Contributions to Nephrology
Editor: C. Ronco
Vol. 160

Proteomics in Nephrology

Towards Clinical Applications

Editor
V. Thongboonkerd

KARGER

Contributions to Nephrology

Vol. 160

Series Editor

Claudio Ronco *Vicenza*

Proteomics in Nephrology – Towards Clinical Applications

Volume Editor

Visith Thongboonkerd *Bangkok*

30 figures, 1 in color, and 8 tables, 2008

KARGER

Basel · Freiburg · Paris · London · New York · Bangalore · Bangkok · Shanghai · Singapore · Tokyo · Sydney

Contributions to Nephrology
(Founded 1975 by Geoffrey M. Berlyne)

Visith Thongboonkerd
Medical Proteomics Unit
Office for Research and Development
Faculty of Medicine Siriraj Hospital
Mahidol University
12th Floor – Adulyadej Vikrom Building
2 Prannok Road, Bangkoknoi
Bangkok 10700 (Thailand)

Library of Congress Cataloging-in-Publication Data

Proteomics in nephrology : towards clinical applications / volume editor, Visith Thongboonkerd.
p. ; cm. – (Contributions to nephrology, ISSN 0302-5144 ; v. 160)
Includes bibliographical references and indexes.
ISBN 978-3-8055-8544-6 (hard cover : alk. paper)
1. Kidneys–Diseases. 2. Proteomics. 3. Biochemical markers. I. Thongboonkerd, Visith. II. Series.
[DNLM: 1. Kidney Diseases–physiopathology. 2. Proteome–analysis. 3. Proteomics–methods. W1 CO778UN v.160 2008 / WJ 301 P9677 2008]
RC903.P76 2008
616.6′1–dc22
2008009966

Bibliographic Indices. This publication is listed in bibliographic services, including Current Contents® and Index Medicus.

www.karger.com
Printed in Switzerland on acid-free and non-aging paper (ISO 9706) by Reinhardt Druck, Basel
ISSN 0302–5144
ISBN 978–3–8055–8544–6

Contents

Preface

An initial phase of proteomics applied to the nephrology field dealt mainly with technical development for analyses of proteomes in urine, kidney, glomeruli, tubules, intrarenal vessels, and various types of individual renal cells. Several years ago, renal and urinary proteomics had a slow progress because of the difficulty to find appropriate and efficient methods and protocols for the high-throughput analyses of kidney and urine proteomes. Recently, several methodologies and protocols for renal and urinary proteome analyses have been continuously developed and provided satisfactory results, allowing the field to move onwards to the next step with a big leap.

After the success of the first volume of 'Proteomics in Nephrology' (*Contributions to Nephrology*, vol. 141) published in 2004 to introduce this emerging subdiscipline of nephrology research, it is obvious that renal and urinary proteomics has been more extensively applied to the nephrology field with ultimate goals to: (i) better understand the renal physiology and pathogenic mechanisms of kidney diseases; (ii) search for novel biomarkers for diagnostics and prognostics, and (iii) define and develop new therapeutic targets and drugs for better therapeutic outcome. While the first volume focused mainly on an overview, technologies and methodologies, this volume of 'Proteomics in Nephrology' highlights successful applications of proteomics to several kidney diseases, including acute kidney injury, nephrotic syndrome, diabetic nephropathy, renal allograft rejection, renal cell carcinoma, obstructive nephropathy, kidney stone disease, uremia, and others.

With such important contents written by acclaimed experts in proteomics and nephrology, this book will be an excellent resource of references for

nephrologists, clinicians, pharmacists, other healthcare professionals, proteomists, physiologists, scientists, and trainees. As the Volume Editor, I wish to thank all the contributors who have made this book possible. Finally, I hope that the knowledge obtained from clinical applications of proteomics to the nephrology field will ultimately lead to an improvement of therapeutic outcome and successful prevention of kidney diseases.

Visith Thongboonkerd
Bangkok

Thongboonkerd V (ed): Proteomics in Nephrology – Towards Clinical Applications.
Contrib Nephrol. Basel, Karger, 2008, vol 160, pp 1–16

Proteomics for the Investigation of Acute Kidney Injury

Prasad Devarajan

Nephrology and Hypertension, Cincinnati Children's Hospital Medical Center, University of Cincinnati, Cincinnati, Ohio, USA

Abstract

Acute kidney injury (AKI), previously referred to as acute renal failure (ARF), represents an important problem in clinical medicine. Despite significant improvements in therapeutics, the mortality and morbidity associated with AKI remain high. The reasons for this include (a) an incomplete understanding of the underlying pathophysiologic mechanisms, and (b) the lack of early markers for AKI, and hence an unacceptable delay in initiating therapy. Fortunately, the application of innovative technologies such as functional genomics and proteomics to human and animal models of AKI has uncovered several novel genes and proteins that are emerging as biomarkers and novel therapeutic targets. Recent advances in proteomics that hold promise in ischemic AKI, the most common and serious subtype of ARF, are chronicled in this article. These include the identification of biomarkers in the plasma (NGAL and cystatin C) and urine (NGAL, KIM-1, IL-18, cystatin C, α_1-microglobulin, fetuin-A, Gro-α, and meprin) for the investigation of AKI. It is likely that the AKI panels will be useful for timing the initial insult and assessing the duration of AKI. Based on the differential expression of the biomarkers, it is also likely that the AKI panels will distinguish between the various etiologies of AKI, and predict clinical outcomes.

Acute kidney injury (AKI), previously referred to as acute renal failure (ARF), represents a significant and devastating problem in clinical medicine [1–4]. The incidence of AKI varies from 5% of hospitalized patients to 30–50% of patients in intensive care units. There is now substantial evidence that the incidence of AKI is rising at an alarming rate, and the associated mortality and morbidity have remained high. While recent advances have suggested novel therapeutic approaches in animal models, translational efforts in humans have yielded disappointing results. The reasons for this include (a) an incomplete understanding of the underlying pathophysiology, and (b) a lack of early markers

for AKI, and hence a delay in initiating therapy [5–7]. In current clinical practice, AKI is typically diagnosed by measuring serum creatinine. Unfortunately, creatinine is an unreliable indicator during acute changes in kidney function [8]. First, serum creatinine concentrations may not change until about 50% of kidney function has already been lost. Second, serum creatinine does not accurately depict kidney function until a steady state has been reached, which may require several days. However, animal studies have shown that while AKI can be prevented and/or treated by several maneuvers, these must be instituted very early after the insult, well before the rise in serum creatinine. The lack of early biomarkers has negatively impacted on a number of landmark clinical trials investigating highly promising therapies for AKI [9, 10].

The quest to improve our knowledge of AKI pathogenesis and early diagnosis is an area of intense contemporary research [11–15]. In recent years, the application of innovative technologies such as functional genomics and proteomics to human and animal models of kidney disease has uncovered several novel candidates that are emerging as biomarkers and therapeutic targets [16–20]. This review will update the reader on current advances in proteomics that hold promise primarily in ischemic AKI, the most common and serious subtype of ARF in hospitalized patients. The reader is referred to other publications that address the role of proteomics following nephrotoxins [21–24], kidney transplantation [25, 26], and glomerulonephritides [27].

Proteomic Analysis in Ischemic AKI – Clues from Transcriptome Profiling

Attempts at unraveling the myriad pathways activated in ischemic AKI have been facilitated by transcriptome profiling technologies. Several investigators have used molecular techniques such as cDNA microarrays [28–31] and subtractive hybridizations [32–34] combined with downstream proteomic analysis to identify novel pathways, biomarkers, and drug targets in ischemic AKI. Findings from these approaches are voluminous, and only those that are potentially pertinent to human AKI at the present time are detailed below.

Supavekin et al. [28] performed detailed mouse kidney microarray analyses at early time-points after ischemia-reperfusion injury to identify consistent patterns of altered gene expression, including transcription factors, growth and regenerative genes, and apoptotic molecules. Prominent among the last category included FADD, DAXX, BAD, BAK, and p53, all of which were confirmed by immunohistochemistry. Mounting evidence now indicates that apoptosis is a major mechanism of early tubule cell death in contemporary clinical AKI [35–38]. Several human models of AKI have consistently demonstrated the

presence of apoptotic changes in tubule cells [39–44]. Importantly, proteomic studies have now identified a multitude of apoptotic pathways, including the intrinsic (Bcl-2 family, cytochrome c and caspase 9), extrinsic (Fas, FADD and caspase 8), and regulatory (p53) factors, that are activated in tubule cells following human AKI [42–44]. As a consequence of these studies, inhibition of apoptosis has emerged as a promising approach in human AKI [45–54]. Cell-permeant caspase inhibitors have provided particularly attractive targets for study [51, 52]. In this regard, an orally active small molecule pan-caspase inhibitor (IDN-6556, Pfizer) has been shown to be effective in preventing injury after lung and liver transplantation in animals [53, 54].

Supavekin et al. [28] also identified neutrophil gelatinase-associated lipocalin (*Ngal*, also known as *lcn2*) as one of the most upregulated transcripts in the early post-ischemic mouse kidney, a finding that has now been confirmed in several other transcriptome profiling studies. Downstream proteomic studies have also revealed NGAL to be one of the earliest and most robustly induced proteins in the kidney after AKI in animal models, and NGAL protein is easily detected in the blood and urine soon after AKI [55–58]. These findings have spawned a number of translational proteomic studies to evaluate NGAL as a novel biomarker of human AKI. In a cross-sectional study, human adults in the intensive care unit with established ARF displayed a greater than 10-fold increase in plasma NGAL and more than a 100-fold increase in urine NGAL by Western blotting when compared to normal controls [57]. Both plasma and urine NGAL correlated highly with serum creatinine levels. Kidney biopsies in these patients showed intense accumulation of immunoreactive NGAL in 50% of the cortical tubules. These results identified NGAL as a widespread and sensitive response to established AKI in humans. In a prospective study of children undergoing cardiopulmonary bypass (CPB), AKI (defined as a 50% increase in serum creatinine) occurred in 28% of the subjects, but the diagnosis using serum creatinine was only possible 1–3 days after surgery [59]. In marked contrast, NGAL measurements by Western blotting and by ELISA revealed a robust 10-fold or more increase in the urine and plasma, within 2–6 h of the surgery in patients who subsequently developed AKI. Both urine and plasma NGAL were powerful independent predictors of AKI, with an outstanding area under the curve (AUC) of 0.998 for the 2-hour urine NGAL and 0.91 for the 2-hour plasma NGAL measurement [59]. Thus, plasma and urine NGAL have emerged as sensitive, specific, and highly predictive early biomarkers of AKI after cardiac surgery in children. These findings have now been confirmed in a prospective study of adults who developed AKI after cardiac surgery, in whom urinary NGAL was significantly elevated by 1–3 h after the operation [60]. NGAL has also been evaluated as a biomarker of AKI in kidney transplantation. Biopsies of kidneys obtained 1 h after vascular anastomosis revealed a significant correlation

between NGAL staining intensity and the subsequent development of delayed graft function [61]. In a prospective multicenter study of children and adults, urine NGAL levels in samples collected on the day of transplant clearly identified cadaveric kidney recipients who subsequently developed delayed graft function and dialysis requirement (which typically occurred 2–4 days later). The receiver-operating characteristic (ROC) curve for prediction of delayed graft function based on urine NGAL at day 0 showed an AUC of 0.9, indicative of an excellent predictive biomarker [62]. In summary, NGAL is emerging as a center-stage player in the AKI field, as a novel predictive biomarker.

Ichimura et al. [32] performed a subtractive hybridization screening of ischemic rat kidneys to identify kidney injury molecule 1 (*Kim-1*) as a gene that is markedly upregulated in ischemic rat kidneys, a finding that has been consistently duplicated in several other transcriptome profiling studies. Downstream proteomic studies have also shown KIM-1 to be one of the most highly induced proteins in the kidney after AKI in animal models, and a proteolytically processed domain is easily detected in the urine soon after AKI [63–65]. In a small human cross-sectional study, KIM-1 was found to be markedly induced in proximal tubules in kidney biopsies from patients with established AKI (primarily ischemic), and urinary KIM-1 measured by ELISA distinguished ischemic AKI from prerenal azotemia and chronic renal disease [63]. Patients with AKI induced by contrast did not have increased urinary KIM-1. Thus, KIM-1 represents a promising candidate for inclusion in the urinary 'AKI panel'. An advantage of KIM-1 over NGAL is that it appears to be more specific to ischemic or nephrotoxic kidney injury, and not significantly affected by chronic kidney disease [66]. It is likely that NGAL and KIM-1 will emerge as tandem biomarkers of AKI, with NGAL being most sensitive at the earliest time-points and KIM-1 adding significant specificity at slightly later time-points.

Gene expression studies have provided several additional clues regarding the AKI proteome, but human data are hitherto lacking. For example, Muramatsu et al. [33] have utilized a subtractive hybridization approach to identify *Cyr61* (also known as *CCN1*) as a markedly upregulated gene in the rat kidney very early after ischemic injury. CYR61 protein was induced in the kidney within 1 h and detectable in the urine at 3–6 h after ischemic injury, but not after volume depletion [33]. However, this detection required a complex bioaffinity purification step with heparin-Sepharose beads, and even after such purification, several cross-reacting peptides were apparent. A more convenient platform for the evaluation of CYR61 as a urinary biomarker in humans is not available. Zahedi et al. [34] described spermidine/spermine N^1-acetyltransferase (SSAT), the rate-limiting enzyme in polyamine catabolism, as a novel early biomarker of tubular cell damage after ischemic injury in rats. SSAT protein appears to play a role in the initiation of oxidant-mediated injury to tubules, raising the possibility of inhibition of

polyamine catabolism as a future therapeutic approach [67]. Tarabishi et al. [68] showed that another maximally induced gene identified very early after ischemic injury in animal models is Zf9, a Kruppel-like transcription factor involved in the regulation of a number of downstream targets. Zf9 protein is markedly upregulated in the post-ischemic tubule cells, along with its major trans-activating factor, TGF-β_1. Gene silencing of Zf9 abrogated TGF-β_1 protein expression and mitigated the apoptotic response to ischemic injury in vitro [68]. These studies have thus identified a novel pathway that may play a critical role in the early tubule cell death that accompanies ischemic renal injury. Thakar et al. [30] have employed transcriptome profiling in rat models to identify thrombospondin 1 (TSP-1), a previously known p53-dependent pro-apoptotic and anti-angiogenic molecule, as another maximally induced gene early after ischemic AKI. The TSP-1 protein product is upregulated in the post-ischemic proximal tubule cells, where it colocalizes with activated caspase-3. TSP-1 null mice were partially protected from ischemic injury, with striking structural preservation of kidney tissue [30]. These results have thus identified yet another previously unknown apoptotic protein that is activated in proximal tubule cells early after ischemic AKI in animals.

Transcripts that have been consistently reported to be either up- or down-regulated in animal models of AKI are listed in tables 1 and 2, respectively. While many of them have now been confirmed by downstream proteomic analysis, the majority of these studies remain in the preclinical research realm, and convincing data attesting to their utility in human AKI are currently unavailable.

Direct Proteomic Profiling in Ischemic AKI

Recent advances in the field of direct proteomic profiling have accelerated the discovery of novel protein biomarkers and therapeutic targets for AKI [16–27]. Of the various methods and platforms available, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) technology has emerged as one of the preferred platforms for urinary protein profiling [84–86]. This approach allows for rapid high-throughput profiling of multiple urine samples, detects low molecular weight biomarkers that are typically missed by other platforms, and even uncovers proteins bound to albumin. The commercial availability of the ProteinChip® Biomarker System and the accompanying bioinformatic software (Ciphergen) has provided investigators with the tools to obtain reproducible results and their statistical interpretation. Previously quoted problems with calibration difficulties and variability of reagents [6] have now been largely resolved by the commercial availability of All-in-1 peptide/protein calibration standards (Ciphergen) and chromatographic solutions (BioSeptra). However, persistent disadvantages of this method include

Table 1. Genes reported to be upregulated in at least three separate transcriptome profiling studies

Gene name	Gene symbol	Protein ref.[1]
Cyclin-dependent kinase inhibitor 1A	*p21/Cip1/WAF*	69
Clusterin	*CLU*	70
A kinase (PRKA) anchor protein (gravin) 12	*AKAP12/SSeCK*	None
Tubulin, β	*TUBB*	71
Heme oxygenase (decycling) 1	*HMOX1*	72
Activating transcription factor 3	*ATF3*	31
Metallothionein 1A	*MT1A*	73
Lectin, galactoside-binding, soluble, 3 (galectin 3)	*LGALS3*	74
Early growth response 1	*EGR1*	75
Claudin 7	*CLDN7*	None
CD68 antigen	*CD68*	76
Lipocalin 2 (neutrophil gelatinase-associated lipocalin)	*LCN2/NGAL*	55
Kidney injury molecule 1	*KIM-1/HAVCR1*	63
c-Fos	*cFos*	77
Annexin A2/calpactin 1	*ANXA2*	29
Heath shock protein 70 kDa	*HSP70*	78
Interleukin-6	*IL6*	79
Chemokine (C-X-C motif) ligand 1	*CXCL1/Gro-1*	77

[1]References of published proteomic studies that have confirmed the induction of the corresponding gene product.

Table 2. Genes reported to be downregulated in at least three separate transcriptome profiling studies

Gene name	Gene symbol	Protein ref.[1]
Epidermal growth factor	*EGF*	80
Afamin/α-albumin	*AFM*	None
Leukemia inhibitory factor receptor	*LIFR*	81
Solute carrier family 9, member 3	*SLC9A3/NHE3*	82
Solute carrier family 16, member 7	*SLC16A7*	None
Uromodulin (Tamm-Horsfall mucoprotein)	*UMOD*	83

[1]References of published proteomic studies that have confirmed the suppression of the corresponding gene product.

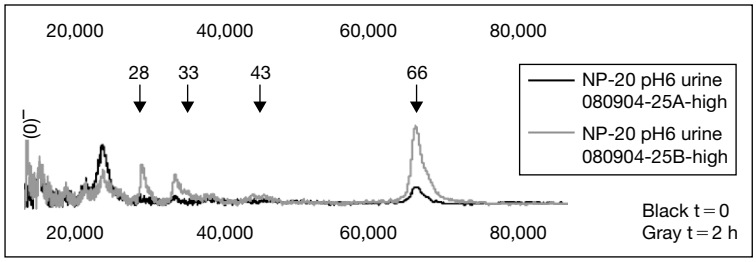


Fig. 1. Overlay of representative SELDI-TOF-MS spectra of urine obtained at baseline and 2 h after CPB from patients who subsequently developed ARF. Marked enhancement of 28-, 33-, 43-, and 66-kDa species is noted in the ARF group at 2 h post-surgery, as highlighted by the arrows. Patients in the control group did not display similar peaks at any time-point post-surgery.

the limited ability to resolve large molecular weight proteins, and the difficulties with identifying the protein peaks. Nguyen et al. [87] have employed SELDI-TOF-MS technology to identify urinary biomarker patterns that predict AKI in patients undergoing CPB. Urine aliquots at baseline (t = 0) and 2 h (t = 2 h) were assigned to control (n = 15) or ARF groups (n = 15). ARF was defined as a 50% or greater increase in serum creatinine. Representative samples of spectra obtained are shown in figure 1. The SELDI-TOF-MS analysis of the ARF group at t = 0 versus t = 2 h consistently showed a marked enhancement of protein biomarkers with m/z of 6.4 (not shown), 28, 43, and 66 kDa. The same biomarkers were significantly different when comparing control versus ARF groups at t = 2 h. No differences were detected in control versus ARF patients at t = 0. It should be noted that the serum creatinine in these patients did not increase until days 2–3 after surgery. Scatterplots revealed a dramatic increase in peak intensity of all four novel biomarkers in the ARF group at baseline (t = 0) versus 2-hour post-CPB, with the AUC of ROC curve in the 0.90–0.98 range, indicative of excellent biomarkers [87]. Thus, this proteomic approach has revealed a distinctive AKI fingerprint comprising of at least four biomarkers that are markedly enhanced within 2 h of CPB in patients who subsequently developed AKI, and has shown that the SELDI-TOF-MS method is sensitive, non-invasive (requiring only microliter quantities of urine), rapid (with no special preparation steps needed), and reproducible. An important limitation to this study is that it represents a single-center analysis involving only children and young adults with congenital heart disease. A second limitation is the exclusion of patients with pre-existing renal insufficiency, diabetes, peripheral vascular disease and nephrotoxin use. While this cohort was intentionally chosen to eliminate confounding variables and comorbid conditions, it is acknowledged that human

ARF is frequently multifactorial, often occurring in a setting where there is a complex interplay of predisposing and precipitating factors. These results therefore need to be validated in a larger population of susceptible patients. It will also be important in future studies to confirm the identity of the four biomarkers uncovered by this study, and to determine their individual and collective robustness for the prediction of AKI.

In another direct proteomic profiling study in humans, Lefler et al. [88] utilized two-dimensional gel electrophoresis (2-DE) followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) or MALDI-TOF/TOF tandem MS to characterize proteins removed by continuous renal replacement therapy for ARF. The 2-DE method allows for good separation and quantitation of individual proteins, and the resolved protein spots are directly amenable to identification by peptide mass fingerprinting (MALDI-TOF-MS) and/or peptide sequencing (MALDI-TOF/TOF). However, gel-based proteomics also has limitations. It is time- and labor-intensive, and there is considerable difficulty in detecting low-abundance proteins and insoluble membrane proteins. Nevertheless, Lefler et al. [88] identified several proteins in the effluent by peptide mass fingerprinting, including albumin, apolipoprotein A-IV, β_2-microglobulin, lithostathine, mannose-binding lectin associated serine protease 2 associated protein, plasma retinol-binding protein, transferrin, transthyretin, vitamin D-binding protein, and Zn-α_2-glycoprotein. Direct sequencing of tryptic peptides confirmed the identity of all except apolipoprotein A-IV, transferrin, transthyretin, and serine protease 2 associated protein. The potential therapeutic or detrimental implications of the identified proteins being removed by renal replacement therapy are unclear at the present time. The identified proteins are known to be present in serum. Given their multiple physiological roles, it is conceivable that loss of albumin, transferrin, and vitamin D-binding protein may contribute to the complex pathophysiology of ARF in dialyzed patients.

Zhou et al. [89] have employed two-dimensional difference gel electrophoresis (2-D DIGE) followed by mass spectrometry (MALDI-TOF/TOF) or liquid chromatography coupled to tandem MS (LC-MS/MS) to examine urinary exosomes in animal models of AKI. Urinary exosomes containing apical membranes and intracellular fluid are normally secreted into the urine from all nephron segments, and contain protein markers of structural and functional renal damage. Exosomes represent a unique source for the discovery of non-invasive urinary biomarkers that can overcome much of the interference from abundant urinary proteins such as albumin, globulin, and Tamm-Horsfall mucoprotein [90, 91]. Zhou et al. [89] initially uncovered 74 peptide spots that showed differential expression by 2-D DIGE of urinary exosomes following nephrotoxic injury with cisplatin. 15 of these proteins were identified by MALDI-TOF/TOF, and an additional 13 detected by LC-MS/MS. Out of these, Western

blotting was able to confirm only two protein expression changes, namely fetuin-A (increased in AKI) and annexin V (decreased in AKI). The very low rate with which differentially expressed proteins were identified and confirmed in this study exemplifies many of the limitations associated with the 2-D DIGE methodology. Nevertheless, the authors subsequently identified fetuin-A within urinary exosomes by immunoelectron microscopy, and validated urinary exosomal fetuin-A to be increased more than 30-fold in the early phase of ischemia-reperfusion injury by Western blotting. Urinary exosomal fetuin-A was also noted to be markedly increased by Western blotting in three patients in the intensive care unit with AKI compared to patients without AKI. This proteomic approach has therefore identified fetuin-A as a potential biomarker for human AKI. Factors that currently limit the widespread clinical testing of fetuin-A include the complex steps required for exosome preparation, and the lack of an easily translatable assay such as an ELISA.

Molls et al. [92] have utilized commercial protein arrays (cytokine multiplex bead-based assays) to measure 18 cytokines and chemokines in mouse kidney homogenates early after ischemia-reperfusion injury. The earliest and most consistent change noted was a rise in kidney keratinocyte-derived chemokine (KC), with a 13-fold increase within 3 h of ischemic injury. By ELISA, serum and urinary KC levels at 3 h post-ischemia were also significantly enhanced in mice that developed an increase in serum creatinine 24 h after the injury. Importantly, in a small cohort of patients, the human analog of KC, namely Gro-α, was markedly upregulated in the urine of deceased donor kidney transplant recipients with delayed graft function, in comparison with recipients with good graft function [92]. Thus, these studies using protein arrays have identified Gro-α as another potential candidate for inclusion in the urinary 'AKI panel'. This approach is obviously hampered by the limited number of candidates that can be detected using a given protein array.

Holly et al. [93] have used 2-D DIGE followed by MALDI-TOF-MS to identify differentially expressed urinary proteins in a rat model of sepsis-induced AKI. Sepsis is one of the most common causes of human ARF, and the resultant renal dysfunction is primarily due to ischemic injury, resulting from a potent combination of renal vasoconstriction and systemic vasodilatation [94]. While initial 2-D DIGE of urine samples identified 97 differentially expressed spots in rats with sepsis-induced AKI, subsequent peptide mass fingerprinting could identify only 30 of those. The few peptides that were upregulated included previously known candidates such as albumin, aminopeptidase, and α_2-microglobulin (also known as lipocalin or NGAL). The majority of the differentially expressed urinary proteins were decreased in sepsis-induced AKI, including uromodulin (Tamm-Horsfall mucoprotein), serum protease inhibitors, and the brush border enzyme meprin-1α. The authors chose to further characterize meprin-1α. By

Western blotting, septic rats with ARF displayed a decrease in meprin. Furthermore, inhibition of meprin with actinonin partially ameliorated sepsis-induced ARF. Thus, despite the limitations described, this proteomic approach has identified meprin not only as a potential urinary biomarker that is repressed in a rat model of sepsis-induced AKI, but also as a therapeutic target. Studies of meprin in human AKI have not been reported to date.

More focused proteomic approaches have recently yielded additional biomarkers for AKI. For example, IL-18 is a pro-inflammatory cytokine that is known to be induced and cleaved in the proximal tubule, and subsequently easily detected in the urine following ischemic AKI in animal models [95]. In a cross-sectional study, urine IL-18 levels measured by ELISA were markedly increased in patients with established AKI, but not in subjects with urinary tract infection, chronic kidney disease, nephritic syndrome, or prerenal failure [96]. Urinary IL-18 was significantly upregulated up to 48 h prior to the increase in serum creatinine in patients with acute respiratory distress syndrome who developed AKI, with an AUC of 0.73, and represented an independent predictor of mortality in this cohort [97]. Urinary IL-18 and NGAL were recently shown to represent early, predictive, sequential AKI biomarkers in children undergoing cardiac surgery [98]. In patients who developed AKI 2–3 days after surgery, urinary NGAL was induced within 2 h and peaked at 6 h whereas urine IL-18 levels increased around 6 h and peaked at over 25-fold at 12 h post-surgery (AUC 0.75). Both IL-18 and NGAL were independently associated with duration of AKI among cases. Urine NGAL and IL-18 have also emerged as predictive biomarkers for delayed graft function following kidney transplantation [62]. In a prospective multicenter study of children and adults, both NGAL and IL-18 in urine samples collected on the day of transplant predicted delayed graft function and dialysis requirement with AUC of 0.9. Thus, IL-18 may also represent a promising candidate for inclusion in the urinary 'AKI panel'. IL-18 is more specific to ischemic AKI, and not affected by nephrotoxins, chronic kidney disease or urinary tract infections. It is likely that NGAL, IL-18 and KIM-1 will emerge as sequential urinary biomarkers of AKI.

Herget-Rosenthal et al. [99] have measured urinary excretion of a number of candidate biomarker proteins (α_1-microglobulin, β_2-microglobulin, cystatin C, retinol-binding protein, α-glutathione *S*-transferase, lactate dehydrogenase, and *N*-acetyl-β-D-glucosaminidase) early in the course of non-oliguric ARF in humans. In this cohort of patients with established ARF (defined as a doubling of serum creatinine) from a variety of causes, urinary excretion of α_1-microglobulin and cystatin C were found to be predictive of severe ARF requiring renal replacement therapy, with an AUC of 0.86 and 0.92 respectively. α_1-Microglobulin is a tubular protein that belongs to the lipocalin superfamily, similar to NGAL. Cystatin C is a cysteine protease inhibitor that is synthesized

and released into the blood at a relatively constant rate by all nucleated cells. It is freely filtered by the glomerulus, normally reabsorbed by the proximal tubule, and not secreted. Both α_1-microglobulin and cystatin C are stable in the urine, and can be easily measured by immunonephelometric methods in most standard clinical chemistry laboratories. The predictive role of these urinary proteins in early AKI remains to be determined.

Since blood levels of cystatin C are not significantly affected by age, gender, race, or muscle mass, it has been proposed as a better predictor of glomerular function than serum creatinine in patients with AKI. In the intensive care setting, a 50% increase in serum cystatin C predicted AKI 1–2 days before the rise in serum creatinine, with an AUC of 0.97 and 0.82, respectively [7]. A recent prospective study compared the ability of serum cystatin C and NGAL in the prediction of AKI following cardiac surgery [100]. Out of 129 patients, 41 developed AKI (defined as a 50% increase in serum creatinine) 1–3 days after CPB. In AKI cases, serum NGAL levels were elevated at 2 h post-surgery, whereas serum cystatin C levels increased only after 12 h. Both NGAL and cystatin C levels at 12 h were strong independent predictors of AKI, but NGAL outperformed cystatin C at earlier time-points. Thus, both NGAL and cystatin C may represent promising tandem biomarker candidates for inclusion in the blood 'AKI panel'.

Conclusions

The tools of contemporary proteomics have provided us with promising novel biomarkers for the investigation of AKI. These include a plasma panel (NGAL and cystatin C) and a urine panel (NGAL, KIM-1, IL-18, cystatin C, α_1-microglobulin, fetuin-A, Gro-α, and meprin). Since they represent tandem biomarkers, it is likely that the AKI panels will be useful for timing the initial insult and assessing the duration of AKI (analogous to the cardiac panel for evaluating chest pain). Based on the differential expression of the biomarkers, it is also likely that the AKI panels will help distinguish between the various types and etiologies of AKI determine the severity of AKI, and predict clinical outcomes [101, 102]. However, they have hitherto been tested only in small studies and in a limited number of clinical situations. It will be important in future studies to validate the sensitivity and specificity of these biomarker panels in clinical samples from large cohorts and from multiple clinical situations. Such studies will be markedly facilitated by the availability of commercial tools for the reliable and reproducible measurement of biomarkers across different laboratories. Ongoing and future proteomic studies will likely yield additional sensitive and specific biomarkers for the investigation of AKI resulting from diverse etiologies. Such tools will be indispensable for the early diagnosis and initiation of timely therapeutic measures.

References

1 Lameire N, Van Biesen W, Vanholder R: Acute renal failure. Lancet 2005;365:417–430.
2 Schrier RW, Wang W, Poole B, Mitra A: Acute renal failure: definitions, diagnosis, pathogenesis, and therapy. J Clin Invest 2004;114:5–14.
3 Uchino S, Kellum JA, Bellomo R, Doig GS, Morimatsu H, Morgera S, Schetz M, Tan I, Bouman C, Macedo E, Gibney N, Tolwani A, Ronco C: for the Beginning and Ending Supportive Therapy for the Kidney (BEST Kidney) Investigators: Acute renal failure in critically ill patients: a multinational, multicenter study. JAMA 2005;294:813–818
4 Devarajan P: Update on mechanisms of ischemic acute kidney injury. J Am Soc Nephrol 2006;17: 1503–1520.
5 Devarajan P: Emerging biomarkers of acute kidney injury. Contrib Nephrol. Basel, Karger, 2007, vol 156, pp 203–212.
6 Hewitt SM, Dear J, Star RA: Discovery of protein biomarkers for renal diseases. J Am Soc Nephrol 2004;15:1677–1689.
7 Herget-Rosenthal S, Marggraf G, Hüsing J, Goring F, Pietruck F, Janssen O, Phillip T, Kribben A: Early detection of acute renal failure by serum cystatin C. Kidney Int 2004;66:1115–1122.
8 Bellomo R, Kellum JA, Ronco C: Defining acute renal failure: physiological principles. Intensive Care Med 2004;30:33–37.
9 Allgren RL, Marbury TC, Rahman SN, Weisberg LS, Fenves AZ, Lafayette RA, Sweet RM, Genter FC, Kurnik BR, Conger JD, Sayegh MH: Anaritide in acute tubule necrosis. Auriculin Anaritide Acute Renal Failure Study Group. N Engl J Med 1997;336:828–834.
10 Hirschberg R, Kopple J, Lipsett P, Benjamin E, Minei J, Albertson T, Munger M, Metzler M, Zaloga G, Murray M, Lowry S, Conger J, McKeown W, O'Shea M, Baughman R, Wood K, Haupt M, Kaiser R, Simms H, Warnock D, Summer W, Hintz R, Myers B, Haenftling K, Capra W, Pike M, Guler H-P: Multicenter clinical trial of recombinant human insulin-like growth factor-1 in patients with acute renal failure. Kidney Int 1999;55:2423–2432.
11 American Society of Nephrology Renal Research Report: J Am Soc Nephrol 2005;16:1886–1893.
12 Devarajan P, Mishra J, Supavekin S, Patterson LT, Potter SS: Gene expression in early ischemic renal injury: clues towards pathogenesis, biomarker discovery, and novel therapeutics. Mol Genet Metab 2003;80:365–376.
13 Han WK, Bonventre JV: Biologic markers for the early detection of acute kidney injury. Curr Opin Crit Care 2004;10:476–482.
14 Zhou H, Hewitt SM, Yuen PST, Star RA: Acute kidney injury biomarkers – needs, present status, and future promise. Nephrology Self Assessment Program, American Society of Nephrology. NephSAP 2006;5:63–71.
15 Perco P, Pleban C, Kainz A, Lukas A, Mayer G, Mayer B, Oberbauer R: Protein biomarkers associated with acute renal failure and chronic kidney disease. Eur J Clin Invest 2006;36: 753–763.
16 Thongboonkerd V: Proteomics in nephrology: current status and future directions. Am J Nephrol 2004;24:360–378.
17 Thongboonkerd V: Proteomic analysis of renal diseases: unraveling the pathophysiology and biomarker discovery. Expert Rev Proteomics 2005;2:349–366.
18 Vidal BC, Bonventre JV, Hsu SI-H: Towards the application of proteomics in renal disease diagnosis. Clin Sci 2005;109:421–430.
19 Pisitkun T, Johnstone R, Knepper MA: Discovery of urinary biomarkers. Mol Cell Proteomics 2006;5:1760–1771.
20 O'Riordan E, Gross SS, Goligorsky MS: Technology insight: renal proteomics – at the crossroads between promise and problems. Nat Clin Pract Nephrol 2006;2:445–458.
21 Witzmann FA, Li J: Proteomics and nephrotoxicity. Contrib Nephrol. Basel, Karger, 2004, vol 141, pp 104–123.
22 Gibbs A: Comparison of the specificity and sensitivity of traditional methods for assessment of nephrotoxicity in the rat with metabolomic and proteomic methodologies. J Appl Toxicol 2005;25:277–295.

