted into cardiomyocyte-like cells after 30 days of induction. The samples collected at different time points were analyzed by the above established method, and the data were processed by PCA. The metabonomic changes during the whole differentiation stage were shown in Fig. 11.21.

As shown in Fig. 11.21, all three drugs (5-aza, components, and ingredients) could induce the stem cells to convert into cardiomyocyte-like cells after 30 days. Among them, the component group exerted the best performance. The result corroborated with the immunoflurescence and pharmacological studies above and showed that the components from SLF could treat MI by promoting the stem cells to convert into cardiomyocyte-like cells through the holistic efficacy of TCM.

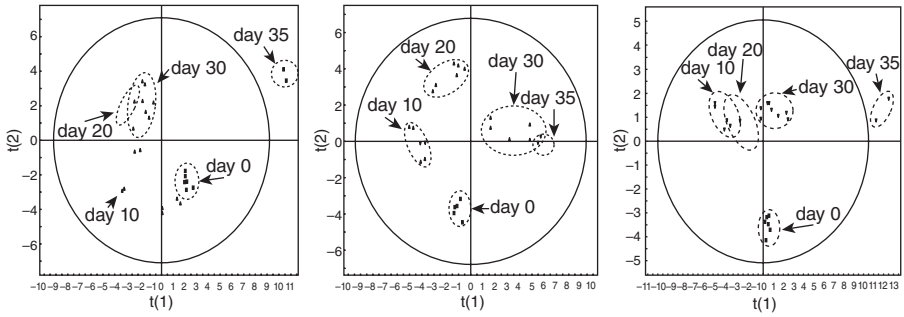

Fig. 11.21 Metabonomic trajectory tracing analysis in the process of stem cells differentiation induced by 5-aza (A), component groups (B), and ingredients (C).

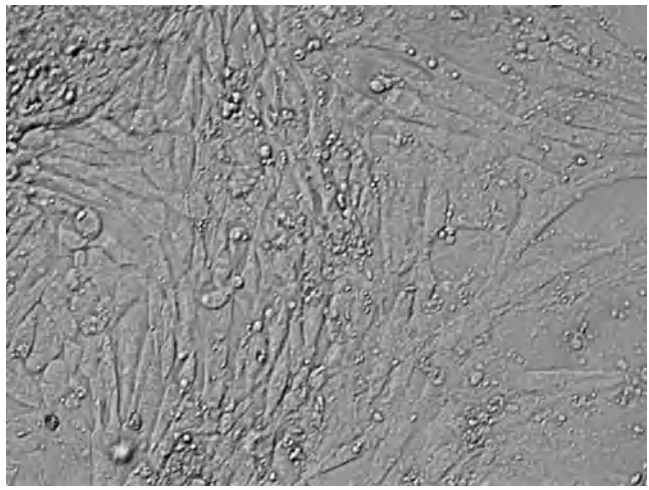

Fig. 11.22 Beating cardiomyocytes differentiated from mESC induced by salvianolic acid B.

11.5 ANTI-MYOCARDIAL INFARCTION EFFECT OF NSLF6

11.5.1 NSLF6 Promotes Cardiomyocytes Renewal

The embryonic bodies (EBs) differentiation model[85] was used to investigate the effect of NSLF6 and its main ingredients on the directional differentiation of mouse embryonic stem cells (mESCs) into cardiomyocytes in vitro. Salvianolic acid B (SAB), as a new stem cell inducer, was previously found to induce the differentiation of mESCs (CGR8 cells) into beating cardiomyocytes (Fig. 11.22). When EBs were treated with 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} mol·L^{-1} SAB, cardiomyocytes differentiated from mESCs ceaselessly increased over the period from d7 + 1 to d7 + 9, and the differentiation rates were

highest at d7 + 9, which were 17.02 ± 1.71%, 81.88 ± 2.49%, 52.45 ± 3.88%, and 23.73 ± 3.19%, respectively. However, the differentiation rates of the positive control (Vc, 10^{-4} mol·L^{-1}) and the blank control were 83.16 ± 1.32% and 19 ± 4.17%, respectively. In particular, with 10^{-4}, 10^{-5} mol·L^{-1} SAB, and 10^{-4} mol·L^{-1} Vc, the inducing effect on the directional differentiation of CGR8 cells into cardiomyocytes was significant compared with the blank control ($P < 0.001$). The differentiation rate was highest when 10^{-4} mol·L^{-1} SAB was adopted. Beating of the cardiomyocytes differentiated from CGR8 cells was first observed on d7 + 1, and obvious from d7 + 3 to d7 + 10. One EB contained one or more beating areas in which the enlarged size, increased contracting strength, and beating frequency were observed during the subsequent differentiation phase. To identify whether the observed cell types derived from CGR8 cells expressed cardiac-specific proteins, indirect immunofluorescence was carried out. The results verified that differentiated beating cardiac cells stained with anti-α-actinin mAb and anti-TnT mAb appeared positively. Western-blot results exhibited that cardiac-specific proteins MHC and TnI were positively expressed on d4 + 10, and the results of the quantitative real-time PCR showed that cardiac-specific gene GATA-4 and MLC-2v were highly expressed on d4 + 7. These results indicated that SAB, as a new stem cell inducer, could induce the differentiation of mESCs into cardiomyocytes. Similarly, NSLF6 could induce rat BMMSCs into myocardial-like cells.[79]

11.5.2 NSLF6 Promotes Human Umbilical Vein Endothelial Cell (HUVEC) Proliferation, Migration, and Angiogenesis

Among NSLF6, TSA, and TGS, NSLF6 (20 μg/mL) exhibited the highest activity in promoting cells proliferation (Fig. 11.23A,B). The determination of cell migration showed that NSLF6, TSA, and TGS significantly enhanced the ability of cell migration (Fig. 11.23C), and NSLF6 was optimal for migration rate, which was increased by 500%. To evaluate the simulative effect on angiogenesis, we treated HUVECs with NSLF6 (10, 20, and 40 μg/mL) for 18 h and examined their ability to attain tubular formation. The 20 μg/mL NSLF6 showed maximum promotion of tubule formation (Fig. 11.23D). The results showed that NSLF6 promotes HUVEC proliferation, migration, and angiogenesis.

11.5.3 NSLF6 Antagonizes Cardiomyocytes Oxidative Damage

The model of cardiomyocytes damaged by H_2O_2 was used to study the protective effect of NSLF6 and its effective ingredients. After cardiomyocytes were cocultured with these drugs for 48 h, 10% H_2O_2–PBS solution was added to the medium, cultured for 2 h. High content screening (HCS) system was used to analyze the health degree of cardiomyocytes.[86] The results showed that the mitochondrial membrane potential of those cardiomyocytes treated with NSLF6 and its effective ingredients was higher, the membrane permeability was lower and the nuclear integrity was better, compared with the model,

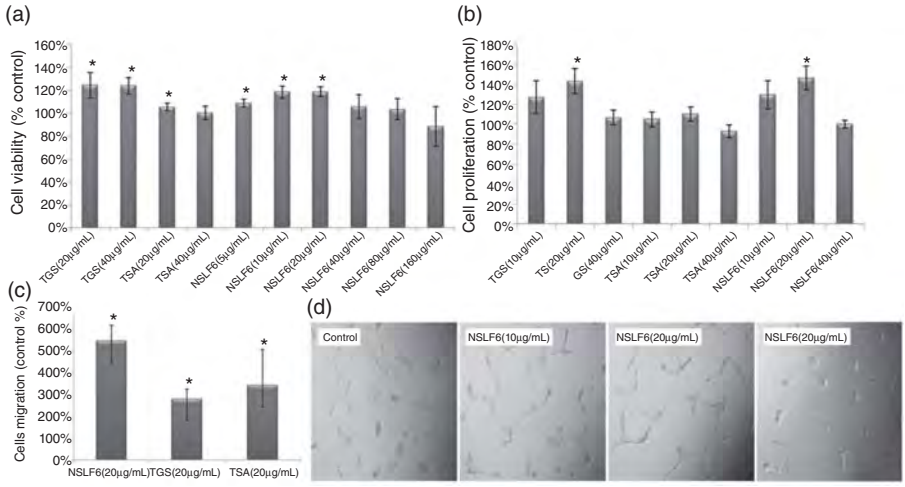

Fig. 11.23 Effects of NSLF6 on HUVECs proliferation, migration, and angiogenesis. A and B cells were incubated for 24 h with GS, TSA, and NSLF6. Cell proliferation was evaluated by MTT assay (A) and cell counting (B). Cell migration was examined by transwell chambers with polycarbonate filters (8.0 μm pore) after incubation for 3 h. D cells were seeded onto matrigel in 24-well plates. After incubated with NSLF6 for 18 h, tube formation was recorded photographically (40×). Results are expressed as percentage of control. $^*P < 0.05$ versus control; $n = 5$.

indicating that NSLF6 had the effect of antagonizing the damage of cardiomyocytes induced by H_2O_2 (Fig. 11.24). SAB and TSA significantly improved the mitochondrial membrane potential of the damaged cardiomyocytes (Fig. 11.24A,B), TGS significantly reduced the membrane permeability, and NSLF6 had an optimized effect on the maintenance of nuclear integrity. Therefore, all of NSLF6 and its effective ingredients could recover the rat cardiomyocytes damaged by H_2O_2. In conclusion, combining the results from three tests of mitochondrial membrane potential, the membrane permeability, and the nuclear integrity, NSLF6 had the best effects and the optimal concentration was 10^{-4} mol/L. Additionally, the investigation of the model of cardiomyocytes damaged by hypoxia/reoxygenation (H/R) also confirmed that NSLF6 had a protective effect on damaged cardiomyocytes, and such a pharmacological action was mainly contributed to by TGS.

11.5.4 Metabolic Target Analysis of the Active Components of SLF for MI

The interaction between the drug molecules and targets is crucial for efficacy. In this section, the interaction between the effective components of SLF and targets was studied using complex network analysis, as shown in Fig. 11.25. All the targets of the 16 major active components of SLF can be found in 16 pathways, which are mainly involved in antiapotosis, angiogenesis, regulating

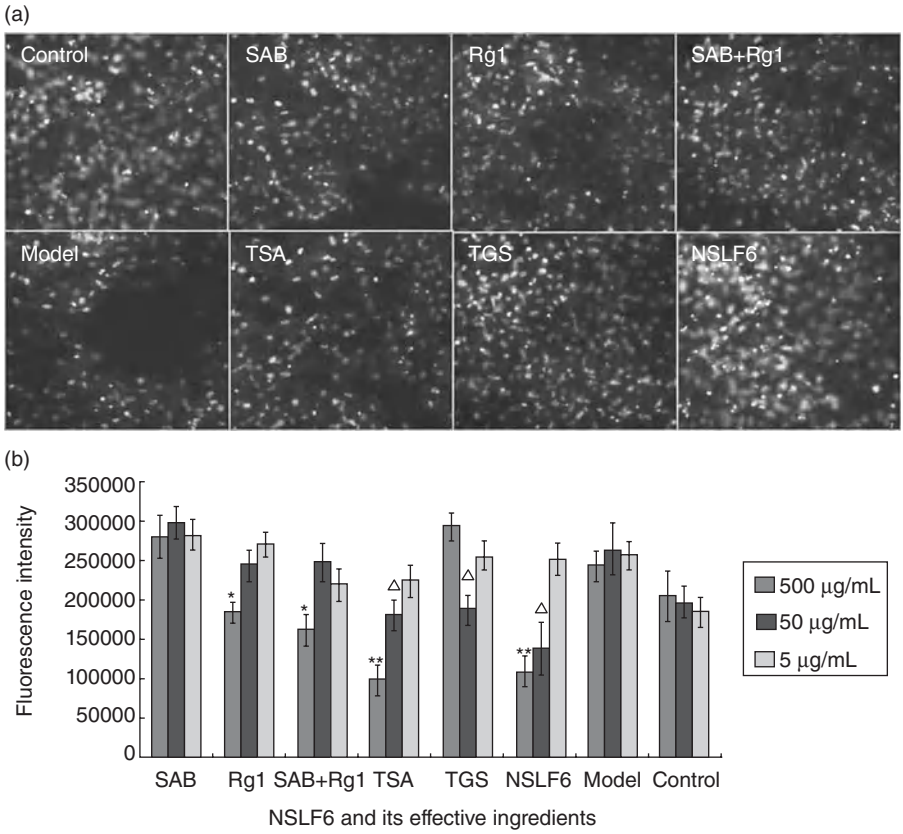

Fig. 11.24 Effect of NSLF6 and its effective ingredients on the membrane permeability of cardiomyocytes by H_2O_2. A: fluorescence expressions of membrane permeability of the damaged cardiomyocytes stimulated by NSLF6 and its effective ingredients. Different fluorescent shows the membrane permeability and nucleus stained by DAPI. B: Comparison of NSLF6 and its effective ingredients to the membrane permeability of cardiomyocytes by H_2O_2. (See color insert.)

enzyme activities, antioxidation, and so on, closely related to acute myocardial infarction. Moreover, the pathways of the 16 active components are almost same as those of the original formula (SLF), indicating that these active components are likely screened out by the complex network analysis which may provide a new method for TCM research and development.

11.6 CONCLUSIONS

In this chapter, based on the integrative system biology model, a new formula, NSLF6, was developed for the traditional Chinese medicine SLF as a clinically effective medicine with comparatively clear phytochemical composition,

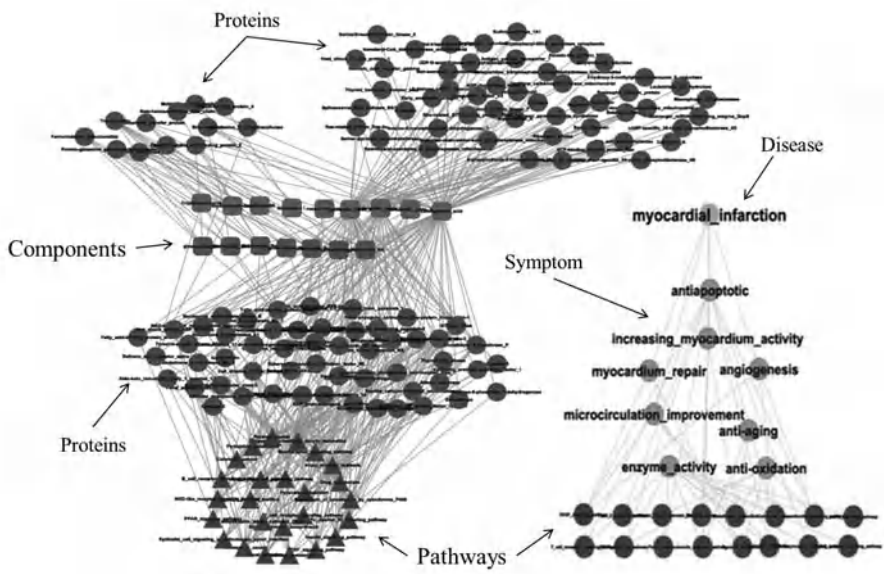

Fig. 11.25 Drug–target–pathway network.

mechanism, and controllable quality. A stringent quality control system has been established using combination of fingerprint and multicomponent determination. NSLF6 proved to be effective on MI by pharmacological test (the four levels of animal, tissue, cell, and molecular), combined with the study of gene, protein, and metabolite networks. The results showed that SLF produced efficacy against MI through synergistic therapeutic efficacies between TGS and TSA. The current S2S model may help with the discovery of new compound medicines.