23 Betton GR, Kenne K, Somers R, Marr A: Protein biomarkers of nephrotoxicity; a review and findings with cyclosporine A, a signal transduction kinase inhibitor and *N*-phenylanthranilic acid. Cancer Biomark 2005;1:59–67.
24 Safirstein RL: Acute renal failure: from renal physiology to the renal transcriptome. Kidney Int 2004;66:S62–S66.
25 Scaub S, Wilkins JA, Rush D, Nickerson P: Developing a tool for noninvasive monitoring of renal allografts. Expert Rev Proteomics 2006;3:497–509.
26 Wishart DS: Metabolomics in monitoring kidney transplants. Curr Opin Nephrol Hypertens 2006;15:637–642.
27 Thongboonkerd V, Klein JB, Jevans AW, McLeish KR: Urinary proteomics and biomarker discovery for glomerular diseases. Contrib Nephrol. Basel, Karger, 2004, vol 141, pp 292–307.
28 Supavekin S, Zhang W, Kucherlapati R, Kaskel FJ, Moore LC, Devarajan P: Differential gene expression following early renal ischemia-reperfusion. Kidney Int 2003;63:1714–1724.
29 Cheng C-W, Rifai A, Ka S-M, Shui H-A, Lin Y-F, Lee W-H, Chen A: Calcium-binding proteins annexin A2 and S100A6 are sensors of tubular injury and recovery in acute renal failure. Kidney Int 2005;68:2694–2703.
30 Thakar CV, Zahedi K, Revelo MP, Wang Z, Burnham CE, Barone S, Bevans S, Lentsch AB, Rabb H, Soleimani M: Identification of thrombospondin 1 (TSP-1) as a novel mediator of cell injury in kidney ischemia. J Clin Invest 2005;115:3451–3459.
31 Yuen PST, Jo S-K, Holly MK, Hu X, Star RA: Ischemic and nephrotoxic acute renal failure are distinguished by their broad transcriptomic responses. Physiol Genomics 2006;25:375–386.
32 Ichimura T, Bonventre JC, Bailly V, Wei H, Hession CA, Cate RL, Sanicola M: Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury. J Biol Chem 1998;273:4135–4142.
33 Muramatsu Y, Tsujie M, Kohda Y, Pham B, Perantoni AO, Zhao H, Jo S-K, Yuen PST, Craig L, Hu X, Star RA: Early detection of cysteine rich protein 61 (CYR61, CCN1) in urine following renal ischemic reperfusion injury. Kidney Int 2002;62:1601–1610.
34 Zahedi K, Wang Z, Barone S, Prada AE, Kelly CN, Casero RA, Yokota N, Porter CW, Rabb H, Soleimani M: Expression of SSAT, a novel biomarker of tubular cell damage, increases in kidney ischemia-reperfusion injury. Am J Physiol Renal Physiol 2003;284:F1046–F1055.
35 Saikumar P, Venkatachalam MA: Role of apoptosis in hypoxic/ischemic damage in the kidney. Semin Nephrol 2003;6:512–521.
36 Kaushal GP, Basnakian AG, Shah SV: Apoptotic pathways in ischemic acute renal failure. Kidney Int 2004;66:500–505.
37 Dagher PC: Apoptosis in ischemic renal injury: roles of GTP depletion and p53. Kidney Int 2004;66:506–509.
38 Del Rio M, Imam A, DeLeon M, G, Mishra J, Ma Q, Parikh S, Devarajan P: The death domain of kidney ankyrin interacts with Fas and promotes Fas-mediated cell death in renal epithelia. J Am Soc Nephrol 2004;15:41–51.
39 Burns AT, Davies DR, McLaren AJ, Cerundolo L, Morris PJ, Fuggle SV: Apoptosis in ischemia/reperfusion injury of human renal allografts. Transplantation 1998;66:872–876.
40 Oberbauer R, Rohrmoser M, Regele H, Muhlbacher F, Mayer G: Apoptosis of tubular epithelial cells in donor kidney biopsies predicts early renal allograft function. J Am Soc Nephrol 1999;10: 2006–2013.
41 Schwarz C, Hauser P, Steininger R, Regele H, Heinze G, Mayer G, Oberbauer R: Failure of Bcl-2 up-regulation in proximal tubular epithelial cells of donor kidney biopsy specimens is associated with apoptosis and delayed graft function. Lab Invest 2002;82:941–948.
42 Hoffman SC, Kampen RL, Amur S, Sharaf MA, Kleiner DE, Hunter K, John Swanson S, Hale DA, Mannon RB, Blair PJ, Kirk AD: Molecular and immunohistochemical characterization of the onset and resolution of human renal allograft ischemia-reperfusion injury. Transplantation 2002;74:916–923.
43 Castaneda MP, Swiatecka-Urban A, Mitsnefes MM, Feuerstein D, Kaskel FJ, Tellis V, Devarajan P: Activation of mitochondrial apoptotic pathways in human renal allografts following ischemia-reperfusion. Transplantation 2003;76:50–54.
44 Hauser P, Schwarz C, Mitterbauer C, Regele HM, Muhlbacher F, Mayer G, Perco P, Mayer B, Meyer TW, Oberbauer R: Genome-wide gene-expression patterns of donor kidney biopsies distinguish primary allograft function. Lab Invest 2004;84:353–361.

45 Ortiz A, Justo P, Sanz A, Lorz C, Egido J: Targeting apoptosis in acute tubular injury. Biochem Pharmacol 2003;66:1589–1594.
46 Fleischer A, Ghadiri A, Dessauge F, Duhamel M, Rebollo MP, Alvarez-Franco F, Rebollo A: Modulating apoptosis as a target for effective therapy. Mol Immunol 2006;43:1065–1079.
47 Faubel S, Edelstein CL: Caspases as drug targets in ischemic organ injury. Curr Drug Targets Immune Endocr Metabol Disord 2005;5:269–287.
48 Green DR, Kroemer G: Pharmacologic manipulation of cell death: clinical applications insight? J Clin Invest 2005;115:2610–2617.
49 Bouchier-Hayes L, Lartigue L, Newmeyer DD: Mitochondria: pharmacological manipulation of cell death. J Clin Invest 2005;115:2640–2647.
50 Letai A: Pharmacologic manipulation of Bcl-2 family members to control cell death. J Clin Invest 2005;115:2648–2655.
51 Lavrik IN, Golks A, Krammer PH: Caspases: pharmacological manipulation of cell death. J Clin Invest 2005;115:2665–2672.
52 Jani A, Ljubanivic D, Faubel SG, Kim J, Mischak R, Edelstein CL: Caspase inhibition prevents the increase in caspase-3, -2, -8 and -9 activity and apoptosis in the cold ischemic mouse kidney. Am J Transplant 2004;8:1246–1254.
53 Hoglen NC, Chen LS, Fisher CD, Hirakawa BP, Groessl T, Contreras PC: Characterization of IDN-6556 (3-[2-(2-tert-butyl-phenylaminooxalyl)-amino]-propionylamino)-4-oxo-5-(2,3,5,6-tetrafluoro-phenoxy)-pentanoic acid): a liver-targeted caspase inhibitor. J Pharmacol Exp Ther 2004;309: 634–640.
54 Quadri SM, Segall L, de Perrot M, Han B, Edwards V, Jones N, Waddell TK, Liu M, Keshavjee S: Caspase inhibition improves ischemia-reperfusion injury after lung transplantation. Am J Transplant 2005;5:292–299.
55 Mishra J, Ma Q, Prada A, Zahedi K, Yang Y, Barasch J, Devarajan P: Identification of neutrophil gelatinase-associated lipocalin as a novel urinary biomarker for ischemic injury. J Am Soc Nephrol 2003;4:2534–2543.
56 Mishra J, Mori K, Ma Q, Kelly C, Barasch J, Devarajan P: Neutrophil gelatinase-associated lipocalin (NGAL): a novel urinary biomarker for cisplatin nephrotoxicity. Am J Nephrol 2004;24:307–315.
57 Mori K, Lee HT, Rapoport D, Drexler I, Foster K, Yang J, Schmidt-Ott, Chen X, Li JY, Weiss S, Mishra J, Cheema FH, Markowitz G, Suganami T, Sawai K, Mukoyama M, Kunis C, D'Agati V, Devarajan P, Barasch J: Endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from ischemia-reperfusion injury. J Clin Invest 2005;115:610–621.
58 Schmitt-Ott KM, Mori K, Kalandadze A, Li J-Y, Paragas N, Nicholas T, Devarajan P, Barasch J: Neutrophil gelatinase-associated lipocalin-mediated iron traffic in kidney epithelia. Curr Opin Nephrol Hypertens 2005;15:442–449.
59 Mishra J, Dent C, Tarabishi R, Mitsnefes MM, Ma Q, Kelly C, Ruff SM, Zahedi K, Shao M, Bean J, Mori K, Barasch J, Devarajan P: Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute renal injury following cardiac surgery. Lancet 2005;365:1231–1238.
60 Wagener G, Jan M, Kim M, Mori K, Barasch JM, Sladen RN, Lee HT: Association between increases in urinary neutrophil gelatinase-associated lipocalin and acute renal dysfunction after adult cardiac surgery. Anesthesiology 2006;105:485–491.
61 Mishra J, Ma Q, Kelly C, Mitsnefes M, Mori K, Barasch J, Devarajan P: Kidney NGAL is a novel early marker of acute injury following transplantation. Pediatr Nephrol 2006;21:856–863.
62 Parikh CR, Jani A, Mishra J, Ma Q, Kelly C, Barasch J, Edelstein CL, Devarajan P: Urine NGAL and IL-18 are predictive biomarkers for delayed graft function following kidney transplantation. Am J Transplant 2006;6:1639–1645.
63 Han WK, Bailly V, Abichandani R, Thadani R, Bonventre JV: Kidney injury molecule-1 (KIM-1): a novel biomarker for human renal proximal tubule injury. Kidney Int 2002;62:237–244.
64 Ichimura T, Hung CC, Yang SA, Stevens JL, Bonventre JV: Kidney injury molecule-1: a tissue and urinary biomarker for nephrotoxicant-induced renal injury. Am J Physiol Renal Physiol 2004;286:F552–F563.
65 Vaidya VS, Ramirez V, Ichimura T, Bobadilla NA, Bonventre JV: Urinary kidney injury molecule-1: a sensitive quantitative biomarker for early detection of kidney tubular injury. Am J Physiol Renal Physiol 2006;290:F517–F529.

66 Mitsnefes M, Kathman T, Mishra J, Kartal J, Khoury P, Nickolas T, Barasch J, Devarajan P: Serum NGAL as a marker of renal function in children with chronic kidney disease. Pediatr Nephrol 2007;22:101–108.
67 Wang Z, Zahedi K, Barone S, Tehrani K, Rabb H, Matlin K, Casero RA, Soleimani M: Overexpression of SSAT in kidney cells recapitulates various phenotypic aspects of kidney ischemia-reperfusion injury. J Am Soc Nephrol 2004;15:1844–1852.
68 Tarabishi R, Zahedi K, Mishra J, Ma Q, Kelly C, Tehrani K, Devarajan P: Induction of Zf9 in the kidney following early ischemia/reperfusion. Kidney Int 2005;68:1511–1519.
69 Hochegger K, Koppelstatter C, Tagwerker A, Huber JM, Heininger D, Mayer G, Rosenkranz AR: p21 and mTERT are novel markers for determining different ischemic time periods in renal ischemia reperfusion injury. Am J Physiol Renal Physiol 2007; in press.
70 Nath KA, Dvergsten J, Correa-Rotter R, Hostetter TH, Manivel JC, Rosenberg ME: Induction of clusterin and chronic oxidative renal disease in the rat and its dissociation from cell injury. Lab Invest 1994;71:209–218.
71 Caron A, Desrosiers RR, Beliveau R: Kidney ischemia-reperfusion regulates expression and distribution of tubulin subunits, β-actin and rho GTPases in proximal tubules. Arch Biochem Biophys 2004;431:31–46.
72 Akagi R, Takahashi T, Sassa S: Cytoprotective effects of heme oxygenase in acute renal failure. Contrib Nephrol. Basel, Karger, 2005, vol 148, pp 70–85.
73 Takahashi T, Itano Y, Noji S, Matsumotot K, Taga N, Mizukawa S, Toda N, Matsumi M, Morita K, Hirakawa M: Induction of renal metallothionein in rats with ischemic renal failure. Res Commun Mol Pathol Pharmacol 2001;110:147–160.
74 Nishiyama J, Kobayashi S, Ishida A, Nakabayashi I, Tajima O, Miura S, Katayama M, Nogami H: Up-regulation of galectin-3 in acute renal failure of the rat. Am J Pathol 2000;157:815–823.
75 Bonventre JV, Sukhatme VP, Bamberger M, Ouellette AJ, Brown D: Localization of the protein product of the immediate early growth response gene, Egr-1, in the kidney after ischemia and reperfusion. Cell Regul 1991;2:251–260.
76 Briscoe DM, Pober JS, Harmon WE, Cotran RS: Expression of vascular cell adhesion molecule-1 in human renal allografts. J Am Soc Nephrol 1992;3:1180–1185.
77 Witzgall R, Brown D, Schwarz C, Bonventre JV: Localization of proliferating cell nuclear antigen, vimentin, c-Fos, and clusterin in the postischemic kidney. Evidence for a heterogeneous genetic response among nephron segments, and a large pool of mitotically active and dedifferentiated cells. J Clin Invest 1994;93:2175–2188.
78 Aufricht C: Heat-shock protein 70: molecular supertool? Pediatr Nephrol 2005;20:707–713.
79 Kielar ML, John R, Bennett M, Richardson JA, Shelton JM, Chen L, Jeyarajah DR, Zhou XJ, Zhou H, Chiquett B, Nagami GT, Lu CY: Maladaptive role of IL-6 in ischemic acute renal failure. J Am Soc Nephrol 2005;16:3315–3325.
80 Gobe G, Zhang ZJ, Wollgoss DA, Schoch E, Hogg NA, Endre ZH: Relationship between expression of Bcl-2 genes and growth factors in ischemic acute renal failure in the rat. J Am Soc Nephrol 2000;11:454–467.
81 Yoshino J, Monkawa T, Tsuji M, Hayashi M, Saruta T: Leukemia inhibitory factor is involved in tubular regeneration after experimental acute renal failure. J Am Soc Nephrol 2003;14: 3090–3101.
82 Du Cheyron D, Daubin C, Poggioli J, Ramakers M, Houillier P, Charbonneau P, Paillard M: Urinary measurement of Na^+/H^+ exchanger isoform 3 (NHE3) protein as new marker of tubule injury in critically ill patients with ARF. Am J Kidney Dis 2003;42:497–506.
83 Nadasdy T, Laszik Z, Blick KE, Johnson DL, Burst-Singer K, Nast C, Cohen AH, Ormos J, Silva FG: Human acute tubular necrosis: a lectin and immunohistochemical study. Hum Pathol 1995; 26:230–239.
84 Devarajan P: Proteomics for biomarker discovery in acute kidney injury. Semin Nephrol 2007; 27:637–651.
85 Schaub S, Rush D, Wilkins J, Gibson IW, Weiler T, Sangster K, Nicolle L, Kappinski M, Jeffery J, Nickerson P: Proteome-based detection of urine proteins associated with acute renal allograft rejection. J Am Soc Nephrol 2004;15:219–227.

86 Schaub S, Wilkins J, Weiler T, Sangster K, Rush D, Nickerson P: Urine protein profiling with surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry. Kidney Int 2004;65: 323–332.
87 Nguyen MT, Ross GF, Dent CL, Devarajan P: Early prediction of acute renal injury using urinary proteomics. Am J Nephrol 2005;25:318–326.
88 Lefler DM, Pafford RG, Black NA, Raymond JR, Arthur JM: Identification of proteins in slow continuous ultrafiltrate by reversed-phase chromatography and proteomics. J Proteome Res 2004;3:1254–1260.
89 Zhou H, Pisitkun T, Aponte A, Yuen PST, Hoffert JD, Yasuda H, Hu X, Chawla L, Shen R-F, Knepper MA, Star RA: Exosomal fetuin-A identified by proteomics: a novel urinary biomarker for detecting acute kidney injury. Kidney Int 2006;70:1847–1857.
90 Pisitkun T, Shen RF, Knepper MA: Identification and proteomic profiling of exosomes in human urine. Proc Natl Acad Sci USA 2004;101:13368–13373.
91 Zhou H, Yuen PS, Pisitkun T, Gonzales PA, Yasuda H, Dear JW, Gross P, Knepper MA, Star RA: Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery. Kidney Int 2006;69:1471–1476.
92 Molls RM, Savransky V, Liu M, Bevans S, Mehta T, Tuder RM, King LS, Rabb H: Keratinocyte-derived chemokine is an early biomarker of ischemic acute kidney injury. Am J Physiol Renal Physiol 2006;290:F1187–F1193.
93 Holly MK, Dear JW, Hu X, Schechter AN, Gladwin MT, Hewitt SM, Yuen PST, Star RA: Biomarker and drug-target discovery using proteomics in a new rat model of sepsis-induced acute renal failure. Kidney Int 2006;70:496–506.
94 Schrier RW, Wang W: Acute renal failure and sepsis. N Engl J Med 2004;351:159–169.
95 Melnikov VY, Ecder T, Fantuzzi G, Siegmund B, Lucia MS, Dinarello CA, Schrier RW, Edelstein CL: Impaired IL-18 processing protects caspase-1 deficient mice from ischemic acute renal failure. J Clin Invest 2001;107:1145–1152.
96 Parikh CR, Jani A, Melnikov VY, Faubel S, Edelstein CL: Urinary interleukin-18 is a marker of human acute tubular necrosis. Am J Kidney Dis 2004;43:405–414.
97 Parikh CR, Abraham E, Ancukiewicz M, Edelstein CL: Urine IL-18 is an early diagnostic marker for acute kidney injury and predicts mortality in the intensive care unit. J Am Soc Nephrol 2005;16:3046–3052.
98 Parikh CR, Mishra J, Thiessen-Philbrook H, Dursun B, Ma Q, Kelly C, Dent C, Devarajan P, Edelstein CL: Urinary IL-18 is an early predictive biomarker of acute kidney injury after cardiac surgery. Kidney Int 2006;70:199–203.
99 Herget-Rosenthal S, Poppen D, Husing J, Marggraf G, Pietruck F, Jakob H-G, Philipp T, Kribben A: Prognostic value of tubular proteinuria and enzymuria in non-oliguric acute tubular necrosis. Clin Chem 2004;50:552–558.
100 VandeVoorde RG, Katlman TI, Ma Q, Kelly C, Mishra J, Dent CA, Mitsnefes MM, Devarajan P: Serum NGAL and cystatin C as predictive biomarkers for acute kidney injury. J Am Soc Nephrol 2006;17:404.
101 Dent CL, Ma Q, Dastrala S, Bennett M, Mitsnefes MM, Barasch J, Devarajan P: Plasma NGAL predicts acute kidney injury, morbidity and mortality after pediatric cardiac surgery: a prospective uncontrolled cohort study. Crit Care 2007;11:R127.
102 Bennett M, Dent CL, Ma Q, Dastrala S, Grenier F, Workman R, Syed H, Ali S, Barasch J, Devarajan P: Urine NGAL predicts severity of acute kidney injury after cardiac surgery: a prospective study. Clin J Am Soc Nephrol 2008, in press.

Prasad Devarajan, MD
Nephrology and Hypertension, MLC 7022
Cincinnati Children's Hospital Medical Center
3333 Burnet Avenue, Cincinnati, OH 45229-3039 (USA)
Tel. +1 513 636 4531, Fax +1 513 636 7407, E-Mail prasad.devarajan@cchmc.org

Thongboonkerd V (ed): Proteomics in Nephrology – Towards Clinical Applications.
Contrib Nephrol. Basel, Karger, 2008, vol 160, pp 17–28

Proteomics of Plasma and Urine in Primary Nephrotic Syndrome in Children

Giovanni Candiano[a], *Luca Musante*[a,d], *Andrea Petretto*[b], *Maurizio Bruschi*[a,d], *Laura Santucci*[a,d], *Andrea Urbani*[e], *Francesco Scolari*[g], *Rosanna Gusmano*[d], *Michele Carraro*[f], *Cristina Zennaro*[d], *Flavio Vincenti*[h], *Gian Marco Ghiggeri*[a]

[a]Laboratory on Pathophysiology of Uremia, [b]Mass Spectrometry Core Facility, [c]Laboratory of Molecular Medicine, G. Gaslini Children Hospital, [d]Renal Child Foundation, Genoa, [e]IRCCS-Fondazione Santa Lucia, Rome, [f]Department of Nephrology, University of Trieste, Trieste, [g]Department of Nephrology, University of Brescia, Brescia, Italy; [h]Transplant Service, University of California, San Francisco, Calif., USA

Abstract

Primary nephrotic syndrome in children, especially the variant with segmental glomerulosclerosis, remains an unsolved clinical problem. In spite of some progress, its pathogenesis is still unknown and the therapy options are confined to gross immune modulation. Indirect evidence based on posttransplant recurrence of the disease suggested an implication of plasma factors, whose characterization remains in course. Besides historical candidates, research is now considering glyco- and lipoderivatives. Structural analysis of plasma and urinary proteins based on proteomics has recently shown an increased proteolysis of major components such as albumin and the implication of α_1-antitrypsin that represents the first-line defense against exogenous and endogenous substances with proteolytic activity. Albumin has also emerged as a major plasma antioxidant, and recent studies have demonstrated that in patients with active focal segmental glomerulosclerosis albumin undergoes massive and stable oxidation with sulfonation of Cys^{34}, formation of an adduct with +48 Da molecular weight, changes of the net charge due to additional negative residues, and loss of free thiol group (SH) titration. Altogether, these data suggest that oxidative stress determines selective protein damages in focal segmental glomerulosclerosis patients with formation of new adducts and fragmentation of plasma proteins. Research should now address whether oxidation of podocyte proteins is important for the maintenance of renal selectivity and is involved in proteinuria.

Primary nephrotic syndrome is a major cause of renal morbidity in children [1]. It is characterized by heavy proteinuria that responds variably to drugs and may progress to renal failure in most resistant and aggressive cases. Clinical variability is due to characteristics that have been only partially recognized and are probably sustained by different pathology variants [2], with possible genetic backgrounds [3–6] and variable response to drugs modifying the long-term outcome [7]. The basic glomerular lesion varies from minimal involvement (minimal-change nephropathy) to focal segmental sclerosis (FSGS), with or without mesangial IgM deposition, making a boundary among them that may be arbitrary in less definite cases [8, 9]. It is still under discussion whether the pathological aspect is representative of separate diseases or underscores different phases or aspects of the same chimeric lesion [10]. Up to now, we have no chance to resolve the dilemma. Repetitive biopsies showing evolution from minimal lesions to mesangial IgM and to FSGS in the same patient [11] suggest homogeneity among different backgrounds and support common pathogenic mechanisms.

Unfortunately, very little is known on the pathogenesis of noninherited nephrotic syndrome in children with the exception of the following consolidated concepts. First, immunological factors related to an abnormal response of T cell have been tentatively considered but they still appear poorly defined and nonspecific [12, 13]. Second, based on posttransplant recurrence, an implication of circulating plasma factors that persist over time and modify glomerular permeability to proteins have been also considered [14–17]. However, their characterizations have not been completed yet and we can only refer them to putative plasma candidates [18, 19]. Recently, the new possibility has been proposed that massive oxidation caused by blood polymorphonuclear leukocytes takes place in response to nonspecific stimuli and alters the podocyte structure [20]. It is noteworthy that plasma proteins are involved in this process as major antioxidant barrier and their characterizations would possibly reveal unexpected aspects. The possibility that free radicals are involved in the pathogenesis of FSGS represents a new frontier of research that requires additional works; even indirect support is given by clinical and experimental data of the literature [21–23].

Proteomics is one of the most appropriate and promising approaches for examining plasma proteins in nephrotic syndrome and awaits wider consideration and applications. Technologies are now available and specific questions are to be clearly addressed. In this chapter, we will review some aspects of plasma and urinary proteins that may be related to the pathogenesis of primary nephrotic syndrome in children and also try to outline the difference, when present, between different pathological backgrounds.

Plasma and Urinary Proteins in Primary Nephrotic Syndrome

There are several reasons to consider plasma and urinary proteins as the relevant factors in nephrotic syndrome. Firstly, albumin is the most abundant protein in nephrotic urine. Hypoalbuminemia represents a clinical parameter of limited significance to the pathogenesis of edema and to hemodynamic aspects. Nevertheless, its role as an antioxidant should stimulate new consideration on the mechanism of podocyte injury. Secondly, other proteins are also involved significantly in nephrotic syndrome. For example, lipoproteins and coagulation factors may be correlated with the disease activity. Thirdly, plasma proteins with antiprotease effects such as α_1-antitrypsin and α_2-macroglobulin may have a direct role in the pathogenesis of nephrotic syndrome [24]. Finally, the characterizations of urinary proteins in nephrotic syndrome with specific proteomic approaches have revealed some previously unexplored aspects that would lead to novel interpretations of proteinuria.

Specific Protein Clusters in Plasma and Urine

The characterization of proteins excreted into urine in nephrotic syndrome has represented for many years a key topic in nephrology. The composition of urinary proteins in glomerular diseases has been utilized not only for consideration on mechanisms of proteinuria but also for a clinical classification that subdivides renal diseases according to loss or maintenance of selectivity towards proteins. It is accepted that the presence in urine of high molecular weight proteins, such as immunoglobulins [25], underscores a more diffuse and probably important pathological process, particularly the presence of immune deposits along the glomerular basement membrane. Behind this simple aspect, it is possible that detailed characterizations of urinary proteins performed with advanced technologies such as two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) can furnish new clinical hints and possibly allow some advances on knowledge of the pathogenesis. Recent descriptions of urinary proteins based on solid proteomic basis represent a good start on this road. Candiano et al. [26] analyzed urine and plasma from 19 patients with idiopathic nephrotic syndrome of different pathologies (FSGS, minimal-change nephropathy and membranous nephropathy) with 2-DE, Western blot analysis and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS. It has been shown that most of urinary protein components corresponded to fragments of albumin (63 peptides were found) and/or of α_1-antitrypsin (9 peptides were found) with several repetitive fragmentation motives. Additionally, few differences among different pathologies were observed (fig. 1). Several urinary fragments (21 out of 72) were also detected, even at a lower concentration, in plasma suggesting a prefer-

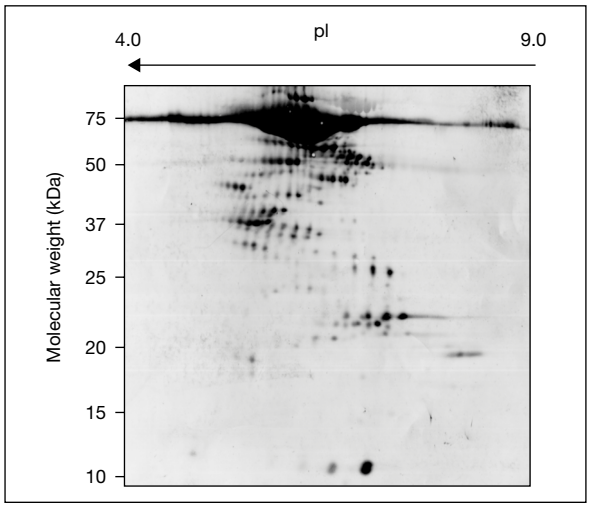


Fig. 1. 2-DE and Western blot analysis using anti-albumin antibodies of fresh random urine obtained in the morning from a child with steroid-dependent nephrotic syndrome. 2-DE was done in soft gels according to Bruschi et al. [61]. Several proteins with a molecular weight of <60 kDa were clearly identified by anti-albumin antibodies and were also characterized by MS as albumin [26].

ential excretion. The bulk of components with low molecular weight were instead detected only in urine suggesting an 'in situ' formation. Moreover, zymograms showed the presence of specific proteases for albumin in the urine of nephrotic patients. Finally, the presence of albumin adducts that harbor both the carboxy (COOH) and amino (NH2) terminal parts of the protein is suggestive of the presence of covalent chemical adducts. The unexpected finding of peptides of albumin and α_1-antitrypsin in the plasma proposes the question of how they form. On the other hand, the enrichment of urine by the same components suggests that they are cleared by the renal filter in nephrotic patients. The mechanism of fragmentation of plasma proteins may hypothetically play a direct pathogenic role in the kidney (see below) and may specifically be implicated in fragmentation of proteins of the podocyte that are involved in maintenance of the cell structure as well as of the ultrafiltration unit that is involved in proteinuria.

A plasma protein of interest in view of an accelerated proteolysis is α_2-macroglobulin since it represents the major circulating defense against exogenous proteolytic enzymes usually of viral or bacterial derivation [27, 28], and acts as a controller of aberrant proteolysis response during normal immunological processes. Metalloproteases [27] and complement components (including the alternative activation pathway such as mannan-binding protein-associated

serine protease or MASP) [28, 29] are examples of proteases that are partially inactivated by α2-macroglobulin. It is also known that plasma levels of α_2-macroglobulin are increased in nephrotic patients [24]; this increase probably represents a compensatory response to inactivation of the protein.

A clear characterization of the fragmentation process may open to a new scenario in the pathological process leading to proteinuria and to FSGS. Overall, new data deriving from the characterization of plasma and urinary proteins by means of proteomic techniques indicate the possibility of an accentuated proteolysis of major plasma components including those proteins that are physiologically deputed to block proteolysis. While the meaning of this occurrence is still obscure, it is possible that it may bring some relation with the mechanism of the renal damage and can also be implicated in the oxidative stress that is discussed below.

Oxidation of Plasma Proteins

Most studies on mechanisms of the antioxidant response in living systems have addressed the intracellular compartment, since it is widely accepted that the cell is the target of free radicals. In recent years, the concept has emerged that the plasma is also exposed to oxidative responses triggered by environmental stress, and that circulating proteins may be seriously damaged by this oxidative stress. How oxidation affects the overall biological balance is now a matter of discussion. Studies that utilized 'in vitro' models convincingly indicated that plasma albumin is oxidized at the unique free thiol groups of Cys^{34}, which is transformed in a sulfonic residue (SO_3^-) [29–35]. 'In vitro' models also suggest that the intermediate step of the reaction is sulfenic acid, which is rather stable 'in vivo' but may also react with free glutathione depending on the accessibility of the pocket that is endowed to form nonmercaptoalbumin or dimers of the protein (fig. 2) [35]. Nonmercaptoalbumin represents 30–35% of total albumin in normal conditions, suggesting an interplay between its presence and oxidation. Recently, our group demonstrated that plasma albumin in patients with active FSGS undergoes massive and stable oxidation with formation of SO_3^- that is the first description of formation of the end product of oxidation 'in vivo' [20, 36]. This change involves some relevant alterations of the protein with formation of an adduct with +48 Da molecular weight, changes of the net charge due to additional negative residues, and loss of free SH titration [37].

Liquid chromatography coupled to electrospray ionization – tandem MS was utilized to characterize plasma albumin in patients with FSGS. In a first approach [20], the analysis of albumin after alkylation showed a fragment with m/z 511.71 (triply charge) and of 1,610.5 (double charge) that corresponded to the sulfonic derivate while control albumin had one fragment with higher m/z (+57 Da), indicating alkylation of Cys^{34}. In the second approach [36], the spectrum of native albumin was evaluated after digestion with trypsin, showing the

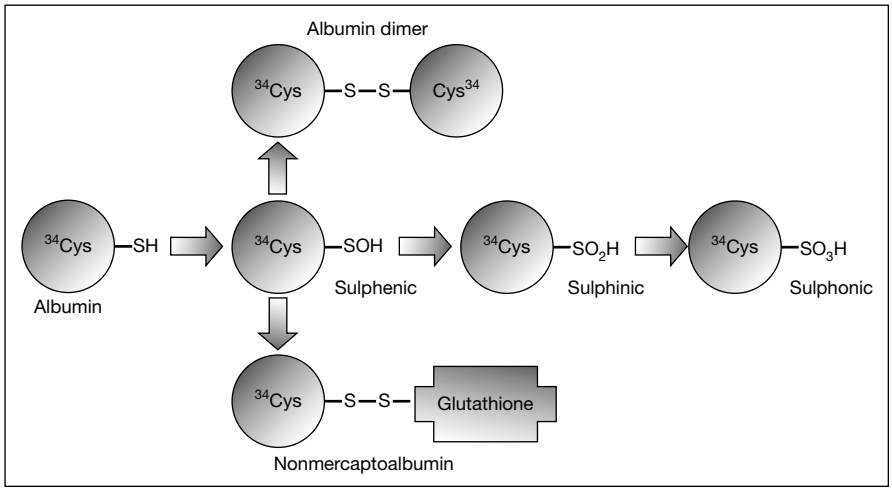


Fig. 2. Chemical transformation steps during oxidation of albumin. Free radicals react with the unique free SH of the entire sequence (^{34}Cys) that is transformed into a sulfenic derivative (SO_3). Alb-SOH is extremely reactive with glutathione to form nonmercaptoalbumin or may undergo further rearrangements to sulfinic (SO_2H) and to sulfonic acid (SO_3H) – the stable end-product of the reaction. Alb- SO_3H was detected in plasma of patients with active FSGS as a unique example of stable oxidation of albumin observed 'in vivo' [20].

presence of a 511.71 m/z ion in triple charge that was consistent with a sequence in which the Cys^{34} brings 3 additional oxygen (O^-) residues. Therefore, 'in vivo' plasma oxidation in patients with FSGS produces a more stable derivative than the one described in 'in vitro' models and, most importantly, SO_3^- does not dimerize but undergoes proteolysis. This can be considered a suicidal effect that is highly reminiscent of what is observed in the urine of patients with FSGS enriched with fragments of the protein (see above). This key observation suggests a few considerations. The first is that albumin, due to its high plasma level, is the major antioxidant substance in plasma. Level of albumin is higher than that of free glutathione by a factor of 100 (0.8 vs. 0.008 mM). Second, massive oxidation of plasma albumin implicates new pathogenic mechanisms of FSGS related to oxidation. Several lines of evidence on both human and experimental FSGS strongly support the implication of free radicals. In fact, all animal models of nongenetic FSGS are based on free radical generation. These include puromycin (PAN) and adriamycin (ADR) nephrosis in rats and Mvp17$^{-/-}$ mice [23, 38–40]. Renal infusion of hydrogen peroxide (H_2O_2) also induces proteinuria in rats and upregulates the expression of factors such as C/EBP homology protein (GADD 153) that are also upregulated in

human FSGS and rat PAN nephrosis [41]. The ADR model is of particular interest since the quinone structure of the molecule allows a direct participation in redox reaction [42, 43] and may directly act as a free radical.

The fragmentation process in nephrotic syndrome is important, as clearly shown by the presence of clusters of albumin and α_1-antitrypsin in the urine. The finding of α_1-antitrypsin seems even more interesting since this protein acts as a major circulating defense against exogenous proteolysis enzymes of viral or bacterial origin [44–46]. Inactivation of α_1-antitrypsin by leukocytes has been extensively reported in literature. This mechanism involves production of free radicals from activated neutrophils and activation of specific proteases [47–49]. Therefore, a possibility of an oxidative damage that results from activated inflammatory cells seems a reasonable explanation of the finding above and new experimental approaches should consider this direction.

Permeability Plasma Factors and Inhibitors

There are several clinical and laboratory observations supporting the existence of plasma factors that potentially alter glomerular permeability of proteins that cause proteinuria. The first and probably most convincing one is that proteinuria may recur within a few hours following transplantation [50, 51] in a significant number of cases (30–50%) depending on different study cohorts. Early treatment of recurrent proteinuria with plasmapheresis [52–54] or immunoadsorption [14] is effective in blunting or abolishing the process, and a pretransplant approach should be scheduled in most serious cases to optimize the therapeutical response. Another observation that reinforces the existence of permeability plasma factors is that the permeability activity may be transmitted from mother with FSGS to fetus and then disappears after birth [55]. So far, we have only an indirect evidence of the presence of plasma substances that alter the dynamic protein transport in the kidney. Therefore, we need to know their identity before considering the intimate (patho)physiological aspects. Unfortunately, the research on the identity of putative plasma factors has not been completed yet and this key point still awaits a clear demonstration. One major problem on the road of purification of circulating plasma factors is that we lack animal models for addressing the activity in experimental conditions, and what we know derives from studies testing permeability activity in a model of ‘isolated glomeruli’. This model has been also extensively utilized as a clinical surrogate of what really happens in the human kidney. It was developed in 1992 by Savin et al. [56] who found that when serum from FSGS is incubated with rat glomeruli perfused with a high oncotic solution (5% bovine serum albumin) and then placed in medium containing 1% BSA, the oncotic gradient determines a flux of water inside and consequent

expansion of glomerular capillaries that is recorded using video microscopy. Calculation of the albumin reflection coefficient (δ alb) and of the conventional permeability (Palb = 1 – δ alb) derives from changes in glomerular volume, where Palb = 1 reflects the maximal activity. Serial evaluations of Palb have been carried out in patients with different glomerulopathies but it was found nonspecific and related to clinical aspects only in patients receiving a renal allograft [15, 54, 57]. A second pitfall in the search of permeability factors is due to the presence of inhibitors in plasma that make it difficult to standardize techniques. Utilizing the 'isolated glomeruli' as a model, Dantal et al. [14] and Sharma et al. [58] tried to define general characteristics, such as molecular weight and thermal stability but produced variable results. Musante et al. [18] purified from FSGS plasma 10 proteins among several hundreds which maintained a permeability activity after their recovery from polyacrylamide and 6 were successfully identified by MS. The list includes the complex apolipoprotein J–vitronectin, a few isoforms of albumin, fibulin, MASP, and fibrinogen-γ. All of these are, for various and different reasons, interesting candidates [59, 60] but it seems unlikely that more than one factor is active in FSGS. Therefore, a critical re-evaluation is now requested. Moreover, apolipoprotein J and albumin have been alternatively found to induce and block permeability in different settings and we have no explanation for this paradox [19]. We speculate that these proteins play different roles depending on their redox status. There are two main pitfalls in utilizing electrophoresis for purifying proteins with biological activity. The first is that denaturation and renaturation could alter protein function and the present methodological approach could lead to a misinterpretation of the phenomenon. The second is that only single components have so far been purified and tested, and it cannot be readily excluded that permeabilization is actually due to a cascade of interacting factors. Therefore, in spite of initial promising results, the research on plasma factors turned out inconclusive and should now be extended to protein derivates such as glycoproteins, lipoproteins and peptides.

What Do We Have to Do to Better Understand Proteinuria?

As summarized in the sections above, we have reasonable evidence that the pathogenesis of proteinuria in children with primary nephrotic syndrome is due to plasma factors that alter the glomerular permeability of proteins. On the other hand, we also have evidence for activation of an oxidative stress in the same patients and chances are that the two lines may coincide at several points. Putative plasma factors responsible for the whole process remain to be identified, and the cellular steps of their devastating action should be also clarified. 'In vitro' data with podocytes treated with partial purification products of

plasma reasonably provide implication of the cytoskeleton and the shedding of nephrin and podocin, which are key components of the slit diaphragm and are directly involved in the maintenance of proper ultrafiltering properties. In spite of some inconclusive remarks, there are few points that encourage research. The first issue is about proteolysis in plasma of patients with nephrotic syndrome that involves specific proteases such as α_1-antitrypsin and α_2-macroglobulin, whose fragmentation can be taken as a direct proof for something related to them. The second aspect is about oxidation; we currently know that oxidation of plasma proteins may be considered as a major index of oxidative stress. Why oxidation is increased in patients with primary nephrotic syndrome and how it mainly affects the kidney is a key point of the puzzle. Both the cellular source and the routes of oxidant production in circulating leukocytes must be carefully checked. It should be clearly ascertained whether oxidants are the correct answer for selective or exaggerated stimuli, or whether they represent the product of an aberrant cell response to trivial triggers. Finally, the question of plasma factors should be further investigated in view of the considerations presented above. The main issue is whether protein fragments and peptides produced by aberrant proteolysis should be considered as new candidates. Their origin and mechanisms of production may furnish essential elements to the pathogenesis of proteinuria and finally explain the link between plasma events and proteinuria.

Acknowledgements

This work was done with the financial support of the Italian Ministry of Health. Authors also acknowledge Fondazione Mara Wilma e Bianca Querci for the financial support to the project 'Nuove evoluzioni sulla multifattorialità della sindrome nefrosica' and the Renal Child Foundation for financial support.

References

1 Braden GL, Mulhern JG, O'Shea MH, Nash SV, Ucci AA Jr, Germain MJ: Changing incidence of glomerular diseases in adults. Am J Kidney Dis 2000;35:878–883.
2 Korbet SM: Primary focal segmental glomerulosclerosis. J Am Soc Nephrol 1998;9:1333–1340.
3 Boute N, Gribouval O, Roselli S, Benessy F, Lee H, Fuchshuber A, Dahan K, Gubler MC, Niaudet P, Antignac C: NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. Nat Genet 2000;24:349–354.
4 Kaplan JM, Kim SH, North KN, Rennke H, Correia LA, Tong HQ, Mathis BJ, Rodriguez-Perez JC, Allen PG, Beggs AH, Pollak MR: Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. Nat Genet 2000;24:251–256.
5 Koziell A, Grech V, Hussain S, Lee G, Lenkkeri U, Tryggvason K, Scambler P: Genotype/phenotype correlations of NPHS1 and NPHS2 mutations in nephrotic syndrome advocate a functional inter-relationship in glomerular filtration. Hum Mol Genet 2002;11:379–388.

6 Caridi G, Bertelli R, Di Duca M, Dagnino M, Emma F, Onetti Muda A, Scolari F, Miglietti N, Mazzucco G, Murer L, Carrea A, Massella L, Rizzoni G, Perfumo F, Ghiggeri GM: Broadening the spectrum of diseases related to podocin mutations. J Am Soc Nephrol 2003;14:1278–1286.
7 Ghiggeri GM, Catarsi P, Scolari F, Caridi G, Bertelli R, Carrea A, Sanna-Cherchi S, Emma F, Allegri L, Cancarini G, Rizzoni GF, Perfumo F: Cyclosporine in patients with steroid-resistant nephrotic syndrome: an open-label, nonrandomized, retrospective study. Clin Ther 2004;26:1411–1418.
8 Border WA: Distinguishing minimal-change disease from mesangial disorders. Kidney Int 1988;34: 419–434.
9 Cohen AH, Border WA, Glassock RJ: Nehprotic syndrome with glomerular mesangial IgM deposits. Lab Invest 1978;38:610–619.
10 McAdams AJ, Valentini RP, Welch TR: The nonspecificity of focal segmental glomerulosclerosis. The defining characteristics of primary focal glomerulosclerosis, mesangial proliferation, and minimal change. Medicine (Baltimore) 1997;76:42–52.
11 Ahmad H, Tejani A: Predictive value of repeat renal biopsies in children with nephrotic syndrome. Nephron 2000;84:342–346.
12 Koyama A, Fujisaki M, Kobayashi M, Igarashi M, Narita M: A glomerular permeability factor produced by human T cell hybridomas. Kidney Int 1991;40:453–460.
13 Sahali D, Pawlak A, Valanciute A, Grimbert P, Lang P, Remy P, Bensman A, Guellaen G: A novel approach to investigation of the pathogenesis of active minimal-change nephrotic syndrome using subtracted cDNA library screening. J Am Soc Nephrol 2002;13:1238–1247.
14 Dantal J, Bigot E, Bogers W, Testa A, Kriaa F, Jacques Y, Hurault de Ligny B, Niaudet P, Charpentier B, Soulillou JP: Effect of plasma protein adsorption on protein excretion in kidney-transplant recipients with recurrent nephrotic syndrome. N Engl J Med 1994;330:7–14.
15 Savin VJ, Sharma R, Sharma M, McCarthy ET, Swan SK, Ellis E, Lovell H, Warady B, Gunwar S, Chonko AM, Artero M, Vincenti F: Circulating factor associated with increased glomerular permeability to albumin in recurrent focal segmental glomerulosclerosis. N Engl J Med 1996;334:878–883.
16 Ghiggeri GM, Carraro M, Vincenti F: Recurrent focal glomerulosclerosis in the era of genetics of podocyte proteins: theory and therapy. Nephrol Dial Transplant 2004;19:1036–1040.
17 Vincenti F, Ghiggeri GM: New insights into the pathogenesis and the therapy of recurrent focal glomerulosclerosis. Am J Transplant 2005;5:1179–1185.
18 Musante L, Candiano G, Bruschi M, Zennaro C, Carraro M, Artero M, Giuffrida MG, Conti A, Santucci A, Ghiggeri GM: Characterization of plasma factors that alter the permeability to albumin within isolated glomeruli. Proteomics 2002;2:197–205.
19 Candiano G, Musante L, Carraro M, Faccini L, Campanacci L, Zennaro C, Artero M, Ginevri F, Perfumo F, Gusmano R, Ghiggeri GM: Apolipoproteins prevent glomerular albumin permeability induced in vitro by serum from patients with focal segmental glomerulosclerosis. J Am Soc Nephrol 2001;12:143–150.
20 Musante L, Bruschi M, Candiano G, Petretto A, Dimasi N, Del Boccio P, Urbani A, Rialdi G, Ghiggeri GM: Characterization of oxidation end product of plasma albumin ‘in vivo’. Biochem Biophys Res Commun 2006;349:668–673.
21 Quinzii C, Naini A, Salviati L, Trevisson E, Navas P, Dimauro S, Hirano M: A Mutation in Para-Hydroxybenzoate-Polyprenyl Transferase (COQ2) Causes Primary Coenzyme Q10 Deficiency. Am J Hum Genet 2006;78:345–349.
22 Ginevri F, Ghiggeri GM, Candiano G, Oleggini R, Bertelli R, Piccardo MT, Perfumo F, Gusmano R: Peroxidative damage of the erythrocyte membrane in children with nephrotic syndrome. Pediatr Nephrol 1989;3:25–32.
23 Ghiggeri GM, Cercignani G, Ginevri F, Bertelli R, Zetta L, Greco F, Candiano G, Trivelli A, Gusmano R: Puromycin aminonucleoside metabolism by glomeruli and glomerular epithelial cells in vitro. Kidney Int 1991;40:35–42.
24 de Sain-van der Velden MG, Rabelink TJ, Reijngoud DJ, Gadellaa MM, Voorbij HA, Stellaard F, Kaysen GA: Plasma alpha 2 macroglobulin is increased in nephrotic patients as a result of increased synthesis alone. Kidney Int 1998;54:530–535.
25 Bazzi C, D’Amico G: The urinary excretion of IgG and alpha1-microglobulin predicts renal outcome and identifies patients deserving treatment in membranous nephropathy. Kidney Int 2002;61:2276.

26 Candiano G, Musante L, Bruschi M, Petretto A, Santucci L, Del Boccio P, Pavone B, Perfumo F, Urbani A, Scolari F, Ghiggeri GM: Repetitive fragmentation products of albumin and alpha1-antitrypsin in glomerular diseases associated with nephrotic syndrome. J Am Soc Nephrol 2006;17:3139–3148.
27 Tortorella MD, Arner EC, Hills R, Easton A, Korte-Sarfaty J, Fok K, Wittwer AJ, Liu RQ, Malfait AM: Alpha2-macroglobulin is a novel substrate for ADAMTS-4 and ADAMTS-5 and represents an endogenous inhibitor of these enzymes. J Biol Chem 2004;279:17554–17561.
28 Meier UC, Boetzel J, Kellermann J, Mann K, Billich A, Stocker W, Schramm HJ: The cleavage of the bait region of alpha 2-macroglobulin by human immunodeficiency virus proteinases and by astacin. Ann N Y Acad Sci 1994;737:431–433.
29 Ambrus G, Gal P, Kojima M, Szilagyi K, Balczer J, Antal J, Graf L, Laich A, Moffatt BE, Schwaeble W, Sim RB, Zavodszky P: Natural substrates and inhibitors of mannan-binding lectin-associated serine protease-1 and -2:a study on recombinant catalytic fragments. J Immunol 2003; 170:1374–1382.
30 Carballal S, Radi R, Kirk MC, Barnes S, Freeman BA, Alvarez B: Sulfenic acid formation in human serum albumin by hydrogen peroxide and peroxynitrite. Biochemistry 2003;42:9906–9914.
31 Lee H, Cha MK, Kim IH: Activation of thiol-dependent antioxidant activity of human serum albumin by alkaline pH is due to the B-like conformational change. Arch Biochem Biophys 2000;380: 309–318.
32 Anraku M, Yamasaki K, Maruyama T, Kragh-Hansen U, Otagiri M: Effect of oxidative stress on the structure and function of human serum albumin. Pharm Res 2001;18:632–639.
33 Salavej P, Spalteholz H, Arnhold J: Modification of amino acid residues in human serum albumin by myeloperoxidase. Free Radic Biol Med 2006;40:516–525.
34 Meucci E, Mordente A, Martorana GE: Metal-catalyzed oxidation of human serum albumin: conformational and functional changes. Implications in protein aging. J Biol Chem 1991;266:4692–4699.
35 Carballal S, Alvarez B, Turell L, Botti H, Freeman BA, Radi R: Sulfenic acid in human serum albumin. Amino Acids 2006.
36 Musante L, Candiano G, Petretto A, Bruschi M, Dimasi N, Caridi G, Pavone B, Del Boccio P, Galliano M, Urbani A, Scolaro F, Vincenti F, Ghiggeri GM: Active focal segmental glomerulosclerosis is associated with massive oxidation of plasma albumin. J Am Soc Nephrol 2007; in press.
37 Bruschi M, Musante L, Candiano G, Santucci L, Zennaro C, Carraro M, Del Boccio P, Gusmano R, Perfumo F, Urbani A, Ghiggeri GM: Transitions of serum albumin in patients with glomerulosclerosis 'in vivo' characterization by electrophoretic titration curves. Electrophoresis 2006;27: 2960–2969.
38 Ginevri F, Gusmano R, Oleggini R, Acerbo S, Bertelli R, Perfumo F, Cercignani G, Allegrini S, D'Allegri F, Ghiggeri G: Renal purine efflux and xanthine oxidase activity during experimental nephrosis in rats: difference between puromycin aminonucleoside and adriamycin nephrosis. Clin Sci (Lond) 1990;78:283–293.
39 Thakur V, Walker PD, Shah SV: Evidence suggesting a role for hydroxyl radical in puromycin aminonucleoside-induced proteinuria. Kidney Int 1988;34:494–499.
40 O'Bryan T, Weiher H, Rennke HG, Kren S, Hostetter TH: Course of renal injury in the Mpv17-deficient transgenic mouse. J Am Soc Nephrol 2000;11:1067–1074.
41 Bek MF, Bayer M, Muller B, Greiber S, Lang D, Schwab A, August C, Springer E, Rohrbach R, Huber TB, Benzing T, Pavenstadt H: Expression and function of C/EBP homology protein (GADD153) in podocytes. Am J Pathol 2006;168:20–32.
42 Doroshow JH, Locker GY, Ifrim I, Myers CE: Prevention of doxorubicin cardiac toxicity in the mouse by N-acetylcysteine. J Clin Invest 1981;68:1053–1064.
43 Bertelli R, Ginevri F, Gusmano R, Ghiggeri GM: Cytotoxic effect of adriamycin and agarose-coupled adriamycin on glomerular epithelial cells: role of free radicals. In Vitro Cell Dev Biol 1991; 27A:799–804.
44 Janciauskiene S, Lindgren S: Human monocyte activation by cleaved form of alpha-1-antitrypsin involvement of the phagocytic pathway. Eur J Biochem 1999;265:875–882.
45 Vaziri ND, Gonzales EC, Shayestehfar B, Barton CH: Plasma levels and urinary excretion of fibrinolytic and protease inhibitory proteins in nephrotic syndrome. J Lab Clin Med 1994;124:118–124.

46 Whiteman M, Tritschler H, Halliwell B: Protection against peroxynitrite-dependent tyrosine nitration and alpha 1-antiproteinase inactivation by oxidized and reduced lipoic acid. FEBS Lett 1996;379:74–76.
47 Ottonello L, Dapino P, Scirocco M, Dallegri F, Sacchetti C: Proteolytic inactivation of alpha-1-antitrypsin by human neutrophils: involvement of multiple and interlinked cell responses to phagocytosable targets. Eur J Clin Invest 1994;24:42–49.
48 Dean RT, Nick HP, Schnebli HP: Free radicals inactivate human neutrophil elastase and its inhibitors with comparable efficiency. Biochem Biophys Res Commun 1989;159:821–827.
49 Wallaert B, Aerts C, Gressier B, Gosset P, Voisin C: Oxidative inactivation of alpha 1-proteinase inhibitor by alveolar epithelial type II cells. J Appl Physiol 1993;75:2376–2382.
50 Ingulli E, Tejani A: Incidence, treatment, and outcome of recurrent focal segmental glomerulosclerosis posttransplantation in 42 allografts in children–a single-center experience. Transplantation 1991;51:401–405.
51 Senggutuvan P, Cameron JS, Hartley RB, Rigden S, Chantler C, Haycock G, Williams DG, Ogg C, Koffman G: Recurrence of focal segmental glomerulosclerosis in transplanted kidneys: analysis of incidence and risk factors in 59 allografts. Pediatr Nephrol 1990;4:21–28.
52 Artero M, Biava C, Amend W, Tomlanovich S, Vincenti F: Recurrent focal glomerulosclerosis: natural history and response to therapy. Am J Med 1992;92:375–383.
53 Broyer M, Selwood N, Brunner F: Recurrence of primary renal disease on kidney graft: a European pediatric experience. J Am Soc Nephrol 1992;2:S255–S257.
54 Dall'Amico R, Ghiggeri G, Carraro M, Artero M, Ghio L, Zamorani E, Zennaro C, Basile G, Montini G, Rivabella L, Cardillo M, Scalamogna M, Ginevri F: Prediction and treatment of recurrent focal segmental glomerulosclerosis after renal transplantation in children. Am J Kidney Dis 1999;34:1048–1055.
55 Kemper MJ, Wolf G, Muller-Wiefel DE: Transmission of glomerular permeability factor from a mother to her child. N Engl J Med 2001;344:386–387.
56 Savin VJ, Sharma R, Lovell HB, Welling DJ: Measurement of albumin reflection coefficient with isolated rat glomeruli. J Am Soc Nephrol 1992;3:1260–1269.
57 Carraro M, Caridi G, Bruschi M, Artero M, Bertelli R, Zennaro C, Musante L, Candiano G, Perfumo F, Ghiggeri GM: Serum Glomerular Permeability Activity in Patients with Podocin Mutations (NPHS2) and Steroid-ResistantNephrotic Syndrome. J Am Soc Nephrol 2002;13: 1946–1952.
58 Sharma M, Sharma R, McCarthy ET, Savin VJ: 'The FSGS factor:' enrichment and in vivo effect of activity from focal segmental glomerulosclerosis plasma. J Am Soc Nephrol 1999;10:552–561.
59 Ghiggeri GM, Candiano G, Ginevri F, Gusmano R, Ciardi MR, Perfumo F, Delfino G, Cuniberti C, Queirolo C: Renal selectivity properties towards endogenous albumin in minimal change nephropathy. Kidney Int 1987;32:69–77.
60 Dixon R, Brunskill NJ: Activation of mitogenic pathways by albumin in kidney proximal tubule epithelial cells: implications for the pathophysiology of proteinuric states. J Am Soc Nephrol 1999;10:1487–1497.
61 Bruschi LM, Musante G, Candiano GM, Ghiggeri B, Herbert F, Antonucci PG: Righetti. Soft immobilized pH gradient gels in proteome analysis: a follow-up. Proteomics 2003;3:821–825.

G.M. Ghiggeri, MD
Laboratory on Pathophysiology of Uremia
G. Gaslini Children Hospital
Largo G. Gaslini 5, IT–16148 Genoa (Italy)
Tel. +39 010 380 742, Fax +39 010 395 214, E-Mail labnefro@ospedale-gaslini.ge.it

Thongboonkerd V (ed): Proteomics in Nephrology – Towards Clinical Applications.
Contrib Nephrol. Basel, Karger, 2008, vol 160, pp 29–36

Urinary Proteome Profiling to Search for Biomarkers in Steroid-Resistant Nephrotic Syndrome

Avram Z. Traum[a,c], *Asher D. Schachter*[b,c]

[a]Pediatric Nephrology Unit, Massachusetts General Hospital,
[b]Division of Nephrology and Children's Hospital Informatics Program,
Children's Hospital Boston, and [c]Harvard Medical School, Boston, Mass., USA

Abstract

Long-term outcomes for patients with nephrotic syndrome (NS) correlate closely with the degree of steroid responsiveness. There are currently no diagnostic tests that accurately predict steroid responsiveness in NS. In children in particular, a prolonged course of daily, high-dose corticosteroid therapy is as much a diagnostic maneuver as it is a treatment. Urine proteomics has been emerging as a potentially rich source of noninvasive yet informative biomarkers of drug responsiveness in NS. In this review, we discuss some of the initial studies of the nephrotic urinary proteome as well as some ongoing and future challenges, including defining the normal urinary proteome, and extracting valuable urinary protein data from an abundance of urinary albumin.

Idiopathic nephrotic syndrome (NS) is the most common glomerular disease in childhood, with an estimated annual incidence of 2/100,000 children. Unlike in adults, where the majority of patients with newly diagnosed NS undergo kidney biopsy, children are assumed to have minimal change disease (MCD) at presentation. This is based on earlier studies of the International Study of Kidney Disease in Childhood (ISKDC) [1, 2]. In this landmark study, all children with new onset NS underwent kidney biopsy at presentation and response to treatment with steroids was correlated with histology. The ISKDC showed that 78% had MCD, the majority of whom responded to steroids. This shaped the practice of pediatric nephrologists to this date. However, growing minorities of children with NS are steroid resistant. Renal histology reveals either MCD or focal segmental glomerulosclerosis (FSGS). However, regardless of histology,

children with steroid-resistant NS (SRNS) often develop chronic kidney disease and constitute the most common acquired cause of pediatric end-stage renal disease [3]. This discrepancy in biopsy findings is likely due to the focal nature of FSGS. That is, a renal biopsy of only approximately 20 glomeruli may be insufficient to diagnose a focal process [4]. Additionally, there are no clinical or biochemical parameters to distinguish children with new onset steroid-sensitive NS (SSNS) from SRNS.

This uncertainty has led a number of groups to search for noninvasive biomarkers that would distinguish between SSNS and SRNS. Such biomarkers would serve a number of important functions. First, children identified with SRNS at diagnosis could avoid ineffective and potentially toxic high-dose steroids. These toxicities are myriad and include such problems as growth impairment, behavior changes, obesity, hypertension, glucose intolerance, bone disease, and others. Second, differentiating biomarkers may offer insight into the pathophysiology of NS. This has been a subject of intense research, with interest in such diverse processes as dysregulated immune function, mutations in podocyte proteins, lipid disorders, and angiogenesis.

Proteomics has provided an ideal platform to identify and study noninvasive biomarkers. In this review, we will cover published efforts to define the 'normal' urine proteome, and to identify biomarkers of steroid resistance in NS. We will also discuss the technical issues related to addressing the high protein content of nephrotic urine.

Characterizing the Normal Urine Proteome

Several groups have attempted to delineate the 'normal human urine proteome' by characterizing all proteins present in urine from healthy individuals, utilizing (a) an undisclosed number of samples of 'normal male urine from a commercial pooled source' [5, 6], or (b) urine from an undisclosed number of healthy volunteers [7], or (c) urine from 20 young, healthy male and female subjects [8], or (d) multiple samples of urine from a healthy male volunteer and a healthy female volunteer [9]. The number of proteins identified in these studies ranged from 47 to 150. Weissinger et al. [10] profiled spot urine samples from 57 healthy individuals, and from 34 individuals with various nephrotic glomerulopathies (including 16 with MCD and 10 with FSGS) and found 173 polypeptides present in 90% of the healthy control samples.

The findings from the studies summarized here are intriguing because the majority of filtered proteins in the normal kidney are processed within the renal parenchyma and largely reabsorbed. Urine proteins undergo intrarenal processing, which can result in urine protein profiles very different from those seen in

serum. However, it is now almost certain that normal urine likely contains more protein information than was previously believed, although much remains unknown about these 'normal' urine profiles. Notably, several of these studies utilized pooled samples and therefore were not able to determine interindividual protein variations within control subjects. As well, none of these studies accounted for important baseline physiologic, anthropomorphic and environmental states that can affect urinary protein excretion. It is also impossible to determine which subjects who are healthy at the time of evaluation will develop proteinuric renal disease in the future.

Podocyte Proteomics and Steroid Therapy

Ransom et al. [11] have reported the results of differential proteomic analysis of proteins induced by steroid therapy in cultured murine podocytes. They reported six proteins that demonstrate differential expression in vitro in response to steroid treatment in comparison with vehicle-treated controls. Five of the proteins demonstrated increased expression in the steroid-treated group, and the investigators were able to use Western blots to confirm their findings in 2 of the 5 upregulated proteins, CNTF and α-B crystallin. However, they acknowledged that their disease model is limited by the challenges involved in isolating significant numbers of murine podocytes for primary culture. Furthermore, there is as yet no known animal model for many forms of NS, and even cultured human podocytes do not reliably form slit diaphragms in vitro. The difficulty in obtaining sufficient numbers of podocytes from mice underscores the difficulty inherent in obtaining sufficient sample material from patients, and underscores the value of a noninvasive source, such as urine.

Proteomics in Idiopathic NS

The selectivity index is based on the findings of more selective proteinuria in SSNS while in those with SRNS, some patients exhibit generalized proteinuria. Ramjee et al. [12] studied 57 children with NS and compared the selectivity index with SDS-PAGE and isoelectric focusing (IEF) to distinguish low molecular weight from high molecular weight proteinuria. While those with selective proteinuria all had SSNS, nonselective proteinuria was found in both groups. In contrast, SDS-PAGE and IEF correctly categorized all subjects into SSNS and SRNS/FSGS based on the selectivity of proteins seen on the gels. The positive and negative predictive values for SDS-PAGE and IEF were 100% for both. While this study is exciting in its application of a noninvasive method

of distinguishing these two groups, no training or test sets were used in these patients to apply this model to a larger cohort. Furthermore, no information is provided on the viral status in this cohort of African children, which may lead to secondary forms of SRNS/FSGS.

Two recent reports have addressed using proteomics to distinguish SSNS and SRNS, both using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) [13, 14]. Woroniecki et al. [14] collected urine samples from a cross-section of 25 children with NS referred for biopsy, all of whom had previously been treated with steroids. Seventeen control samples were also used and included healthy children, and 3 each with asthma and orthostatic proteinuria. SSNS patients were in remission at the time of sample collection; some had immune complex-mediated forms of NS, such as membranoproliferative GN and IgA nephropathy. Samples were depleted of albumin and spotted in duplicate onto four chip types (Q10, CM10, H50, and IMAC30). Spectra were analyzed using two approaches within a proprietary package known as Biomarker Pattern Software (Ciphergen). First, a tree-based classification algorithm was applied based on the highest intensity peaks, followed by a boosting algorithm by categorizing the strength of various peaks as classifiers. This approach identified a single m/z peak at 4,144 on the Q10 chip as the strongest predictor of SRNS, followed by a series of other peaks on the CM10 chip. Unfortunately, none of these peaks were further identified, but the approach was successful in distinguishing the two groups.

We recently reported a similar experience in a cross-section of children with idiopathic NS [13]. A total of 44 children were recruited including 14 with SSNS in relapse, 5 with SRNS in relapse, 19 with SSNS in remission, and 6 with orthostatic proteinuria. No depletion protocol was used, and SELDI-TOF MS was used on CM10 and IMAC30 chips with urine samples spotted in duplicate. We then used two separate bioinformatics algorithms tailored for this approach. The first technique began with a noise reduction algorithm that determined the nadir between the bimodal distributions of m/z intensity values of each spectrum and defined the m/z values below the nadir as noise while those above were defined as peaks. This removed potential bias of applying a random signal to noise ratio, and used each spot's distribution of m/z values to determine a distinct, data-driven threshold for each spot. Once we had defined peaks, we then subtracted the peaks found in (a) subjects with SSNS in remission and (b) subjects with orthostatic proteinuria with the rationale that proteins found in the urine of subjects in those two groups were passively filtered and not related to glomerular disease. Finally, we used a genetic algorithm to search for the group of up to 10 peaks that distinguished between SSNS and SRNS with an accuracy of 95% or greater. After 2,000 generations, the genetic algorithm identified a single peak at an m/z of 11,117.4 that distinguished the two groups.

Since any threshold-based algorithm that forces discrete value from continuous data, such as our noise reduction algorithm, risks losing low intensity but potentially biological important information, we used principal component analysis on the normalized, nondiscrete spectra from the relapsed groups. A genetic algorithm was then used to identify the peaks that provided the maximal separation between SSNS and SRNS in the top three components (dimensions) of principal component space. Five peaks were identified including 11,117.4. Since this peak was found by both methods, it was identified using a series of fractionation steps followed by matrix-assisted laser desorption/ionization (MALDI)-TOF/TOF tandem MS as β_2-microglobulin, and validated by immunodepletion using a monoclonal antibody against β_2-microglobulin. The peak at m/z 11,117.4 was likely not intact β_2-microglobulin, but rather a nontryptic cleavage product due to tubular injury from chronic kidney disease in the SRNS subjects. Nonetheless, these two papers demonstrate that even lower resolution approaches like SELDI can be useful for distinguishing SSNS from SRNS.

Candiano et al. [15] studied urine protein patterns from 10 children with SSNS and MCD, 7 children with SRNS and SRNS/FSGS, and 6 adolescents and adults with membranous nephropathy. Using two-dimensional gel electrophoresis, followed by MALDI-TOF MS and peptide mass fingerprinting, they identified fragments of albumin and α_1-antityrpsin not seen in urine from healthy controls. Interestingly, some of these fragments were also found in plasma, suggesting that fragmentation is not merely due to urinary proteases. However, they do not clearly distinguish between the categories of NS in this study.

Technical Issues of Nephrotic Urine

A main limitation imposed by the use of urine is the harsh environment (mostly due to the extreme range of pH values and concentration of solutes found in normal urine), which likely alters excreted protein characteristics. For example, high-abundance urinary substances such as urea, uric acid, and ammonium can modify proteins in ways and extent not seen in the serum. Urea can induce carbamylation [16, 17], phosphorylation [18], and methylation [19] of specific amino acid residues. Urate may protect against oxidation of lipoproteins, depending upon the concurrent concentration of copper, Cu^{2+} [20, 21]. Ammonia and ammonium are involved in modulating phosphorylation [22, 23], nitration [23], and oxidation [24–26]. The use of serum-based proteomic methods to profile urine proteins requires innovative adjustments that take into account the harsh urinary environment.