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CHAPTER 12

DEMONSTRATIVE RESEARCH ON THE SAFETY EVALUATION OF *LIUSHEN* PILLS

12.1 INTRODUCTION

Liushen pills (LSP) are made up of a compound that is composed of *Zhenzhufen* (Margarita), *Niuhuang* (Bovis Calculus), *Shexiang* (Moschus), *Bingpian* (Borneolum Syntheticum), *Chansu* (toad venom), and *Xionghuang* (realgar). LSP has various medical effects such as clearing heat and detoxification, detumescence and odynolysis, strengthening the heart, and strengthening immunity. However, *Chansu* and *Xionghuang* in LSP are somewhat toxic; and there have been some reports of adverse reactions.[1–2] The content of arsenic is very high in *Xionghuang*. Long-term use of arsenic could lead to its accumulation in the body, which may lead to hepatic or renal impairment. Bufogenin, toad ligands, and some other toad toxin compounds are both effective and toxic components. Long-term use of *Chansu* may lead to circulation, digestion, and nervous system poisoning, while an overdose of *Chansu* can lead cardiac injury.[3–4] In the study mentioned in 12.2, we found that *Chansu* was toxic, but LSP as a TCM formula has been used for more than 250 years and is widely used in clinical practices nowadays. Thus LSP is considered safe when used as clinically directed. The former safety evaluation of LSP was based on clinical experience but lacked experimental data. The safety reevaluation of LSP was carried out and its chemical substance and action mechanism were disclosed to some degrees.

This chapter takes LSP as the research subject to explore the relationship between toxic herbs and TCM formulas. We adopted modern technological

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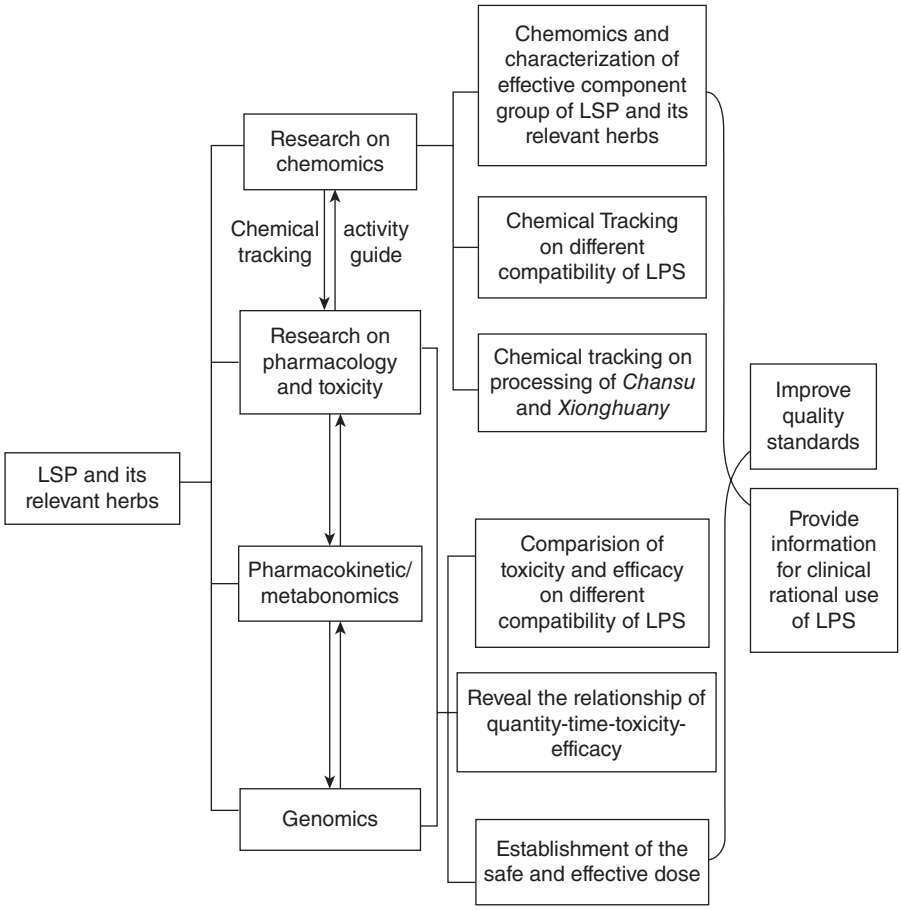


Fig. 12.1 Research frame of the safety evaluation of LSP.

analysis combined with toxicology to explore the relationship of quantity–time–toxicity–efficacy from the perspectives of active herbs ("effective" herbs and "toxic" herbs) and components ("effective" components and "toxic" components). The study is helpful for the safety reevaluation of LSP and raw herbs in it, and has great importance for clinic research. The concept that combines chemomics with pharmacological and toxicological research is essential and provides a model for the safety evaluation of TCM. A brief instruction of the research frame for this chapter is shown in Fig. 12.1.

12.2 TOXICITY STUDY OF *CHANSU*

In this section, the acute toxicity of *Chansu*, metabonomics studies on rats orally administered C*hansu*, and genomics research on rat hearts were examined.

12.2.1 Acute Toxicity of *Chansu*

Sprague-Dawley rats (180–210 g in weight) were purchased from the Centre of Laboratory Animals, Tsinghua University (Beijing, China). The experimental animals were housed for 2 days, then 40 rats were randomly divided into 5 groups, half males and half females in each group. The experimental groups took *chansu* orally at the doses of 128, 64, 32, 16 $mg \cdot kg^{-1}$. The control group received saline buffer at equal quantity. The MPA multichannel biological signal analysis system was used to measure and analyze ECG changes before and after drug administration of the rats. The animals were fasted for 12 h, anesthetized with urethane (1.3 g/kg, IP), and fixed and positioned on their backs on a pad, and the MPA multichannel biological signal analysis system was linked to record the ECG for 5 min. Rats with unusual ECG were discarded. The ECG were measured before and 0.5, 1, 1.5, and 2 h after drug administration. ECG measurements were taken after heartbeat became steady for 2–3 min.

A stable period of 15–30 sec of ECG from each rat was selected for the analysis. Average data within the selected time period could be seen as the heart rate of that rat, and then the average heart rate of the same group with the same administration time could be taken. Taking the value of the heart rate before drug administration as 1, relative heart rates with the ratio of heart rate of other time points to the heart rate before administration of *chansu* are shown in Fig. 12.2.

From Fig. 12.2, we can see that the heart rates after administration of *chansu* in each group were lower to varying degrees, with greater dose corresponding to lower heart rate. The medicine started working right after drug administration, and reached a maximum between 1 and 1.5 h and then had an obvious trend of recovery stage. The total time of efficacy was up to 2 h. The heart rate data was identified with t-fit, from which we can see that efficacy appeared earlier and longer as the dose of *chansu* increased.

In the high dose *Chansu* group, the PR interval was significantly prolonged as heart rate slowed down noticeably. PR intervals of other groups were

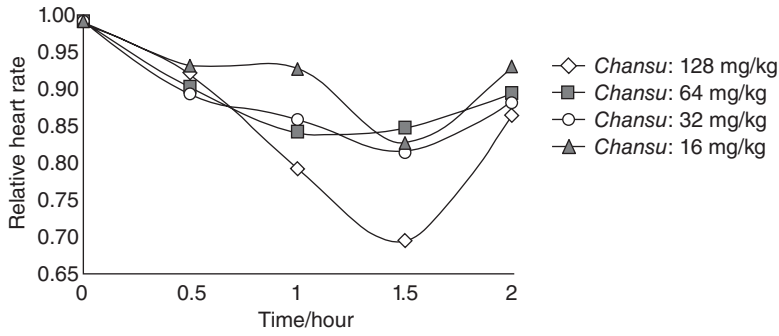


Fig. 12.2 Effect of *Chansu* on heart rates of rats at different time points.

significantly shorter, which is associated with increased heart rate arrhythmia. We can get the same result from electrocardiograph (ECG) of each group. Two hours later, the PR interval of the high dose group returned to the level before administration.

12.2.2 Metabonomics Studies After Administration of *Chansu* to Rats

12.2.2.1 Metabolic Fingerprints of Rat Serum We established metabolic fingerprints of rat serum based on UPLC-TOF/MS.[5] The MS spectra were first processed using the MarkerLynx Applications Manager version 4.1 (Waters Corp., Manchester, UK). The processed data list was then exported for partial least squares-discriminant analysis (PLS-DA) in the software package SIMCA-P 11.5 version (Umetrics AB, Umeå, Sweden).

12.2.2.2 Results and Discussion

12.2.2.2.1 PLS-DA Processing of UPLC-MS Data The ion intensities for each peak detected were then normalized within each sample, then the software SIMCA-P (11.5 version) was used for PLS-DA analysis to distinguish between the classes that were expected to show metabolic differences. Results of PLS-DA analysis are shown in Fig. 12.3.

As shown in Fig. 12.3, the *Chansu* group and the control group obviously separated from each other, indicating that *Chansu* disturbed the endogenous

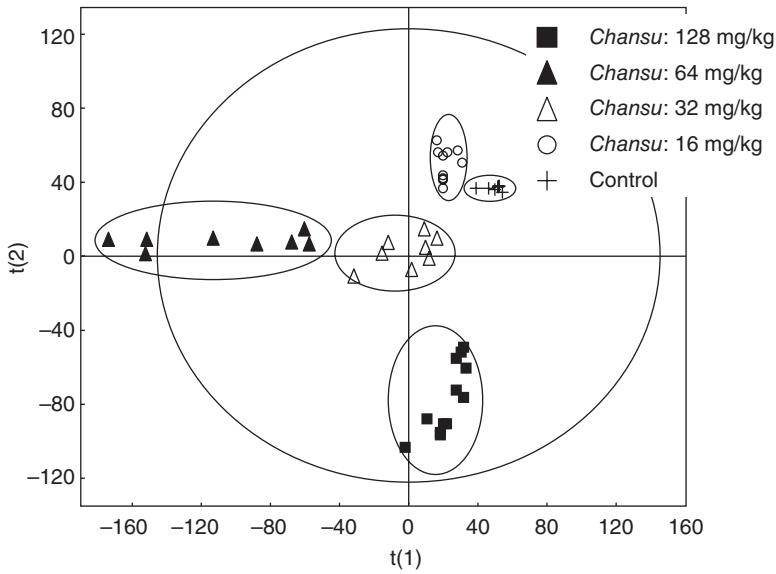


Fig. 12.3 PLS-DA score plot of rat serum fingerprints from different groups.

substance metabolism and significantly altered the metabolic fingerprints of rat serum compared with the normal state; and furthermore, the metabolic fingerprints of rat serum were much further deviated from the normal group, indicating that a greater dose of *chansu* led to greater metabolic alteration, and the toxicity of *Chansu* is dose-dependent.

12.2.2.2.2 Identification of Potential Biomarkers Variables (metabolites) that significantly contributed to clustering and discrimination were identified according to a threshold of variable importance in the projection (VIP) values (VIP > 1), which could be generated after PLS-DA processing. In order to select potential biomarkers worthy of preferential study in the next step, these differential metabolites were validated using a student's t-test. The critical *p*-value was set to 0.05 for significantly differential variables in this study. Then, the structural information was obtained by searching the freely accessible databases of KEGG (http://www.genome.jp) and HMDB (http://www.hmdb.ca) utilizing detected molecular weights and elemental compositions. As a result, significantly differential endogenous metabolites were selected for further study. Identification of these metabolites was then carried out as follows, and the results are listed in Table 12.1.

These potential biomarkers were all Lyso-PCs or fatty acids, which indicated that most Lyso-PC biomarkers significantly decreased in *Chansu* groups compared with the control group. Fatty acids, such as arachidonic acid, were also altered to varying degrees. A greater dose of *Chansu* corresponded to greater deviation of potential biomarker levels from the normal levels.

12.2.2.2.3 Biological Significance of the Potential Biomarkers Lyso-PCs are very different from highly lipophilic phospholipids, as Lyso-PCs are balanced between hydrophilic and lipophilic, and tend to distribute in the aqueous–nonaqueous interface. The special solubility of Lyso-PCs increases their purifying ability and accelerates the rate of exchange between cell membrane and cytoplasm. Therefore, Lyso-PCs can be potentially damaging to red blood cells.

Lyso-PCs are associated with many diseases since they can accelerate inflammatory response. Lyso-PCs can bind to glucose transport proteins to inhibit glucose transport. Moreover, Lyso-PCs can enhance PLA2 activity through activating the PKC-α pathway. PLA2 is an important phospholipase in vivo, as it catalyzes the cleavage of phospholipids to produce free arachidonic acids (AA).[6] Under normal circumstances, AAs in vivo are mainly stored in the plasma membrane in the form of fatty acyl groups, particularly as phosphatidylcholines (PC), while the concentration of free AAs in a cell is close to none. The activated PKC-α pathway further activates PLA2, which quickly hydrolyzes membrane phospholipids, resulting in increased levels of phospholipids and increased levels of AAs. Linoleic acid is a metabolite of AA and the precursor of AA biosynthesis.[7] Free AAs increase in vivo after drug

TABLE 12.1 Identification of Significantly Differential Endogenous Metabolites in the Rat Serum

				Intensities(Mean ± SD, n = 8)				
t_R/min	m/z	Component	Identification Result	Control	TV (16 mg/kg)	TV (32 mg/kg)	TV (64 mg/kg)	TV (128 mg/kg)
10.14	568.3572	$C_{27}H_{55}NO_9P$	Lyso-PC (C18:0)	407.26 ± 15.53	425.03 ± 51.27	438.06 ± 45.32	452.16 ± 47.58	458.74 ± 30.64
7.60	540.3269	$C_{25}H_{51}NO_9P$	Lyso-PC (C16:0)	472.19 ± 19.86	449.52 ± 24.74	435.38 ± 37.30	408.73 ± 34.85	414.47 ± 29.49
6.76	564.3284	$C_{27}H_{51}NO_9P$	Lyso-PC (C18:2)	203.33 ± 7.35	173.03 ± 18.43	179.59 ± 13.52	169.71 ± 21.43	152.78 ± 15.04
6.77	588.3274	$C_{29}H_{51}NO_9P$	Lyso-PC (C20:4)	207.49 ± 6.95	185.16 ± 13.33	190.71 ± 24.4	154.9 ± 12.5	157.7 ± 28.4
8.18	566.3452	$C_{27}H_{53}NO_9P$	Lyso-PC (C18:1)	117.93 ± 4.76	89.52 ± 9.06	90.64 ± 25.32	77.05 ± 10.35	78.16 ± 2.77
6.06	538.3153	$C_{24}H_{48}NO_7P$	Lyso-PC(16:1(9Z))	30.04 ± 0.60	23.23 ± 0.19	15.07 ± 13.21	14.70 ± 2.62	9.73 ± 0.79
12.44	303.2329	$C_{20}H_{31}O_2$	Arachidonic acid	38.73 ± 1.36	40.86 ± 0.34	51.88 ± 5.07	64.87 ± 5.24	76.58 ± 4.25
12.73	279.2329	$C_{18}H_{31}O_2$	Linoleic acid	65.43 ± 2.87	38.72 ± 1.56	31.03 ± 3,72	35.49 ± 5.89	37.15 ± 1.99
14.73	281.2438	$C_{18}H_{33}O_2$	Oleinic acid	45.24 ± 3.25	24.21 ± 2.14	33.12 ± 2.13	30.07 ± 3.21	15.77 ± 1.18

administration, and they may inhibit the formation of linoleic acid, resulting in a certain degree of reduction of linoleic acids.

In this study, for animals in the *Chansu* group, both AA and linoleic acid levels were reduced, which indicated that *Chansu* might inhibit the biosynthesis of AA as a side effect. Metabolic abnormalities of AA have a great impact on cardiac functions, which includes decreased myocardial contractility and cardiac output, and can even induce myocardial infarction and angina pectoris.[8]

In this section, the metabonomics method coupled with ECG was applied to the study of the acute toxicity of *Chansu*. The profiles of serum samples were obtained with UPLC-TOF/MS. The potential biomarkers were screened out according to the biological functions of the obtained potential biomarkers. It was deduced that the cardiac injuries may be induced by a perturbed lipid metabolism through hampering the reacylation of free fatty acids or activating protein kinase pathways. This study provides a new approach to discovering the underlying mechanism of *Chansu* toxicity.