There is little published about the protein content of nephrotic urine. The protein content of plasma and serum has been studied extensively, with the albumin

Table 1. Proteins identified in unfractionated nephrotic urine in relapse

SSNS		SRNS	
score	protein name	score	protein name
43.06	albumin	72.89	albumin
37.04	transferrin	29.05	transferrin
36.57	ceruloplasmin	15.32	α_1-antitrypsin
32.36	α_1-antitrypsin	14.65	haptoglobin
19.38	α_1-antichymotrypsin	13.07	complement C3
14.96	α_{1B}-glycoprotein	10.21	apolipoprotein A-1
12.37	α_2-glycoprotein 1, zinc	10.2	Ig γ_1-chain C region
10.75	haptoglobin	9.45	orosomucoid 1
8.49	uromodulin	7.91	α_{1B}-glycoprotein
8.43	thyroxine-binding globulin	6.85	ceruloplasmin

Proteins shown with top 10 scores from each sample are listed.

constituting approximately half of the plasma proteome. The next most abundant proteins make up another 40% of plasma proteins, while another 12 proteins constitute the next 9%. Thus, 22 proteins make up 99% of the plasma proteome, while the remaining 1% constitutes low abundant proteins that are a potential rich source of biomarkers. A number of approaches have been published to mine these low-abundance proteins. The most commonly used method makes use of columns that deplete high-abundance proteins, such as albumin, IgG, transferrin and others. This approach has gained wide acceptance in plasma proteomics and is highly effective in depletion of high-abundance proteins. However, others have raised concerns about the loss of data that then occurs. Many low-abundance proteins are also of low-molecular weight (LMW) and travel in plasma bound to carrier proteins such as albumin. Thus, depletion methods risk losing these LMW proteins and along with them potential biomarkers. A more recent approach has successfully used centrifugal ultrafiltration to enrich the LMW plasma proteome [27].

We have applied some of these approaches to the study of nephrotic urine. We first performed liquid chromatography coupled to tandem MS (LC-MS/MS) on unfractionated nephrotic urine from one patient each with SSNS and SRNS. The proteins with the top 10 highest scores are displayed in table 1, which shows that nephrotic urine mirrors the protein content of plasma. We then used the approach of Sutton [27] using filters with molecular weight cut-off at 30 kDa with centrifugal ultrafiltration. The flow-throughs were then subjected to trypsin digestion and LC-MS/MS. Unfortunately, the chromatograms were plagued by contamination with polyethylene glycol (PEG) as manifested

by multiple peaks separated by 22 Da on multiple scans. We suspected that the source of contamination was from the original urine containers used in the clinic setting prior to transfer to MS-friendly cryotubes. This problem would not necessarily have manifested in plasma studies, as blood is collected in glass tubes where PEG is not a concern.

We next focused on removing the contaminating PEG. We felt that dialysis of the samples would have given inconsistent removal and also was counter to our goal of developing a high-throughput to working with nephrotic urine. Next, we performed SDS-PAGE on nephrotic urine samples that were controlled for protein mass. Gel lanes were cut below albumin and designated the LMW fraction. After in-gel tryptic digestion, peptides were extracted and underwent LC-MS/MS. The resulting spectra were free of PEG contamination and gave interpretable results with Mascot.

Conclusions

The noninvasive yet informative nature of urine proteomic profiling suggests that this approach to biomarker discovery will continue to be developed, particularly for diseases that have a relatively large impact in children, such as NS. One important remaining challenge is the establishment of publicly available benchmark datasets for normal or control urine proteomic profiles, preferably including extensive clinical data to allow detailed and comprehensive characterization of the urinary proteome. The high albumin content in nephrotic urine is a hurdle that will likely be resolved in the near future.

References

1 Nephrotic syndrome in children: prediction of histopathology from clinical and laboratory characteristics at time of diagnosis. A report of the International Study of Kidney Disease in Children. Kidney Int 1978;13:159–165.

2 The primary nephrotic syndrome in children. Identification of patients with minimal change nephrotic syndrome from initial response to prednisone. A report of the International Study of Kidney Disease in Children. J Pediatr 1981;98:561–564.

3 NAPRTCS 2006 Annual Report, in North American Pediatric Renal Trials and Collaborative Studies, 2006.

4 Schachter AD: Computational simulation of renal biopsy accuracy in focal segmental glomerulosclerosis. Pediatr Nephrol 2006;21:953–957.

5 Spahr CS, Davis MT, McGinley MD, et al: Towards defining the urinary proteome using liquid chromatography-tandem mass spectrometry. I. Profiling an unfractionated tryptic digest. Proteomics 2001;1:93–107.

6 Davis MT, Spahr CS, McGinley MD, et al: Towards defining the urinary proteome using liquid chromatography-tandem mass spectrometry. II. Limitations of complex mixture analyses. Proteomics 2001;1:108–117.

7 Thongboonkerd V, McLeish KR, Arthur JM, et al: Proteomic analysis of normal human urinary proteins isolated by acetone precipitation or ultracentrifugation. Kidney Int 2002;62:1461–1469.

8 Oh J, Pyo JH, Jo EH, et al: Establishment of a near-standard two-dimensional human urine proteomic map. Proteomics 2004;4:3485–3497.
9 Pieper R, Gatlin CL, McGrath AM, et al: Characterization of the human urinary proteome: a method for high-resolution display of urinary proteins on two-dimensional electrophoresis gels with a yield of nearly 1400 distinct protein spots. Proteomics 2004;4:1159–1174.
10 Weissinger EM, Wittke S, Kaiser T, et al: Proteomic patterns established with capillary electrophoresis and mass spectrometry for diagnostic purposes. Kidney Int 2004;65:2426–2434.
11 Ransom RF, Lam NG, Hallett MA, et al: Glucocorticoids protect and enhance recovery of cultured murine podocytes via actin filament stabilization. Kidney Int 2005;68:2473–2483.
12 Ramjee G, Coovadia HM, Adhikari M: Comparison of noninvasive methods for distinguishing steroid-sensitive nephrotic syndrome from focal glomerulosclerosis. J Lab Clin Med 1997;129:47–52.
13 Khurana M, Traum AZ, Aivado M, et al: Urine proteomic profiling of pediatric nephrotic syndrome. Pediatr Nephrol 2006;21:1257–1265.
14 Woroniecki RP, Orlova TN, Mendelev N, et al: Urinary proteome of steroid-sensitive and steroid-resistant idiopathic nephrotic syndrome of childhood. Am J Nephrol 2006;26:258–267.
15 Candiano G, Musante L, Bruschi M, et al: Repetitive fragmentation products of albumin and alpha1-antitrypsin in glomerular diseases associated with nephrotic syndrome. J Am Soc Nephrol 2006;17:3139–3148.
16 Erill S, du Souich P, Courteau H: Carbamylation of proteins and sulfacetamide free fraction in serum in experimentally-induced high blood urea states. Res Commun Chem Pathol Pharmacol 1985;50:45–56.
17 Lippincott J, Apostol I: Carbamylation of cysteine: a potential artifact in peptide mapping of hemoglobins in the presence of urea. Anal Biochem 1999;267:57–64.
18 Osterberg R, Orgel LE, Lohrmann R: Further studies of urea-catalyzed phosphorylation reactions. J Mol Evol 1973;2:231–234.
19 Strak GR, Link TP: Preparation and properties of three specific active derivatives of ribonuclease A obtained by methylation of methionine residues in 8 M urea. Biochemistry 1975;14:4576–4581.
20 Filipe P, Haigle J, Freitas J, et al: Anti- and pro-oxidant effects of urate in copper-induced low-density lipoprotein oxidation. Eur J Biochem 2002;269:5474–5483.
21 Schlotte V, Sevanian A, Hochstein P, et al: Effect of uric acid and chemical analogues on oxidation of human low density lipoprotein in vitro. Free Radic Biol Med 1998;25:839–847.
22 Llansola M, Erceg S, Felipo V: Chronic exposure to ammonia alters the modulation of phosphorylation of microtubule-associated protein 2 by metabotropic glutamate receptors 1 and 5 in cerebellar neurons in culture. Neuroscience 2005;133:185–191.
23 Schliess F, Gorg B, Fischer R, et al: Ammonia induces MK-801-sensitive nitration and phosphorylation of protein tyrosine residues in rat astrocytes. Faseb J 2002;16:739–741.
24 Murthy CR, Hertz L: Acute effect of ammonia on branched-chain amino acid oxidation and incorporation into proteins in astrocytes and in neurons in primary cultures. J Neurochem 1987;49:735–741.
25 Murthy CR, Hertz L: Comparison between acute and chronic effects of ammonia on branched-chain amino acid oxidation and incorporation into protein in primary cultures of astrocytes and of neurons. J Neurosci Res 1987;17:271–276.
26 Reaich D, Channon SM, Scrimgeour CM, et al: Ammonium chloride-induced acidosis increases protein breakdown and amino acid oxidation in humans. Am J Physiol 1992;263:E735–E739.
27 Harper RG, Workman SR, Schuetzner S, et al: Low-molecular-weight human serum proteome using ultrafiltration, isoelectric focusing, and mass spectrometry. Electrophoresis 2004;25:1299–1306.

Avram Z. Traum, MD
Pediatric Nephrology Unit
Massachusetts General Hospital
55 Fruit Street, Boston, MA 02114 (USA)
Tel. +1 617 726 2908, Fax +1 617 724 0581, E-Mail atraum@partners.org

Thongboonkerd V (ed): Proteomics in Nephrology – Towards Clinical Applications.
Contrib Nephrol. Basel, Karger, 2008, vol 160, pp 37–52

Searching for Novel Biomarkers and New Therapeutic Targets of Diabetic Nephropathy Using Proteomics Approaches

Visith Thongboonkerd

Medical Proteomics Unit and Medical Molecular Biology Unit,
Office for Research and Development, Faculty of Medicine Siriraj Hospital,
Mahidol University, Bangkok, Thailand

Abstract

Diabetic nephropathy is a major complication of diabetes and remains a common health problem worldwide. It is the dominant cause of incident end-stage renal disease. Currently, microalbuminuria is an only noninvasive marker available for the diagnosis of diabetic nephropathy. However, some patients with microalbuminuria have advanced renal pathological changes for which therapy is less effective than earlier stages of the disease. Additionally, the immunoassay to measure microalbuminuria can detect only immunoreactive forms of albumin, whereas immunounreactive forms are undetectable by this conventional method. Therefore, novel biomarkers for earlier diagnosis of diabetic nephropathy are crucially required. Moreover, the incidence rate of diabetes-induced end-stage renal disease remains high despite good control of blood sugar levels and adequate treatment with appropriate regimens, indicating the need of new therapeutic targets for better therapeutic outcome and successful prevention of diabetic nephropathy in diabetes patients. During the proteomic era, proteomics has become a powerful tool for unraveling the disease pathophysiology and for biomarker discovery. This chapter summarizes recent studies that applied proteomics for the investigation of diabetic nephropathy with major aims to search for novel biomarkers and new therapeutic targets.

Diabetic nephropathy remains a common health problem worldwide. Based on the United States Renal Data System reported in 2007 [1], diabetes is the dominant cause of incident end-stage renal disease (ESRD) with an incidence rate of 148.8 cases per million in 2004. More than 45% of patients who

receive renal replacement therapy (RRT) have diabetes as a primary diagnosis at the initiation of RRT [1]. Although treatment with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers successfully reduces proteinuria and can slow the progression rate of diabetic renal injury, renal failure remains the major diabetic complication. At an initial phase (stage I), primary functional changes in diabetic kidney include hyperperfusion and hyperfiltration. Pathological changes, including thickening of glomerular basement membranes and mesangial expansion, are subsequently observed after a few years of the onset of diabetes (stage II). Patients with these two stages of diabetic nephropathy usually have normal urinary albumin excretion (normoalbuminuria; $<$20 mg/l). Microalbuminuria (20–200 mg/l) occurs after more than 7 years of diabetes (stage III). To date, microalbuminuria is the best noninvasive marker available for the detection of diabetic nephropathy [2]. However, some patients with microalbuminuria have quite advanced renal pathological changes, for which therapy is less effective than earlier stages of the disease [3–5]. Additionally, the immunoassay to measure microalbuminuria recognizes just immunoreactive forms of albumin [6]. Immunounreactive forms of albumin are thus undetectable by this conventional method, which unfortunately remains the standard. Therefore, an alternative method that can detect all forms of albumin, rather than only immunoreactive albumin, is crucially required. Urgent attention is also needed to define novel biomarkers for earlier detection of diabetic nephropathy and to develop new therapeutic targets for better therapeutic outcome as well as for successful prevention of diabetic nephropathy.

Conventional methods for protein study have mainly involved immunological techniques, i.e. Western blotting, enzyme-linked immunosorbent assay, and radioimmunoassay. These methods, however, have some limitations. Only a relatively small number of proteins can be examined in a single experiment (it may take months to years for a complete analysis of a large number of proteins using these conventional methods). In addition, specific antibodies for the proteins to be examined must be existing and available. Furthermore, the proteins of interest are based on a priori assumption and the detection must rely on epitopes of the immunogens used for antibody production. To successfully search for novel biomarkers and new therapeutic targets of diabetic nephropathy, an effective method for global analysis of proteins is required. This ideal technique should be capable of simultaneously exploring both known (previously determined) and unknown (previously undetermined) components of the 'protein universe' in cells, tissues, organs, and/or biofluids [7]. In the postgenomic era, proteomics has been continuously developed for unbiased, high-throughput analysis of proteins on the genomic scale. Recently, Candiano et al. [8] identified numerous fragments of albumin in the urine using an unbiased proteomic methodology. Proteomics, thus, can be an optimistic approach to globally characterize both

immunoreactive and immunounreactive forms of albumin and other proteins in the urine. Additionally, proteomics also offers opportunities to search for novel biomarkers and new therapeutic targets of diseases. Therefore, proteomics seems to be an ideal approach in current and future diabetes research.

During the past 5 years, renal and urinary proteomics has been widely applied to the nephrology field [9–14]. Proteomics has been extensively applied also for the investigation of diabetes [15–22] and diabetic nephropathy [23–27], following an encouragement of the National Institute of Diabetes and Digestive and Kidney Diseases [28]. The main objectives of proteomic applications to diabetes are not only to better understand the pathogenic mechanisms and pathophysiology, but also to discover novel biomarkers and new therapeutic targets of this metabolic disorder and its complications. This chapter summarizes the data obtained from recent proteomic studies for the investigation of diabetic nephropathy.

Recent Proteomic Studies for the Investigation of Diabetic Nephropathy

For diabetic complications, either macrovascular or microvascular ones, proteomics has been mostly applied to diabetic nephropathy [23–27], which is a serious complication leading to ESRD. The following sections, therefore, highly focus on proteomic applications to diabetic nephropathy. Recent proteomic studies, which offer significant impact to the field, are summarized and discussed. Proteomic technologies, which were commonly used in these studies, include two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), two-dimensional difference gel electrophoresis (2-D DIGE), capillary electrophoresis coupled to mass spectrometry (CE-MS), and surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS). Additionally, a microfluidic technology on a chip, which is the most recent proteomic technology just implemented to the investigation of diabetic nephropathy and other glomerular disorders, is also discussed.

Two-Dimensional Polyacrylamide Gel Electrophoresis

Among these studies, 2-D PAGE is the most commonly employed proteomic method. The first dimension of 2-D PAGE separates proteins by differential pH or charges, whereas the second dimensional separation is based on differential molecular masses [29]. Resolved proteins in a 2-D gel can then be visualized by various stains or radiolabeling. The spots of interest can be identified mostly by

peptide mass fingerprinting following matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), by other MS methods, and also by immunoblotting for the known proteins.

2-D PAGE Analysis of Altered Renal Proteome in Animal Models of Type 1 and Type 2 Diabetes

Recently, the author and colleagues performed a classical, gel-based, proteomic analysis of diabetic nephropathy in the OVE26 transgenic mouse model of type 1 diabetes [23, 30, 31] and in the *db/db* mouse model of type 2 diabetes [32]. Differential proteomics was performed using these two mouse models compared to their respective background strains. A total of 41 (30 identified and 11 unidentified) and 39 (20 identified and 19 unidentified) proteins were differentially expressed in diabetic kidneys of type 1 and type 2 diabetes, respectively [23, 30, 32]. These altered proteins included proteases, protease inhibitors, apoptosis-associated proteins, regulators for oxidative tolerance, Ca^{2+}-binding proteins, transport regulators, cell-signaling proteins, and smooth muscle contractile elements. Some of the altered proteins had been previously shown to be regulated during diabetes, while roles for other altered proteins had not been established, suggesting that they may involve in the novel mechanisms of diabetic nephropathy. Alterations in renal elastin-elastase system and upregulated renal calbindin-D28k, which may play important roles in the pathophysiology of diabetic nephropathy, were highlighted in these studies [30, 31].

Subsequently, Tilton et al. [33] evaluated changes in renal cortical proteome of *db/db* mice. They identified 147 cortical renal proteins whose levels were altered (either increase or decrease) in diabetic animals, particularly those involved in catalytic, oxidoreductase and transferase activities, and nucleotide and ATP bindings. Interestingly, approximately 1/4 of these altered proteins were typical mitochondrial proteins. Additionally, bioinformatic analysis revealed peroxisome proliferator-activated receptor-α (PPARα) as a common node of interaction of several metabolic enzymes altered in diabetic nephropathy.

More recently, Ramachandra Rao et al. [34] performed subproteome analysis of human mesangial cells to identify altered membrane and cytosolic proteins after 7 days of exposure to high-glucose condition. Differential analysis showed 4 proteins in the membrane fraction whose levels were significantly decreased by the high-glucose environment, including BiP protein, calreticulin precursor, 63-kDa transmembrane protein from endoplasmic reticulum/Golgi intermediate, and protein disulfide isomerase. In the cytosolic fraction, there were 3 proteins (enolase 1, annexin VI and γ_2-actin) whose levels were significantly decreased, whereas 2 proteins (heat shock protein 70 kDa and calmodulin) had increased levels after the exposure to the high-glucose condition.

2-D PAGE Analysis of Altered Glomerular Proteome in Diabetic Glomerulopathy

Barati et al. [35] utilized a classical 2-D PAGE approach to analyze glomerular proteome of *db/db* diabetic mice compared to normal controls. They identified 40 glomerular proteins whose levels were significantly altered in diabetic glomerulopathy. Among these, antioxidative enzymes peroxiredoxin 1 and 3, glutathione peroxidase 1, and superoxide dismutase 1 had significantly increased levels in the diabetic glomeruli, suggesting altered cellular redox pathways in diabetic glomerulopathy. Additionally, they also found that glyoxalase I had an increased level in the diabetic glomeruli, but had a declined activity in the renal cortex of the diabetic kidney.

2-D PAGE Analysis of Altered Urinary Proteome in Patients with Diabetic Nephropathy

Analysis of urinary proteome would lead to the discovery of novel noninvasive biomarkers for earlier detection of diabetic nephropathy. Recently, the author and colleagues [36] applied a classical 2-D PAGE approach to compare human urinary proteomes of various glomerular disorders, including diabetic nephropathy (n = 11), focal segmental glomerulosclerosis (n = 4) and lupus nephritis class V (n = 5) compared to normal healthy individuals (n = 5). Quantitative intensity analyses, with and without normalization using urine creatinine levels, were performed and ANOVA with Tukey post-hoc multiple comparisons revealed a set of urinary proteins whose levels significantly differed between diabetic nephropathy and normal urine. These significantly differed proteins included albumin and its fragments, transferrin precursor, kininogen, E-cadherin, phorbolin 3, and hypothetical protein XP_006848. Unfortunately, no single disease-specific markers were identified in diabetic nephropathy group when compared to other glomerular disorders. This could be the fact that too small number of samples was used in this study. Moreover, an evaluation of molecular signature or panel of multiple biomarkers may be essential.

Thereafter, Varghese et al. [37] performed a similar study analyzing urine samples collected from patients with diabetic nephropathy (n = 4), focal segmental glomerulosclerosis (n = 4), lupus nephritis (n = 4) and membranous nephropathy (n = 4). Initial analysis showed similar results to our previous study, which demonstrated that there were no single markers that could differentiate groups of diseases found. However, these authors applied an intelligent artificial neural network to create a prediction algorithm and to define disease-specific urinary proteome profile or molecular signature containing multiple markers that could differentiate groups of the diseases. They successfully determined such molecular signature, which provided a sensitivity of 75–86% and a

specificity of 67–92% to predict the presence of diseases in a validation set (n = 4, 4, 7 and 1 for diabetic nephropathy, focal segmental glomerulosclerosis, lupus nephritis and membranous nephropathy, respectively). Totally 21 protein spots were most important for the determination of this molecular signature to differentiate groups of the diseases. These proteins were subsequently identified as orosomucoid, transferrin, α_1-microglobulin, zinc-α_2-glycoprotein, α_1-antitrypsin, complement factor B, haptoglobin, transthyretin, retinol-binding protein, albumin, and hemopexin.

2-D PAGE Analysis of Altered Serum Proteome in Patients with Diabetic Nephropathy

Serum proteomics was also employed for the discovery of novel biomarkers in diabetic nephropathy. Kim et al. [38] performed 2-D PAGE analysis of sera obtained from type 2 diabetes patients with normoalbuminuria (n = 30), microalbuminuria (n = 29) and ESRD (n = 31). They found that serum levels of C-type lectin domain family 3, apolipoprotein CIII, apolipoprotein E, proapo A-I, retinol-binding protein 4, ficolin 3 precursor, haptoglobin-related protein precursor, hemopexin precursor, complement factor I precursor, sex hormone-binding globulin, and glutathione peroxidase precursor were decreased, whereas levels of pigment epithelium-derived factor, complement component C4B3 and C4A, adiponectin precursor, and β_2-microglobulin were increased in diabetic patients with microalbuminuria or ESRD. Comparing between microalbuminuric and normoalbuminuric patients, they observed that the microalbuminuric patients had lower levels of complement C4A, proapo A-I, MASP-2-related protein, retinol-binding protein, glutathione peroxidase precursor, ficolin 3 precursor, and haptoglobin-related protein precursor, but had higher levels of pigment epithelium-derived factor, complex-forming glycoprotein HC, vitamin D-binding protein precursor, and complement factor H-related 1 protein [39].

Two-Dimensional Difference Gel Electrophoresis

The concept of 2-D DIGE has been recently introduced to reduce gel-to-gel variability [40, 41]. Briefly, each of two samples (or sample pools) is differentially labeled with fluorescent dye (Cy3 or Cy5). The two differentially labeled samples are then mixed and resolved simultaneously within the same 2-D gel. An internal standard labeled with a third dye (i.e. Cy2) can be also incorporated, resulting in more accurately quantitative analysis. The spots of interest can be identified mostly by peptide mass fingerprinting following MALDI-TOF MS or by other MS methods.

Recently, Sharma et al. [42] adopted 2-D DIGE to evaluate the urinary proteome profile of 3 patients with longstanding diabetes, impaired renal function and overt proteinuria (>200 mg/l). Urinary excretion levels of 63 proteins were significantly increased in the urine of diabetic patients, while those of 36 proteins were decreased as compared to 5 healthy controls. One spot that was increased 19-fold in the diabetic urine was identified as α_1-antitrypsin. ELISA of urine samples from an independent group of 19 patients and 20 healthy controls confirmed a marked increase in urinary α_1-antitrypsin in diabetic patients. Immunostaining of human diabetic kidneys also revealed an increased level of tissue α_1-antitrypsin, particularly at the fibrotic area.

Another study by Rao et al. [43] also employed a similar strategy for urinary proteome profiling of type 2 diabetes patients with normoalbuminuria (n = 10), microalbuminuria (n = 13), and macroalbuminuria (or overt proteinuria; n = 10) compared to control subjects (n = 9). 2-D DIGE analysis revealed significantly differed levels of several proteins among groups. Of these, vitamin D-binding protein, leucine-rich α_2-glycoprotein, hemopexin, Zn-α_2-glycoprotein, α_{1B}-glycoprotein, α_{2HS}-glycoprotein precursor, and calgranulin B were found to have progressively increased levels related to the degree of proteinuria, whereas ceruloplasmin precursor, hepatocellular carcinoma-associated protein TB6, α_1-microglobulin/bikunin precursor, and retinol-binding protein precursor had progressively declined levels in association with the degree of proteinuria.

Capillary Electrophoresis Coupled to Mass Spectrometry

CE-MS is a powerful tool for proteome/peptidome profiling. The system uses capillary loops for protein separation and a mass spectrometer for analyzing protein/polypeptide ions. A variety of MS-coupling techniques can be used for CE-MS [44, 45]. The most commonly used ionization method for CE-MS is ESI, whereas MALDI has been used as well [44, 45]. Modern ESI-TOF mass analyzers can record up to 20 spectra per second and provide the high resolution and high accuracy. More details on methodology of CE-MS can be found in other chapters of this volume by Zürbig and Mischak [pp. 107–126], Decramer et al. [pp. 127–141] and Schiffer et al. [pp. 159–171].

CE-MS was utilized by Mischak and colleagues to differentiate urinary polypeptide profiles of patients with type 1 [46] and type 2 [47] diabetes from those of age-matched healthy controls. The urinary polypeptide pattern of patients with diabetes significantly differed from that of the normal controls. Moreover, there was a specific polypeptide pattern of ‘diabetic renal damage’ in patients with high-grade albuminuria. These data underscore the usefulness of

the proteomics approach in clinical diagnostics and biomarker discovery of diabetic nephropathy.

Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

SELDI-TOF MS is an easy-to-use system for examination of human body fluids. SELDI-TOFMS combines MALDI-TOF-MS with surface retentate chromatography [48–51]. The sample is first applied onto a chip surface specifically designed to retain particular group of proteins. After incubation, unbound proteins are removed and the bound proteins are analyzed by TOF mass spectrometer. This approach reduces the complexity of proteins in the sample being analyzed by selecting only a subset of particular functionality or property.

Recently, Cho et al. [52] utilized SELDI-TOF MS to search for potential biomarkers in sera and kidneys of 26 streptozotocin-induced diabetic rats compared to control animals. They observed 8 potential biomarkers in the serum (one of which was identified as C-reactive protein), whereas only 1 potential biomarker was found in the kidney lysate.

Dihazi et al. [53] performed SELDI proteome profiling of urine samples obtained from type 2 diabetes patients with microalbuminuria or overt proteinuria (n = 38), compared to the profiles of type 2 diabetes patients with normoalbuminuria (n = 45), proteinuric patients with nondiabetic causes (n = 34), and healthy controls (n = 45). Using this large sample size (considerably large in previous proteomics studies), they identified a peak with mass/charge (m/z) of 14,766 that was selectively excreted into the urine of diabetic patients with proteinuria and was subsequently identified as UbA52, a ubiquitin ribosomal fusion protein, which could serve as a potential diagnostic biomarker of diabetic nephropathy.

More recently, Otu et al. [54] conducted a SELDI-TOF MS study of baseline urine samples collected from Pima Indians with type 2 diabetes before microalbuminuria occurred. SELDI-TOF MS was performed to compare the baseline samples of patients who developed diabetic nephropathy 10 years later (n = 31) to those who remained normoalbuminuric after 10 years of the first sample collection (n = 31). They identified a molecular signature containing 12 SELDI peaks that could differentiate patients who subsequently developed diabetic nephropathy from those who remained normoalbuminuric with 93% sensitivity and 86% specificity in the training set. This molecular signature provided 71% sensitivity and 76% specificity for prediction of diabetic nephropathy in an independent validation set. This is a very nice work that yielded significant data to predict diabetic nephropathy in a long-term follow-up study.

Microfluidic Technology on a Chip

During the past decade, miniaturization with microfluidics has gained a wide acceptance for clinical applications [55, 56]. Comparing to conventional methods, advantages of microfluidic technology include smaller volume of samples required, reduced reagent consumption, higher sensitivity, decreased analytical time, higher throughput and automation, and smaller footprints of analytical devices [57, 58]. The microfluidic technology on a chip is one among currently available microfluidic devices that require a tiny amount (down to 10^{-18} l) of biological fluids to be analyzed on multichannels chip with dimension of tens to hundreds of micrometers [59, 60]. Therefore, this lab on a chip is feasible for bedside applications, particularly for clinical diagnostics, prognostics, and prediction of therapeutic response.

The author and colleagues recently applied a microfluidic technology on a chip to proteome profiling of human urine from 31 normal healthy individuals, 6 patients with diabetic nephropathy and 4 patients with IgA nephropathy [61]. Using only 4 μl of untreated urine, automated separation of proteins/peptides was achieved. Multiple comparative analyses revealed 9 spectra, of which amplitudes significantly differed between normal and diabetic nephropathy (diabetic nephropathy/normal amplitude ratios ranged from 2.9 to 3,102.7). Moreover, the results also showed that 3 spectra (with molecular masses of 12–15, 27–28, and 34–35 kDa) were significantly different between diabetic nephropathy and IgA nephropathy (diabetic nephropathy/IgA nephropathy amplitude ratios ranged from 3.9 to 7.4). Frequencies of some spectra could differentiate the normal from the diseased urine, but could not distinguish between diabetic nephropathy and IgA nephropathy. These data underscore the clinical applicability of the microfluidic technology on a chip in diagnostics and biomarker discovery.

Proteomic Analysis of Posttranslationally Modified Proteins in Diabetic Nephropathy

Glycation of mitochondrial proteins was recently examined by Rosca et al. [62] using a proteomics approach. They applied 2-D Western blot analysis (using anti-methylglyoxal-derived imidazole antibody) to detect methylglyoxal-modified renal cortical mitochondrial proteins that underwent glycation in streptozotocin-induced diabetic rats. Methylglyoxal could react with arginine, lysine, and sulfhydryl groups of proteins, inducing the formation of a variety of advanced glycation end products [63, 64]. These immunoreactive spots or glycated mitochondrial proteins were identified by liquid chromatography coupled to MS

(LC-MS) including ubiquinol-cytochrome C reductase core protein I, cytochrome C1, NADH-ubiquinone oxidoreductase 30-kDa subunit, F1-ATPase chain G, enoyl-CoA hydratase, and electron flavoprotein β-subunit. These modified proteins might explain the excess oxidative stress in the diabetic kidney.

Similarly, Schmitt et al. [65] also applied 2-D Western blot analysis (using anti-carboxymethyl lysine antibody) to identify carboxymethyl lysine-induced glycated proteins in hemodialysate fluid of patients with diabetic ESRD who underwent RRT (n = 40). These immunoreactive spots or glycated hemodialysate proteins were identified by Edman sequencing, including albumin, immunoglobulin κ-chain, prostaglandin D2 synthase, lysozyme C, retinol-binding protein, and β_2-microglobulin.

Pharmacoproteomics in Diabetes Research: Drug Discovery and Investigation of Drug Action, Response, Toxicity and Resistance

After screening for novel therapeutic targets and validation, the next step is to discover novel drug compounds by designing their molecular structures to fit into the functional parts of protein molecules that are the therapeutic targets. Bioinformatics plays a crucial role in such design. Proteomics applied to pharmaceutical purposes is named 'pharmacoproteomics', which involves almost all of basic methodologies aforementioned. Indeed, various MS methods, including electrospray and nanospray ionization, atmospheric pressure chemical ionization, photoionization, and their interface with LC have been utilized to measure levels of drugs and their metabolites in the plasma and urine for quite some time [66]. Recent advances in high-performance LC coupled to tandem MS (HPLC-ESI-MS/MS) make the identification of drug compounds more effective with a better resolution. A high-throughput capability of HPLC-ESI-MS/MS, with or without stable isotope labeling, facilitates the studies of in vitro and in vivo drug metabolisms, examination of metabolite activities, identification and characterization of impurities in the pharmaceuticals, analysis of chiral impurities in drug substances, and drug discovery [67–70].

Pharmacoproteomics can be also applied for prediction of therapeutic responses to a specific drug. However, responses to a particular drug may not be easily predictable because of the interindividual variability [71], which is partly due to genetic factors [72]. Hence, combination of pharmacoproteomics and pharmacogenomics is essential for predicting the therapeutic response as well as for evaluating the genetically and biochemically dynamic processes during treatment [73]. Proteomic technologies are not used alone for drug design and discovery as well as for other pharmaceutical purposes, but rather they are integrated with genomic and other chemical methodologies.

Some examples of pharmacoproteomics studies in diabetes research are given as follows. Edvardsson et al. [74] performed a differential proteomics study on livers of lean mice, obese (*ob/ob*) mice without treatment, and *ob/ob* mice treated with WY14643 (PPARα agonist) or rosiglitazone (PPARγ agonist). PPARs are ligand-activated transcription factors that modulate lipid and glucose homeostasis. PPARα and PPARγ agonists can thus be useful for treatment of hypertriglyceridemia and insulin resistance, respectively. The investigators found that livers from *ob/ob* mice displayed higher levels of enzymes involving in fatty acid oxidation and lipogenesis compared to lean mice, and these differences were further amplified by treatment with both PPAR activators. WY14643 could normalize expression levels of several enzymes involved in glycolysis, gluconeogenesis and amino acid metabolism in *ob/ob* mice to the basal levels of lean mice, whereas rosiglitazone only partially normalized the levels of enzymes involved in amino acid metabolism. The data provide some new insights into the molecular mechanisms or the therapeutic actions of these two drugs.

Sanchez et al. [75, 76] evaluated effects of rosiglitazone, an insulin sensitizer, on protein expression in pancreatic islets, liver, adipose tissues, and muscles of obese C57BL/6J *lep/lep* mice compared to lean littermates. Rosiglitazone could normalize the impaired glucose tolerance in *lep/lep* mice but had no significant effect on glucose tolerance in the lean littermates. This insulin sensitizer was also shown to bind and activate PPARγ1 in adipocytes and PPARγ2 in hepatocytes. The identification of new molecular targets associated with fatty acid oxidation and PPARγ nuclear receptor regulation in insulin resistance tissues was one of key research goals. In pancreatic islets, 9 proteins were differentially expressed between *lep/lep* and lean mice, and 4 were significantly modulated by rosiglitazone treatment of the obese mice. These differentially expressed proteins were identified by MS analysis and provided evidence that differential expression of actin-binding proteins might be an important aspect of defective islet function. For liver, adipose tissues and muscles, 34 polypeptides were differentially expressed between *lep/lep* and lean mice and 11 were significantly modulated by rosiglitazone treatment of the obese mice. None of these proteins was modulated by rosiglitazone treatment in the lean mice. These differentially expressed proteins were identified using tandem MS/MS analysis and revealed components of fatty acid and carbohydrate metabolisms, as well as proteins with unknown function. Rosiglitazone increased carboxypeptidase B expression in both *lep/lep* and normal mice, suggesting that it might be an independent effect of rosiglitazone that contributes to improved insulin processing.