12.2.3 Genomics Research on Rat Hearts After Administration of *Chansu*

In order to study the cardiac toxicity of *Chansu*, the rats in the experiment were divided into two groups, high dose of *Chansu* group (HDG) and low dose of *Chansu* group (LDG). By comparing the differences in gene expressions in the high dose group and low dose group, one might be able to explain the acute cardiac toxicity caused by *Chansu*. The experiment was conducted with 12 adult SD rats (200 ± 20 g in weight), equal in males and females, which were purchased from the PLA Military Academy of Medical Sciences Laboratory Animal Center (Certificate No.: SCXK-[Army] 2007-004).

12.2.3.1 Microarray Analysis Total RNA was extracted using Trizol (Invitrogen, USA) according to the manufacturer's directions. RNA was then further purified by NucleoSpin RNA clean-up kit (MN, Germany). Total RNA concentration and integrity were separately determined by Biophotomete (Eppendorf, Germany) and agarose gel electrophoresis.

Total RNA was reverse transcribed into cDNA. Cy5-dCTP was used to label the *Chansu* group, and Cy3-dCTP was used to label the saline control group. Three sets of chips were used to ensure repeatability. Hybridization was done on a Rattus norvegicus genome oligonucleotide set (version 3.0), which consists of 70-per probes representing 26962 genes (CapitalBio, China); and measurement of signal intensity was done with a LuxScan™ 10K Microarray Scanner (CapitalBio). The microarray procedures were carried out according to the methods provided by CapitalBio.

Analysis of microarray hybridization data was performed with assistance from CapitalBio. Raw microarray data was first normalized and bad spots were excluded. Statistically significant changes in gene expression ($p < 0.05$) induced

TABLE 12.2 Differentially Expressed Genes

Group of Experiments	Upregulation	Downregulation	Total
Chansu HDG (128 mg/kg)	56	73	129
Chansu LDG (16 mg/kg)	48	25	73

TABLE 12.3 Pathways Related to Both High and Low Dose of *Chansu* Groups

No.	GO No.	Pathways Related to Both *Chansu* HDG and LDG
1	GO:0009628	Response to endogenous stimulus
2	GO:0050801	Ion homeostais
3	GO:0055080	Cation homeostais
4	GO:0030036	Actin organization
5	GO:0006006	Protein import into nucleus translocation
6	GO:0009725	Response to hormone stimulus

by *Chansu* treatment relative to control rats were then analyzed using a software package (provided by CapitalBio). Genes with signal intensity (Cy3 or Cy5) greater than 800 were regarded as being expressed. Lastly, the following thresholds were used to define the differentially expressed probe sets: a transcript was considered to be differentially expressed in the *Chansu* group when it was at least 2.0 fold higher than that in the control group. In order to reveal the expression changes of a subset of biologically important genes, microarray data were subject to hierarchical clustering (Cluster 3.0). Differentially expressed genes were screened out from gene cluster as shown in Table 12.2.

12.2.3.2 GO Analysis of Differentially Expressed Genes DAVID bioinformatics resources were used to conduct gene ontology analysis of differentially expressed genes.[9, 10] The significant biological pathways ($P < 0.01$) were investigated among the differentially expressed genes. Table 12.3 lists the pathways associated with both *Chansu* HDG and LDG, indicating that *Chansu* might influence ion homeostasis in rat hearts, and the disturbance of ion channels might lead to arrhythmia and other common *Chansu* toxicity. The GO analysis results also indicated that *Chansu* could also affect actin composition and cause stress reactions to interfere with normal cardiac functions.

Biological pathways related to *Chansu* high dose group are listed in Table 12.4. The results indicated that high doses of *Chansu* can damage ion homeostasis and energy metabolism, and even induce apoptosis and cause cell necrosis in the heart.

Biological pathways related to low doses of *Chansu* are listed in Table 12.5. The results indicated that low doses of *Chansu* cause anti-apoptotic actions and promote the proliferation of cardiac cells. It can also cause a series of reactions on elimination. All these changes can be seen as defensive actions caused by the low dose of *Chansu*.

TABLE 12.4 Pathways Related to High Dose of *Chansu* Group

No.	GO No.	Pathways Related to *Chansu* HDG
1	GO:0042981	Apoptosis regulation
2	GO:0042592	Homeostatic process
3	GO:0055072	Iron ion homeostasis
4	GO:0018106	Phosphorylation
5	GO:0006006	Glucose metabolic process
6	GO:0055114	Oxidation reduction

TABLE 12.5 Pathways Related to Low Dose of *Chansu* Group

No.	GO No.	Pathways Related to *Chansu* LDG
1	GO:0045767	Regulation of anti-apoptosis
2	GO:0042127	Regulation of cell proliferation
3	GO:0048009	Insulin-like growth factor receptor signaling pathway
4	GO:0030198	Extracellular matrix organization
5	GO:0046164	Alcohol catabolic process
6	GO:0030155	Regulation of cell adhesion
7	GO:0010565	Regulation of cellular ketone metabolic process

TABLE 12.6 KEGG Pathway Analysis of Differentially Expressed Genes of *Chansu* Groups

Pathway	TV HDG	TV LDG
Antigen processing and presentation	rno04612	rno04612
Cell adhesion molecules (CAMs)	rno04514	rno04514
Asthma	rno05310	rno05310
Adipocytokine signaling pathway	rno04920	–
Focal adhesion	–	rno04510
Type II diabetes mellitus	–	rno04930
Arachidonic acid metabolism	–	rno00590
Linoleic acid metabolism	–	rno00591

12.2.3.3 KEGG Pathway Analysis of Differentially Expressed Genes

The Kyoto Encyclopedia of Genes and Genomes (KEGG) database can be used to conduct pathway enrichment analysis on differentially expressed genes. Nowadays, a lot of databases, including DAVID, can carry out pathway enrichment analysis. The result of pathway enrichment analysis is shown in Table 12.6.

The results from KEGG analysis showed that high and low doses of *Chansu* were both associated with the asthma pathway, which is consistent with the report that *Chansu* can cause respiratory failure.[11] It has been suggested that *Chansu* might cause respiratory failure through affecting muscle contraction.

In addition, the main enrichment pathway was fatty acid metabolism for the low dose group, suggesting that low doses of *Chansu* can lead to enhanced myocardial contraction and the body's defensive reactions, including anti-apoptotic actions, thereby accelerating energy consumption, while there was no corresponding high energy metabolism for high dose of *Chansu* group, suggesting that high doses of *Chansu* had higher cardiac toxicity than low doses of *Chansu*, which led to structural damages and eventually caused cardiac cell apoptosis.

All the above genomic research results showed that low doses of *Chansu* can disturb ion homeostasis in rat hearts and the composition of actin. It can also cause anti-apoptotic actions and boost elimination and some other defensive actions to resist the interference from *Chansu*. As the dose of *Chansu* increased, *Chansu* not only disturbed ion homeostasis in rat hearts and the composition of actin, but also caused oxidative stress responses and some redox reactions, this would produce a series of irreversible heart damages, and ultimately cause apoptosis.

12.3 CHEMOMICS STUDY OF LSP

The main components of *Chansu* in LSP are bufadienolides and bufotenines. Cholic acids are the main component in *Niuhuang*. In addition, there are mainly volatile components in *Shexiang* and *Bingpian*, while *Xionghuang* and *Zhenzhufen* are mineral drugs. HPLC-MS, GC-MS, and ICP-MS were adopted according to the characteristics of medicinal materials in LSP for chemical genomics research to reveal the physical basis of safety evaluation.

12.3.1. Multicomponent Quantification of LSP

This section first obtained multidimensional fingerprint of LSP by HPLC/UV-ELSD. The UV fingerprint was obviously different from the ELSD fingerprint; and this difference was caused by cholic acid. There were 16 common peaks in the UV-fingerprint, and 22 common peaks in the ELSD-fingerprint. The results showed that the number of theoretical plates was more than 8000 when calculated by three types of cholic acid (cholic acid, deoxycholic acid, and chenodeoxycholic acid) and four types of bufadienolide (hydroxycinobufagin, bufalin, cinobufagin, and resibufogenin) as target compounds. The separation between adjacent peaks is greater than 1.5, and the symmetry factor is in the range of 0.95–1.05. The repeatability, precision, and sample stability studies showed that the total peak area RSD were all less than 5%, indicating that this method was reliable to be used for quality control for nonvolatile materials in LSP. Contents of three samples of LSP are shown in Table 12.7.

12.3.1.1 Analysis of Nonvolatile Components in LSP by HPLC-MS The representative HPLC-MS chromatograms of LSP are shown in Fig. 12.4.

TABLE 12.7 Contents of Seven Compounds in LSP (mg/g)

Sample No.	040408	040305	041201
Cholic acid	3.234	3.256	3.345
Deoxycholic acid	1.653	1.667	1.537
Chenodeoxycholic acid	0.840	0.826	0.980
Hydroxycinobufagin	1.418	1.438	1.424
Bufalin	2.398	2.840	2.275
Cinobufagin	2.840	2.771	3.266
Resibufogenin	2.275	2.536	3.424

Sample no. was batch number of LSP.

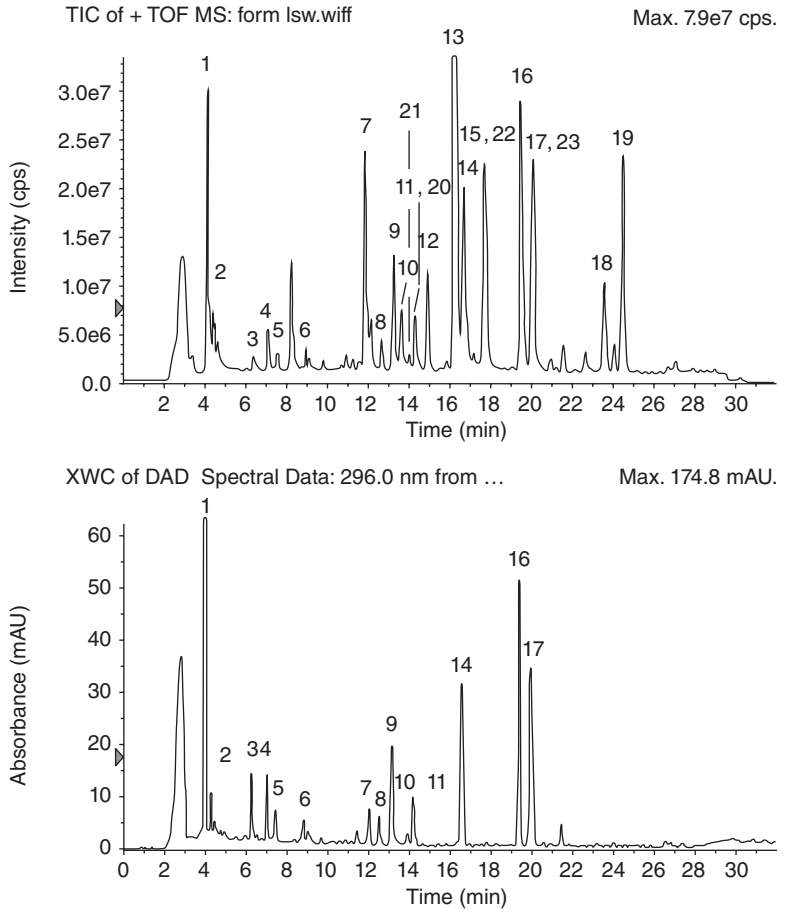


Fig. 12.4 TIC chromatogram by HPLC-TOF/MS (A) and HPLC/UV chromatogram at 296 nm (B) of LSP (identification of No. 1–23 peaks is shown in Table 12.8).

TABLE 12.8 Hydrophobic Compounds in LSP Identified by HPLC-TOF/MS

No	t_R	Selected Ion	Formula	Measured Mass (m/z)	Calculated Mass (m/z)	Error mDa	Error ppm	Compound
1	4.01	$[M + H]^+$	$C_{13}H_{18}N_2O$	219.1484	219.15502	–1.7869	–8.1542	Bufolenidine
2	4.35	$[M + H]^+$	$C_{10}H_{12}N_2O$	177.1104	177.1033	7.0632	39.8805	5-HT
3	6.37	$[M + H]^+$	$C_{24}H_{33}O_6$	417.2257	417.2282	–2.4627	–5.9026	Ψ-Bufarenogin
4	7.12	$[M + H]^+$	$C_{24}H_{35}O_5$	403.2473	403.2489	–1.5981	–3.9632	Gamabufotalin
5	7.57	$[M + H]^+$	$C_{24}H_{33}O_6$	417.2265	417.2282	–1.6627	–3.9852	Bufarenogin
6	8.90	$[M + H]^+$	$C_{24}H_{33}O_6$	417.2263	417.2282	–1.8627	–4.4645	Arenobufagin
7	12.12	$[M + H]^+$	$C_{24}H_{35}O_5$	403.2473	403.2489	–1.5981	–3.9632	Telocinobufagin
8	12.65	$[M + H]^+$	$C_{24}H_{33}O_5$	401.2315	401.2322	–0.6509	–1.6223	Deacetylcinobufagin
9	13.25	$[M + H]^+$	$C_{26}H_{37}O_6$	445.2601	445.2584	1.7342	3.8949	Bufotalin
10	13.98	$[M + H]^+$	$C_{25}H_{33}O_7$	457.2204	457.2220	–1.5802	–3.4561	19-Oxo-cinobufagin
11	14.30	$[M + H]^+$	$C_{26}H_{35}O_7$	459.2378	459.2377	0.0696	0.1517	Cinobufotalin
12	14.93	$[M + H]^+$	$C_{24}H_{41}O_5$	409.2989	409.2948	4.1487	10.1361	Hyocholic acid
13	16.25	$[M\text{-}20H_2O+H^+$	$C_{24}H_{37}O_3$	373.2731	373.2737	–0.6218	–1.6658	Cholic acid
14	16.68	$[M + H]^+$	$C_{24}H_{35}O_4$	387.2621	387.2529	9.2136	23.7916	Bufalin
15	17.72	$[M\text{-}20H_2O+H^+$	$C_{24}H_{37}O_2$	357.2854	357.2788	6.5928	18.4525	Deoxycholic acid
16	19.50	$[M + H]^+$	$C_{26}H_{35}O_6$	443.2642	443.2428	21.3843	48.2428	Cinobufagin
17	20.10	$[M + H]^+$	$C_{24}H_{33}O_4$	385.2844	385.2373	11.4636	29.7565	Resibufagin
18	23.60	$[M\text{-}20H_2O+H^+$	$C_{24}H_{37}O_2$	357.2789	357.2788	0.0928	0.2597	Chenodeoxy cholic acid
19	24.45	$[M\text{-}20H_2O+H^+$	$C_{24}H_{37}O_2$	357.2787	357.2788	–0.1071	–0.2999	Ursodeoxycholic acid
20	14.50	$[M + H]^+$	$C_{24}H_{33}O_5$	401.2324	401.2322	0.249	0.6207	Marinobufagin
21	14.01	$[M + H]^+$	$C_{24}H_{31}O_5$	399.2155	399.2166	–1.1008	–2.7575	Resibufagin
22	17.9	$[M + H]^+$	$C_{19}H_{29}O_2$	289.2735	289.2677	0.3938	0.9293	Testosterone
23	20.1	$[M\text{-}20H_2O+H^+$	$C_{19}H_{29}O_2$	271.2537	271.2512	0.2737	0.3847	Dehydrotestosterone

Combining HPLC-TOF/MS and HPLC-ion Trap/MS identified 23 compounds from *Chansu*, *Niuhuang*, and *Shexiang*. The results shown in Table 12.8 laid the foundation for clarifying safety and efficacy of LSP.

12.3.1.2 Analysis of Volatile Components in LSP by GC-MS and GC-MS/MS The volatile components of LSP mainly came from *Shexiang* and *Bingpian*. Through first-order spectrum, characteristic ions of *Shexiang* and *Bingpian* all had m/z of 95 (100%) and 67 (22%) and 41 (11%). There was not much difference between *Shexiang* and *Bingpian*, so a second-order mass spectrum was needed. We gradually increased the voltage of the selected parent ion until the most suitable collision voltage was found (0.5 V was selected as the CID voltage). Through second-order mass spectrum and taking m/z 95 as the parent ion, characteristic ions of *Shexiang* were m/z 67 (99%), m/z 93 (19.6%), m/z 91 (12.1%), and m/z 65 (7.8%), while the characteristic ions of iso-*Bingpian* were m/z 67 (99%), m/z 95 (26.3%), m/z 91 (18.4%), and m/z 81 (14.3%). These results indicated that iso-*Bingpian* and *Bingpian* had noticeable differences in GC-MS/MS spectrum, so that this method could be used to qualitatively distinguish between two isomers. A quantitative method for *Bingpian* and muscone was also established. The detection limits of *Shexiang* and ketone were 4 ng/mL and 0.1 ng/mL, respectively, by GC-MS, while the limits were 0.2 ng/mL and 4 ug/mL, respectively, by GC-MS/MS. Repeatability and precision investigation showed that the RSD percentage of the peak area was less than 5 by the two methods, but when GC-MS/MS is compared with GC-MS, the RSD percentage of repeatability and accuracy was much smaller. The recovery concentrations were from 98% to 103%. Although the content results of the two methods did not have significant differences, GC-MS/MS improved the selectivity, reduced the chemical noise interference, and had higher sensitivity and lower detection limits. Therefore GC-MS/MS had an advantage in the analysis of complex systems.[12]

12.3.1.3 Analysis of Arsenic in LSP and* Xionghuang *by ICP-MS The toxicity and biological importance of arsenic are mainly dependent on its chemical form. Inorganic arsenic is the most toxic, while methylated arsenic is less toxic, and AsB and AsC can be considered nontoxic. Studies have shown the order of toxicity in vivo is: MMA^{III}, $DMA^{III} > As^{III} > As^{V} > MMA^{V}$, DMA^{V}.[13, 14]

The total arsenic content was 60.8 mg/g in LSP by Agilent 7500C ICP-MS. HPLC-ICP/MS was adopted to analyze the species of arsenic. AN optimized chromatogram is shown in Fig. 12.5. The detection limit of this method was 0.20 $ng \cdot mL^{-1}$. In addition, RSD of MMA peak was 3.11%, while the others were less than 2%.

The main constituent in *Xionghuang* is insoluble As_4S_4, accounting for more than 90% of *Xionghuang*. *Xionghuang* also contains small amounts of As^{III} and As^{V}, which are soluble and inorganic. There are four kinds of herbal forms of arsenic: As^{III}, As^{V}, MMA^{V}, and DMA^{V}. We found that our samples mainly

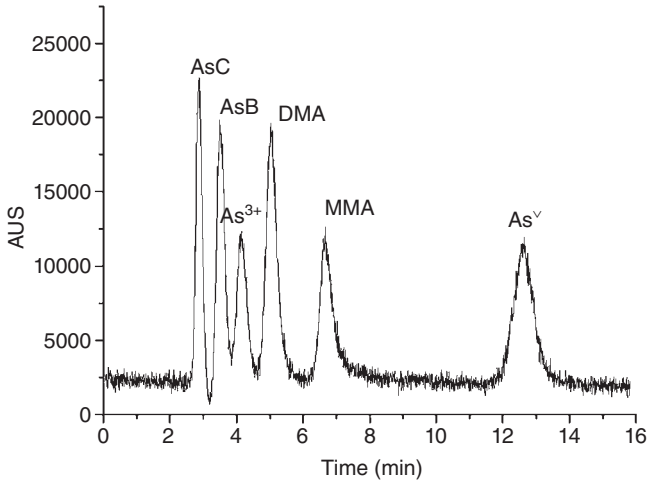


Fig. 12.5 HPLC-ICP/MS chromatogram of the six species of arsenic spiked in tissue sample (concentration: 40 $ng \cdot g^{-1}$).

contain two major forms of inorganic arsenic, and DMA^V, MMA^V, AsC^V, and AsB^V were not detected during in vitro studies (processing of *Xionghuang* and research of chemomics). Organic arsenic was detected in vivo studies (body distribution and research of metabonomics). The results above are also consistent with what has been reported.[15]

12.3.2 Detoxicity Effect of Processing of *Chansu* and *Xionghuang*

***12.3.2.1 Processing of* Chansu** Processing as a major feature for medicine could play a role effecting detoxification. This section studies the effects of processing from two aspects: processing method and amount of auxiliary material. The types of *Chansu* studied include original *Chansu*, alcohol processed *Chansu*, milk processed *Chansu*, and talcum powder processed *Chansu*. For alcohol processed toad venom and milk processed *Chansu*, the effect on four components (hydroxycinobufagin, bufalin, cinobufagin, and resibufogenin) in *Chansu* caused by the amount (0 times, original *Chansu*, 2 times, 4 times, and 10 times) of auxiliary material was studied.

12.3.2.1.1 Establishment of Fingerprint and Multicomponent Quantification Method HPLC-ionTrap/MS spectrometry was adopted for component analysis on *Chansu* according to references.[16] HPLC chromatogram of *Chansu* is shown in Fig. 12.6, and the chemical components were identified from the chromatographic peaks, which is shown in Table 12.9. The four components (hydroxycinobufagin, bufalin, cinobufagin, and resibufogenin) in *Chansu* had a good linear relationship within the standard curve in (1–50) μg/mL. Precision

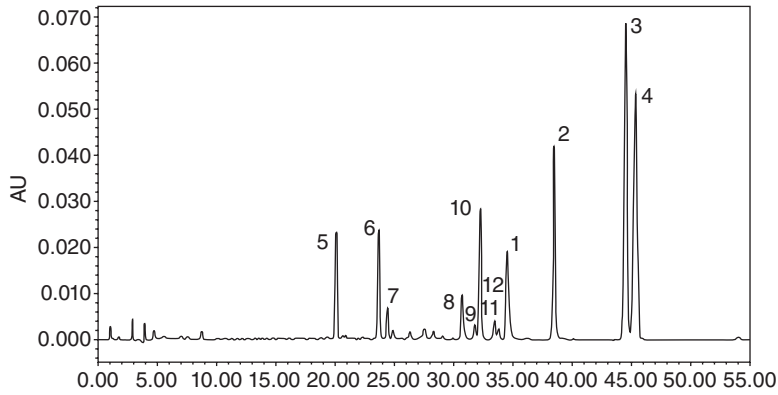


Fig. 12.6 HPLC chromatogram of *Chansu* (identification of No. 1–12 peaks was shown in Table 12.9).

TABLE 12.9 Identification of the Components of *Chansu*

No.	Rt (min)	Mass	MS^2	MS^3	Formula	Name
1	34.54	459.1	363.2	345.0	$C_{26}H_{35}O_7$	Cinobufotalin
2	38.49	387.2	351.1	333.3	$C_{24}H_{35}O_4$	Bufalin
3	44.56	443.2	365.2	347.0	$C_{26}H_{35}O_6$	Cinobufagin
4	45.36	385.1	367.3	349.0	$C_{24}H_{33}O_4$	Resibufogenin
5	20.09	403.1	349.1	331.0	$C_{24}H_{35}O_5$	Gamabufotalin
6	23.66	417.2	399.3	317.1	$C_{24}H_{33}O_6$	Bufarenogin
7	24.40	417.1	335.2	317.0	$C_{24}H_{33}O_6$	Arenobufagin
8	30.71	403.1	349.3	331.0	$C_{24}H_{35}O_5$	Telocinobufagin
9	31.78	401.1	365.1	347.1	$C_{24}H_{33}O_5$	Deacetylcinobufagin
10	32.24	445.2	349.3	330.9	$C_{26}H_{37}O_6$	Bufotalin
11	33.44	399.3	257.3	238.9	$C_{24}H_{31}O_5$	Resibufagin
12	33.80	457.4	333.0	305.0	$C_{26}H_{33}O_7$	19-Oxo-cinobufagin

RSDs were all less than 5% for five repeated trials, which indicated that this method could satisfy the requirements for quantitative analysis.

12.3.2.1.2 The Effects of Processing on the Components of Chansu The content of the four hydrophobic components of original *Chansu*, alcohol processed *Chansu*, milk processed *Chansu*, and talcum powder processed *Chansu* were determined; their chromatograms are shown as Fig. 12.7. The values of the content of the four hydrophobic components provided by drug company were taken as reference values. Hydroxycinobufagin and bufalin had favorable local anesthesia effects, while cinobufagin and resibufogenin had cardiotonic effects.[17]

According to Chinese Pharmacopoeia,[18] the content of cinobufagin and resibufogenin should not be less than 6.0% (60 mg/g), so we took the contents

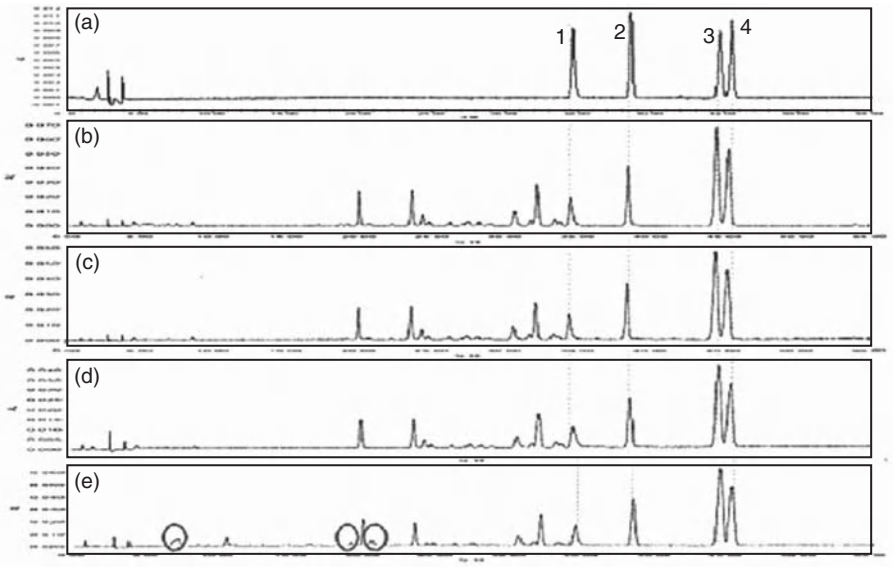


Fig. 12.7 Chromatograms of standard mixture (A), original *Chansu* (B), alcohol processed *Chansu* (C), milk processed *Chansu* (D), and talcum powder processed *Chansu* (E).

TABLE 12.10 Content of the Four Hydrophobic Components in *Chansu* by Different Processing Methods (mg·g^{-1})

	Original TV	Alcohol Processed TV	Milk Processed TV	Talcum Powder Processed TV	TV Processed by Drug Company
Hydroxycinobufagin (1)	13.65	12.31	12.57	11.50	7.949
Bufalin (2)	21.56	20.06	22.05	20.75	22.86
Cinobufagin (3)	46.53	41.29	46.28	42.93	37.00
Resibufogenin (4)	30.98	28.36	31.52	29.63	39.25
(3)+(4)	77.51	69.65	77.80	72.56	76.25
(1)+(2)+(3)+(4)	112.7	102.0	112.4	104.8	107.1

of cinobufagin and resibufogenin as references. The contents of the four hydrophobic components in *Chansu* by different processing methods are shown in Table 12.10.

It can be seen from the results that all the above processing methods could meet the standard of Chinese Pharmacopoeia. There were not any distinct differences between the original *Chansu* and processed *Chansu*, except processed *Chansu* were much easier to process and smash than original *Chansu*.

Chansu contains exogenous protein, epinephrine, and alkaloids, belongs to animal drugs, and might have side effects. Alcohol can denature proteins. Milk processed *Chansu* contains amino acids as milk is added to it. Amino acids in milk would complexate with alkaloids in medicine, and reduce protein bindings in the human body. The temperature of talcum powder C*hansu* processing also could denature proteins. Therefore, these three methods are acceptable.

As shown in Fig. 12.7, alcohol processed *Chansu* and milk processed *Chansu* were very similar in chromatogram. Their peaks were at fixed percentages, indicating that components of *Chansu* did not significantly change after processing. However, the chromatogram of talcum powder processed *Chansu* was obviously changed at 7.3 min, 19.3 min, and 20.7 min. Compared with original *Chansu*, the contents of bufadienolides in talcum powder processed *Chansu* reduced significantly. We suggested that bufadienolides might convert to anti-o-hydroxy cinnamic acid salt in heated conditions.[19] The contents of hydroxycinobufagin and cinobufagin were also significantly lower than these in the original *Chansu*, so we suggested that two compounds both had $OCOCH_3$ but lost -$OCOCH_3$ after processing. In addition, chromatogram of talcum powder processed *chansu* was also obviously changed at 23.66 min and 24.40 min according to Table 12.10; and the two compounds were identified to be bufarenogin and arenobufagin. The contents of bufarenogin and arenobufagin in talcum powder processed *Chansu* decreased, indicating that the two compounds could decompose when heated during processing.

***12.3.2.2 Processing of* Xionghuang** Traditional processing methods of *Xionghuang* are the refining powder with water method and dry grinding method. Pickling process, caustic method, vinegar washing method, yogurt refining powder with water method, and other methods were later developed. As_2S_2, one of components in *Xionghuang*, is insoluble in water, acids, or bases, while As_2O_3 is soluble in acids and bases, and slightly soluble in water. As for a human's digestive juices, the pH value of gastric juice is about 1.35, which is acidic, while the pH value of intestinal juice is between 7.3 and 7.5, which was alkaline.

In order to examine the effects on detoxification and dissolution of arsenic, water, artificial gastric juice, and artificial intestinal fluid were used to extract soluble arsenic in *Xionghuang* and LSP. HPLC-ICP-MS was adopted for content and speciation analysis for arsenic; the results are shown in Table 12.11. It could be seen that the caustic method was better than pickling process and the refining powder with water method for processing of *Xionghuang*. In other words, the human digestive juice better promoted the dissolution of arsenic than water did.

12.3.3 Chemical Research on the Compatibility of LSP

12.3.3.1 Multicomponent Fingerprint Comparison Study on Different Formulas of LSP and* Chansu *by HPLC-MS/MS We established the HPLC/UV method in Section 12.3.2.1 for the determination of the contents

TABLE 12.11 Content of Soluble Arsenic in *Xionghuang* ($n = 3$)

Extracting Solvent	As^{III} ($mg \cdot g^{-1}$)	As^{V} ($mg \cdot g^{-1}$)	Soluble Arsenic[a] ($mg \cdot g^{-1}$)	Soluble Arsenic in Total Arsenic (w%)	RSD (%)
Water	23.3	0	23.3	3.96%	6.09
Artificial gastric	28.4	0	28.4	4.82%	5.59
Artificial intestinal	35.3	1.70	37.0	6.28%	6.66

[a]The content of soluble arsenic was the sum of As^{III} and As^{V}.

TABLE 12.12 Content of Four Bufadienolides in Different Formulas of LSP ($n = 3$)

Sample	Hydroxycinobufagin ($mg \cdot g^{-1}$)	Bufalin ($mg \cdot g^{-1}$)	Cinobufagin ($mg \cdot g^{-1}$)	Resibufogenin ($mg \cdot g^{-1}$)
Only TV and *Xionghuang*	1.667	2.810	3.298	4.398
Only *Xionghuang*	0	0	0	0
Only TV	1.641	2.755	3.238	4.322
Original LSP	1.726	3.137	3.594	4.684
LSP removed *Xionghuang*	1.611	2.847	3.327	4.429
LSP removed TV	0	0	0	0
LSP removed *Xionghuang* and TV	0	0	0	0

of four bufadienolides (hydroxycinobufagin, bufalin, cinobufagin, and resibufogenin) in different formulas of LSP. The results are shown in Table 12.12. The content of the original formula of LSP was higher than that of the other formulas, which indicated that the compatibility of LSP might have some solubilization effect on some chemical components of *chansu*. In other words, it accelerated the release of effective component groups.