For diabetic nephropathy, Rossing et al. [77] recently applied CE-MS not only to define the urinary polypeptide pattern specific for diabetic nephropathy,

but also to study effects of an angiotensin II receptor blocker (candesartan) on the urinary polypeptide pattern. They found that candesartan could effectively return urinary levels of some polypeptides to their normal levels. More recently, Diao et al. [78] examined changes in levels of proteins in serum, liver and kidney of type 1 diabetic mice induced with alloxan. They found alterations in levels (either increase or decrease) of 43 proteins in the serum, liver and kidney of the alloxan-induced type 1 diabetic mice. After an insulin gene transfer of naked plasmid by electroporation into sural muscles of these diabetic animals, their blood glucose levels declined to normal. As a consequence of this gene transfer therapy, levels of 7 proteins in the serum, 5 proteins in the liver, and 5 proteins in the kidney returned to their basal levels. These proteins, which were recovered by the gene transfer therapy, included those involved in lipid and glucose metabolism, phosphorylation, signal transduction, oxidation and immune mediation. These studies underline the value of proteomics in the discovery of novel biomarkers and new therapeutic targets of diabetes and diabetic nephropathy.

Conclusions

During the postgenomic era, proteomics has become an important tool for the investigation of diabetic nephropathy. With more extensive applications in the coming years, proteomics will add up a wealth of useful information and knowledge, and the ultimate goals of earlier diagnosis, better therapeutic outcome and successful prevention of diabetic nephropathy will then be achievable.

Acknowledgements

This work was supported by Siriraj Grant for Research and Development, Mahidol University, Vejdusit Foundation, Thailand Research Fund, Commission on Higher Education, National Center for Genetic Engineering and Biotechnology, and National Research Council of Thailand.

References

1 Foley RN, Collins AJ: End-stage renal disease in the United States: an update from the United States Renal Data System. J Am Soc Nephrol 2007;18:2644–2648.
2 Caramori ML, Fioretto P, Mauer M: The need for early predictors of diabetic nephropathy risk: is albumin excretion rate sufficient? Diabetes 2000;49:1399–1408.
3 Chavers BM, Bilous RW, Ellis EN, Steffes MW, Mauer SM: Glomerular lesions and urinary albumin excretion in type I diabetes without overt proteinuria. N Engl J Med 1989;320:966–970.
4 Fioretto P, Steffes MW, Mauer M: Glomerular structure in nonproteinuric IDDM patients with various levels of albuminuria. Diabetes 1994;43:1358–1364.

5 Bangstad HJ, Osterby R, Dahl-Jorgensen K, Berg KJ, Hartmann A, Nyberg G, Frahm BS, Hanssen KF: Early glomerulopathy is present in young, type 1 (insulin-dependent) diabetic patients with microalbuminuria. Diabetologia 1993;36:523–529.
6 Comper WD, Osicka TM, Jerums G: High prevalence of immuno-unreactive intact albumin in urine of diabetic patients. Am J Kidney Dis 2003;41:336–342.
7 Aebersold R: Constellations in a cellular universe. Nature 2003;422:115–116.
8 Candiano G, Musante L, Bruschi M, Petretto A, Santucci L, Del Boccio P, Pavone B, Perfumo F, Urbani A, Scolari F, Ghiggeri GM: Repetitive fragmentation products of albumin and alpha1-antitrypsin in glomerular diseases associated with nephrotic syndrome. J Am Soc Nephrol 2006;17:3139–3148.
9 Thongboonkerd V, Klein JB (eds): Proteomics in Nephrology. Contrib Nephrol. Basel, Karger, 2004, vol 141, pp 1–327.
10 Thongboonkerd V: Proteomics in Nephrology: Current Status and Future Directions. Am J Nephrol 2004;24:360–378.
11 Thongboonkerd V, Malasit P: Renal and urinary proteomics: Current applications and challenges. Proteomics 2005;5:1033–1042.
12 Thongboonkerd V: Proteomic analysis of renal diseases: Unraveling the pathophysiology and biomarker discovery. Expert Rev Proteomics 2005;2:349–366.
13 Thongboonkerd V: Recent progress in urinary proteomics. Proteomics Clin Appl 2007;1:780–791.
14 Thongboonkerd V: Practical points in urinary proteomics. J Proteome Res 2007;6:3881–3890.
15 Korc M: Diabetes mellitus in the era of proteomics. Mol Cell Proteomics 2003;2:399–404.
16 Sparre T, Bergholdt R, Nerup J, Pociot F: Application of genomics and proteomics in Type 1 diabetes pathogenesis research. Expert Rev Mol Diagn 2003;3:743–757.
17 Korc M: Update on diabetes mellitus. Dis Markers 2004;20:161–165.
18 Sparre T, Larsen MR, Heding PE, Karlsen AE, Jensen ON, Pociot F: Unraveling the pathogenesis of type 1 diabetes with proteomics: present and future directions. Mol Cell Proteomics 2005;4:441–457.
19 Basso D, Greco E, Fogar P, Pucci P, Flagiello A, Baldo G, Giunco S, Valerio A, Navaglia F, Zambon CF, Pedrazzoli S, Plebani M: Pancreatic cancer-associated diabetes mellitus: an open field for proteomic applications. Clin Chim Acta 2005;357:184–189.
20 Scott EM, Carter AM, Findlay JB: The application of proteomics to diabetes. Diab Vasc Dis Res 2005;2:54–60.
21 D'Hertog W, Mathieu C, Overbergh L: Type 1 diabetes: entering the proteomic era. Expert Rev Proteomics 2006;3:223–236.
22 Thongboonkerd V: Proteomic for diabetes research: An update and future perspectives. Expert Rev Endocrinol Metab 2006;1:507–515.
23 Thongboonkerd V, Barati MT, McLeish KR, Pierce WM, Epstein PN, Klein JB: Proteomics and diabetic nephropathy. Contrib Nephrol 2004;141:142–154.
24 Merchant ML, Klein JB: Proteomics and diabetic nephropathy. Curr Diab Rep 2005;5:464–469.
25 Rossing K: Progression and remission of nephropathy in type 2 diabetes: new strategies of treatment and monitoring. Dan Med Bull 2007;54:79–98.
26 Susztak K, Bottinger EP: Diabetic nephropathy: a frontier for personalized medicine. J Am Soc Nephrol 2006;17:361–367.
27 Merchant ML, Klein JB: Proteomics and diabetic nephropathy. Semin Nephrol 2007;27:627–636.
28 Fradkin J: NIDDK Encourages Technology Research in Diabetes. Diabetes Technol Ther 2002;4:713–716.
29 Klein E, Klein JB, Thongboonkerd V: Two-dimensional gel electrophoresis: a fundamental tool for expression proteomics studies. Contrib Nephrol 2004;141:25–39.
30 Thongboonkerd V, Barati MT, McLeish KR, Benarafa C, Remold-O'Donnell E, Zheng S, Rovin BH, Pierce WM, Epstein PN, Klein JB: Alterations in the renal elastin-elastase system in Type 1 diabetic nephropathy identified by proteomic analysis. J Am Soc Nephrol 2004;15:650–662.
31 Thongboonkerd V, Zheng S, McLeish KR, Epstein PN, Klein JB: Proteomic identification and immunolocalization of increased renal calbindin-D28k expression in OVE26 diabetic mice. Rev Diab Stud 2005;2:17–24.

32 Thongboonkerd V, Barati MT, McLeish KR, Rovin BH, Pierce WM, Epstein PN, Klein JB: Altered elastase inhibitor and elastin expression in Type 2 diabetic kidneys defined by proteomic analysis. J Am Soc Nephrol 2003;14(suppl):600A.
33 Tilton RG, Haidacher SJ, Lejeune WS, Zhang X, Zhao Y, Kurosky A, Brasier AR, Denner L: Diabetes-induced changes in the renal cortical proteome assessed with two-dimensional gel electrophoresis and mass spectrometry. Proteomics 2007;7:1729–1742.
34 Ramachandra Rao SP, Wassell R, Shaw MA, Sharma K: Profiling of human mesangial cell subproteomes reveals a role for calmodulin in glucose uptake. Am J Physiol Renal Physiol 2007;292: F1182–F1189.
35 Barati MT, Merchant ML, Kain AB, Jevans AW, McLeish KR, Klein JB: Proteomic analysis defines altered cellular redox pathways and advanced glycation end-product metabolism in glomeruli of db/db diabetic mice. Am J Physiol Renal Physiol 2007;293:F1157–F1165.
36 Thongboonkerd V, Klein JB, Jevans AW, McLeish KR: Urinary proteomics and biomarker discovery for glomerular diseases. Contrib Nephrol 2004;141:292–307.
37 Varghese SA, Powell TB, Budisavljevic MN, Oates JC, Raymond JR, Almeida JS, Arthur JM: Urine biomarkers predict the cause of glomerular disease. J Am Soc Nephrol 2007;18:913–922.
38 Kim HJ, Cho EH, Yoo JH, Kim PK, Shin JS, Kim MR, Kim CW: Proteome analysis of serum from type 2 diabetics with nephropathy. J Proteome Res 2007;6:735–743.
39 Cho EH, Kim MR, Kim HJ, Lee DY, Kim PK, Choi KM, Ryu OH, Kim CW: The discovery of biomarkers for type 2 diabetic nephropathy by serum proteome analysis. Proteomics Clin Appl 2007;1:352–361.
40 Lilley KS, Friedman DB: All about DIGE: quantification technology for differential-display 2D-gel proteomics. Expert Rev Proteomics 2004;1:401–409.
41 Marouga R, David S, Hawkins E: The development of the DIGE system: 2D fluorescence difference gel analysis technology. Anal Bioanal Chem 2005;382:669–678.
42 Sharma K, Lee S, Han S, Lee S, Francos B, McCue P, Wassell R, Shaw MA, RamachandraRao SP: Two-dimensional fluorescence difference gel electrophoresis analysis of the urine proteome in human diabetic nephropathy. Proteomics 2005;5:2648–2655.
43 Rao PV, Lu X, Standley M, Pattee P, Neelima G, Girisesh G, Dakshinamurthy KV, Roberts CT Jr, Nagalla SR: Proteomic identification of urinary biomarkers of diabetic nephropathy. Diabetes Care 2007;30:629–637.
44 Kolch W, Neususs C, Pelzing M, Mischak H: Capillary electrophoresis-mass spectrometry as a powerful tool in clinical diagnosis and biomarker discovery. Mass Spectrom Rev 2005;24: 959–977.
45 Simpson DC, Smith RD: Combining capillary electrophoresis with mass spectrometry for applications in proteomics. Electrophoresis 2005;26:1291–1305.
46 Meier M, Kaiser T, Herrmann A, Knueppel S, Hillmann M, Koester P, Danne T, Haller H, Fliser D, Mischak H: Identification of urinary protein pattern in type 1 diabetic adolescents with early diabetic nephropathy by a novel combined proteome analysis. J Diabetes Complications 2005;19:223–232.
47 Mischak H, Kaiser T, Walden M, Hillmann M, Wittke S, Herrmann A, Knueppel S, Haller H, Fliser D: Proteomic analysis for the assessment of diabetic renal damage in humans. Clin Sci (Lond) 2004;107:485–495.
48 Fung E, Diamond D, Simonsesn AH, Weinberger SR: The use of SELDI ProteinChip array technology in renal disease research. Methods Mol Med 2003;86:295–312.
49 Petricoin EF, Liotta LA: Clinical proteomics: application at the bedside. Contrib Nephrol 2004;141:93–103.
50 Papale M, Pedicillo MC, Thatcher BJ, Di Paolo S, Lo ML, Bufo P, Rocchetti MT, Centra M, Ranieri E, Gesualdo L: Urine profiling by SELDI-TOF/MS: monitoring of the critical steps in sample collection, handling and analysis. J Chromatogr B Analyt Technol Biomed Life Sci 2007;856:205–213.
51 Poon TC: Opportunities and limitations of SELDI-TOF-MS in biomedical research: practical advices. Expert Rev Proteomics 2007;4:51–65.
52 Cho WC, Yip TT, Chung WS, Leung AW, Cheng CH, Yue KK: Differential expression of proteins in kidney, eye, aorta, and serum of diabetic and non-diabetic rats. J Cell Biochem 2006;99: 256–268.

53 Dihazi H, Muller GA, Lindner S, Meyer M, Asif AR, Oellerich M, Strutz F: Characterization of diabetic nephropathy by urinary proteomic analysis: identification of a processed ubiquitin form as a differentially excreted protein in diabetic nephropathy patients. Clin Chem 2007;53: 1636–1645.
54 Otu HH, Can H, Spentzos D, Nelson RG, Hanson RL, Looker HC, Knowler WC, Monroy M, Libermann TA, Karumanchi SA, Thadhani R: Prediction of diabetic nephropathy using urine proteomic profiling 10 years prior to development of nephropathy. Diabetes Care 2007;30:638–643.
55 Tudos AJ, Besselink GJ, Schasfoort RB: Trends in miniaturized total analysis systems for point-of-care testing in clinical chemistry. Lab Chip 2001;1:83–95.
56 Verpoorte E: Microfluidic chips for clinical and forensic analysis. Electrophoresis 2002;23: 677–712.
57 Whitesides GM: The origins and the future of microfluidics. Nature 2006;442:368–373.
58 Lion N, Reymond F, Girault HH, Rossier JS: Why the move to microfluidics for protein analysis? Curr Opin Biotechnol 2004;15:31–37.
59 Srinivasan V, Pamula VK, Fair RB: An integrated digital microfluidic lab-on-a-chip for clinical diagnostics on human physiological fluids. Lab Chip 2004;4:310–315.
60 Minas G, Wolffenbuttel RF, Correia JH: A lab-on-a-chip for spectrophotometric analysis of biological fluids. Lab Chip 2005;5:1303–1309.
61 Thongboonkerd V, Songtawee N, Sritippayawan S: Urinary proteome profiling using microfluidic technology on a chip. J Proteome Res 2007;6:2011–2018.
62 Rosca MG, Mustata TG, Kinter MT, Ozdemir AM, Kern TS, Szweda LI, Brownlee M, Monnier VM, Weiss MF: Glycation of mitochondrial proteins from diabetic rat kidney is associated with excess superoxide formation. Am J Physiol Renal Physiol 2005;289:F420–F430.
63 Lo TW, Westwood ME, McLellan AC, Selwood T, Thornalley PJ: Binding and modification of proteins by methylglyoxal under physiological conditions. A kinetic and mechanistic study with N alpha-acetylarginine, N alpha-acetylcysteine, and N alpha-acetyllysine, and bovine serum albumin. J Biol Chem 1994;269:32299–32305.
64 Thornalley PJ, Westwood M, Lo TW, McLellan AC: Formation of methylglyoxal-modified proteins in vitro and in vivo and their involvement in AGE-related processes. Contrib Nephrol 1995;112:24–31.
65 Schmitt S, Linder M, Standker L, Hammes HP, Preissner KT: Identification of CML-modified Proteins in Hemofiltrate of Diabetic Patients by Proteome Analysis. Exp Clin Endocrinol Diabetes 2007;116:26–34.
66 Lim CK, Lord G: Current developments in LC-MS for pharmaceutical analysis. Biol Pharm Bull 2002;25:547–557.
67 Iyer RA, Malhotra B, Khan S, Mitroka J, Bonacorsi S Jr, Waller SC, Rinehart JK, Kripalani K: Comparative Biotransformation of Radiolabeled [(14)C]Omapatrilat and Stable-labeled [(13)C(2)]Omapatrilat after Oral Administration to Rats, Dogs, and Humans. Drug Metab Dispos 2003;31:67–75.
68 Kim Y, Hwang J, Kim M, Lee W: Determination of Tripamide in human urine by high-performance liquid chromatography and high-performance liquid chromatography/electrospray ionization tandem mass spectrometry. Rapid Commun Mass Spectrom 2003;17:301–306.
69 Garzotti M: Lacidipine, a potential peroxynitrite scavenger: investigation of activity by liquid chromatography and mass spectrometry. Rapid Commun Mass Spectrom 2003;17:272–278.
70 Jain KK: Proteomics and drug discovery. Contrib Nephrol 2004;141:308–327.
71 Chapman AB, Schwartz GL, Boerwinkle E, Turner ST: Predictors of antihypertensive response to a standard dose of hydrochlorothiazide for essential hypertension. Kidney Int 2002;61: 1047–1055.
72 Trotta R, Donati MB, Iacoviello L: Trends in pharmacogenomics of drugs acting on hypertension. Pharmacol Res 2004;49:351–356.
73 Cadman PE, O'Connor DT: Pharmacogenomics of hypertension. Curr Opin Nephrol Hypertens 2003;12:61–70.
74 Edvardsson U, Brockenhuus VL, Panfilov O, Nystrom AC, Nilsson F, Dahllof B: Hepatic protein expression of lean mice and obese diabetic mice treated with peroxisome proliferator-activated receptor activators. Proteomics 2003;3:468–478.

75 Sanchez JC, Converset V, Nolan A, Schmid G, Wang S, Heller M, Sennitt MV, Hochstrasser DF, Cawthorne MA: Effect of rosiglitazone on the differential expression of diabetes- associated proteins in pancreatic islets of C57Bl/6 lep/lep mice. Mol Cell Proteomics 2002;1:509–516.
76 Sanchez JC, Converset V, Nolan A, Schmid G, Wang S, Heller M, Sennitt MV, Hochstrasser DF, Cawthorne MA: Effect of rosiglitazone on the differential expression of obesity and insulin resistance associated proteins in lep/lep mice. Proteomics 2003;3:1500–1520.
77 Rossing K, Mischak H, Walden M, Parving HH, Christensen CK, Hillmann M, Kaiser T: The impact of diabetic nephropathy and angiotensin II receptor blocker treatment on urinary polypeptide patterns in type 2 diabetic patients. Kidney Int 2005;68:193–205.
78 Diao WF, Chen WQ, Wu Y, Liu P, Xie XL, Li S, Shen PP, Ji J: Serum, liver, and kidney proteomic analysis for the alloxan-induced type I diabetic mice after insulin gene transfer of naked plasmid through electroporation. Proteomics 2006;6:5837–5845.

Visith Thongboonkerd, MD, FRCPT
Medical Proteomics Unit and Medical Molecular Biology Unit
Office for Research and Development
12th Floor Adulyadej Vikrom Building, Siriraj Hospital
2 Prannok Road, Bangkoknoi, Bangkok 10700 (Thailand)
Tel./Fax +66 2 418 4793, E-Mail thongboonkerd@dr.com

Thongboonkerd V (ed): Proteomics in Nephrology – Towards Clinical Applications.
Contrib Nephrol. Basel, Karger, 2008, vol 160, pp 53–64

Diagnostic and Prognostic Biomarkers in Acute Renal Failure

John M. Arthur, Michael G. Janech, Sanju A. Varghese, Jonas S. Almeida, T. Brian Powell

Ralph H. Johnson VA Medical Center and Medical University of South Carolina, Charleston, S.C., and University of Texas MD Anderson Cancer Center, Houston, Tex., USA

Abstract

Acute kidney injury (AKI) is a process that can lead to renal failure. No biological markers are available for predicting the cause or prognosis of AKI. Tests that can predict which patients will need renal replacement therapy (RRT) are needed. In this chapter, we review the recent literature for proteomic analysis in AKI and identify new candidate markers to predict the need for RRT. We also used artificial neural network (ANN) analysis of urine protein data obtained by two-dimensional gel electrophoresis from 19 patients with acute tubular necrosis to identify a set of proteins that can predict whether a patient will require RRT. Ten patients were randomly selected to train an ANN algorithm. The remaining 9 patients were withheld to serve as an independent validation set. The ANN algorithm correctly predicted the renal prognosis of all 10 patients in the training set. In the validation set, the test correctly predicted the future course of renal failure in 7 of the 9 patients (78% accuracy) including 3 of 4 patients who would require RRT (75% sensitivity) and 4 of 5 who would not (80% specificity). Combinations of urine proteins can be used to predict which patients will require RRT.

Acute renal failure (ARF) is a common condition among hospitalized patients. Mortality associated with ARF in the hospital is approximately 50% in spite of advances in renal replacement therapy (RRT) [1, 2]. The term acute kidney injury (AKI) has been used to emphasize the renal injury which is responsible for ARF. Diagnosis of the initiating event of the kidney injury can be difficult. Biomarkers associated with injury will help guide treatment. However, even the definition of ARF has been controversial and over 35 different definitions have been used [3]. Recently, the Acute Dialysis Quality

Initiative published a consensus definition [4]. The criteria use a set of descriptors termed RIFLE (Risk, Injury, Failure, Loss, and End stage). The criteria for the three acute categories are well-defined and describe progressively larger increases in serum creatinine or decreases in urine output. The criteria have provided a standardized set of definitions that can help improve our understanding of ARF. Moreover, in recent studies there was a nearly linear correlation between the RIFLE class and mortality both in an ICU setting [5] and in a large university-affiliated hospital [6]. Standardized definitions can facilitate biomarker discovery by limiting one source of variability. This will facilitate the development of both prognostic and diagnostic biomarkers. While the definition of renal failure has become more defined, our ability to treat it is still extremely limited. RRT is used to remove waste products and manage electrolytes and volume status, but the optimal dose and timing of this treatment in ARF is not known. Furthermore, no pharmacologic therapies have been shown to prevent or slow development of ARF. A major reason for the slow progress in developing treatments is the lack of tools for early diagnosis and prognosis associated with the renal injury. Biomarkers will aid in designing and interpreting new studies as well as facilitating treatment of individual patients.

Proteomics in ARF

Proteomic analysis has been used to analyze kidney tissue and to better understand the pathophysiology of renal injury. It has also been used to identify tissue and fluid biomarkers associated with kidney injury. Proteomic studies identified changes in renal proteins associated with exposure to toxins such as lead [7–9], gentamicin [10], and other agents [11–13]. These studies used two-dimensional gel electrophoresis (2-DE) to visualize renal proteins that are differentially expressed following toxin exposure. Although these studies have identified proteins that change in response to toxic injury, the relevance of these proteins in other forms of AKI has not been determined.

A modification of the 2-DE technique, called difference gel electrophoresis (DIGE), uses fluorescent labeling of two protein samples that are separated in the same gel along with a third fluorescent label, which is used as an internal standard. DIGE has been used in a rat model of cecal ligation and puncture to study urine protein changes in sepsis-induced ARF [14]. Thirty urinary proteins were changed in rats that developed multi-organ injury and ARF. The authors further investigated the role of one of these altered proteins, the brush border membrane protein, meprin-1α. They tested the effects of actinonin, an inhibitor of brush border membranes on the development of renal failure in a mouse model. Actinonin reduced the rise in serum creatinine. This demonstrates how

changes identified using proteomic analysis can be followed up with more traditional techniques to validate the findings and to identify novel treatment strategies. Urine level of meprin-1α could potentially be used as a marker for renal tubular injury.

Increases in serum creatinine occur late relative to kidney injury and are unreliable during acute injury for a number of reasons. Therefore, early diagnosis of kidney injury is difficult using commonly available methods. An early marker of injury that appears in the urine would help to diagnose AKI when treatment may be more successful. Furthermore, the absence of a predictive marker in AKI has slowed development of new therapies since patients who would benefit the most from new treatments can not be identified until well into the course of the disease. Markers that can both diagnose the injury and predict the magnitude of damage of the renal parenchyma would facilitate testing of new therapies. Zhou et al. [15] used DIGE to analyze changes in urinary exosome proteins associated with renal injury. Exosomes are membrane vesicles that are secreted into the urine from the apical surface of all cells exposed to the luminal surface of the nephron. They contain membrane proteins and intracellular fluid [16, 17]. Urinary exosomal fetuin-A was increased in a cisplatin-induced and an ischemia reperfusion model of AKI. Fetuin-A is synthesized in the liver and is a negative acute phase response protein. The relevance of renal fetuin-A in AKI is not clear but the authors showed that the protein was in the fraction containing the exosomes, it could not be washed from the surface of the exosomes and it was located inside the exosomes by immunoelectron microscopy. In the cisplatin model, urinary fetuin-A was increased 2 days prior to the increase in serum creatinine concentration. To determine if it was a potential biomarker of injury in patients, fetuin A was measured in 3 ICU patients with AKI. The levels were increased in patients with AKI compared to normal control subjects or ICU patients without AKI. Further validation will be required to determine the sensitivity and specificity of fetuin-A in patients with multiple coexisting diseases. Markers identified in this way will need to be validated in larger numbers of patients using techniques that can be used clinically. It is unlikely that a single protein will serve as a universal marker of tubular injury but fetuin-A and other proteins could be members of a panel of markers to diagnose renal injury and predict the magnitude of the injury.

Proteomic analysis has also been used to identify polypeptides that can predict renal injury in the early stages. Nguyen and colleagues used SELDI to search for early markers of AKI in urine [18]. Urine was collected from 60 patients 2 and 6 h after cardiopulmonary bypass (CPB). Mass peaks that correlated with a 50% increase in serum creatinine within 3 days after CPB were found at 6.4, 28.5, 43 and 66 kDa. The identity of these polypeptides was not determined. This study shows the potential of an unbiased approach to biomarker

discovery. In order to be useful for diagnostic or research purposes, the peaks will need to be identified and the findings validated in a different and larger set of patients, preferably using a methodology that is widely available. Identification of early markers such as these that can predict the presence of renal injury and the magnitude of the injury will be very useful.

These studies showed individual proteins that may be useful biomarkers in uncomplicated situations. The proteins have not yet been tested in groups of patients with related or confounding diseases. Other studies that have looked at complex clinical situations have not been able to find individual proteins that can differentiate a single disease from a group of diseases. When Anderson and Anderson [19] published an assessment of the state of the art in proteomics in 2002, they described the rapid increase in the number of proteins that could be resolved by improving proteomics techniques. They contrasted this with the decrease in the number of approvals of diagnostic protein assays by the FDA over the 10-year period of rapid growth in the number of proteins resolved. They attributed much of the difficulty to the large dynamic range of abundance of proteins (at least ten orders of magnitude). While the dynamic range issue is certainly a component of the failure to identify usable biomarkers, another aspect is an underappreciation of the importance of multiple markers to diagnose complex diseases.

The importance of multiple markers may be particularly relevant in ARF. The injury and host responses to kidney injury are dynamic processes. Proteins that correlate with these processes will change over time. Concentrations of urine proteins associated with early injury may increase and then decrease before other proteins associated with recovery from injury have even begun to increase. Neutrophil gelatinase-associated lipocalin is a protein that appears in the urine early after a well-defined acute event such as injury that occurs during CPB [20]. An example of the changes in a protein that reflects acute injury is shown by the pink line (Injury 1) in figure 1. The abundance of the protein increases and decreases rapidly. The maximum abundance of the protein may reflect the magnitude of the injury but a single measurement is unlikely to capture the concentration at its peak. Therefore, a single measurement of the protein will not be informative about the prognosis if the time of the measurement relative to the injury is not known. In most clinical situations, the time factor is not known. Measurement of the protein at slightly different time points will result in large differences in the value obtained. In addition, most renal injuries occur over a period of time rather than at a single discrete time, which further confounds the interpretation of urinary abundances of acute injury proteins. Other proteins may increase later after injury because they require synthesis of new protein. The yellow line (Injury 2) in the figure is representative of this type of protein. Combined analysis of these two proteins will improve the interpretation

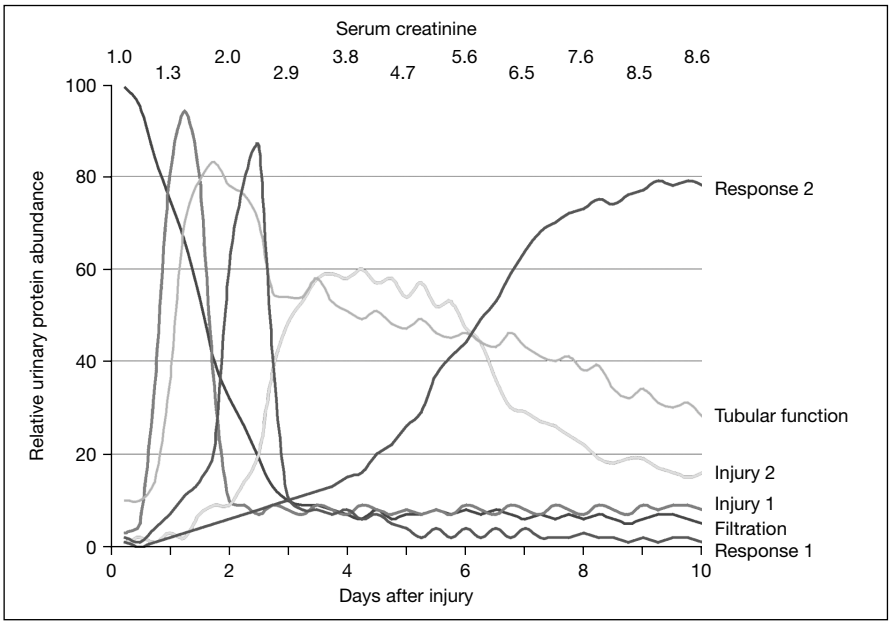


Fig. 1. Theoretical changes in urine proteins over time in arbitrary units. Abundances of individual urine proteins change relative to the time that injury occurred. The abundance of an individual protein can reflect a snapshot of the response to one facet of the disease. For instance, one protein may be increased by injury to the tubule while another may increase in association with the response of the tubule to the injury. Measurement of a single protein cannot predict the outcome of the patient without knowledge of what is happening to other markers of the injury response. In the example shown here, the patient's serum creatinine (mg/dl) increases over several days after the initial injury. Proteins reflecting two different types of injury increase at different times as do two different injury response proteins. Proteins that reflect glomerular filtration and tubular function are also shown.

of the injury. Another group of proteins reflects the inflammatory response to the injury, which may have a detrimental effect. An example of this type of protein is shown by the orange (Response 1) line in the figure. Still, other proteins may reflect a protective response to the injury and be associated with an improved outcome. An example of a protein in this group may be hepatocyte growth factor represented by the brown line (Response 2). Finally, other groups of proteins may reflect glomerular filtration (dark blue line) or tubular function (light blue line). An analysis that uses proteins which reflect multiple components of the injury and response to injury can more accurately diagnose the magnitude of the injury and predict the prognosis of the patient.

Analysis of these types of data can be difficult even if one knows the effect that is being measured with each protein since the values of each protein are independent of the others and do not have a linear correlation with other protein abundance values. Because of the nonlinear relationship of the proteins, informatic algorithms are required for analysis. Two recently published studies have used combinations of urine biomarkers to predict renal diseases. Liangos et al. [21] prospectively examined the ability of a combination of two markers to predict adverse outcomes (dialysis or death) in patients with ARF. They measured urinary activity of the brush border enzyme N-acetyl-β-glucosaminidase (NAG) and urinary concentrations of kidney injury molecule 1 (KIM-1). NAG activity is associated with tubular damage and KIM-1 level is associated with tubular cell dedifferentiation and injury. Area under the ROC curve (AUC) is a standard assessment of the quality of a test. The combination of KIM-1 and NAG produced an AUC of 0.71. The combination of KIM-1 and NAG with four clinical variables produced an AUC of 0.80. This demonstrates that combinations of biomarkers (and clinical information) can lead to improved outcomes. We have used 2-DE and informatic analysis to identify a set of proteins that can predict which of four glomerular diseases is present in a patient with proteinuria [22]. In this study, twenty-one protein spots were most important for the differentiation of patients with focal segmental glomerulosclerosis, diabetic nephropathy, lupus nephritis and membranous nephropathy. The accuracy of the prediction decreased rapidly when fewer proteins were included in the analysis demonstrating the importance of multiple proteins. The spots were identified by mass spectrometry as charge forms of eleven plasma proteins: orosomucoid, transferrin, α_1-microglobulin, Zn-α_2-glycoprotein, α_1-antitrypsin, complement factor B, haptoglobin, transthyretin, plasma retinol-binding protein, albumin and hemopexin. These studies suggest that combination of abundances of multiple proteins or their pattern will be necessary to differentiate complex diseases or predict prognoses.

We performed a study using 2-DE and informatics analysis to differentiate between patients with increases in serum creatinine by two causes of ARF, acute tubular necrosis (ATN) or prerenal azotemia (PRA) [unpubl. data]. Urine proteins from 19 patients with ATN and 19 patients with PRA were separated by 2-DE. We used artificial neural networks (ANN) to identify sets of protein markers that could predict the disease. The ANN algorithm was tested in a novel validation set of patients with ARF. An ROC curve was generated for this validation set with a total AUC of 0.88. A nonlinear relationship (called an XOR interdependency) of two proteins was found to be responsible for the accuracy of the test. We have published the complete data set on the web through the AGML database [23, 24]. Images of all the gels and raw abundance data for all spots across all gels can be viewed in the database of the public login area of

AGML central at http://www.agml.org. A list of publicly available data can be found by clicking on the 'Database' tab at the top of the screen. The file is called 'Acute renal failure analysis_Two_Diseases_ATN_PRA'. Deposition of the data in the AGML Central repository makes it available so that the quality of the data can be analyzed, the findings can be confirmed using the raw data, and further analysis of the data is possible by other investigators accessing the repository. All of the information about the experiment can be accessed via the tools. Representations of the images with spot IDs can be obtained using the 'AGML visualizer'. Complete details for the protocol can be obtained under 'View Protocol'. All images can be viewed and downloaded as TIF images under 'Images'. The protein abundances for all spots can be downloaded as a comma delimited text file by clicking on the label 'spreadsheet.csv'. The complete list of all protein information can be downloaded as an AGML file in XML format under 'AGML XML'. This data repository allows any user to download and analyze the data. We have further analyzed these data to identify prognostic markers in ARF.