12.3.3.2 Comparison Study on Different Formulas of LSP and* Xionghuang *about the Content of Soluble Arsenic The median lethal doses LD50 (mg/kg) of major arsenic are: arsenic trioxide (As^{III}) 34.5, arsenite (As^{III}) 14, arsenate (As^{V}) 20. In the previous study, we found that the soluble components in *Xionghuang* were mainly arsenite (As^{III}) and arsenate (As^{V}). A comparison study on different formulas of LSP and *Xionghuang* about the content of soluble arsenic and a study on the mechanism of action of *Xionghuang* and amino acids were conducted to reveal the attenuation mechanism of different LSP formulas and *Xionghuang*.

12.3.3.2.1 Comparison Study on Different Formulas of LSP and Xionghuang *about the Content of Soluble Arsenic* In order to examine changes

TABLE 12.13 Content of Soluble Arsenic and Total Arsenic of Different Technologies for Extraction ($n = 3$)

Groups	Total Arsenic ($mg \cdot g^{-1}$)	As^{III} ($mg \cdot g^{-1}$)	As^{V} ($mg \cdot g^{-1}$)	Soluble Arsenic[a] ($mg \cdot g^{-1}$)	Soluble Arsenic W%
Xionghuang	141.6	1.15 ± 0.26	0	1.15 ± 0.26	0.81
LSP	126.1	0.73 ± 0.05	0.09 ± 0.01	0.82 ± 0.06	0.65
LSP (removed TV)	138.1	0.62 ± 0.03	0.07 ± 0.01	0.69 ± 0.04	0.50
Xionghuang (artificial gastric fluid)	141.6	1.71 ± 0.34	0.32 ± 0.08	2.03 ± 0.42	1.43
Xionghuang (artificial intestinal liquid)	141.6	5.49 ± 0.89	0.55 ± 0.17	6.04 ± 1.06	4.27
LSP (artificial gastric fluid)	126.1	0.26 ± 0.03	0.84 ± 0.35	1.10 ± 0.38	0.87
LSP (artificial intestinal liquid)	126.1	4.19 ± 0.43	3.08 ± 0.07	7.27 ± 0.50	5.77

[a]The content of souble arsenic was the sum of As^{III} and As^{V}.

in the content of soluble arsenic of *Xionghuang* in LSP, water, artificial gastric fluid, and artificial intestinal liquid were used as solvents for the extraction of *Xionghuang* and LSP (containing the same amount of *Xionghuang* as *Xionghuang*) by ultrasonic methods. The content of total arsenic was determined by ICP/MS, and HPLC-ICP/MS was adopted to analyze the species of arsenic. The results are shown in Table 12.13.

From the results in Table 12.13, we can see that, comparing LSP and LSP without *Chansu* with *Xionghuang*, the content of soluble arsenic significantly decreased, indicating that the formula of LSP could reduce the dissolution of *Xionghuang* in artificial gastric fluid. For extraction by artificial intestinal liquid, the content of soluble arsenic did not have apparent changes but As^{V} proportion increased significantly, as As^{III} is more toxic than As^{V}. Above all, the results showed that the compatibility of LSP could play a role in attenuation.

12.3.3.2.2 Compatibility Research on Xionghuang *with Other Related Raw Herbs in LSP* To further examine the attenuation ability after compatibility of *Xionghuang* with LSP, we first mixed *Xionghuang* with other medicinal materials in LSP together and extracted them, then determined the content and species of the arsenic. The effects of other medicinal materials in LSP on the dissolution of arsenic in *Xionghuang* were studied. The results are shown in Table 12.14.

From these numbers, it could seem that the content of soluble arsenic was reduced for all groups after compatibility of *Xionghuang* with other medicinal materials, especially with *Chansu*, *Shexiang* and *Niuhuang*. These three medicinal materials as animal ingredients had many compounds containing

TABLE 12.14 Content of Arsenic in Compatibility of *Xionghuang* with Other Raw Herbs ($n = 3$)

Group	As^{III}(mg·g^{-1})	As^{V} (mg·g^{-1})	Soluble Arsenic[a] (mg·g^{-1})
Xionghuang	28.91 ± 1.19	0	28.91 ± 1.19
Xionghuang + TV	1.70 ± 0.27	0.16 ± 0.04	1.86 ± 0.31
Xionghuang + *Shexiang*	8.30 ± 0.45	0	8.30 ± 0.45
Xionghuang + *Niuhuang*	3.75 ± 0.39	0.19 ± 0.02	3.94 ± 0.41
Xionghuang + *Bingpian*	20.92 ± 1.62	0	20.92 ± 1.62
Xionghuang + *Zhenzhufen*	24.65 ± 4.21	0	24.65 ± 4.21

[a]The content of souble arsenic was the sum of As^{III} and As^{V}.

TABLE 12.15 Retention Time and Peak Area of *Xionghuang* Extract, Cysteine in Water Solution, and a Mixture of Them

	Peak 1		Peak 2	
	t_R/min	Area	t_R/min	Area
Xionghuang	–	–	14.958	2854956
Cysteine	–	–	15.127	959932
Mixture	13.709	468329	15.119	1405372

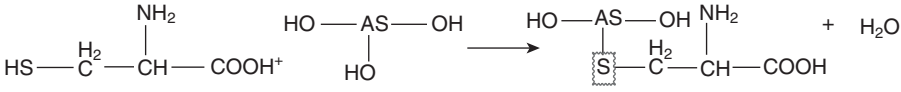
sulfhydryl and hydroxyl, which could form complexes with As^{III}, thereby reducing the dissolution of inorganic arsenic and the toxicity of *Xionghuang*.

12.3.3.2.3 Compatibility Research on Xionghuang *with Amino Acids* As^{III} could easily form complexes with sulfhydryl to form less toxic organoarsenic compounds, while cysteine is a common amino acid containing sulphur groups. *Chansu*, *Shexiang*, and *Niuhuang* all contain cysteine, so we suggested that cysteine might be involved in the mechanism of decreasing toxicity.

Xionghuang extract, cysteine in water solution, and a mixture of them were analyzed by HPLC-ELSD; the results are shown in Table 12.15.

Compared with chromatograms of *Xionghuang* and cysteine, a new unknown peak was found in the chromatogram of the mixture at 13.709 min, while the content of arsenic reduced 50% after mixing with cysteine. We suggested this might be involved in the mechanism of decreasing toxicity.

As^{III} could easily form complexes with sulfhydryl to form organoarsenic compounds. The possible reaction equation is:



In the negative ion monitoring mode of mass spectrometry, an unknown [MH] ion with m/z 228 formed. Extracting the target ion with m/z = 228, we found that a new compound formed when the extract of *Xionghuang* is mixed

with cystenine, which did not exist in either the extract of *Xionghuang* or cysteine water solutions, further confirming that it was a product of the reaction between arsenic and cysteine. However, because the retention times of the unknown compound and As^{III} were nearly the same, the unknown compound was not detected in total ion chromatogram in the previous study. Increasing the amount of cysteine to 10:1 and 50:1 (cysteine : *Xionghuang*) enabled us to find that the content of the unknown compound increased as the amount of cysteine increased, indicating that the amount of the unknown compound related to the ratio of *Xionghuang* to cysteine.

Molecular weight matching of 228 (m/z) was conducted with TOF-MS, confirming the molecular formula of the unknown compound was $C_3H_7NO_4SAs$. We concluded that the compound was formed by dehydration of cysteine and As^{III}. The results from this study supported the attenuation mechanism of *Xionghuang* with compatibility of LSP from the perspective of chemical transformation.

12.4 ASSESSMENT OF EFFECTIVENESS AND SAFETY OF LSP

12.4.1 Pharmacological Research of LSP

The clinical efficacy of LSP is mainly from its anti-inflammatory, analgesic, and detoxification effects. Previous studies have shown that *Chansu* containing dienol and catecholamine compounds had efficacies in cardiotonic areas, anti-inflammatory and analgesic effects, and so on, and *Xionghuang* containing As_2S_3 had efficacies in bacteriostat and improving immunologic function. According to the indications and efficacy of LSP and potential efficacies of *Chansu* and *Xionghuang*, we observed four aspects (anti-inflammatory, analgesic, antibacterial, and immunologic function) to evaluate the efficacies of *Chansu* and *Xionghuang* in LSP. The pharmacodynamic study of LSP was done by the Shanghai Institute of Chinese Medicine.

For comprehensive and thorough study of compatible mechanism, low, middle, and high dose LSP groups without *Chansu*, *Chansu* groups, *Xionghuang* groups, separate positive control groups, and control groups were designed. Group 1 (LSP), group 2 (LSP without *Chansu*), group 3 (LSP without *Xionghuang*), group 4 (*Chansu*), and group 5 (*Xionghuang*) were dissolved into distilled water containing 1% CMC to obtain the drugs with desired concentrations. Injections of Fenbid, hydrocortisone, and penicillin potassium were used as positive control.

The anti-inflammatory effect of LSP was demonstrated by its effects on auricle edema induced by xylene in mice and ankle swelling induced by diagonal fork dish glue in rats. The analgesic activity of LSP is demonstrated by how many times the mice twist their waists inducing by acetic acid. The immunologic function was demonstrated using carbon grains purging in mice blood. The antibacterial effect was demonstrated using the mortality rate in rats

TABLE 12.16 Results of Pharmacological Study

	Groups				
Experimental Method	1	2	3	4	5
Auricle edema induced by xylene	+++	+		++	
Ankle swelling induced by diagonal fork dish glue in rats	++	+		++	
Back pain induced by aceticacid	++	++		+	
Carbon grains purging in mice blood	++	–		+	–
Infection of coccus of golden yellow grape in enterocoelia	+	+	–	+	++

+: ($p < 0.05$ or $P < 0.01$); –: ($P > 0.05$).

caused by infection of coccus of golden yellow grape in enterocoelia. Results of the efficacy study are summarized in Table 12.16.

The results show that groups 1 and 4 had obvious efficacies in anti-inflammatory, analgesic, antibacterial, and immunologic functions; groups 2 and 5 displayed obvious efficacy in bacteriostat, and the antibacterial activity of group 3 was not significant. All of the above indicated that the efficacy against infection of coccus of golden yellow grape in enterocoelia was closely related with *Xionghuang*; the efficacy of group 1 in analgesic functions was significantly better than those of groups 2 and 4. The results showed that the compatibility of LSP had some synergistic effect.

12.4.2 Acute Toxicity Research of LSP

Research in this section about animals, biochemistry, and pathology detection was carried out by the drug safety evaluation center in accordance with GLP standards. Research on toxicokinetics and metabolomics was conducted by us.

12.4.2.1 Research on Acute Toxicity of LSP on Mice *Kunming* mice were used for research on acute toxicity by intragastric administration. The results showed that animals first appeared to be quiet, had less activity, were in prone positions, and had their eyes closed after administration. Some animal behaviors showed peritoneal irritation and short of breath. When doses greater than 900 $mg \cdot kg^{-1}$ were administered, animal death generally happened within 30 min after administration. The greater the dose, the faster the animal died after administration. Dead animals showed symptoms of stiff backs and limbs and twitching bodies. The LD50 of LSP was 600–650 $mg \cdot kg^{-1}$ by intragastric administration.

Research of maximum tolerance was performed in Wistar mice. The results showed that as the dose of LSP increased, animals had adverse reactions such as reduced activities (living in a quiet corner, unsteady gait), short and

uneven breath, reduced stimulatory responses, and so on, but would return to normal in an hour. The maximum tolerated dose of LSP was greater than 8000 $mg \cdot kg^{-1}$.

Toxic sensitivity research was carried out by different strains of rats. Animals had similar symptoms to those of the Wistar rats after administration by gastric perfusion at a dose of 1000 $mg \cdot kg^{-1}$ for SD rats and F344 rats. Female rats had more serious toxic responses than the male rats, but all animals could return to normal in an hour without death.

All the results indicated that rats became desensitized to LSP, and LSP was well tolerated. The maximum tolerated dose of LSP was greater than 8000 $mg \cdot kg^{-1}$, and there was no significant difference among different strains of rats.

12.4.2.2 Research on Acute Toxicity of LSP on Beagle Dogs The accumulated dose method was used on 5-month-old beagle dogs, with starting dose of 0.2 $mg \cdot kg^{-1}$ and terminal dose of 48.6 $mg \cdot kg^{-1}$. LSP, *Chansu*, and *Chansu*-removed LSP groups were designed. On one hand, reassessment on toxicity was carried out at a single dose of LSP, on the other hand, the relationship between attenuation and compatibility was examined for *Chansu*.

After each administration, the symptoms, behaviors, and appearances of animals were observed, such as posture, gait, attitude, appetite, hair, eyes, mouth, nose, ears, limbs, breathing, excrement, and so on. At different time points, ECG, hematology, and serum biological were examined simultaneously.

Clinical investigations indicated that animals in the *Chansu*-removed LSP group did not show obvious abnormal behaviors under doses of 1.8–48.6 $mg \cdot kg^{-1}$, but they showed symptoms such as vomiting and reduced activities. A female beagle dog had reduced volume and weight; a male beagle dog died after 102 min at a dose of 48.6 $mg \cdot kg^{-1}$. As dose of LSP increased to 5.4 $mg \cdot kg^{-1}$, animals of the *Chansu* group showed symptoms such as vomiting, diarrhea, and reduced activity.

The results of ECG showed that LSP and *Chansu* groups showed a dose-dependent effect on lowered heart rate, decreased S-T amplitude, and T-wave inversion. We suggest that these could be related to toxic ingredients in *Chansu*, but Q-wave amplitude increased at dose of 16.2 and 48.6 $mg \cdot kg^{-1}$. We suggest that it might be related to some other ingredients in LSP.

From the results of hematological study, the number and HTC of erythrocytes increased, hemoglobin and platelet counts went up, and lymphocyte decreased at doses of 16.2 and 48.6 $mg \cdot kg^{-1}$ in the animals. We suggest that all these could be related to toxic ingredients in *Chansu.*

From the serum biochemical study, serum alanine aminotransferase, albumin, and cholesterol levels were elevated at doses of 16.2 and 48.6 $mg \cdot kg^{-1}$. We suggest that this may be related to toxic ingredients in *Chansu*. The study also found that creatine kinase level was elevated for the LSP group at doses from 0.2 to 48.6 $mg \cdot kg^{-1}$. *Chansu* completely inhibited the increase of creatine

kinase, while LSP reduced this effect caused by *Chansu*; this might be related to pharmacological effects of *Chansu*.

We found the dose without obvious poisonous reactions was 0.6 mg/kg^{-1}, which is equivalent to a clinical dose on a human, on a 5-month-old beagle with the accumulated dose method. The minimum lethal dose was 48.6 mg·kg^{-1}, which is equivalent to 93.5 times of a human clinical dose.

12.4.3 Research on Chronic Toxicity of LSP

12.4.3.1 Experimental Clinical usage and dose of LSP for adults is that an adult can take ten of these pills twice a day, or three times a day if symptoms are severe. The maximum dose for an adult in one day is 30 pills (3.125 mg/pill), which is equivalent to a dose 1.5 mg·kg^{-1} for a beagle dog according to calculations of body surface area.

Animal preliminary experiments found that animals vomit as dose of *chansu* increases. Considering the tolerated dose of animals, we used 1.0 mg·kg^{-1} as the low dose and 3.0 mg·kg^{-1} as the medium dose for safety evaluation of clinical dose. In order to avoid animal vomiting and taking into account that the purpose of the study was the effect of accumulated toxicity of *Xionghuang* and animals' scavenging capability to toxic accumulation, we used *Chansu*-removed LSP and dose of 30.0 mg·kg^{-1} as the high dose group. We had a blank control group, low, medium, and high dose of LSP groups, and medium and high dose of *Xionghuang* groups. Medium and high dose of *Xionghuang* groups had an equivalent amount of *Xionghuang* compared with the medium and high dose of LSP groups, respectively.

We chose oral administration as the route of administration, same as clinical administration. The aim of this study was the evaluation of chronic toxicity, thus the treatment cycle was three times, as in a clinical setting.

12.4.3.2 Research on Neural Symptoms and Pathophysiology

12.4.3.2.1 Observations of Animal Symptoms Medium and high dose of *Xionghuang* groups showed obvious symptoms, particularly the high dose group. Animals of these two groups had diluted stool, salivation, reduced activity, dim conjunctiva, and other severe clinical symptoms. Two dogs died on day 19 after administration.

12.4.3.2.2 Results of Pathological Physiology *Effect on weight reduction*: The results of weight determination showed that the weights of animals in the high dose *Xionghuang* group were lower than those of the animals in the other groups, indicating that high dose of *Xionghuang* noticeably affected animal body weight. During the later recovery period, weights of animals in the high dose *Xionghuang* group significantly increased, but were still much lower than the other groups.

Weight of animal reproductive organs: For weights of reproductive organs, those of animals in the *Xionghuang* groups were lower than the other groups

during either administration or recovery periods, indicating that *Xionghuang* had reproductive and developmental toxicity. In addition, these *Xionghuang* animals were very difficult to restore to normal even after drug administration was stopped.

ECG of animals: ECG of animals showed that the PR interval was significantly prolonged for animals in high dose of *Xionghuang* group on day 27 after administration. Animals of the *Xionghuang* group removed a high dose of LSP and *Xionghuang* groups all showed decreased amplitude of T wave and T wave inversion, which have biological significance. The *Xionghuang* group had greater change than the *Xionghuang*-removed LSP group, and PR interval of high doses of *Xionghuang* prolonged significantly.

Pathological examination: Animals administered high doses of *Xionghuang* (including dead animals) showed sporadic bleeding points on duodenum.

Analysis of biochemistry indices: Bone marrow cell count results: administering high dose of LSP and *Xionghuang* could affect bone marrow cells of the animals. The effect of the medium dose of *Xionghuang* was stronger than the effect of LSP; and high dose of *Xionghuang* had a stronger effect than high dose of LSP.

Effects on urinary biochemistry: Compared with the blank control group, pH value of high dose of *Xionghuang* group reduced significantly on day 27 after administration.

Blood test results: Compared with the blank control group, ALP (alkaline phosphatase) of high dose of *Xionghuang* increased significantly on day 28 after administration. Observing animals in the high dose of *Xionghuang* group, we found that ALP levels of MD116 animals were higher, indicating that these animals might have liver damage. Compared with the blank control group, percentage of BASO (basophilic) in medium dose of *Xionghuang* significantly decreased on day 28 after administration. For high dose of *Xionghuang*, percentage of white blood cell count (WBC) and mononuclear cells (MONO) increased significantly, activated partial thromboplastin time (APTT) prolonged significantly, red blood cell volume distribution width (RDW) reduced, and red blood cell count (RBC), hemoglobin (HGB), mean corpuscular hemoglobin (HCT), and reticulocyte percentage and absolute number (RET) all decreased with the dose dependency of *Xionghuang*. Therefore, we suggested that all these changes could be related to the toxicity of administered drugs. After the recovery period, compared with the blank control group, mean corpuscular hemoglobin concentration (CHCM) of the high dose of *Xionghuang* group decreased with a statistically significant difference, but the average difference between the two groups was less than 5%. This change of CHCM was without biological meaning. RDW of the high dose of *Xionghuang* group increased, but the change was not significant and contrasted with the results after administration, so the change of RDW was also without biological meaning. Other hematological indices did not show significant differences. Therefore, we considered hematological indices as being restored to normal after three weeks of recovery.

12.5 IN VIVO DISTRIBUTION AND METABONOMICS OF LSP AND *XIONGHUANG*

12.5.1 In Vivo Distribution in Various Tissues after Administration of LSP and *Xionghuang* to Beagles

This section used LSP and *Xionghuang* as subjects to discuss the absorption and distribution in beagles in order to study the action features of *Xionghuang*, safety assessments for *Xionghuang* and LSP, and to provide evidences for rational clinical uses of LSP.[20]

12.5.1.1 Toxicokinetics Curves of Total Arsenic ICP-MS method was established for determination of total arsenic concentration. For details of the method please refer to Section 12.3.1.3, and for experimental design methods please refer to Section 12.4.3.1. A chronic toxicity experiment was conducted to study the distribution of total arsenic, cumulative toxicity, target organs, and reversibility in beagles by repeated administrations, and in order to further research the attenuation mechanism of compatibility of *Xionghuang* in LSP.

Beagle dogs were administered according to the designed doses. Four beagle dogs of each group were euthanized after administration for 28 days, and the other two dogs of each group were euthanized after the recovery stage. Brain, cerebellum, heart, liver, bile, spleen, lung, kidney, ovary or testis, spinal cord, blood, urine, pancreas, muscle, feces, and fat were obtained from the euthanized beagles. We first observed the pathomorphological changes then determined the total arsenic. The results of total arsenic of 14 tissues and excretion from 32 beagle dogs are shown in Figs. 12.8 and 12.9.

Fig. 12.8 shows that the total arsenic in the spinal cord of the high dose of *Xionghuang* group was much higher, while the total arsenic of high dose of LSP group was close to the blank control group. The spinal cord is important for conducting the important pathways of sensory and motor nerve impulses. Studies have shown that arsenic is a nerve poison,[21] so we suggested that the neurotoxicity of *Xionghuang* may be related to the accumulation of arsenic in the spinal cord and neurotoxicity could be reduced after comparability.

Toxicological and physiological testing showed that dogs in the high dose of *Xionghuang* group were vomiting (containing bubbles, mucous, and so on). This lasted from the first day of administration to the day of death. From the second week, animals showed diluted stool, salivation, reduced activity, dim conjunctiva, and other severe clinical symptoms. Two dogs died on day 19 after administration. Total arsenic levels in tissues, especially in liver, kidney, muscle, pancreas, and fats, of the two dead dogs were much higher than in the others. For a toxicological standpoint, we can say that *Xionghuang* without compatibility was highly toxic and led to animal death.

Fig. 12.9 showed that after 14 days of recovery, arsenic in most tissues of dogs restored to the normal level, indicating that the tissue distribution of arsenic was reversible and could be cleared within 14 days. However, liver

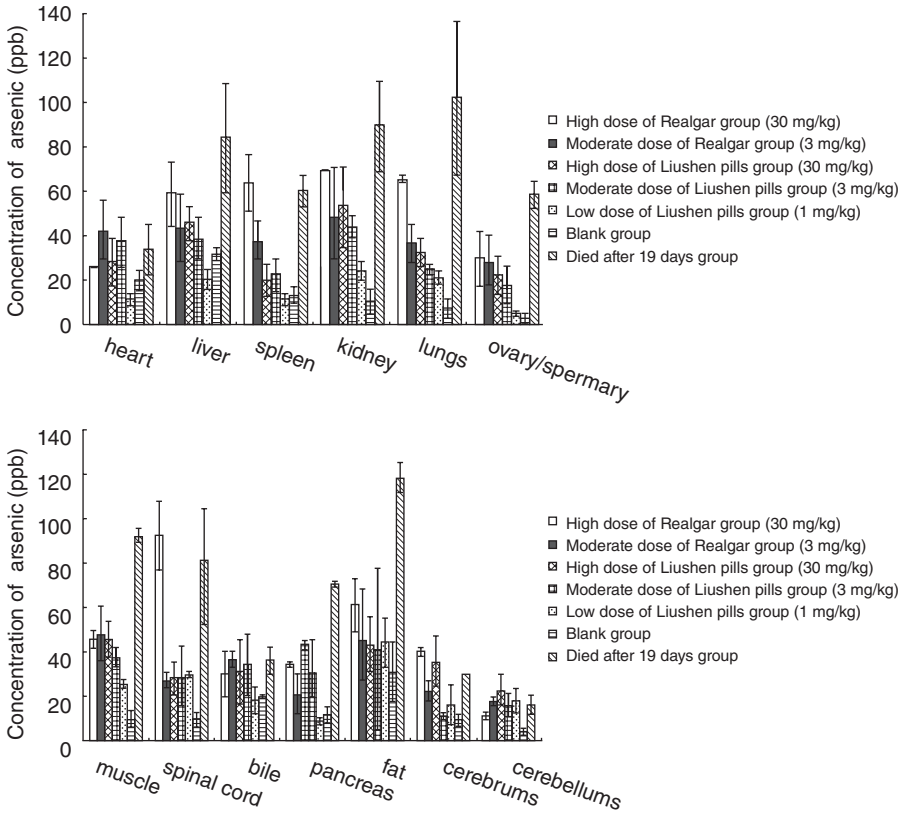


Fig. 12.8 Concentration of total arsenic in various tissues of different dose groups after administration of LSP and realgar.

and genital distribution of arsenic in medium and high dose of *Xionghuang* groups and high dose of LSP group did not return to the level of the control group. We suggested that the liver, an important detoxifying organ, contains a large number of sulfhydryl groups, which could easily form complex compounds with As^{III}, so that arsenic in liver still could not be cleared after 14 days of recovery. Arsenic in the reproductive organs was difficult to clear, which indicated that arsenic in *Xionghuang* was toxic to reproductive organs. The result about reproductive toxicity was also consistent with other literature reports.[22]

Compared with the *Xionghuang* groups, the contents of total arsenic were all much lower. The differences were the most dramatic in liver, spleen, kidney, lung, and spinal cord tissue when comparing the high dose of *Xionghuang* group with the high dose of LSP group. All the differences indicated that tissue distribution of arsenic was reduced after compatibility. A low dose of LSP was equivalent to the clinical dose. Arsenic levels of this group were close to those

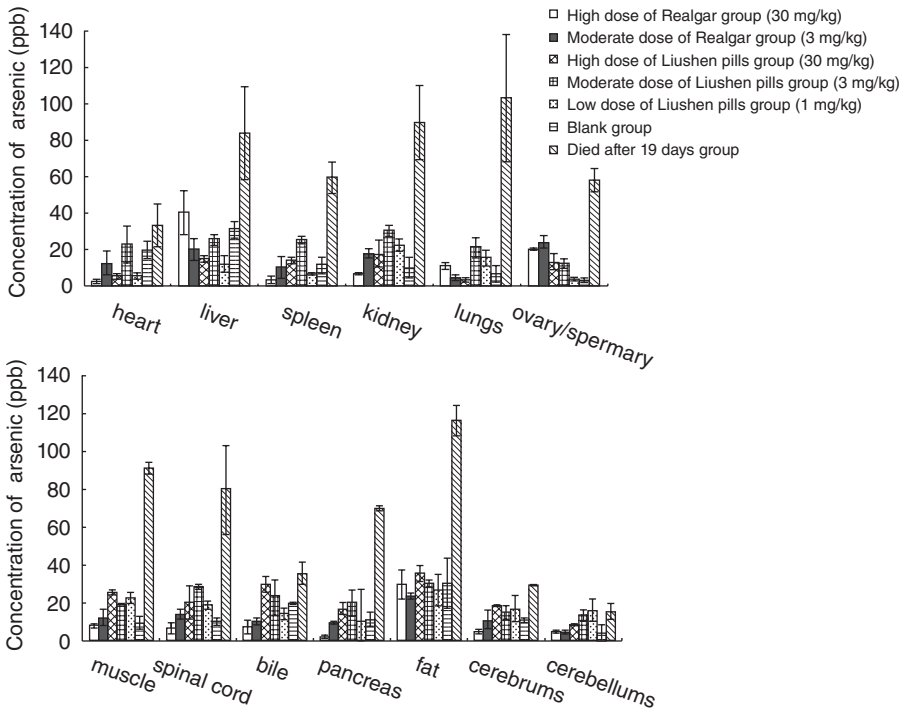


Fig. 12.9 Concentration of total arsenic in various tissues of different dose groups of LSP and realgar with 14-day recovery.

of the blank control group in both the administration and recovery stages, indicating that there was not visible effect on humans taking LSP at clinical doses even for 28 days. In other words, LSP is safe for clinical use.

12.5.1.2 Deformation and Distribution Analysis of Arsenic in Multiple Tissues of Beagles After Oral Administration of LSP and Xionghuang

A HPLC-ICP/MS method was established for the determination of six forms of arsenic (Fig. 12.5). They all showed good linearity ($r > 0.99$) in the range of 0–200 ppb. Except for the recoveries of AsC and MMA^{v}, all the others were more that 70%. The accuracies (RSD) of AsC, AsB, As^{III} + DMA^{v}, MMA^{v}, and As^{v} were 6.33%, 6.63%, 1.49%, 1.03%, and 5.76%, respectively.

All the drugs were processed and prepared by Shanghai Lei's Pharmaceutical Limited Company in accordance with proportions of the confidential formula of LSP, and the missing medicinal herbs in the prescriptions were replaced with excipients. In particular, for the *Xionghuang* group, all the other five medicinal herbs except *Xionghuang* were replaced by excipients. Due to the irritation of *Chansu* and induced intolerance of high dose LSP in beagles, *Chansu* was removed and replaced with an excipient in the preparation of

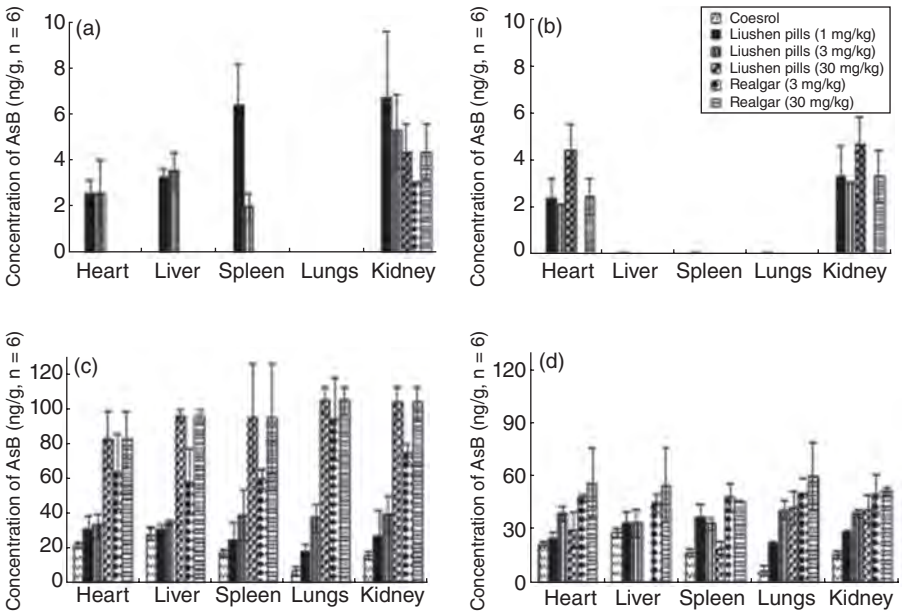


Fig. 12.10 Concentration of arsenic species in the main distribution organs ($n = 6$). A: concentration of AsB on day 28; B: concentration of AsB after 14-day recovery; C: concentration of (As^{III} + DMA^{V}) on day 28; D: concentration of (As^{III} + DMA^{V}) after 14-day recovery.

high-dose LSP. The dose of *Xionghuang* in the low dose of LSP (1 mg·kg^{-1}) was as the same as is used in clinical settings. In the medium and high dose groups, the doses were 3 mg·kg^{-1} and 30 mg·kg^{-1}, which were the same as the low and high dose of *Xionghuang* groups, respectively. According to the content of total arsenic and pathological section results, heart, liver, spleen, lung, and kidney were confirmed to be the main distribution organs. The results of species analysis of arsenic in the main distribution organs are shown in Fig. 12.10. AsC and MMA^{v} were not detected.