Prediction of Prognosis in AKI by Urine Protein Patterns

The clinical course of ARF is highly variable. Early in the course of the disease, it is difficult to determine which patients will require some form of RRT such as hemodialysis or continuous venovenous hemofiltration. No assays are available currently that can predict who will need RRT and who will not. The lack of markers makes treatment of individual patients more difficult and limits the ability to test new therapies. Prognostic markers are urgently needed to identify groups of patients who are at higher risk of developing renal failure in order to test new therapies. To determine if patterns of urine protein abundance as measured by 2-DE could predict the prognosis of a patient with AKI, we analyzed protein abundances from 19 patients with ATN that we previously used to identify biomarkers that predict the cause of disease. RRT requirement was determined by review of the chart to identify those patients that had any form of RRT during the admission. Figure 2 shows the serum creatinine at the time the samples were collected in the group of patients who required RRT and the group that did not. Serum creatinine values were not statistically different between the groups. The figure shows that there is a large amount of overlap between groups and that serum creatinine at the time the sample was collected could not be used to estimate which patients would require RRT. Urine was collected at the time of consult by the nephrology service. Ten patients from the group were randomly assigned to a set used to train an ANN, while the data from the remaining 9 patients was kept separate to validate the results after the

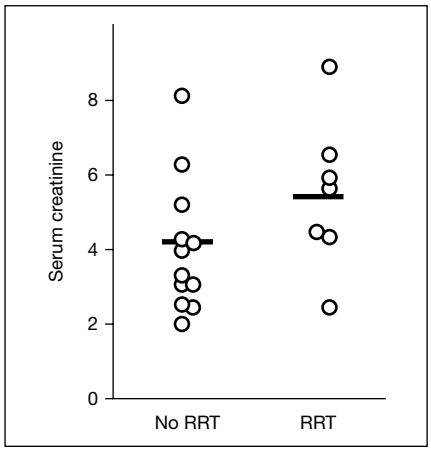


Fig. 2. Serum creatinine at time of consult. Circles show individual creatinine values (mg/dl) for the 11 patients in the group that did not require RRT and the 7 patients in the group that did require it. The solid bars show the mean serum creatinine values for the two groups.

networks were trained. The abundance levels of 232 spots were used to train the network to predict whether the patient would require RRT or not. The training set contained 7 patients that did not require RRT and 3 patients that did require RRT. The network was trained as we previously described using a cross-validation fraction of 1/9 [22]. Within the training set, all 10 patients were predicted correctly. Seventeen protein spots contributed 1% or more of the sensitivity to the analysis (fig. 3). Seven of the spots were identified by mass spectrometry, including α_1-microglobulin (3 spots), Zn-α_2-glycoprotein, α_1-antitrypsin (2 spots) and gelsolin. Validation of the findings of the ANN analysis is important to ensure that the prediction was not based on overfitting of the algorithm to the data. The purpose of the randomly selected validation set was to provide an unbiased assessment of the accuracy of the diagnostic algorithm. The data from the randomly selected, independent test set were applied to the ANN algorithm to test the validity of the algorithm in a novel set. The test set contained 9 patients with ATN of whom four required RRT. Table 1 lists the characteristics of the patients in the independent test set. The serum creatinine and urine sodium values were determined at the time the urine samples were collected. Some of the serum creatinine values are relatively high since the samples were originally obtained to attempt to predict the disease present and not whether the patients would require RRT. The test correctly predicted the future course of renal failure in 7 of the patients (78% accuracy). The test correctly predicted 3 of the 4 patients who would require RRT (75% sensitivity) and 4 of the 5 who would not (80% specificity; fig. 4). These data provide evidence that patterns of urine proteins on 2-D gels can predict the requirement for RRT.

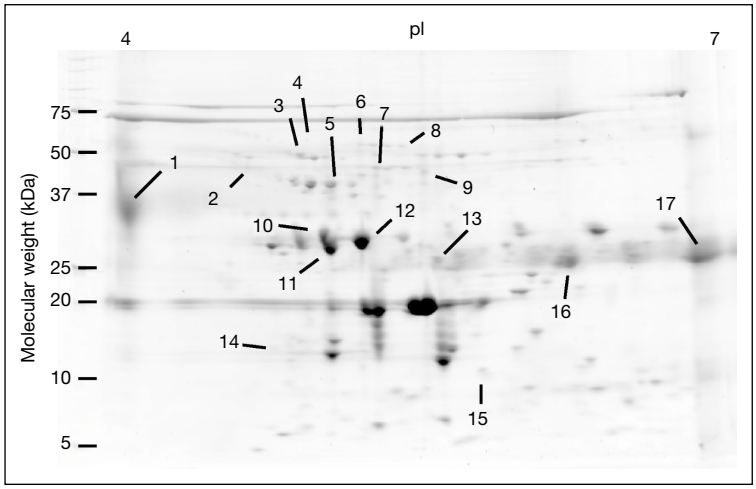


Fig. 3. Two-dimensional gel image of proteins that predict the need for RRT in patients with ATN. Seventeen proteins that contributed 1% or more of the predictability of the ANN analysis are shown. We identified seven of these proteins; including α_1-antitrypsin (spots 3 and 4), Zn-α_2-glycoprotein (spot 5), gelsolin (spot 7), and α_1-microglobulin (spots 10–12).

Table 1. Characteristics of patients in the independent test set used to predict the need for dialysis in AKI

Patient no.	Age, years	Sex	Race	Dialysis		Serum creatine mEq/l	Urine sodium mg/dl
				required	predicted		
1	39	M	Caucasian	no	yes	3	89
2	31	M	African-American	no	no	2	95
3	45	F	African-American	no	no	5.2	40
4	68	M	Caucasian	no	no	4	46
5	47	F	African-American	no	no	4.3	52
6	22	F	African-American	yes	yes	5.9	40
7	21	M	African-American	yes	yes	4.3	78
8	55	M	African-American	yes	no	4.4	13
9	82	M	Caucasian	yes	yes	6.5	34

Conclusions

AKI is an important clinical problem with a high mortality rate. Although currently there are no good tests to predict which patients will require RRT, the few studies that have been done offer hope that we will be able to predict

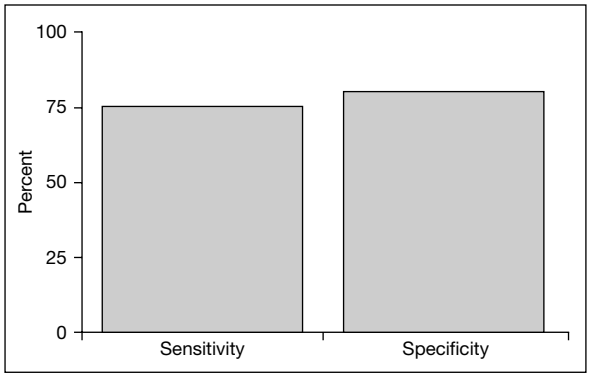


Fig. 4. Accuracy of predictive markers for RRT in patients with ATN. The markers algorithm was trained in a set of 10 patients and validated in a second set of 9 patients that had not been used to train the network. The test correctly predicted the future course of renal failure in 7 of the 9 patients in the validation set (78% accuracy) including 3 of the 4 patients who would require RRT (75% sensitivity) and 4 of the 5 who would not (80% specificity).

diagnosis and prognosis in the not too distant future. Recent studies have used cDNA microarrays and proteomics to identify a number of new candidate markers. Because of the heterogeneity of diseases and the changes over time in urine proteins after injury, single proteins are unlikely to be good markers. Combinations of proteomic methods to identify multiple proteins and bioinformatic techniques to analyze them offer the best chance of identifying new markers. Discovery of these markers will enable combinations of them to be used in new diagnostic and prognostic tests.

Acknowledgements

Support for this project came from the Department of Veterans Affairs and grants from Dialysis Clinics, Inc., and the NHLBI Proteomics Initiative from the National Heart, Lung, and Blood Institute, National Institutes of Health, under contract No. N01-HV-28181.

References

1 Obialo CI, Okonofua EC, Tayade AS, Riley LJ: Epidemiology of de novo acute renal failure in hospitalized African Americans: comparing community-acquired vs hospital-acquired disease. Arch Intern Med 2000;160:1309–1313.

2 Brivet FG, Kleinknecht DJ, Loirat P, Landais PJ: Acute renal failure in intensive care units–causes, outcome, and prognostic factors of hospital mortality; a prospective, multicenter study. French Study Group on Acute Renal Failure. Crit Care Med 1996;24:192–198.

3 Kellum JA, Levin N, Bouman C, Lameire N: Developing a consensus classification system for acute renal failure. Curr Opin Crit Care 2002;8:509–514.
4 Bellomo R, Ronco C, Kellum JA, Mehta RL, Palevsky P: Acute renal failure – definition, outcome measures, animal models, fluid therapy and information technology needs: the Second International Consensus Conference of the Acute Dialysis Quality Initiative (ADQI) Group. Crit Care 2004;8:R204–R212.
5 Hoste EA, Clermont G, Kersten A, Venkataraman R, Angus DC, De Bacquer D, Kellum JA: RIFLE criteria for acute kidney injury are associated with hospital mortality in critically ill patients: a cohort analysis. Crit Care 2006;10:R73.
6 Uchino S, Bellomo R, Goldsmith D, Bates S, Ronco C: An assessment of the RIFLE criteria for acute renal failure in hospitalized patients. Crit Care Med 2006;34:1913–1917.
7 Witzmann FA, Fultz CD, Grant RA, Wright LS, Kornguth SE, Siegel FL: Regional protein alterations in rat kidneys induced by lead exposure. Electrophoresis 1999;20:943–951.
8 Kanitz MH, Witzmann FA, Zhu H, Fultz CD, Skaggs S, Moorman WJ, Savage RE Jr: Alterations in rabbit kidney protein expression following lead exposure as analyzed by two-dimensional gel electrophoresis. Electrophoresis 1999;20:2977–2985.
9 Witzmann FA, Daggett DA, Fultz CD, Nelson SA, Wright LS, Kornguth SE, Siegel FL: Glutathione S-transferases: two-dimensional electrophoretic protein markers of lead exposure. Electrophoresis 1998;19:1332–1335.
10 Charlwood J, Skehel JM, King N, Camilleri P, Lord P, Bugelski P, Atif U: Proteomic analysis of rat kidney cortex following treatment with gentamicin. J Proteome Res 2002;1:73–82.
11 Witzmann FA, Bauer MD, Fieno AM, Grant RA, Keough TW, Lacey MP, Sun Y, Witten ML, Young RS: Proteomic analysis of the renal effects of simulated occupational jet fuel exposure. Electrophoresis 2000;21:976–984.
12 Witzmann FA, Carpenter RL, Ritchie GD, Wilson CL, Nordholm AF, Rossi J III: Toxicity of chemical mixtures: proteomic analysis of persisting liver and kidney protein alterations induced by repeated exposure of rats to JP-8 jet fuel vapor. Electrophoresis 2000;21:2138–2147.
13 Xu H, Hu LS, Chang M, Jing L, Zhang XY, Li GS: Proteomic analysis of kidney in fluoride-treated rat. Toxicol Lett 2005.
14 Holly MK, Dear JW, Hu X, Schechter AN, Gladwin MT, Hewitt SM, Yuen PS, Star RA: Biomarker and drug-target discovery using proteomics in a new rat model of sepsis-induced acute renal failure. Kidney Int 2006;70:496–506.
15 Zhou H, Pisitkun T, Aponte A, Yuen PS, Hoffert JD, Yasuda H, Hu X, Chawla L, Shen RF, Knepper MA, Star RA: Exosomal Fetuin-A identified by proteomics: a novel urinary biomarker for detecting acute kidney injury. Kidney Int 2006;70:1847–1857.
16 Pisitkun T, Shen RF, Knepper MA: Identification and proteomic profiling of exosomes in human urine. Proc Natl Acad Sci USA 2004;101:13368–13373.
17 Zhou H, Yuen PS, Pisitkun T, Gonzales PA, Yasuda H, Dear JW, Gross P, Knepper MA, Star RA: Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery. Kidney Int 2006;69:1471–1476.
18 Nguyen MT, Ross GF, Dent CL, Devarajan P: Early prediction of acute renal injury using urinary proteomics. Am J Nephrol 2005;25:318–326.
19 Anderson NL, Anderson NG: The Human Plasma Proteome: History, Character, and Diagnostic Prospects. Mol Cell Proteomics 2002;1:845–867.
20 Mishra J, Ma Q, Prada A, Mitsnefes M, Zahedi K, Yang J, Barasch J, Devarajan P: Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. J Am Soc Nephrol 2003;14:2534–2543.
21 Liangos O, Perianayagam MC, Vaidya VS, Han WK, Wald R, Tighiouart H, Mackinnon RW, Li L, Balakrishnan VS, Pereira BJ, Bonventre JV, Jaber BL: Urinary N-Acetyl-{beta}-(D)-Glucosaminidase Activity and Kidney Injury Molecule-1 Level Are Associated with Adverse Outcomes in Acute Renal Failure. J Am Soc Nephrol 2007;18:904–912.
22 Varghese SA, Powell TB, Budisavljevic MN, Oates JC, Raymond JR, Almeida JS, Arthur JM: Urine biomarkers predict the cause of glomerular disease. J Am Soc Nephrol 2007;18:913–922.

23 Stanislaus R, Jiang LH, Swartz M, Arthur JM, Almeida JS: An XML standard for the dissemination of annotated 2D gel electrophoresis data complemented with mass spectrometry results. BMC Bioinformatics 2004;5:9.
24 Stanislaus R, Chen C, Franklin J, Arthur JM, Almeida JS: AGML central: web based gel proteomic infrastructure. Bioinformatics 2005;21:1754–1757.

John M. Arthur
Division of Nephrology, Medical University of South Carolina
Charleston, SC, 96 Jonathan Lucas Street
PO Box 250623, Charleston, SC 29425 (USA)
Tel. +1 843 792 4123, Fax +1 843 792 8399, E-Mail arthurj@musc.edu

Thongboonkerd V (ed): Proteomics in Nephrology – Towards Clinical Applications.
Contrib Nephrol. Basel, Karger, 2008, vol 160, pp 65–75

Proteomics and Renal Transplantation: Searching for Novel Biomarkers and Therapeutic Targets

Stefan Schaub[a], *John A. Wilkins*[b], *Peter Nickerson*[b]

[a]Department for Transplantation Immunology and Nephrology, University Hospital Basel, Basel, Switzerland; [b]Sections of Biomedical Proteomic and Rheumatology, Manitoba Centre for Proteomics and Systems Biology, Faculty of Medicine, University of Manitoba, Winnipeg, Man., Canada

Abstract

Renal transplantation has emerged as the preferred option for many patients with end-stage renal failure. While significant progress has been achieved in short-term outcomes, long-term survival has only marginally improved. Adaptation of immunosuppressive drugs to the individual needs of every patient at every time point after transplant will be essential to improve long-term outcomes. Thus, assays are required that detect allograft injury very early, which implies frequent noninvasive measurements (e.g. in urine or serum). In this review, we describe important general aspects in urine biomarker discovery using proteomics and discuss currently published studies. Although proteomics has the potential to provide insights into complex pathophysiological processes and reveal novel diagnostic biomarkers as well as therapeutic drug targets, the actual status of urine proteomic activities in renal transplantation is still far from reaching these ambitious goals.

Current Problems in Renal Transplantation

Although short-term renal allograft survival has continuously improved over the last two decades and acute clinical rejection episodes have been significantly reduced, long-term outcome became only marginally better [1, 2]. There are several possible interpretations for this contradictory finding. First, due to the growing gap between the increasing number of patients waiting for a deceased donor organ and the decreasing availability of organs with excellent quality, more marginal donors with pre-existing kidney pathologies have been used in recent years

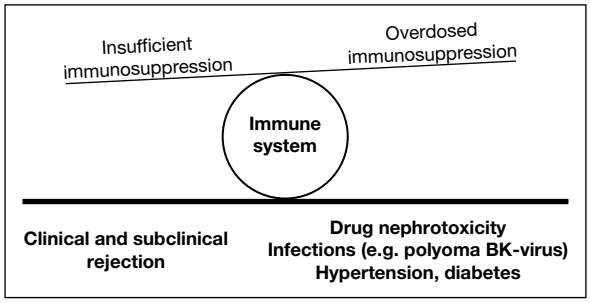


Fig. 1. Balancing the immunosuppression is a key element for successful transplantation.

(i.e. expanded criteria donors). Such organs can provide comparable survival rates in the short-term, but their inferior mass of functional kidney tissue may limit longevity. Second, the benefit of fewer clinical rejection episodes may be partially off-set by side effects of more potent immunosuppression (e.g. drug nephrotoxicity, polyoma BK virus nephropathy). Third, there is still a significant proportion of patients who have undetected subclinical rejection which damages the allograft over years. Therefore, adjusting the level of immunosuppression to the individual patient in order to balance the risk for rejection and overimmunosuppression is essential to improve long-term allograft survival (fig. 1).

Clearly, the major goal in transplantation is to reduce injuries to the allograft. While pre-existing organ damage cannot be influenced, all other insults should be limited. The effects of these insults (i.e. ischemia-reperfusion, rejection, drug-induced nephrotoxicity, infections and hypertension) accumulate over time and lead to progressive destruction of the allograft (fig. 2) [3, 4]. Several studies have shown that even subtle injuries detectable only by protocol allograft biopsies are a risk factor for subsequent deterioration of allograft function and graft loss [5, 6]. Indeed, repeated protocol allograft biopsies would be advisable for patient management and adaptation of the immunosuppressive therapy. However, this strategy is hampered by the small but inherent risk of allograft biopsies (e.g. bleeding, arteriovenous fistula, and infection), the associated costs, and the inconvenience for patients. Therefore, noninvasive biomarkers that allow for early detection of allograft injury and correlate with allograft histology would be helpful.

Diagnostic Requirements to Improve Patient Management

Currently, noninvasive monitoring of renal allograft relies mainly on measurement of serum creatinine. However, several studies have demonstrated that

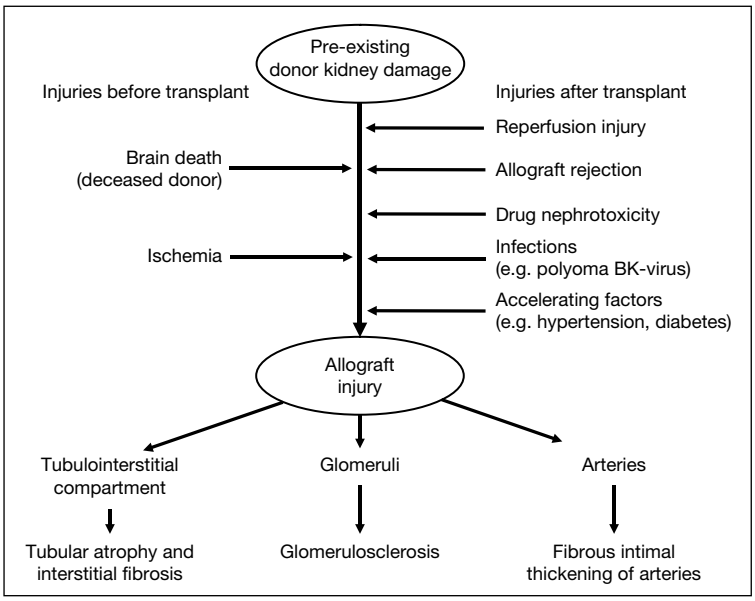


Fig. 2. Overview of factors that can lead to allograft injury. These injuries can be restricted to mainly one compartment of the allograft or can affect all compartments (i.e. tubulointerstitial compartment, glomeruli, arteries). Independent of the disease causing the injury, the final common consequence is tubular atrophy, interstitial fibrosis, glomerulosclerosis and fibrous intimal thickening of arteries, which all represent irreversible damage.

serum creatinine is not sensitive enough to detect clinically important allograft pathologies which can progress to irreversible allograft damage [5, 6]. Therefore, assays are required that detect allograft pathologies before organ damage is severe enough to impact serum creatinine.

As detailed in figure 2, various insults (e.g. rejection, drug toxicity) can injure the allograft. In addition, these insults can affect one or more compartments of the allograft (e.g. tubulointerstitial compartment, glomeruli, arteries). For example, renal allograft rejection can present as tubulointerstitial inflammation (i.e. cellular rejection Banff Ia [7]) or can be restricted to glomeruli and arteries (i.e. antibody-mediated rejection [7]). Calcineurin inhibitors can lead to damage of small vessels or the tubulointerstitial compartment. Independent of the underlying process, the common consequence of the injury is development of irreversible tubular atrophy, interstitial fibrosis, glomerulosclerosis, and fibrous intimal thickening of arteries.

In general, noninvasive biomarkers can be used to monitor the immune response, to assess tissue injury in the three compartments of the renal allograft, or to monitor specific diseases (e.g. polyoma BK virus nephropathy). While immune and injury monitoring have their specific limitations, combining both may enhance the accuracy of noninvasive monitoring [8].

Concept of an Unbiased Proteomics-Based Approach to Develop Novel Biomarkers in Renal Transplantation

Although many noninvasive biomarkers for renal allograft rejection have been proposed, none has found wide clinical application [8]. This highlights that the search for biomarkers enhancing noninvasive monitoring beyond serum creatinine is a difficult task [9]. With the continuously improving proteomic technology, it becomes possible to screen for novel biomarkers in an unbiased way on a broad protein level.

An unbiased proteomics-based approach to develop noninvasive biomarkers involves four steps: (a) establishment of a reproducible technological platform for analysis and determination of sample-related confounders, (b) biomarker discovery phase using well-defined clinical phenotypes, (c) biomarker validation in a strictly independent sample set, and (d) high-throughput assay development.

The first step also involves the decision as to which source (i.e. allograft tissue, serum, urine) for biomarker development will be used. Clearly, allograft tissue offers the potential to analyze the proteins of all cells involved in the investigated process and is therefore an ideal source for biomarker discovery. However, comparative analysis may be complicated by differences in the cellular composition of individual biopsies (e.g. percentage of cortex and medulla) which do not necessarily reflect the process. The use of laser-capture microdissection to select distinct compartments (e.g. glomeruli, tubules, vessels) can circumvent this confounding factor. Once a potential biomarker has been detected and identified in the tissue, it has to be measurable in urine or serum, and these levels have to correlate with the concentrations in the tissue in order to become a useful noninvasive biomarker. Serum and urine as sources for biomarker development have the advantage that collection of sufficient material is not a major issue. However, proteomic analysis in serum is hampered by its complexity. Ten high-abundance proteins (e.g. albumin, immunoglobulins) account for $>95\%$ of the total protein content [10]. These proteins, which are unlikely to provide any useful information regarding the allograft, must be removed to allow detection of the remaining lower-abundance proteins. Urine as a specimen for proteomic analysis may offer some potential

advantages because (a) it is in direct contact with main targets of rejection and other harmful processes (i.e. tubular epithelial cells) and (b) it may represent the whole kidney allograft. However, urine has variable and changing physicochemical properties (dilution, pH) and cellular components (epithelial cells, leukocytes, red blood cells), which can affect its protein content [11]. Furthermore, stability of proteins under these changing conditions may be impaired [12].

As biomarker discovery is often performed with few samples, it is essential that these samples are carefully selected and that they represent a distinct and clinically important phenotype, and include equally well-defined control groups to enhance the significance of the detected biomarkers. In the biomarker validation step, a larger but clearly independent sample set should be used. If known biomarkers for the investigated disease/process already exist, they should be analyzed in parallel to determine the diagnostic value of the novel biomarker in comparison with existing biomarkers. Most efforts are currently concentrated in the biomarker discovery phase; however, the validation phase is critical and only few potential biomarkers have undergone this step.

Current Status of Proteomic Studies in Renal Transplantation

General Aspects

Currently published proteomic investigations in human renal transplantation are limited to studies aiming to detect novel urine biomarkers for specific pathologies (i.e. allograft rejection, polyoma BK virus nephropathy) [13–17]. Urine might be a valuable source for biomarker development of processes primarily affecting the tubulointerstitial compartment (e.g. tubulointerstitial rejection, polyoma BK virus nephropathy, drug toxicity), because urine is the only biological fluid that is in direct contact with tubular epithelial cells. In addition, urine may reflect the whole allograft overcoming the inherent limitation of allograft biopsies to miss focal processes due to sampling error [18, 19]. Indeed, urine protein analysis might be of particular interest to screen for early and subtle processes targeting the tubulointerstitial compartment.

Although there are several different proteomic platforms, high-throughput technologies such as surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) [13–15, 17] and capillary electrophoresis coupled to mass spectrometry (CE-MS) [16] were used in all currently published studies searching for novel urine biomarkers in renal transplantation. Therefore, in the following paragraphs these two platforms will be described and discussed in more detail, while referring to recently published

reviews regarding advantages and limitations of other proteomic approaches [20, 21].

SELDI-TOF MS combines matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) with surface retentate chromatography. Specifically, a sample is applied to a chip surface carrying a functional group (e.g. normal phase, hydrophobic, cation or anion exchange). After incubation, proteins that do not bind to the surface are removed by a simple wash step, and bound peptides/proteins are analyzed by mass spectrometry. This approach reduces the complexity of the sample being analyzed by selecting only a subset of the total proteins. Spectra of samples from different groups (e.g. acute rejection vs. no rejection) can now be analyzed for differences in their respective proteomes. The advantages of SELDI-TOF MS are its user friendliness and high-throughput capabilities [11]. The major disadvantages are a limited sensitivity to detect proteins and a low resolution and mass accuracy of the generated spectra [8, 22]. Therefore, only a restricted part of the proteome is accessible for analysis by SELDI-TOF MS.

CE-MS combines protein separation by electrophoresis coupled to an electrospray source for on-line mass spectrometric analysis. This platform provides fast analysis with high resolution and good mass accuracy of peptides/proteins smaller than 10 kDa. Limitations of CE-MS are the restriction of the investigation to small proteins and a limited sensitivity to detect proteins because only a small sample volume can be injected into the capillary. Both SELDI-TOF-MS and CE-MS were criticized because most detected potential biomarkers were not identified. Notably, the generated peptide/protein pattern analyzed with sophisticated bioinformatics can be used itself as a diagnostic assay (protein pattern diagnostic), or significantly different expressed proteins can be identified, which allows to develop quantitative, high-throughput assays (i.e. ELISA). It is unknown at this point which approach (protein pattern diagnostics or protein identification/ELISA assay development) will reveal more robust diagnostic markers that can be utilized in a clinical setting [23, 24].

Published Studies Using Proteomics in Human Transplantation

Table 1 summarizes all studies published until January 2007. Four research groups aimed to detect urine proteins associated with renal allograft rejection (in most cases tubulointerstitial rejection), one group investigated urine proteins associated with polyoma BK virus nephropathy. Although these are two different pathological processes, both lead to injury in the tubulointerstitial compartment with a subsequent tissue response, which might be a common feature. Interestingly, each group found a different set of urinary proteins that are associated with the investigated process. To understand these apparent discrepancies, one must consider that in each study disease definition, sample collection,

Table 1. Published studies using proteomics in human renal transplantation

Reference	Proteomic platform	Discovery of bio-markers for	Use of bio-informatics	Peptides/proteins detected	Biomarker identified	Independent validation performed	Validation outcome
Clarke et al. [13]	SELDI-TOF MS	Allograft rejection	Yes	6,500, 6,600, 6,700, 7,100, 13,400 Da	No	No	
Schaub et al. [14, 22, 25]	SELDI-TOF MS	Allograft rejection	No	Three peak clusters at 5,270–5,550, 7,050-7,360, and 10,530-11,100 Da	Cleaved β_2-microglobulin	Yes	Biomarker is confounded by urine pH Not specific for rejection Similar performance as other tubular injury biomarkers
O'Riordan et al. [15, 26]	SELDI-TOF MS	Allograft rejection	Yes	2,003, 2,802, 4,756, 5,872, 6,990, 19,018, 25,665 Da	β-Defensin 1, fragment of α_1-antichymotrypsin	No	
Wittke et al. [16]	CE-MS	Allograft rejection	Yes	16 peptides (5 upregulated): 1,168, 1,707, 2,078, 2,121, 3,359 Da	No	Yes	66% correctly classified as rejection
Jahnukainen et al. [17]	SELDI-TOF MS	Polyoma-nephropathy	Partially	5,872, 11,311, 11,929, 12,727, 13,349 Da	No	No	

sample handling, protocol for protein separation/visualization, and data analysis were not identical. This complicates direct comparison of these studies and highlights the need for some standardization in disease definition, preanalytical sample handling, and sample analysis.

Using SELDI-TOF MS, O'Riordan et al. [15, 26] found that decreasing levels of urinary β-defensin-1 and increasing levels of a fragment of α_1-antichymotrypsin were associated with renal allograft rejection. Although we identified a different protein as a potential biomarker for renal allograft rejection (i.e. cleaved β_2-microglobulin), in both studies fragments of a protein were predictive for the pathology. Low molecular weight fragments of a protein may simply be waste products that are even less informative than the intact protein form. However, they may also indicate increased protease activity associated with the allograft rejection process and may therefore provide important information. Clearly, the significance of protein fragments in the urine as biomarkers requires thorough investigation of the fragment, the intact protein, the responsible proteases and factors that activate them.

Protein identification of a potential biomarker is essential for several reasons. First, knowing the protein/peptide can help to understand their pathophysiology in the investigated process. Indeed, in our study we identified the previously detected potential biomarker for tubulointerstitial renal allograft rejection as cleaved β_2-microglobulin. As intact β_2-microglobulin is a well-known biomarker for tubular injury, it became obvious that cleaved β_2-microglobulin was unlikely to be specific for rejection but rather an indicator of tubular injury [27]. O'Riordan et al. [26] identified β-defensin-1 and a fragment of α_1-antichymotrypsin as their previously detected biomarkers for renal allograft rejection, which are both involved in inflammatory processes. The other three groups have not yet identified their potential biomarkers (table 1) [13, 16, 17]. The second important reason for protein identification is that it allows one to select adequate control groups for a subsequent validation study and to identify major confounding factors (e.g. urine pH, urine cell components, high proteinuria).

As already discussed above, validation in an independent sample set is the next critical step after detection of a potential biomarker. So far, only two of the five groups have performed a validation study. Wittke et al. [16] used CE-MS to analyze urines regarding peptide pattern associated with renal allograft rejection. In a small validation set, they could correctly classify 66% of samples as rejection. Our group used a validation sample set that was obtained in another center with refined control groups and side-by-side evaluation of comparable biomarkers. In fact, we could confirm the prevalence of cleaved β_2-microglobulin in patients with clinical tubulointerstitial rejection and stable transplants with normal tubular histology. However, the validation study revealed that cleaved

β_2-microglobulin is (a) expectedly not specific for rejection, (b) unable to distinguish normal tubular histology from subclinical tubulointerstitial rejection, (c) similar to the other investigated biomarkers for tubular injury (retinol-binding protein, neutrophil-gelatinase-associated lipocalin, and α_1-microglobulin), and (d) confounded by urine pH restricting its clinical usefulness [25]. These two studies highlight that validation is a key element in biomarker development and that many identified potential biomarkers will not pass this step.

Conclusions

With the low rejection and high short-term allograft survival rates that have been achieved in renal transplantation, the focus will shift to improve long-term outcomes. A major goal will be to tailor immunosuppression to the individual needs of every patient at every time point to balance risk for rejection and over-immunosuppression. To achieve this, novel biomarkers are necessary to detect subtle forms of allograft rejection and allograft injury, and to allow adapting immunosuppression before irreversible damage to the allograft has occurred.

Unbiased proteomics-based approaches raise the hope to reveal molecular mechanisms of allograft rejection and injury, which could translate into novel biomarkers. So far, no biomarker identified by an unbiased proteomics-based approach has found a clinical application. As detailed above, the currently published approaches were restricted to analysis of urine using high-throughput technology (i.e. CE-MS, SELDI-TOF MS), which can only assess a limited part of the proteome. The fast and continuous developments in the field of proteomics including more sensitive mass spectrometers with higher mass accuracy, differential protein expression technology (e.g. stable isotope labeling), and analysis of allograft tissue parts selected by laser-capture microdissection may allow gaining deeper insights into changes of the proteome associated with renal allograft rejection and/or injury. Eventually, these data may also reveal potential targets for future drug development.

References

1 Meier-Kriesche HU, Schold JD, Kaplan B: Long-term renal allograft survival: have we made significant progress or is it time to rethink our analytic and therapeutic strategies? Am J Transplant 2004;4:1289–1295.

2 Meier-Kriesche HU, Schold JD, Srinivas TR, Kaplan B: Lack of improvement in renal allograft survival despite a marked decrease in acute rejection rates over the most recent era. Am J Transplant 2004;4:378–383.