Results showed that the concentration of As^{III} + DM^{V} increased as the dose increased, showing a dose dependency; the concentration of As^{III} + DM^{V} in the recovery period was lower than that in the administration period, indicating beagles could eliminate some arsenic on their own; the concentrations of As^{III} + DM^{V} of all organs in LSP groups were more than those in the *Xionghuang* groups with the same dose of *Xionghuang*, while the concentration of AsB in liver was higher. Because the toxicities of As^{III} and DMA^{V} are higher than that of AsB and the toxicity of As^{III} + DM^{V} is higher than that of AsB, these above results demonstrated that more As compounds were converted into lower toxicity forms when LSP was used than when *Xionghuang* alone was used, and further pointed out that LSP can reduce the toxicity of *Xionghuang* through the compatibility of medicines.

12.5.2 Toxicokinetics Research of Total Arsenic in Plasma of Beagles

Male and female beagles were administered with 3, 30 mg·kg^{-1} LSP or *Xionghuang* for 28 days. Toxicokinetics research was conducted on day 1 and day 28 to evaluate the dose–time–toxicity relationship and the toxicity reducing effect of LSP.

12.5.2.1 Toxicokinetics Curves of Total Arsenic Inductively coupled plasma mass spectrometry (ICP-MS, Perkin Elmer) was used to determinate the total arsenic concentration in plasma-assisted digestion.[21] The method had good linearity in 1–200 ppb and the detection limit was 0.01 ppb. Because the sample was diluted 10 times during the preparation, the minimum detection concentration of total arsenic in plasma was 0.1 ppb. The recovery was between 94.4% and 104.8%; and the interday and intraday accuracies were both good. These all showed that this method could be used for the determination of total arsenic in plasma.

As mentioned earlier, in the high dose of LSP group, *Chansu* was removed and replaced with an excipient. The doses of *Xionghuang* in medium and high dose of LSP groups were 3 mg·kg^{-1} and 30 mg·kg^{-1}, which were the same as the dose in the medium and high dose of *Xionghuang* groups, respectively. It was administered to 24 beagles intragastrically, and their blood samples were collected at 0, 10, 20, 40, 60, 80, 100, 120, 150, 180, 200, and 220 min and 4, 5, 6, 8, 12, and 24 h after the administration on both days 1 and 28. The plasma samples were analyzed by the established method, and the results are shown in Fig. 12.11.

When comparing day 28 with day 1, we found that the total arsenic concentration, as well as C_{max} and the area under the concentration–time curve (AUC), in plasma increased in the *Xionghuang* high dose group, indicating slower elimination and enhanced distribution of total arsenic in blood. This unusual phenomenon in the high dose of *Xionghuang* group may be because

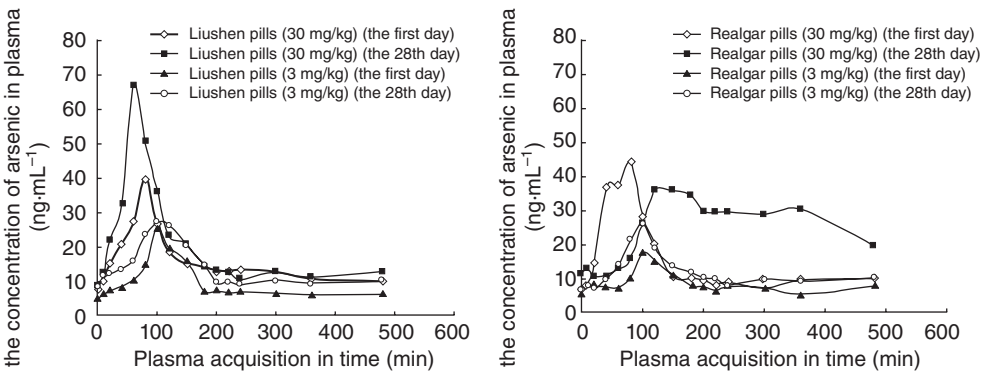


Fig. 12.11 Concentration–time curves of total arsenic in beagle plasma.

TABLE 12.17 Results of Species Analysis of Arsenic ($n = 6$, ppb)

No.	Group	AsB	DMA^V	As^V
1	*Xionghuang* HDG (30 mg/kg)	4.29 ± 1.58	10.42 ± 3.21	17.07 ± 2.16
2	LSP HDG (30 mg/kg)	4.34 ± 1.37	18.73 ± 4.10*	6.27 ± 2.68**
3	*Xionghuang* MDG (3 mg/kg)	3.58 ± 1.42	5.78 ± 2.50	5.67 ± 2.50
4	LSP MDG (3 mg/kg)	2.80 ± 1.01	7.48 ± 3.11##	5.50 ± 1.50

*$p < 0.05$,
**$p < 0.01$, group 2 versus group1;
##$p < 0.01$, group 4 versus group3.

the dogs were vomiting the whole time and thus the results were affected. Besides, the AUCs of the LSP groups were all lower than those in the corresponding *Xionghuang* groups, showing lower distribution of total arsenic in blood. The mean residence time (MRT) of the high dose of *Xionghuang* group was significantly longer than that in the high dose of LSP group, and the MRT of the medium dose of *Xionghuang* group was also longer than that in the medium dose of LSP group, showing that arsenic was easier to eliminate by compatibility of medicines. Moreover, as the dose increased, Tmax decreased, and peak concentration and AUC increased in all the groups.

12.5.2.2 Morphological Analysis of Arsenic In order to evaluate the toxicity of *Xionghuang* in LSP and its toxicokinetics, the deformation analysis of arsenic was conducted by the methods mentioned in the last section; the results are listed in Table 12.17.

The concentration of DMA^V was higher in the LSP group than in the corresponding *Xionghuang* group, while the concentration of As^V was lower. In addition, the difference was more significant in the high dose groups. Because of the low toxicity of DMA^V, these results showed that more compounds were converted into low toxicity forms when using LSP rather than single *Xionghuang*. AsB is inherent in plasma, and the concentrations in all the groups showed no significant difference. The concentrations of DMA^V and As^V increased as the dose increased, showing a dose dependency.

12.6 METABONOMIC RESEARCH OF LSP

The metabolomics tool can help to evaluate the safety and toxicity of TCM prescriptions, establish the evaluation system, and develop rational dose schedules for clinical uses.

12.6.1 Metabolomic Research on Cardiac Tissue

There were 48 mice (13–15 g) randomly divided into four groups (12 mice in each group, 6 male, 6 female). Three LSP groups were given LSP at a dose of

150, 240, and 320 mg/kg, respectively; and the other group was given 2.5% CMC-2Na as control. Both the chloroform and water extracts were analyzed as described in the references.

12.6.1.1 Metabolic Fingerprint and Profile Analysis The metabolic profile extract showed much more compound information than the one of aqueous extract, and thus fulfilled the requirements of metabolomic research.

PCA results showed that the samples of the control and administration groups cannot be distinguished from each other. This may be due to two reasons. First, there was no significant difference between the metabolic profiles of these two groups. Second, the chosen principal compositions were not appropriate. The peak intensities of many compounds were decreased when compared with the administration group. Especially when comparing high dose groups with the control, there was a significant metabolic change. Therefore, the proper cause of the unsatisfactory results may be the bad choice for principal composition. The concentrations of some high peaks after 8.5 min reduced significantly as the dose increased. Then these compounds were found to have the same neural loss of m/z 44 by using MS^n. Considering that the in vivo compounds affected by drugs are always of the same class, the compounds with the same m/z 44 neutral loss were selected by difference analysis of mass spectrometry as the principal composition for PCA analysis.

12.6.1.2 Data Processing Using Differential Mass Spectrometry First, the MS^3 analysis of heart chloroform extract was performed by the same HPLC separation and MS detection conditions in the full scan mode; then, difference analysis of mass spectra was used to screen the compounds with neutral loss m/z 44 to select 18 compounds; last, a matrix containing the retention time and peak intensity of these compounds was set up for PCA. Although the control, low dose, and high dose groups still could not be distinguished clearly, high dose of LSP group and the control could be significantly separated into two independent parts. In the biological samples, compounds with neutral loss m/z 44 were likely endogenous fatty acids, which mainly participate in energy metabolism in myocardium. Therefore, there might be some fatty acid metabolism disorder in the hearts after 11 days of administration of high dose of LSP. After the loading analysis, five compounds were found to have important contributions to the classification, and were identified as potential biomarkers (listed in Table 12.18).

12.6.1.3 Analysis of Metabolic Markers and Pathways The fingerprint profiles of the administration group and the control group could be successfully distinguished after difference analysis, which is quite helpful to the identification of potential biomarkers.

Fatty acids are the main energy source in myocardium. When comparing the high dose of LSP group with the control, the concentrations of five fatty acid biomarkers all decreased, showing a metabolic disorder of fatty acid in

TABLE 12.18 Identification of Potential Biomarkers

RT (min)	$[M-H]^-$/Da Measured Mass	$[M-H]^-$/Da Theoretical Mass	MS3	Elemental Composition	Identification
9.3	327.2324	327.2329	MS^2: 283, 229; 191; MS^3: 283-191, 93	$C_{22}H_{31}O_2$	Docosahexaenoic acid
9.8	303.2324	303.2329	MS^2: 259, 285; 205; MS^3: 259-93, 206	$C_{20}H_{31}O_2$	Arachidonic acid
10.5	279.2332	279.2329	MS^2: 261, MS^3: 261-243, 97	$C_{18}H_{31}O_2$	Linoleic acid
12.6	255.2336	255.2339	MS^2: 235, 209;	$C_{16}H_{31}O_2$	Palmitic acid
13.2	281.2480	281.2486	MS^2: 263, MS^3: 263-109	$C_{18}H_{33}O_2$	Oleic acid

myocardium. In addition, the results of clinical observation pointed out that during the late administration period, animals in the high dose group displayed some toxicity symptoms, such as reduced activities, unstable gait, and shortness of breath, which means that long term use of high dose LSP could lead to a certain degree of toxic side effects.

The concentrations of arachidonic acid and linoleic acid were both reduced in the high dose group, indicating that the toxicity of high dose LSP may inhibit the biosynthesis of arachidonic acid. Arachidonic acid metabolism disorder[23] has a great impact on normal cardiac function, and leads to decreased contraction and cardiac output, or even the induction or aggravation of myocardial infarction and angina pectoris.[24–25] The effective component group of venenum bufonis is bufotoxin, which was proven to be harmful for heart tissues when overdosed.[1] The results showed that the heart may be the target organ of the toxic side effects of high doses of LSP. Moreover, when the doses were below 240 mg/kg, there was no significant difference between the control and administration groups, explaining the safety and rationality of the clinical dose of LSP.

12.6.2 Urine Metabonomic Research

Beagle dogs were separately given low, medium, and high doses of LSP, and medium and high doses of *Xionghuang*. The grouping and administration information were the same as described in Section 12.4.3.1. After 28 days of administration and 14 days of recovery, urine samples were collected three times (0–6 h, 6–12 h, 12–24 h) per day on days 0, 1, 6, 13, and 27 during the administration period and days 1, 7, and 13 during the recovery period, and then analyzed.

12.6.2.1 HPLC-TOF/MS Metabolic Fingerprint In order to evaluate the long-term toxicity and reversibility of LSP and *Xionghuang*, unsupervised

learning method PCA was applied to the data mining of LC-MS fingerprint data obtained from 570 urine samples.

The ionization mode was used to get optimal results. As most of the compounds in urine are products from energy metabolism and are anions,[26] they showed better responses in the negative mode, which was chosen as the detection mode. A urine sample collected at 0–6 h on day 1 was chosen as the quality control (QC) sample to evaluate the reproducibility and stability of the method and instruments during the entire analysis. The QC samples were prepared and analyzed every day at a regular interval; and the total sample number was 17. The results showed that the RSD of retention time and peak intensity were all less than 10%. Therefore, the established LC-TOF/MS method had good reproducibility and stability, and can meet the metabonomic requirements.

There were certain differences in metabolic fingerprints between the control and each administration group, especially on day 27 (0–6 h) in the administration period. The difference between the low dose of LSP group and the control was mostly focused on 7–11 min, while the difference between the high dose of LSP group and the control was mostly focused on 10–14 min. Furthermore, the fingerprints of the *Xionghuang* group and the control had more differences. Besides, although the fingerprints of the medium and high dose of *Xionghuang* groups showed differences at 2–4 min and 9–14 min, they had certain similarities, indicating that these two groups might have similar impacts on endogenous metabolic substances.

12.6.2.2 Metabonomic Research of LSP Long-Term Toxicity The low, medium, and high dose groups were studied to evaluate the long-term toxicity of LSP. After PCA analysis of the urine samples on three time periods of days 0, 1, 6, 13, and 27, the results of 12–24 h showed that in the low dose group, the samples before and after administration could not be distinguished. Additionally, as the dose increased, the classification tendency became clearer; finally the samples could be perfectly distinguished in the high dose group. The changes of urine metabolic fingerprint indicated that the administration of LSP disturbed the metabolism of some endogenous substances; and the fact that higher dose group had better classification performance suggested a dose-dependent relationship in the impact on endogenous substances of LSP.

Urine samples that were collected at the same period (6–12 h) of days 0, 1, 6, 13 and 27 were analyzed separately to study the effects of different doses of LSP on metabolism in vivo. The results showed that these three groups could not be classified on days 1 and 6; on day 13, the high dose group could be distinguished from the low and medium dose groups; then on day 27, the high dose group could be clearly distinguished from the other groups except for one sample, probably because the individual differences among all the animals. As a conclusion, on early days of the administration period (days 1 and 6), these three groups displayed similar effects on endogenous substances, but as time passed, the difference of their effects showed up gradually on days 13 and 27.

TABLE 12.19 Potential Biomarkers of High Dose LSP Group

RT (min)	$[M-H]^-$/Da Measured Value	Elemental Composition	Identification
8.4	275.012	$C_6H_{12}O_{10}P$	6-Phosphogluconate (↑)
2.0	78.960		(↓)
3.8	139.006	$C_3H_7O_4S$	(↓)
2.3	191.023	$C_6H_8O_7$	Citric acid (↓)
8.2	244.994	$C_9H_9O_6S$	3-Hydroxyphenyl propionic acid sulfate (↓)
11.6	248.095	$C_{10}H_{18}NO_4S$	N-(tert-Butoxycarbonyl)-L-methionine (↓)
11.1	199.092	$C_{10}H_{15}O_4$	(↑)
9.7	211.991	$C_4H_7NO_7P$	L-Aspartate-4-phosphate (↑)

"↑" represents increased concentration after 27 days; "↓" represents decreased concentration after 27 days.

This metabolic fingerprint difference for the high dose group from the other two groups and its relationship with the administration time indicated that long-term administration of high doses of *chansu*-removed LSP may lead to drug accumulation, and thus greatly affect normal physiological metabolism. Five potential biomarkers (see Table 12.19) were found based on the loading chart of PCA analysis before and after administration in the high dose LSP group, and were identified using standards and consulting related literatures.[27–30]

Taking citric acid as an example to study the concentration changes during the administration period, results showed that the concentration first increased at days 1 and 6, then decreased at day 13; after 27 days, it was lower than the level before administration. This tendency was probably related to the regulation of in vivo steady state balance. After high doses of medication, the internal environment was changed greatly; therefore, organisms would generate compensatory secretions to maintain the balance.[31] Then after a period of administration, the cumulative toxicity of the drug would destroy this compensatory secretion, resulting in the reduction of citric acid. Similar analyses were done for other potential biomarkers. The changes of their concentrations were also nonlinear. The physiological significance of these potential biomarkers and associated metabolic pathways will be discussed later.

To sum up, the results of differential metabolomic research of 28 days of administration of different doses of LSP indicated a dependent relationship between LSP dose and its impact on in vivo metabolism. More specifically, the high dose group displayed a significant difference from the other groups. Combining the results of pathophysiology and total arsenic and arsenic form determination, we speculated that this metabolic disorder was caused by the toxicity of *Xionghuang* in LSP. In addition, the results of metabonomic analysis showed that the physiological status of the high dose group was obviously

different from the low and medium dose groups, revealing that the appearance of this toxicity in high doses of LSP was a process of accumulation overtime starting on day 13. However, histopathological examination of the high dose group showed no pathological changes, pointing out that metabolomics could help to monitor the toxicity when no histopathological change was found. By PCA analysis, five toxicity related potential biomarkers were identified, including two elevated compounds (6-phosphogluconate and L-aspartate-4-phosphate) and three declined compounds (citric acid, 3-hydroxyphenyl propionic acid sulfate, and N-(tert-butoxycarbonyl)-L-methionine). Moreover, neither metabolomics nor pathophysiology research found any toxicity or side effects in the low and medium dose groups, demonstrating the safety of these two doses for clinical uses.

12.6.2.3 Metabonomic Research of Xionghuang *Long-Term Toxicity*

Urine samples of the medium and high dose *Xionghuang* groups were processed with PCA and the results of the 12–24 h segment showed that the samples before and after administration could be clearly classified into two classes in both groups, revealing a significant interference in metabolism after medication. Except for one sample, the distance between the two classes in high dose groups was farther than that in the medium dose group. Therefore, the high dose realgar group displayed a more significant impact on in vivo metabolism, indicating a dependent relationship between doses and the interfering effect of *Xionghuang* on endogenous substances.

In order to further investigate the effect of different doses of *Xionghuang* on physiological metabolism, samples collected at the same time segments every day of the high and medium dose groups were processed with PCA. Similar to the results of LSP, these two groups could not be distinguished on days 1 and 6, while they could be successfully classified into two classes on days 13 and 27. As a conclusion, during the early days of the administration period (days 1 and 6), these two groups displayed similar effects on endogenous substances, but as time progressed (after two weeks), the differences of their effects showed up gradually. In addition, the samples that were given high doses of *Xionghuang* were more dispersed when compared to those that were given medium doses of realgar. This was probably because a higher dose would produce more toxicity, and therefore lead to elevated individual differences. Potential biomarkers from the medium and high dose groups were found and identified separately to study the related toxicity pathways. Additionally, most of them are the same and are listed in Tables 12.20 and 12.21.

We used hippuric acid as an example by which to study the concentration changes during the administration period. Its variation tendency was nonlinear, similar to the trend of potential biomarkers of the high dose LSP. After administration, the concentration first decreased on days 1 and 6, then increased on day 13; after 27 days, it was higher than the level before administration. Tables 12.20 and 12.21 list the concentration changes of all the potential bio-

TABLE 12.20 Potential Biomarkers of Medium Dose of *Xionghuang* Group

RT (min)	$[M-H]^-$/Da Measured Value	Elemental Composition	Identification
8.4	275.012	$C_6H_{12}O_{10}P$	6-Phosphogluconate (↑)
9.7	211.991	$C_4H_7NO_7P$	L-Aspartate-4-phosphate (↑)
2.3	191.023	$C_6H_8O_7$	Citric acid (↓)
3.8	139.006	$C_3H_7O_4S$	(↓)
9.2	272.991		(↓)
8.3	260.996		(↑)
8.8	244.998	$C_9H_9O_6S$	3-Hydroxyphenyl propionic acid sulfate(↓)
7.1	178.036	$C_9H_9NO_3$	Hippuric acid (↑)
12.1	248.097	$C_{10}H_{18}NO_4S$	N-(tert-Butoxycarbonyl)-L-methionine (↓)

"↑" represents increased concentration after 27 days; "↓" represents decreased concentration after 27 days.

TABLE 12.21 Potential Biomarkers of High Dose of *Xionghuang* Group

RT (min)	$[M-H]^-$/Da Measured Value	Elemental Composition	Identification
7.1	178.039	$C_9H_9NO_3$	Hippuric acid (↑)
5.4	208.054		(↓)
11.8	514.283		(↑)
8.4	275.012	$C_6H_{12}O_{10}P$	6-Phosphogluconate (↑)
11.6	248.095	$C_{10}H_{18}NO_4S$	N-(tert-Butoxycarbonyl)-L-methionine (↓)
8.8	244.998	$C_9H_9O_6S$	3-Hydroxyphenyl propionic acid sulfate (↓)
2.3	191.023	$C_6H_8O_7$	Citric acid (↓)

"↑" represents increased concentration after 27 days; "↓" represents decreased concentration after 27 days.

markers on day 27. The physiological significance of these potential biomarkers and associated metabolic pathways will be discussed later.

In summary, the results of differential metabonomic research for 28 days of administration of different doses of *Xionghuang* indicated that long-term medication of *Xionghuang* alone would produce a cumulative toxicity in the body and significantly interfere with normal physiological metabolism. This cumulative toxicity was dependent on the dose, as the greater the dose was, the more serious the side effects were. This dose induced toxicity difference, which became significant after 13 days of administration. By PCA analysis, the potential biomarkers of the medium and high dose of *Xionghuang* groups were found and identified and most of them were the same for the two groups.

The levels of hippuric acid, 6-phosphogluconate and L-aspartate-4-phosphate were elevated, while the levels of citric acid, 3-hydroxyphenyl propionic acid sulfate, and N-(tert-Butoxycarbonyl)-L-methionine were reduced.

***12.6.2.4 Comparison of the Long-Term Toxicities of LSP and* Xionghuang** With the purpose of studying the possible compatibility attenuation of LSP, the long term toxicities of LSP and *Xionghuang* were discussed and compared to find the differences of their impacts on physiological metabolism.

Some potential biomarkers of high doses of LSP and realgar were the same, such as 6-phosphogluconate and L-aspartate-4-phosphate, which meant that they had a similar impact on in vivo metabolic pathways. However, some different potential biomarkers were also found, such as hippuric acid, showing that there were also some differences between their interference mechanisms.

After comparing the urine samples of the LSP and *Xionghuang* groups at the same doses with PCA, results showed that the medium dose LSP and *Xionghuang* groups could be clearly distinguished after 13 and 27 days of administration; and the high dose LSP and *Xionghuang* groups could be distinguished starting on day 6. All these results revealed that the difference of their impacts on physiological metabolism showed up earlier when a higher dose was used.

In the long-term toxicity analysis of LSP and *Xionghuang*, the two high dose groups were both found to have cumulative toxicity, which would disturb normal metabolism. However, the degrees of disturbance were not the same between the LSP and corresponding *Xionghuang* groups. This was probably because the pharmacological and other properties were changed after compatibility of medicines in LSP, which displayed a certain attenuation effect.

The determination results of total arsenic in tissues showed that there was significantly more cumulative arsenic in the *Xionghuang* groups than in the LSP groups, especially for the high dose group. The deformation analysis results revealed that the concentration of high toxicity from As^{III} + DMA was higher in the *Xionghuang* groups than in the LSP groups, especially for the high dose group. These all indicated that compatibility of medicines could promote the transformation of arsenic compounds into lower toxicity ones. These results corresponded to the compatibility attenuation results in the metabonomic research.

Moreover, pathophysiological study results also proved the compatibility attenuation effect of LSP. In the study of differential count of bone marrow cells, medium doses of *Xionghuang* showed a stronger effect than medium doses of LSP, while high doses of *Xionghuang* showed a stronger effect than high doses of LSP. In the ECG research, both of the high dose groups showed significantly decreased or even inverted T wave amplitude; and the high dose *Xionghuang* group displayed more variation in amplitude than the high dose group of LSP. Furthermore, the PR interphase was significantly extended in

the high dose of *Xionghuang* group, indicating that these ECG changes were caused by *Xionghuang*. After compatibility of medicines in LSP, the reduced perturbations on ECG revealed a certain compatibility attenuation.

In the metabonomic research, especially toxicology metabonomics, the metabolic trajectory method can be used to evaluate the development of and recovery from toxicity.[31–33] This method was used in the differential metabonomic study of toxicity evaluation of LSP to reveal the metabolic differences among different doses and compatibility groups as well as to monitor its dynamic trend over time. After 28 days of administration, one female and one male animal was selected to continue a 2 week recovery study, focusing on the high dose of LSP group, and the medium and low dose of *Xionghuang* groups that appeared to have toxic reactions.

Urine samples collected at 0–6 h on day 1 (R1), day 7 (R7) and day 13 (R13) of the recovery period were analyzed and then processed by PCA. All the samples were connected in chronological order to draw the metabolic trajectory (shown in Fig. 12.12) and investigate the development and recovery of metabolic disorders.

As shown in Fig. 12.12A, from day 6, samples started to shift away from day 0 samples, indicating the metabolic levels of endogenous substances in urine deviated from the normal state. By day 13, samples returned to levels of day 0 samples as a result of the regulatory effect of the in vivo steady state balance. From day 13 to day 27, samples continued to diverge from the samples before drug administration, and reached the highest point by day 27, indicating the most significant difference from day 0 samples and the greatest interference. After drug administration was stopped, samples gradually recovered from day 1, and finally on day 13, they were close to the levels of day 0 samples. This meant that the metabolic levels of urinous endogenous substances could

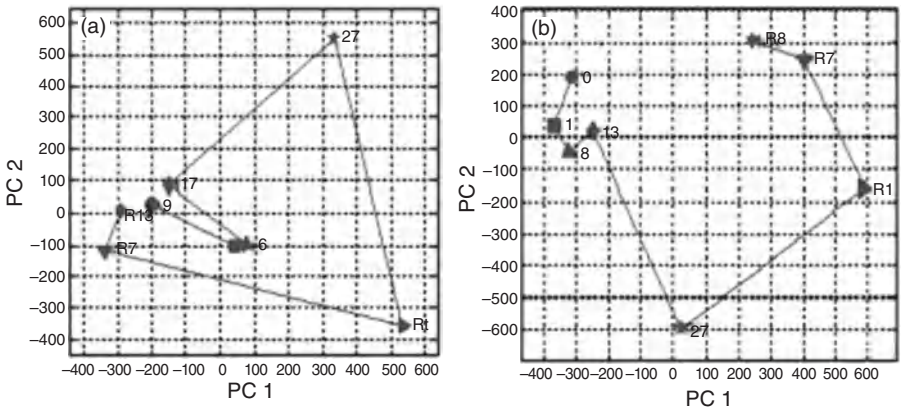


Fig. 12.12 Trajectory tracing of high dose LSP group (A) and high dose *Xionghuang* group (B).

recover after termination of medication. The situation of high dose of *Xionghuang* showed a similar phenomenon (Fig. 12.12B). However, after 13 days of recovery, samples were still far away from day 0 samples, showing that the metabolic disorders in high dose of *Xionghuang* group could not fully recover after the termination of medication. This metabolic trajectory analysis validated the detoxifying effect of LSP by compatibility again.

12.6.2.5 Analysis of Metabolic Markers and Pathways After long-term administration of high doses of LSP and medium and high doses of *Xionghuang*, toxicity and side effects were both found in vivo and may be induced by the cumulative toxicity of *Xionghuang*. By PCA analysis, six related potential biomarkers were identified, including 6-phosphogluconate, L-aspartate-4-phosphate, hippuric acid, citric acid, 3-hydroxyphenyl propionic acid sulfate, and N-(tert-Butoxycarbonyl)-L-methionine. Through the biological significance analysis of these potential biomarkers, we could find and explain metabolic pathways related the toxicity. 6-Phosphogluconate is an intermediate product of the pentose phosphate pathway in glucose degradation, and it is ultimately metabolized into pyruvic acid, which enters into the TCA, an important energy metabolic process. L-aspartate-4-phosphate is an intermediate product of the biosynthesis of glycine, homoserine, threonine, and lysine.[34]

Fig. 12.13 shows the homoserine biosynthesis pathway. L-aspartate-4-phosphate is the production of aspartic acid phosphorylation. It is metabolized into L-aspartic-semialdehyde, which is then converted to homoserine and enters the threonine biosynthesis pathway. L-aspartate-4-phosphate in urine has been previously reported as a potential biomarker of lung cancer[28] and renal toxicity.[35]

Hippuric acid (N-benzoyl glycine) was found as a potential biomarker in the medium and high dose of *Xionghuang* groups, but not in the high dose of LSP group, indicating some toxicity differences among these three groups. Hippuric acid metabolic disorder is an internal sign that glomerular filtration and recovery functions are disturbed. Therefore, normal kidney functions

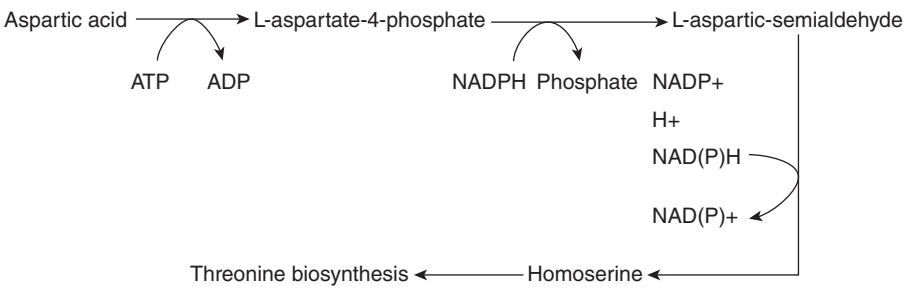


Fig. 12.13 Homoserine biosynthesis pathway.

might be damaged in both of the two *Xionghuang* groups. Arsenic is the main element of *Xionghuang*, and it may accumulate in the kidney after long-term medication of *Xionghuang* and lead to kidney toxicity. The analytical results of high toxicity As^{III} + DMA showed that after 28 days, the concentrations of two *Xionghuang* groups (medium dose: 74.2 ± 5.2 ng/g; high dose: 102.8 ± 9.5 ng/g) were much higher than those of high dose of LSP group (54.9 ± 12.9 ng/g), revealing that more arsenic with high toxicity accumulated in the kidney after *Xionghuang* was administered, and normal kidney functions were affected. This may be the reason why hippuric acid appeared in the *Xionghuang* groups and not in the high dose of LSP group.

Citric acid was found as a potential biomarker in all three of the toxic groups. Citric acid is an intermediate product of the citric acid cycle, and its metabolic disorder is closely related with energy metabolism. In addition, citric acid in urine has been reported as a potential biomarker of liver toxicity[29] and lung cancer.[36]

All the research above showed that the toxicity of *Xionghuang* mainly interfered with energy metabolism and amino acid metabolic pathways, including the pentose phosphate pathway of glucose degradation, citric acid metabolism in energy metabolism, and glycine, homoserine, threonine, and lysine biosynthesis in amino acid metabolism.

12.7 CONCLUSIONS

In this chapter, a chemomics-integrated global systems biology method was applied to the safety evaluation study of a TCM formula, LSP. First, acute toxicity, metabonomics, and heart genomics research on *chansu* was introduced in Section 12.2 to prove its toxicity. Then in Section 12.3, fingerprint and multiple component quantification of LSP were combined in chemomics research to clarify the chemical material base of LSP. Especially for the two toxic herbs, *Chansu* and *Xionghuang*, the related detoxifying effects of processing and compatibility were examined. In Section 12.4, the GLP results showed that the clinical dose of LSP was safe. In vivo distribution and metabolism research of arsenic in LSP and *Xionghuang* were mainly described in Section 12.5. After the compatibility of raw herbs, the concentrations of total arsenic in tissues were reduced and more arsenic was transformed into its less toxic forms, proving the safety of clinical dose of LSP and clearly explaining the attenuation mechanisms. The differential metabolomic research in Section 12.6 identified some related potential biomarkers and pathways for *Xionghuang* safety evaluation. After compatibility, the toxicity of *Xionghuang* was significantly reduced; and there were differences between the LSP and *Xionghuang* groups. In summary, this applied case integrated omics and general GLP research, combined overall with local features, and provided a new model and approach for the safety evaluation of TCM formulas.

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