3 Pascual M, Theruvath T, Kawai T, Tolkoff-Rubin N, Cosimi AB: Strategies to improve long-term outcomes after renal transplantation. N Engl J Med 2002;346:580–590.
4 Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Allen RD, Chapman JR: The natural history of chronic allograft nephropathy. N Engl J Med 2003;349:2326–2333.
5 Rush D: Protocol transplant biopsies: an underutilized tool in kidney transplantation. Clin J Am Soc Nephrol 2006;1:138–143.
6 Moreso F, Ibernon M, Goma M, Carrera M, Fulladosa X, Hueso M, et al: Subclinical rejection associated with chronic allograft nephropathy in protocol biopsies as a risk factor for late graft loss. Am J Transplant 2006;6:747–752.
7 Racusen LC, Colvin RB, Solez K, Mihatsch MJ, Halloran PF, Campbell PM, et al: Antibody-mediated rejection criteria – an addition to the Banff 97 classification of renal allograft rejection. Am J Transplant 2003;3:708–714.
8 Schaub S, Wilkins JA, Rush D, Nickerson P: Developing a tool for noninvasive monitoring of renal allografts. Expert Rev Proteomics 2006;3:497–509.
9 Hewitt SM, Dear J, Star RA: Discovery of protein biomarkers for renal diseases. J Am Soc Nephrol 2004;15:1677–1689.
10 Tirumalai RS, Chan KC, Prieto DA, Issaq HJ, Conrads TP, Veenstra TD: Characterization of the low molecular weight human serum proteome. Mol Cell Proteomics 2003;2:1096–1103.
11 Schaub S, Wilkins J, Weiler T, Sangster K, Rush D, Nickerson P: Urine protein profiling with surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry. Kidney Int 2004;65: 323–332.
12 Donaldson MD, Chambers RE, Woolridge MW, Whicher JT: Stability of α1-microglobulin, β2-microglobulin and retinol binding protein in urine. Clin Chim Acta 1989;179:73–77.
13 Clarke W, Silverman BC, Zhang Z, Chan DW, Klein AS, Molmenti EP: Characterization of renal allograft rejection by urinary proteomic analysis. Ann Surg 2003;237:660–665.
14 Schaub S, Rush D, Wilkins J, Gibson IW, Weiler T, Sangster K, et al: Proteomic-based detection of urine proteins associated with acute renal allograft rejection. J Am Soc Nephrol 2004;15:219–227.
15 O'Riordan E, Orlova TN, Mei JJ, Butt K, Chander PM, Rahman S, et al: Bioinformatic analysis of the urine proteome of acute allograft rejection. J Am Soc Nephrol 2004;15:3240–3248.
16 Wittke S, Haubitz M, Walden M, Rohde F, Schwarz A, Mengel M, et al: Detection of acute tubulointerstitial rejection by proteomic analysis of urinary samples in renal transplant recipients. Am J Transplant 2005;5:2479–2488.
17 Jahnukainen T, Malehorn D, Sun M, Lyons-Weiler J, Bigbee W, Gupta G, et al: Proteomic analysis of urine in kidney transplant patients with BK virus nephropathy. J Am Soc Nephrol 2006;17: 3248–3256.
18 Sorof JM, Vartanian RK, Olson JL, Tomlanovich SJ, Vincenti FG, Amend WJ: Histopathological concordance of paired renal allograft biopsy cores. Effect on the diagnosis and management of acute rejection. Transplantation 1995;60:1215–1219.
19 Nicholson ML, Wheatley TJ, Doughman TM, White SA, Morgan JD, Veitch PS, et al: A prospective randomized trial of three different sizes of core-cutting needle for renal transplant biopsy. Kidney Int 2000;58:390–395.
20 Baggerman G, Vierstraete E, De Loof A, Schoofs L: Gel-based versus gel-free proteomics: a review. Comb Chem High Throughput Screen 2005;8:669–677.
21 Steel LF, Haab BB, Hanash SM: Methods of comparative proteomic profiling for disease diagnostics. J Chromatogr B Analyt Technol Biomed Life Sci 2005;815:275–284.
22 Schaub S, Wilkins JA, Antonovici M, Krokhin O, Weiler T, Rush D, et al: Proteomic-Based Identification of Cleaved Urinary β2-microglobulin as a Potential Marker for Acute Tubular Injury in Renal Allografts. Am J Transplant 2005;5:729–738.
23 Zolg W: The proteomic search for diagnostic biomarkers: lost in translation? Mol Cell Proteomics 2006;5:1720–1726.
24 Diamandis EP: Mass spectrometry as a diagnostic and a cancer biomarker discovery tool: opportunities and potential limitations. Mol Cell Proteomics 2004;3:367–378.
25 Schaub S, Mayr M, Hönger G, Bestland J, Steiger J, Regeniter A, et al: Detection of Subclinical Tubular Injury after Renal Transplantation: Comparison of Urine Protein Analysis with Allograft Histopathology. Transplantation 2007; in press.

26 O'Riordan E, Orlova TN, Podust VN, Chander PN, Yanagi S, Nakazato M, et al: Characterization of urinary Peptide biomarkers of acute rejection in renal allografts. Am J Transplant 2007;7: 930–940.
27 Schardijn GH, Statius van Eps LW: β2-microglobulin: its significance in the evaluation of renal function. Kidney Int 1987;32:635–641.

Stefan Schaub, MD, MSc
Department for Transplantation Immunology and Nephrology, University Hospital Basel
Petersgraben 4
CH–4031 Basel (Switzerland)
Tel. +41 61 265 4533, Fax +41 61 265 2410, E-Mail schaubs@uhbs.ch

Thongboonkerd V (ed): Proteomics in Nephrology – Towards Clinical Applications.
Contrib Nephrol. Basel, Karger, 2008, vol 160, pp 76–87

Metabolomics: A Complementary Tool in Renal Transplantation

David S. Wishart

Departments of Biological Sciences and Computing Science, University of Alberta, National Research Council, National Institute for Nanotechnology, Edmonton, Alta., Canada

Abstract

Renal transplant success is closely tied to the ability to monitor transplant recipients and responsively change their medications. However, transplant monitoring still depends on relatively dated technologies – serum creatinine levels, urine output, and histopathology of biopsy samples. These techniques do not offer sufficient specificity, sensitivity, or accuracy for appropriate and timely interventions. As a result, more specific diagnostic techniques, based on proteomics, genomics and metabolomics are being sought. Metabolomics (the high-throughput measurement and analysis of metabolites) may make it possible to monitor transplants more effectively and specifically. Changes in the concentration profiles of a number of small molecule metabolites found in either blood or urine can be used to localize kidney damage, assess organs at risk of rejection, assess kidneys suffering from ischemia-reperfusion injury or identify organs that have been damaged by immunosuppressive drugs. The application of metabolomics to kidney transplant monitoring is still in its early stages. Nevertheless, there are a number of easily measured metabolites in both urine and serum that can provide reliable indications of kidney function, kidney injury, and immunosuppressive drug toxicity. Metabolomics could serve as a good complement to existing proteomic and genomic technologies.

The first kidney transplant was performed by Joseph Murray at the Peter Bent Brigham Hospital in Boston in 1954 [1]. In the intervening 50 years, kidney transplantation has become the most common and most successful organ transplant operation performed today. Of course, the remarkable success and widespread application of renal transplantation would not be possible without carefully controlled immunosuppression. Before modern immunosuppressive drugs were developed, 1-year graft survival was less than 65% [2]. Thanks to

the development of calcineurin inhibitors, such as tacrolimus and cyclosporin, 1-year organ survival now approaches 90% [3]. However, long-term organ survival is not yet optimal. About 25% of all kidney transplants fail within 5 years after transplantation [4, 5].

Transplants can fail for any number of reasons including pre-operative organ stress, surgical complications, postsurgical infection, acute rejection, or immunosuppressive nephrotoxicity. Organ loss and organ failure are not the only concerns in renal transplantation. Because of the need for long-term immunosuppressive therapies, transplant patients also face increased risks for developing atherosclerosis, bone disease, chronic viral infections (HBV, CMV or BK virus), diabetes, hyperlipidemia, hypertension and lymphoma [6, 7]. To reduce of the risks of postengraftment failure and to mitigate other long-term health complications, kidney transplant recipients must be monitored closely. In particular, their renal and cardiac functions must be checked regularly for signs of infection and immunosuppressive drug toxicity.

However, renal transplant patients continue to be monitored using relatively simplistic clinical measurements – serum creatinine levels, total urine output, body temperature, blood pressure or blood glucose. In many cases, these single-compound assays do not offer the specificity, sensitivity, or accuracy for appropriate and timely interventions. As a result, invasive follow-up biopsies and time-consuming histopathological measurements are often needed to make definitive diagnoses. Recent studies, however, suggest that even these 'gold-standard' histology assays can be problematic [8]. Given these limitations, more and more transplant specialists are looking to the emerging fields of genomics, proteomics and, most recently, metabolomics to improve the current situation.

The hope is that these high-throughput 'omic' techniques could help identify combinations of biomarkers that might be used to inexpensively and noninvasively identify transplantation problems earlier and more robustly than currently possible. As described in other chapters in this book, proteomic methods are increasingly being used to identify urinary protein biomarkers specific to acute rejection [9, 10]. Genomic methods have also found a role in renal transplant monitoring. In fact, a number of rejection-specific genes or transcripts have been identified from kidney biopsies using microarray experiments [11]. However, as we shall see later, small molecule metabolites (i.e. metabolomics) may well prove to be the most useful biomarkers for monitoring kidney function and detecting adverse renal events. This is because the kidney is specifically designed to concentrate or filter small molecule metabolites and small molecule toxins. As a result, one would expect changes in metabolite levels in blood or urine to be more detectable and reflective of kidney function than subtle changes to the kidney proteome or transcriptome [12].

In this chapter, I will describe how measurements of metabolites or metabolic profiles can be used to monitor posttransplant kidney function. Specifically, I will explore three areas in which metabolite measurements or metabolomic studies are beginning to affect the practice of renal transplantation. These include applying metabolomics towards: (1) monitoring ischemia-reperfusion (I/R) injury; (2) monitoring immunosuppressive drug toxicity, and (3) assessing transplant organ function and localizing organ damage. However, before delving into these specific applications, it is perhaps worthwhile to give readers a brief introduction to the field of metabolomics.

A Brief Metabolomics Tutorial

Metabolomics is a newly emerging field of 'omics' research concerned with the high-throughput identification and quantification of the small molecule (<1,000 Da) metabolites in the metabolome. The metabolome is defined as the collection of all small molecule metabolites (endogenous or exogenous) that can be found in a cell, organ or organism. Metabolomics is a relatively new term, having been officially coined in 2000 [13]. Metabolomics is also known as metabonomics [14] or metabolic profiling [15]. As with genomics and proteomics, metabolomics only became possible as a result of recent technological breakthroughs in small molecule separation and identification. These include robust, high-resolution mass spectrometry (MS) instruments for precise mass determination, high-resolution, high-throughput nuclear magnetic resonance (NMR) spectrometers for accelerated compound identification, capillary electrophoresis), high-pressure liquid chromatography (HPLC), and ultra-high pressure liquid chromatography systems for rapid compound separation, and new software programs to rapidly process spectral or chromatographic patterns [16]. These hardware and software innovations have made it possible to identify and quantify not just one or two small molecules at the same time (as is done with clinical chemistry), but dozens of small molecule metabolites in just minutes [16–18].

In the relatively short time that metabolomics has been around, two very distinct schools of thought about how to analyze metabolomic data have emerged. In one version, called chemometrics, compounds are not formally identified; only their spectral patterns and intensities are recorded, compared and used to make diagnoses, identify phenotypes or draw conclusions [14, 17]. In the other version, called targeted profiling, the compounds are formally identified and quantified. The resulting list of compounds and concentrations (a metabolic profile) is then used to make diagnoses, identify phenotypes or draw conclusions [15, 16, 18]. Figure 1 illustrates the conceptual differences

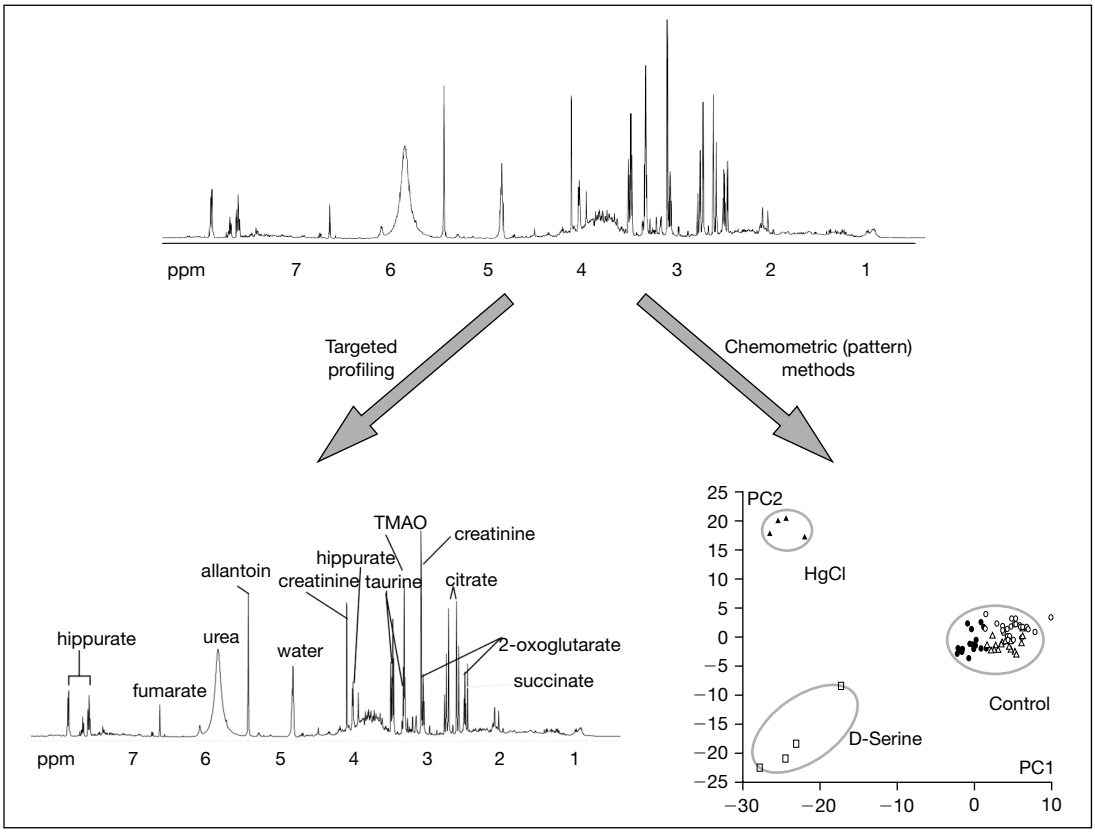


Fig. 1. A graphical illustration showing the difference between chemometric approaches to metabolomics and targeted profiling. In chemometric methods, multiple spectra (MS or NMR) from different samples are compared and the spectral features are clustered using statistical techniques such as principal component analysis. In targeted profiling methods, individual spectra (MS or NMR) are analyzed so that individual peaks are identified and quantified using spectral libraries of known compounds – much as is done with proteomics.

between the two techniques. Both methods have their advantages and disadvantages, although there is a growing preference for targeted profiling in many biomedical applications. Targeted profiling has also been made much easier due to the recent release of the Human Metabolome Database or HMDB [19]. This database is the metabolomic equivalent of GenBank. The HMDB is a web-accessible electronic resource that provides reference NMR and MS spectra (for rapid compound identification), metabolite-disease associations, metabolic

pathway data and reference metabolite concentrations (for diagnostic comparisons) for hundreds of human metabolites from many different biofluids.

Metabolites are normally associated with specific pathways and processes, just as genes and proteins are. As might be expected, most of the small molecule metabolites measured by today's metabolomic techniques are associated with generic metabolic processes (glycolysis, gluconeogenesis, lipid metabolism) found in all living cells. Changes in the relative concentrations of certain 'universal' metabolites such as glucose, citrate, lactate, α-ketoglutarate and others can reflect changes in cell viability (apoptosis), levels of oxygenation (anoxia, ischemia, oxidative stress), local pH, general homeostasis and so on [5]. These molecules can provide useful information about cell function or cell stress and organ function. Other kinds of metabolites are specifically associated with tissue remodeling, muscle atrophy and muscle breakdown, such as methyl-histidine, creatine, taurine and glycine. By noting changes in the levels of these metabolites, it is possible to determine the extent of tissue repair or tissue damage [14, 17]. Some compounds, such as trimethylamine-N-oxide (TMAO), act as buffers to stabilize serum proteins from the effects of accumulated waste products [20]. In short, each metabolite tells a unique story. The challenge for the physician and the scientist is to accurately interpret each one.

Monitoring I/R Injury via Metabolomics

Kidney transplantation is particularly traumatic to a healthy donor organ because the organ must be temporarily removed from a stable blood (and oxygen) supply. The time period without oxygen is known as 'ischemia time'. Obviously, the shorter the ischemia time the better the chance the organ has to recover and function properly. Longer periods of ischemia time can cause serious kidney damage [21]. Ischemia is not the only source of organ damage. Kidney tissue can be further damaged by the reoxygenation or reperfusion process after the transplanted organ is connected to the recipient's blood supply. Reperfusion injury is a term used to describe tissue damage caused when the blood supply returns to the transplanted organ after an extended period of ischemia. This damage is typically caused by white blood cells, inflammatory proteins and free radicals flowing back into the organ during the reperfusion process.

Identifying I/R injury in newly transplanted kidneys is especially challenging. Current methods are relatively simple, using such nonspecific measures as serum creatinine, urine output and biopsies [21]. Because of their limited diagnostic potential, there has been a growing interest in developing more effective biomarkers and less invasive procedures – including metabolomic methods. Most metabolomic studies on I/R injury have been performed on rat models

[21–23] although more recent studies have been extended to humans [24]. In these NMR-based investigations, the more severe the I/R injury, the higher the levels of urinary citrate, dimethylamine, lactate and acetate. Substantially increased levels of allantoin (50–100× normal) and TMAO were also found in the blood where I/R injury had occurred. Allantoin, an oxidative product of uric acid, is a common marker of oxidative cell stress. TMAO is a homeostatic 'rescue' compound that allows blood proteins to handle increased concentrations of urea and guanidine (both strong protein denaturants) that arise during renal failure or renal stress [20, 25]. TMAO is also known to be a marker of renal medullar injury. What is surprising is that serum creatinine levels – which have long been used as an injury marker – have not been found to correlate with the level of ischemia/reperfusion damage [21]. Because of the close similarity between rat and human metabolism, it is likely that the metabolomic findings in these rat models will likely translate well to humans.

In a related study with human subjects suffering I/R injuries, metabolic profiling identified the presence of significantly elevated serum levels of hypoxanthine and inosine (hypoxanthine nucleoside) after kidney reperfusion [24]. Hypoxanthine and inosine are both well-known markers of ischemia and oxidative damage. Both molecules are typically formed as breakdown products of ATP. They are also common by-products of the enzyme xanthine oxidoreductase, an enzyme that converts hypoxanthine to xanthine and then to uric acid. As an oxidase, xanthine oxidoreductase naturally generates superoxide radicals along with other reactive oxygen products, which upon reperfusion and reoxygenation can lead to further oxidative tissue damage. While this I/R injury study did not correlate the levels of I/R injury or graft function with hypoxanthine levels, it does suggest that there are much better metabolic markers for I/R injury than serum creatinine or urine output. Collectively, these studies illustrate that metabolomic methods could significantly improve the monitoring of I/R injury and help us to better understand the effects of ischemia and reperfusion after renal transplantation.

Monitoring Immunosuppressive Drug Toxicity via Metabolomics

Kidney transplant would not be possible without modern immunosuppressive therapies. However, many of today's immunosuppressive drugs are known to be nephrotoxic. Furthermore, extended periods of immunosuppression lead to elevated risks for cardiovascular disease (CVD), diabetes and cancer. It is particularly challenging to detect and monitor these adverse drug effects, because relatively few tests exist for measuring immunosuppressive drug or drug metabolite levels. Indeed no single FDA-approved test exists for detecting

the wide range of known adverse drug effects. Metabolomics may be able to address these challenges. A key advantage of metabolomics over other 'omic' approaches is that it is ideally suited for monitoring small molecule drugs and drug metabolites as well as for tracking the drug-induced changes to organ function and organ metabolism. Monitoring drug toxicity is particularly important for the immunosuppressive drugs cyclosporin, sirolimus and tacrolimus because of their narrow therapeutic index. Furthermore, the efficacy of these potent drugs varies considerably from one individual to the next.

Two cytochrome P450 variants known as CYP3A4 and CYP3A5 metabolize cyclosporin, sirolimus and tacrolimus. Polymorphisms in these enzymes can lead to 'ultrafast' or 'ultraslow' metabolizers of these immunosuppressive drugs. Differences in drug metabolism may have significant effects on organ function and patient health [26]. To help address this potential problem, metabolomic techniques (HPLC-MS) have been developed to rapidly track serum concentrations of cyclosporin (CsA) and five of its known metabolites among transplant recipients [27]. Interestingly, the concentration of one particular CsA metabolite, known as AM19, was found to correlate strongly with several inflammatory and atherosclerotic markers. These data suggest that adverse immunosuppressive drug effects may be predicted and mitigated by using metabolomic techniques to track certain CsA metabolite concentrations. Similar HPLC-MS methods have also been used to develop effective blood assays to monitor the concentrations of mycophenolic acid (another common immunosuppressant) and its metabolites [28].

In addition to these drug profiling studies, there have been several NMR and MS-based metabolomic studies describing the consequences of CsA on endogenous metabolites [29, 30]. These effects, which were initially studied in rat models, included elevated levels of urinary acetate, glucose, succinate and trimethylamine along with reduced levels of urinary TMAO, kynurenate, xanthurenate, citrate and riboflavin [29]. A more recent study focusing on serum instead of urinary metabolites found that both CsA and sirolimus led to elevated serum levels of creatine, creatinine, hydroxybutyrate, glucose, TMAO and cholesterol along with reduced concentrations of glutathione [30]. These results are consistent with many of the calcineurin inhibitor complications seen in human patients such as diabetes (increased glucose and hydroxybutyrate in urine and blood), heightened CVD risk (reduced riboflavin, elevated cholesterol), medullar damage (elevated serum TMAO and creatinine levels), increased incidence of kidney stones (low levels of citrate), proximal tubule damage (reduced concentrations of kynurenate and xanthurenate) and general oxidative stress (elevated levels of acetate and succinate, reduced glutathione). Metabolomic studies in humans have shown comparable CsA toxicity profiles including reduced citrate and increased oxalate levels [31], increased cholesterol or LDL

levels [32], increased malondialdehyde (a marker for oxidative stress) [33] and glucose intolerance [34]. Other human metabolic profiling studies aimed at assessing CsA and tacrolimus toxicity have shown increased levels of serum uric acid (a well-known nephrotoxin) [35–37] as well as increased levels of homocysteine and other CVD risk markers [38]. Overall, these results suggest that metabolomics holds considerable promise in being able to comprehensively monitor or assess immunosuppressive drug toxicity. In particular, metabolomics appears to be flexible enough to allow for the noninvasive tracking of drug and drug metabolite levels (i.e. exogenous metabolites) as well as the noninvasive tracking of endogenous metabolite levels.

Assessing Kidney Function and Localizing Kidney Damage Using Metabolomics

Post-transplant monitoring of organ function is particularly important for identifying signs of renal stress or dysfunction. This kind of monitoring is also useful for localizing organ damage or detecting the early stages of acute rejection. Close monitoring can allow pre-emptive or corrective action to be implemented before the organ is irreparably damaged. However, outside of serum creatinine measurements to assess generally function and protocol biopsies to help localize organ damage, relatively few alternative tests are being used. Given the demonstrated potential of metabolomic measurements to track organ function and localize organ damage, this is somewhat surprising. Indeed, over the past 20 years more than 30 papers have been published describing a surprising number of urinary and serum metabolite markers associated with posttransplantation function, acute rejection, subclinical rejection and localized organ damage. One feature common to almost all of these studies is the substantial (3- to 4-fold) increase seen in both urine and serum concentrations of TMAO [39, 40]. As noted before, this metabolite is an endogenous buffer that helps stabilize serum proteins from the effects of accumulated waste products. In addition to reports of elevated levels of TMAO, other organic amines (trimethylamine, dimethylamine) and amino acids (glycine, alanine) have also been detected. Metabolomic studies of transplanted, dysfunctional or rejected kidneys have detected elevated (2- to 5-fold) serum levels of nephrotoxins such as hippuric acid and uric acid [36, 41]. Kidney dysfunction is also associated with increased levels of nitric oxide synthase inhibitors such as phenylacetic acid [42] and dimethylarginine [43] in the serum. These inhibitors are known to lead to significantly reduced nitric oxide production [44]. Reduced nitric oxide levels are often correlated with hypertension and cardiovascular complications, both of which tend to further diminish kidney function. Damaged kidneys also

appear to have increase serum and urinary levels of lactate, acetate, succinate, citrate and urea, which are generally considered to be markers of Kreb's cycle (i.e. metabolic) distress, increased anaerobic metabolism and tubular acidosis [39, 40]. The identification of these previously unidentified metabolite imbalances is leading to therapeutic and dietary interventions that appear to have some benefit [45, 46].

Noninvasive (i.e. biopsy-free) approaches to localize organ damage are another area where metabolomic approaches may eventually find some clinical utility. A growing body of research is showing that it is possible to correlate localized kidney damage with distinct metabolite patterns [47]. For example, using rat models and various site-specific nephrotoxins researchers have found that damage to the proximal straight tubules (via the toxin D-serine) is typically associated with increased concentrations of lactate along with elevated levels of the amino acids phenylalanine, tryptophan, tyrosine and valine [48]. Straight tubule injury is also manifested by reduced levels of methylsuccinic, sebacic and xanthurenic acid. Meanwhile damage to the proximal convoluted tubules (via the toxin gentamicin) is generally associated with elevated levels of urinary glucose and reduced levels of TMAO, xanthurenic acid and kynurenic acid [49]. On the other hand, it has been noted that renal papillary and medullar injury (via bromoethaneamide) is characterized by increased urinary concentrations of glutaric acid, creatine and adipic acid along with reduced levels of citrate, succinate, oxoglutarate and TMAO [17]. In contrast, renal cortical damage (induced via mercuric chloride) is associated with increased urinary glucose, alanine, valine, lactate, hippurate and decreased citrate, succinate and oxoglutarate [50]. While it may be some time before these animal model results can be translated to humans in the transplant clinic, the possibility of using simple metabolic profiles to noninvasively characterize the foci of organ damage is obviously quite appealing.

Conclusions

The application of metabolomics to renal transplant monitoring is still at its earliest stages. However, it is clear that there are a surprising number of metabolites in both urine and serum that seem to provide reliable indications of organ function, organ injury, and immunosuppressive drug toxicity. As the field of metabolomics advances, it is likely that more metabolite markers or more specific metabolic profiles will be discovered and clinically validated, allowing even more precise diagnostic determinations. While metabolomics clearly offers a number of exciting prospects, one must always remember that metabolites are only a small part of the molecular picture needed to understand renal

transplantation. Indeed, the full picture must also include the detailed measurements of genes and proteins. In other words, metabolomics should always be regarded as a complementary tool to both proteomics and genomics.

Acknowledgements

The author wishes to thank Genome Alberta (a division of Genome Canada), the National Institute for Nanotechnology and the Canada Foundation for Innovation for financial support.

References

1 Murray JE, Merrill JP, Harrison JH: Renal homotransplantation in identical twins. Surg Forum 1955;6:432–437.
2 Murray JE, Barnes BA, Atkinson JC: Fifth Report of the Human Kidney Transplant Registry. Transplantation 1967;5:752–755.
3 Cecka JM: The UNOS Scientific Renal Transplant Registry–2000. Clin Transpl 2000:1–18.
4 Cecka JM: The OPTN/UNOS renal transplant registry. Clin Transpl 2004:1–16.
5 Oberholzer J, Testa G, Sankary H, John E, Thielke J, Benedetti E: Kidney transplantation at the University of Illinois at Chicago from 1988–2004. Clin Transpl 2004:143–149.
6 Djamali A, Premasathian N, Pirsch JD: Outcomes in kidney transplantation. Semin Nephrol 2003;23:306–316.
7 Hariharan S: BK virus nephritis after renal transplantation. Kidney Int 2006;69:655–662.
8 Veronese FV, Manfro RC, Roman FR, Edelweiss MI, Rush DN, Dancea S, Goldberg J, Goncalves LF: Reproducibility of the Banff classification in subclinical kidney transplant rejection. Clin Transplant 2005;19:518–521.
9 Schaub S, Wilkins JA, Antonovici M, Krokhin O, Weiler T, Rush D, Nickerson P: Proteomic-based detection of urine proteins associated with acute renal allograft rejection. J Am Soc Nephrol 2004;15:219–227.
10 O'Riordan E, Goligorsky MS: Emerging studies of the urinary proteome: the end of the beginning? Curr Opin Nephrol Hypertens 2005;14:579–585.
11 Eikmans M, Roos-van Groningen MC, Sijpkens YW, Ehrchen J, Roth J, Baelde HJ, Bajema IM, de Fijter JW, de Heer E, Bruijn JA: Expression of surfactant protein-C, S100A8, S100A9, and B cell markers in renal allografts: investigation of the prognostic value. J Am Soc Nephrol 2005;16: 3771–3786.
12 Wishart DS: Metabolomics: the principles and applications to transplantation. Am J Transplant 2005;5:2814–2820.
13 Drysdale R, Bayraktaroglu L: Current awareness. Yeast 2000;17:159–166.
14 Nicholson JK, Lindon JC, Holmes E: 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica 1999;29:1181–1189.
15 Thompson JA, Markey SP: Quantitative metabolic profiling of urinary organic acids by gas chromatography-mass spectrometry: comparison of isolation methods. Anal Chem 1975;47: 1313–1321.
16 Dunn WB, Bailey NJ, Johnson HE: Measuring the metabolome: current analytical technologies. Analyst 2005;130:606–625.
17 Lindon JC, Holmes E, Bollard ME, Stanley EG, Nicholson JK: Metabonomics technologies and their applications in physiological monitoring, drug safety assessment and disease diagnosis. Biomarkers 2004;9:1–31.

18 Wishart DS, Querengesser LMM, Lefebvre BA, Epstein NA, Greiner R, Newton JB: Magnetic Resonance Diagnostics: A new technology for high-throughput clinical diagnostics. Clin Chem 2001;47:1918–1921.
19 Wishart DS, Tzur D, Knox C, Eisner R, Guo AC, Young N, Cheng D, Jewell K, Arndt D, Sawhney S, Fung C, Nikolai L, Lewis M, Coutouly MA, Forsythe I, Tang P, Shrivastava S, Jeroncic K, Stothard P, Amegbey G, Block D, Hau DD, Wagner J, Miniaci J, Clements M, Gebremedhin M, Guo N, Zhang Y, Duggan GE, Macinnis GD, Weljie AM, Dowlatabadi R, Bamforth F, Clive D, Greiner R, Li L, Marrie T, Sykes BD, Vogel HJ, Querengesser L: the Human Metabolome Database. Nucleic Acids Res 2007;35:D521–D526.
20 Bell JD, Lee JA, Lee HA, Sadler PJ, Wilkie DR, Woodham RH: Nuclear magnetic resonance studies of blood plasma and urine from subjects with chronic renal failure: identification of trimethylamine-N-oxide. Biochim Biophys Acta 1991;1096:101–107.
21 Serkova N, Fuller TF, Klawitter J, Freise CE, Niemann CU: H-NMR-based metabolic signatures of mild and severe ischemia/reperfusion injury in rat kidney transplants. Kidney Int 2005;67: 1142–1151.
22 Fuller TF, Serkova N, Niemann CU, Freise CE: Influence of donor pretreatment with N-acetylcysteine on ischemia/reperfusion injury in rat kidney grafts. J Urol 2004;171:1296–1300.
23 Hauet T, Baumert H, Gibelin H, Hameury F, Goujon JM, Carretier M, Eugene M: Noninvasive monitoring of citrate, acetate, lactate and renal medullary osmolyte excretion in urine as biomarkers of exposure to ischemic reperfusion injury. Cryobiology 2000;41:280–291.
24 Domanski L, Safranow K, Dolegowska B, Rozanski J, Myslak M, Ciechanowski K, Jakubowska K, Dziedziejko V, Romanowski M, Sulikowski T, Sienko J, Kaminski M, Ostrowski M, Domanski M, Pawlik A, Rac ME, Chlubek D: Hypoxanthine as a graft ischemia marker stimulates catalase activity in the renal vein during reperfusion in humans. Transplant Proc 2006;38:35–38.
25 Al Banchaabouchi M, Marescau B, D'Hooge R, Van Marck E, De Deyn PP: Biochemical and histopathological changes in nephrectomized mice. Metabolism 1998;47:355–361.
26 Thervet E, Legendre C, Beaune P, Anglicheau D: Cytochrome P450 3A polymorphisms and immunosuppressive drugs. Pharmacogenomics 2005;6:37–47.
27 Vollenbroeker B, Koch JH, Fobker M, Suwelack B, Hohage H, Muller U: Determination of cyclosporine and its metabolites in blood via HPLC-MS and correlation to clinically important parameters. Transplant Proc 2005;37:1741–1744.
28 Annesley TM, Clayton LT: Quantification of mycophenolic acid and glucuronide metabolite in human serum by HPLC-tandem mass spectrometry. Clin Chem 2005;51:872–877.
29 Lenz EM, Bright J, Knight R, Wilson ID, Major H: Cyclosporin A-induced changes in endogenous metabolites in rat urine: a metabonomic investigation using high field 1H NMR spectroscopy, HPLC-TOF/MS and chemometrics. J Pharm Biomed Anal 2004;35:599–608.
30 Serkova NJ, Christians U: Biomarkers for toxidynamic monitoring of immunosuppressants: NMR-based quantitative metabonomics of the blood. Ther Drug Monit 2005;27:733–737.
31 Stapenhorst L, Sassen R, Beck B, Laube N, Hesse A, Hoppe B: Hypocitraturia as a risk factor for nephrocalcinosis after kidney transplantation. Pediatr Nephrol 2005;20:652–656.
32 Tse KC, Lam MF, Yip PS, Li FK, Lai KN, Chan TM: A long-term study on hyperlipidemia in stable renal transplant recipients. Clin Transplant 2004;18:274–280.
33 Moreno JM, Ruiz MC, Ruiz N, Gomez I, Vargas F, Asensio C, Osuna A: Modulation factors of oxidative status in stable renal transplantation. Transplant Proc 2005;37:1428–1430.
34 Teutonico A, Schena PF, Di Paolo S: Glucose metabolism in renal transplant recipients: effect of calcineurin inhibitor withdrawal and conversion to sirolimus. J Am Soc Nephrol 2005;16: 3128–3135.
35 Kanbay M, Akcay A, Huddam B, Usluogullari CA, Arat Z, Ozdemir FN, Haberal M: Influence of cyclosporine and tacrolimus on serum uric acid levels in stable kidney transplant recipients. Transplant Proc 2005;37:3119–3120.
36 Perico N, Codreanu I, Caruso M, Remuzzi G: Hyperuricemia in kidney transplantation. Contrib Nephrol 2005;147:124–131.
37 Armstrong KA, Johnson DW, Campbell SB, Isbel NM, Hawley CM: Does uric acid have a pathogenetic role in graft dysfunction and hypertension in renal transplant recipients? Transplantation 2005;80:1565–1571.

38 Wong W, Tolkoff-Rubin N, Delmonico FL, Cardarelli F, Saidman SL, Farrell ML, Shih V, Winkelmayer WC, Cosimi AB, Pascual M: Analysis of the cardiovascular risk profile in stable kidney transplant recipients after 50% cyclosporine reduction. Clin Transplant 2004;18:341–348.
39 Foxall PJ, Mellotte GJ, Bending MR, Lindon JC, Nicholson JK: NMR spectroscopy as a novel approach to the monitoring of renal transplant function. Kidney Int 1993;43:234–245.
40 Le Moyec L, Pruna A, Eugene M, Bedrossian J, Idatte JM, Huneau JF, Tome D: Proton nuclear magnetic resonance spectroscopy of urine and plasma in renal transplantation follow-up. Nephron 1993;65:433–439.
41 Knoflach A, Binswanger U: Serum hippuric acid concentration in renal allograft rejection, ureter obstruction and tubular necrosis. Transpl Int 1994;7:17–21.
42 Jankowski J, van der Giet M, Jankowski V, Schmidt S, Hemeier M, Mahn B, Giebing G, Tolle M, Luftmann H, Schluter H, Zidek W, Tepel M: Increased plasma phenylacetic acid in patients with end-stage renal failure inhibits iNOS expression. J Clin Invest 2003;112:256–264.
43 Zoccali C, Kielstein JT: Asymmetric dimethylarginine: a new player in the pathogenesis of renal disease? Curr Opin Nephrol Hypertens 2006;15:314–320.
44 Dedeoglu IO, Feld LG: Decreased urinary excretion of nitric oxide in acute rejection episodes in pediatric renal allograft recipients. Transplantation 1996;62:1936–1968.
45 Yatzidis H: Oral supplement of six selective amino acids arrest progression renal failure in uremic patients. Int Urol Nephrol 2004;36:591–598.
46 Albrecht EW, van Goor H, Smit-van Oosten A, Stegeman CA: Long-term dietary L-arginine supplementation attenuates proteinuria and focal glomerulosclerosis in experimental chronic renal transplant failure. Nitric Oxide 2003;8:53–58.
47 Holmes E, Nicholls AW, Lindon JC, Connor SC, Connelly JC, Haselden JN, Damment SJ, Spraul M, Neidig P, Nicholson JK: Chemometric models for toxicity classification based on NMR spectra of biofluids. Chem Res Toxicol 2000;13:471–478.
48 Williams RE, Major H, Lock EA, Lenz EM, Wilson ID: D-Serine-induced nephrotoxicity: a HPLC-TOF/MS-based metabonomics approach. Toxicology 2005;207:179–190.
49 Lenz EM, Bright J, Knight R, Westwood FR, Davies D, Major H, Wilson ID: Metabonomics with 1H-NMR spectroscopy and liquid chromatography-mass spectrometry applied to the investigation of metabolic changes caused by gentamicin-induced nephrotoxicity in the rat. Biomarkers 2005;10:173–187.
50 Lenz EM, Bright J, Knight R, Wilson ID, Major H: A metabonomic investigation of the biochemical effects of mercuric chloride in the rat using 1H NMR and HPLC-TOF/MS: time dependent changes in the urinary profile of endogenous metabolites as a result of nephrotoxicity. Analyst 2004;129:535–541.

David S. Wishart
2–21 Athabasca Hall
University of Alberta
Edmonton, AB T6G 2E8 (Canada)
Tel. +1 780 492 0383, Fax +1 780 492 1071, E-Mail david.wishart@ualberta.ca

Thongboonkerd V (ed): Proteomics in Nephrology – Towards Clinical Applications.
Contrib Nephrol. Basel, Karger, 2008, vol 160, pp 88–106

Understanding and Managing Renal Cell Carcinoma: Can Proteomic Studies Contribute to Clinical Practice?

Rachel A. Craven, Rosamonde E. Banks

Cancer Research UK Clinical Centre, St James's University Hospital, Leeds, UK

Abstract

Renal cell carcinoma (RCC) is associated with a poor prognosis and there is a need for biomarkers to assist at all stages of disease management including diagnosis, prognosis, monitoring for relapse and predicting response to therapy. Additionally, identification of new therapeutic targets is a priority. Increased understanding of disease pathogenesis and the molecular changes underlying tumour formation is essential to assist in the rational design of such molecules. As the technologies underlying proteomics-based research have developed, they have been applied extensively to the analysis of cancers including RCC, with tissues, cell lines and biological fluids being used for analysis. A number of approaches have been adopted including two-dimensional polyacrylamide gel electrophoresis and mass spectrometry profiling of intact proteins, shotgun mass spectrometry-based profiling at the peptide level, antibody arrays and strategies analysing the immune response to tumours with a view to identifying tumour-associated antigens. Although these studies are still at a relatively early stage, promising results have been reported with some being taken forward to preliminary validation. The challenge now is to build on these initial efforts, focusing particularly on interrogating the less readily accessible, lower abundance proteome and implementing large-scale validation studies to develop potential markers, antigens and targets and facilitate translation of suitable findings into the clinic.

Renal Cell Carcinoma and the Need for Novel Biomarkers and Targets

Renal cell carcinoma (RCC) is the most common cancer of the kidney, encompassing several distinct histological subtypes, the most common of which are clear cell (conventional), papillary and chromophobe RCC that

account for around 75, 15 and 5% of cases, respectively [1]. Although often treated as a single entity, the different subtypes of RCC have different underlying genetic changes and molecular mechanisms that accompany tumour formation; biological variation is also seen within histological subtypes, thus a group of patients with RCC can potentially be very diverse.

RCC currently accounts for ~3% of adult malignancies and its incidence is increasing. Localised RCC can be successfully treated by surgical removal of the primary tumour and although a significant number of patients (~30%) with apparently localised disease go on to develop metastases, patients with low stage disease have a relatively good prognosis. However, as symptoms are generally non-specific and often do not appear until relatively late in the course of disease progression, many patients have locally advanced or metastatic disease at the time of diagnosis. This, combined with the lack of response of RCC to standard chemotherapy and radiotherapy regimens which limits treatment options that can be used in combination with surgery, accounts for the poor survival rates of patients with advanced RCC. Until recently the best treatment option for patients with metastatic RCC was cytokine therapy (interleukin-2 and interferon-α) but the benefits of such therapy was limited, with response rates of only ~20%. New therapies including strategies based on molecular targeting (see below) are giving promising results and it is hoped they will transform the treatment of RCC patients.

There are currently no biomarkers for RCC that have been translated into routine clinical use; in the clinical setting, tumour stage and grade are still the most widely used predictors of outcome for RCC patients. There is a clear need for new markers to facilitate early detection of disease, to monitor patients for relapse and to allow stratification of patients into prognostic groups and predict response to therapy on an individual basis. A number of studies have described potential new markers with independent prognostic value and as in other cancers, the concept of using multiple biomarkers together with clinical information in more complex staging systems, is being explored [2]. Similarly, identification of targets that can be exploited in novel therapeutic strategies is an area of intense research; this includes investigation of strategies exploiting the immunological nature of RCC and targeted approaches based on the molecular changes that occur in tumour development.

Exploitation of Changes in Biological Pathways

In the multi-step process of tumour development and metastasis, mutations and epigenetic changes leading to changes in gene expression are acquired as cancer cells evolve. In RCC, the principal example of how knowledge of a

biological pathway can be exploited in the development of new clinical assays and therapeutic approaches is provided by the von Hippel-Lindau (VHL) tumour suppressor gene. In clear cell RCC, loss of the tumour suppressor gene *VHL* is central to tumour formation. The most well-documented function of VHL is as the substrate recognition domain of a multi-subunit E3 ubiquitin ligase formed with elongins B/C, Cul2 and Rbx1 that targets hypoxia-inducible factor (HIF)-α subunits for ubiquitination and degradation by the proteasome. For HIFα to bind to VHL it must be hydroxylated on proline residues in its oxygen-dependent degradation domain and this modification, which is seen in normoxic but not hypoxic conditions, regulates recruitment of HIFα to the E3 ubiquitin ligase, thus controlling its turnover. Loss of VHL function leads to stabilisation of HIFα in normoxia leading to increased expression of proteins characteristic of a hypoxic response such as carbonic anhydrase IX (CAIX or G250), glucose transporter 1 (GLUT-1), glycolytic enzymes, transforming growth factor-α, platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF). CAIX remains one of the best biomarkers currently identified for RCC and is also being investigated as a therapeutic target.

Trials of therapies designed to target aspects of the HIF pathway, such as VEGF and PDGF and their downstream signalling pathways, have shown extremely promising results both in renal and other cancers. These are illustrated by the receptor tyrosine kinase (RTK) inhibitors sunitinib and sorafenib. Sunitinib (SU11248) is a broad-spectrum RTK inhibitor, whose targets include VEGF and PDGF receptors. In phase II clinical trials as a second-line therapy in patients with metastatic RCC who had progressed on cytokine therapy, sunitinib showed good efficacy and was well tolerated [3, 4]. A phase III clinical trial comparing sunitinib with IFN-α as a first-line treatment for patients with metastatic clear cell RCC showed an improvement in progression-free survival and objective response rate [5] and this is likely to become the new standard of care for advanced renal cancer. Sorafenib (BAY 43-9006), a Raf-1 kinase inhibitor that also affects some RTKs including receptors for VEGF and PDGF, has also performed well in phase II and III clinical trials and has received FDA approval for treatment of patients with advanced RCC [6–8].

Proteomics and Biomarker Discovery Studies: General Considerations

Investigation of specific molecules or biological pathways can give insight into tumorigenesis and thereby identify potential biomarkers or targets but the approach most commonly adopted in biomarker discovery experiments is that of global, untargeted comparative analyses of samples in different clinical groups. Gene expression profiling of tissues and cell lines at the mRNA level

using arrays has been carried out extensively in RCC, resulting in the development of preliminary expression signatures that allow diagnostic or prognostic classification of samples and identification of molecules with potential clinical utility [9]. Such mRNA-based analysis is high throughput and many thousands of gene products can be analysed in a single experiment, making global expression profiling of large numbers of samples a feasible strategy.

Protein profiling offers a complementary approach, which has several advantages despite being unable to compete with the coverage and throughput of analysis of mRNA; studies at the protein level overcome the lack of correlation between mRNA and protein and can also demonstrate the presence of post-translational modifications such as glycosylation and phosphorylation, which can impact enormously on protein function and activity. Analysis of proteins also lends itself to the study of biological fluids for the direct identification of circulating markers that may form the basis of non-invasive assays.

Biomarker programmes can be divided into several stages. The initial discovery step identifies potential biomarkers by comparative analysis of different sample groups. Downstream studies aimed at validating these findings are then required, often confirming the results using alternative assays but also using further samples. Following comprehensive validation the ultimate step is translating laboratory findings into the clinic [10]. The different phases have very different needs, which are reflected in the approaches that can be used to achieve them. Discovery requires profiling of large numbers of disparate protein species in order to achieve good proteome coverage and can rely on the use of relatively small numbers of samples if they are carefully selected and matched and the protein profiling technique used is sufficiently reproducible. Validation studies, on the other hand, focus on a small number of molecules found in initial screening and must be large scale to fully test their potential so often make use of higher throughput, generally antibody-based techniques including immunohistochemistry using tissue microarrays, ELISA, and reverse-phase protein arrays.

Samples for Proteomic Analysis

Tumour tissue is a valuable resource for studies aiming to identify biomarkers and further understanding of disease pathogenesis. In RCC, patient-matched normal kidney is generally available in addition to tumour tissue, therefore allowing simple pairwise comparisons. However, analysis of whole tissue extracts can be subject to problems associated with tissue heterogeneity, with normal kidney cortex containing multiple cell types in addition to the epithelial cells of the proximal tubules which are most often assumed to be the normal cell

type giving rise to RCC, and even areas of tumour tissue that are free from necrosis and haemorrhage containing areas of stroma and large numbers of infiltrating cells and blood vessels in addition to tumour cells. Purification of cell types of interest using antibodies can be employed to overcome this problem [11, 12] but these are not straightforward, requiring a single cell suspension as a starting material and specific antibodies to select or deplete particular cell types. Strategies such as laser capture microdissection have also been employed but are subject to limitations on material available for analysis [13]. Cell lines present a source of enriched epithelial cell populations that can be used for comparing normal and disease states, and act as a model to study processes such as the effect of drug treatment. Although cell lines are subject to potential in vitro artefacts resulting from short- or long-term culture, primary cell lines have been shown to maintain differential gene expression changes characteristic for RCC [14–16] and established cell lines have a proven track record as a model system for studying changes underlying disease pathogenesis, particularly in the study of VHL where cell line pairs generated from VHL-defective RCCs and transfected with vector control or *VHL* have proved invaluable.

Serum and plasma offer a rich source of disease biomarkers produced by the tumour or reflecting secondary systemic changes. Samples from patients with RCC can be compared with age- and sex-matched healthy controls or patients with benign urological conditions. Alternatively, longitudinal samples from the same patient can be compared, for example pre- and post-nephrectomy or treatment. Serum and plasma have a high protein concentration ($>$50 g/l) but are dominated by a relatively small number of protein species; the dynamic range of protein expression exceeds twelve orders of magnitude but ten proteins make up $>$90% of the total protein content. Removal of the more abundant proteins by immunodepletion or alternative pre-fractionation strategies is therefore necessary if proteins shed or secreted from tumour cells are to be detected and profiled. Urine is an obvious alternative biological fluid that may represent an enriched source of biomarkers for RCC, containing filtered serum proteins and proteins derived directly from the kidney by processes including secretion, shedding or release of exosomes. Analysis of urine is complicated by its low protein concentration and the influence of several factors including hydration state and potential protease content.

The study of tumour interstitial fluid to allow the direct analysis of proteins released into interstitial space by all cell types within a tumour, prior to dilution in the systemic circulation, has also received attention although not as yet in RCC. The potential of this is shown in a preliminary study of interstitial fluid from invasive breast carcinomas where 267 primary translation products were identified, many of which were not detected in the plasma/serum proteome [17].

The most important resource underlying any biomarker discovery programme is a good quality sample bank, collected using well-defined, robust protocols for sample collection, handling and processing to reduce pre-analytical factors that will compromise downstream analysis. The value of such samples is also dependent on the accompanying clinical data to allow selection of appropriate samples for analysis and interpretation of results.

Techniques for Protein Profiling

Various proteomic profiling approaches can be adopted, with protein separation and quantitation being achieved at either the protein ('top-down') or peptide level following tryptic digestion ('bottom-up'). Each has inherent advantages and limitations, while complementing each other in terms of the results and type of information achieved. It is now readily apparent from many studies however that whichever approach is adopted, sample pre-fractionation is necessary to allow either detection of lower abundance proteins for example by focussing on particular subcellular organelles or specific subproteomes such as the phosphoproteome.

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) remains a central tool for the separation of proteins in protein profiling studies [18] and the use of 2-D difference gel electrophoresis (2-D DIGE), which allows simultaneous analysis of two fluorescently labelled samples and a reference in a single gel thereby simplifying gel analysis, is becoming more routine practice [19]. Proteome coverage can be improved by the use of zoom gel technology using more narrow range pH gradients for the isoelectric focussing to allow the study of less abundant molecules. Although allowing visualisation of different protein forms, for example due to post-translational modifications, hydrophobic proteins such as membrane proteins and proteins at the extremes of pI and molecular weight are still under-represented. More recently, multidimensional liquid chromatography separations of intact proteins, for example using PF2D from Beckman Coulter which separates proteins by chromatographic focussing followed by reverse phase, has started to show its potential in several studies, complementing results achieved by 2-D PAGE [20].

Matrix-assisted laser desorption/ionisation time-of-flight MS (MALDI-TOF-MS) can also be used to profile intact proteins or peptides, complementing 2-D PAGE in terms of being more optimal in lower mass ranges ($<$30–50 kDa). This is illustrated by the profiling of whole plasma by MALDI-TOF-MS [21] with 58 reproducible peaks being visible in the 4,000–160,000 range. In most studies, especially those analysing biological fluids, fractionation is needed to some extent prior to analysis to enhance profiling of less abundant proteins and, concomitantly, remove sample components that interfere with

downstream MS. Surface-enhanced laser desorption/ionisation (SELDI)-TOF-MS, essentially based on MALDI-TOF but using ProteinChip arrays with standard chromatographic surfaces to selectively capture subsets of proteins from samples of interest prior to profiling offers some degree of sample fractionation. Many studies have been published which show the potential of this approach in generating sets of markers or signatures which classify samples when combined with data analysis methods such as neural networks and hierarchical clustering. A number of reports emphasise the need for standardisation and ongoing quality control [22] and indeed, sample collection and processing can introduce more variability than the biological process under investigation [23]. However, multi-site studies have shown that the SELDI-TOF-MS platform has the potential to generate reproducible and consistent data [24, 25]. A similar sample chromatographic pre-fractionation using magnetic beads with functionalised surfaces for protein selection with eluted proteins being subsequently profiled by MS has also been developed [26, 27].

Imaging MS, which analyses tissue sections directly by MS thereby integrating histology with protein profiling, provides a means to produce high-resolution protein distribution within tissues [28]. Profiling using this technique can generate patterns with clinical utility for diagnosis and prognosis and questions can also be asked regarding tumour architecture, for example comparing the viable cells found at the rim of a tumour with the necrotic core or examining normal-tumour boundaries and surgical margins.

Tandem mass spectrometry has been combined with multidimensional chromatography to profile protein mixtures from biological samples following tryptic digestion, an approach termed shotgun proteomics. Studies can employ label-free quantitation [29] or incorporate strategies such as in vitro or in vivo labelling with stable isotopes [30] or iTRAQ (isobaric tags for relative and absolute quantitation) [31] to facilitate comparative analysis. Such approaches are very powerful with many hundreds of proteins being identified in a single experiment, but such bottom-up approaches do not provide information about the intact protein in terms of size and modifications/isoforms, as the connection between peptide and protein is lost.

Global protein profiling can also be complemented by antibody-based proteomic strategies. The development of antibody arrays, particularly for the profiling of cytokines which often fall below the sensitivity of other proteomic profiling techniques is of particular note. The database of protein expression patterns (www.proteinatlas.org) generated by large-scale antibody production and staining tissue microarrays of normal tissues and common cancers [32, 33] is also likely to have a significant impact on biomarker research.

A different strategy for biomarker discovery is the identification of antigens that lead to an antibody-based response in patients. SEREX (SERological

identification of antigens by recombinant EXpression cloning) is based on the probing of cDNA expression libraries with autologous antibodies. Proteomics-based adaptations of this approach, variously called SERPA (SERological Proteomic Analysis), SPEAR (Serological and Proteomic Evaluation of Antibody Responses) and PROTEOMEX use 2-D PAGE-based Western blotting to identify tumour antigens recognised specifically by patient sera. Alternatively, recombinant proteins have been used to generate protein arrays for antibody binding and potential antigens have been precipitated from phage display libraries [34]. In the case of T-lymphocyte-mediated anti-tumour responses, efforts have focussed on identification of processed and presented peptides bound to MHC molecules [35]. MHC-bound peptides can be eluted from MHC molecules purified from cell lines or tissues by immunoprecipitation and characterised by MS/MS. This strategy has been successfully applied in a number of studies including comparative analyses [36, 37].

Proteomics and RCC

Identification of Biomarkers by Analysis of Tissues and Cell Lines

A strategy that has been used by several groups for identifying biomarkers for RCC is the comparative analysis of patient-matched normal and tumour tissues by 2-D PAGE [11, 38–46] with subsequent validation by immunohistochemistry as exemplified in figure 1. Studies vary with some selecting homogeneous tumours based on pathology and others using more heterogeneous sample sets, but with a predominance of clear cell tumours. All the studies analysed proteins extracted from total tissue samples with one exception where extracts were prepared following removal of B and T lymphocytes from tissue samples by immunodepletion [11]. There was significant overlap between the findings of different groups and a number of differentially expressed protein species have been identified, highlighting a number of important changes that occur in RCC. Changes in metabolic pathways were particularly prevalent [42, 45], including increases in the levels of glycolytic enzymes and concomitant decreases in enzymes involved in gluconeogenesis and a number of mitochondrial proteins, the latter reflecting a more generalised loss of mitochondrial proteins seen in RCC. Several of these changes have been confirmed in preliminary validation studies including loss of the mitochondrial proteins agmatinase [40] and GRIM-19 [46], increases in the level of annexin IV [41] and changes in the expression pattern of fatty acid binding proteins [44] and two of these changes have been followed up by functional studies. Depletion of GRIM-19 using siRNA was shown to promote growth of ACHN cells in culture and tumour formation in athymic nude mice, whilst over-expression

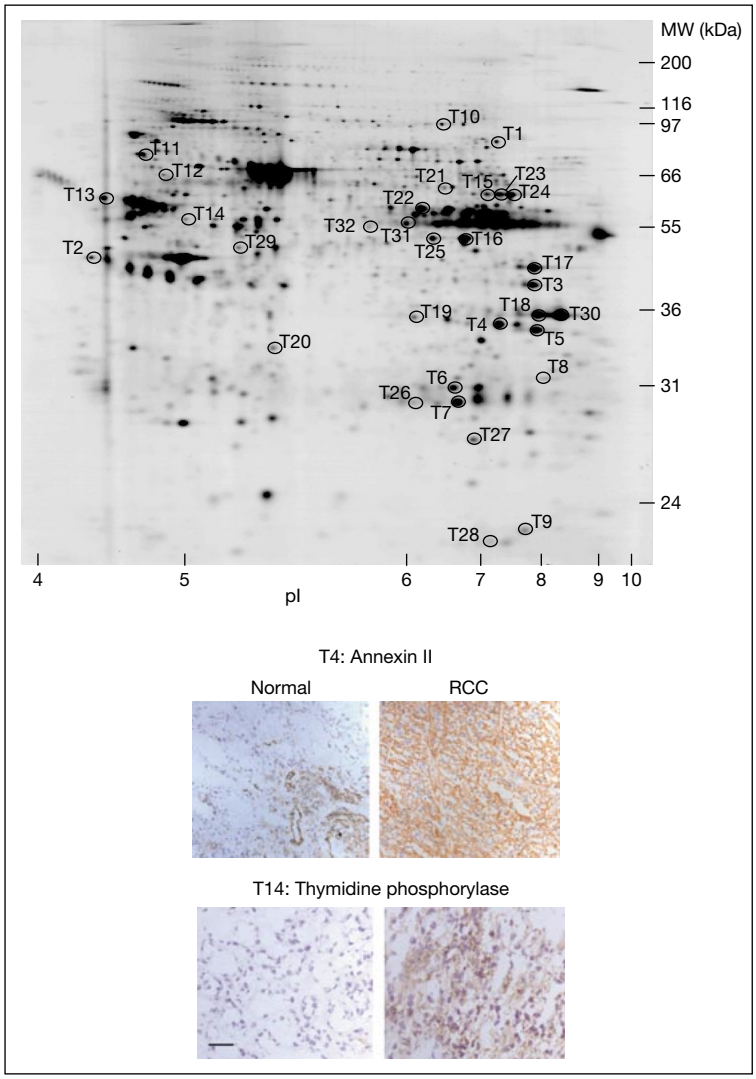


Fig. 1. Analysis of RCC using 2-D PAGE. 2-D PAGE with broad range (pH3–10NL) IPG strips was used to compare the protein profiles of six pairs of patient-matched normal and tumour tissues. A silver-stained gel of protein extracted from a tumour tissue showing the proteins up-regulated in at least 4/6 patients is shown, together with representative images of normal and tumour tissues included in the initial validation of two differentially expressed proteins by immunohistochemistry (Bar = 50 μm). Adapted from Unwin et al. [42].

induced apoptosis [46] and annexin IV was shown to promote cell migration in an assay for integrin $\alpha\nu\beta5$-mediated migration on vitronectin, albeit using a breast cancer cell line [41].

Primary and established cell lines have also been employed in 2-D PAGE-based studies looking for proteins that are differentially expressed in RCC. Analysis of 11 pairs of primary cell lines derived from patient-matched normal and malignant kidney samples identified 21 protein spots that were increased and 8 that were decreased in RCC. Of particular note was up-regulation of 3 proteins, namely αβ-crystallin, MnSOD (3 isoforms) and annexin IV (4 isoforms), which were found in >50% RCC cell lines [14]. A similar study using 5 pairs of primary cell lines from patients with clear cell RCC identified 43 protein spots that were increased and 29 that were decreased in at least 3/5 RCC-derived cell lines [16]; several of the changes identified had been previously associated with RCC but others were novel including increased expression of fascin, which was confirmed by Western blotting and immunohistochemistry in tissue samples. Analysis of VHL-defective 786-0 cells transfected with vector control or VHL has also been successful, showing that VHL contributes to loss of mitochondrial proteins seen in RCC and identifying VHL-dependent changes in expression of septin 2 [47].

The study of cell lines is complemented by investigation of proteins released by cells, i.e. conditioned medium. In a screen looking for proteins that were differentially secreted by 786-0 cells in response to VHL status, 1-D and 2-D PAGE were used to compare proteins in conditioned medium from 786-0 cells transfected with vector control or VHL [48]. This analysis showed cells lacking VHL (corresponding to the malignant phenotype), secreted increased amounts of insulin-like growth factor binding protein 3 (IGFBP3) and plasminogen activator inhibitor-1, proteins known to be the product of HIF-responsive genes, but secreted decreased amounts of clusterin. This latter change was also apparent in cells at the mRNA level but was independent of the HIF pathway. Reduced clusterin staining was also seen in VHL-defective tumour tissues compared to tumours with no VHL defect.

2-D PAGE has also been used to profile clear cell and papillary RCC, oncocytoma and Wilms' tumour using manual microdissection to select tumour-rich areas free from inflammation, necrosis and haemorrhage for analysis [49]. Individual protein profiles were successfully obtained for each tumour type and a number of discriminating proteins identified. A similar strategy was employed to facilitate diagnosis of metastatic adenocarcinomas with unknown primary site. The protein profiles of adenocarcinomas from kidney, lung, colon, breast, ovary and stomach were compared by 2-D PAGE. Artificial neural networks trained using discriminating sets of proteins achieved average predictive accuracy of 82% in leave-one-out cross-validation [50].

Generally, these studies have used 2-D PAGE conservatively, using broad-range pH gradients and standard methods for protein detection. An exception is a recent feasibility study in which a single pair of microdissected normal and tumour tissue samples were labelled with ^{125}I or ^{131}I, co-separated on contiguous IPGs (pH 4–5, 5–6 and 6–9) and analysed using a multiplex differential radioactive imaging approach termed 'ProteoTope'. Less than 4 μg material was required and 29 differentially expressed proteins were identified, including several known to be disease-associated. Generation of further data using more extensive series of patients is underway [51].

A small proof of principle study has assessed the potential of SELDI-TOF-MS in distinguishing normal and tumour tissues. Two peaks at m/z 11950 and 12,020 were found to be higher in tumour tissue samples using reverse-phase ProteinChip arrays and highlighted as being of potential interest [52]. Similarly, an initial study using SELDI with archival cytological samples generated disease-specific fingerprints for RCC, melanoma and sarcoma, which showed good categorisation of further blind samples [53]. More recently, analysis of clear cell RCC and normal renal tissues using ion exchange ProteinChip arrays and incorporating more elaborate data analysis using rule-based data mining identified seven protein peaks in a training set of tissues which performed well on a subsequent test set of samples, giving values for sensitivity and specificity of 77% and 100% respectively [54]. SELDI-TOF-MS has also been applied to the problem of looking for markers of response to treatment by profiling proteins in IFN-α sensitive and resistant cell lines chosen for their response to drug in vitro [55]. These investigations are all at a preliminary stage, involving relatively small sample numbers and no protein identities have been determined, however the results are promising, endorsing the need for further studies.

The first study in RCC analysing a subcellular fraction focussed on the plasma membrane, with the aim of cataloguing cell surface proteins on tumour cells and thereby identifying potential molecules for use in antibody-targeted cytotoxic therapies [56]. Plasma membrane-enriched fractions were separated by 1-D PAGE and proteins identified by MS/MS before subsequent comparative analysis of selected proteins in downstream studies. One finding was that the type II membrane protein CD70 (TNF ligand superfamily member 7), a co-stimulatory molecule of the TNF superfamily, was expressed at high levels on the cell surface of a number of RCC cell lines. Immunohistochemistry showed strong staining in 16/20 clear cell tumours and 8/11 metastases whilst normal kidney showed no staining. The potential of CD70 as a target for toxin-conjugated antibody-based therapy was shown by the internalisation of a CD70-antibody complex by cell lines, with killing of approaching 50% being mediated via a saporin-conjugated secondary antibody. The

potential of CD70 has also been implicated in a number of other recent studies [57–59]. Although the study described here did not involve any comparative analysis initially, it does highlight the potential of subcellular fractionation in proteomic profiling.

A novel 'pre-fractionation' strategy to identify tumour-associated markers focussed on accessing the vasculature and other structures accessible from the bloodstream in tumour and normal tissue. Ex vivo perfusion of resected kidneys with sulfo-NHS-LC-biotin to allow biotinylation of accessible structures followed by purification of biotinylated proteins, tryptic digestion and analysis of resulting peptides by LC and MALDI-TOF/TOF-MS identified 637 proteins in samples from 3 patients, 184 of which were only found in the analysis of the tumour samples. Several markers were subsequently confirmed by PCR of cDNA libraries and immunohistochemistry, including up-regulation of periostin, versican and melanoma-associated antigen MG50 [60].

Imaging MS is still in its infancy and not yet widely available however a study that included a single case of RCC, has already raised interesting questions [61]. Differences were found between the protein profiles of normal and tumour tissues and heterogeneity within the tumour, but at the tumour-normal border, tumour-associated expression patterns were present in some histologically normal tissue, clearly showing the importance of being able to directly link protein expression patterns to tissue morphology.

Identification of Biomarkers by Analysis of Biological Fluids

The number of studies using plasma/serum and urine to identify RCC-associated biomarkers are small and have involved relatively small numbers of samples but have highlighted the potential of proteomic analysis of biological fluids. One of the most extensive characterisations of the urinary proteome reported to date used 2-D PAGE to analyse urine following depletion of albumin and IgG and resolved 1,400 distinct protein spots of which ~420 were successfully identified; these corresponded to 150 proteins which included only 50 classical plasma proteins [62]. This study went on to examine the urine of an RCC patient pre- and post-nephrectomy and was able to identify a small number of changes in the protein profile, including down-regulation of kininogen and mannan-binding lectin serine protease 2 after nephrectomy [62].

Using SELDI-TOF-MS with weak cation exchange ProteinChip arrays to compare serum samples from RCC patients with those from healthy controls and patients with other urological conditions, a decision tree classification strategy based on 5 biomarkers at m/z 3900, 4107, 4153, 5352 and 5987 could distinguish the sample groups with specificity and sensitivity >80% [63]. A similar study comparing albumin-depleted sera from healthy controls and RCC patients identified peaks at m/z ~9200, 10840 and a cluster at ~11400–11700

as being disease-related. Using 1-D and 2-D PAGE and MS the peak at ~9200 was identified as haptoglobin 1-α and the cluster at ~11400–11700 as variants of the acute phase protein serum amyloid α-1 (SAA-1) [64]. Good correlation was found between the SAA-1 triplet assessed by SELDI and levels of SAA measured by ELISA, with the ELISA providing a more sensitive assay.

Similar comparative analysis of 218 urine samples from patients with clear cell RCC and healthy controls or patients with benign urological diseases by SELDI-TOF-MS using weak cation exchange ProteinChip arrays produced neural network models which achieved sensitivity and specificity values >80% in an initial blind test set [65]. Subsequent analysis of a further test set several months later failed to reproduce such promising results, highlighting problems at that time with long-term robustness and the need for more rigorous quality control, improvements that are now more routinely incorporated into such profiling studies. Several peaks were found to be increased in either the RCC or control group and these await identification.

Profiling of sera from patients undergoing high-dose IL-2 therapy has also been carried out to further understanding of its mechanism of action and the basis of its toxicity. Whole sera or fractions generated by anion exchange chromatography were profiled on strong anion exchange ProteinChip arrays and a doublet of peaks at ~11500 and ~11700 and a further peak at ~23000 were observed to be present after therapy. These were putatively identified as isoforms of SAA and C-reactive protein respectively, which was confirmed by SELDI-TOF-MS-based immunoassays [66]. This analysis was complemented by a parallel study using antibody arrays with the serum levels of 68 soluble factors measured in 10 patients undergoing high-dose IL-2 therapy [67] showing many to be dramatically altered and the authors now plan to extend this work with the aim of being able to predict outcome and toxicity.

Identification of Antigens Based on Immune Response

In the first study using 2-D PAGE-based Western blotting to identify immunogenic proteins in RCC samples that have elicited an antibody response, five RCC-specific spots, which reacted with patient but not control sera, were identified. Two of these proteins were identified as carbonic anhydrase I (CAI) and smooth muscle protein 22α (SM22-α) and validation studies using recombinant proteins showed that 5/11 and 3/11 patient sera recognised SM22-α and CAI respectively [68] whilst control sera did not react. In a similar study focussing on anti-heat-shock protein immunoreactivity, extracts from three RCC and one normal kidney cell line were probed with autologous and allogeneic patient sera or with control sera and a significant number of candidate patient specific spots were identified. The reactivity of control and patient sera with heat-shock proteins was complex and also varied depending on which cell line was used as source of

antigens [69]. In an extension of this work, results for two other groups of proteins have been reported, namely cytoskeletal proteins and metabolic enzymes [70, 71] and again complex patterns of reactivity were obtained but no patient-specific antigens were identified.

A later study using 2-D immunoblot analyses of normal and RCC tissue as the antigen source and probing with autologous patient sera identified a number of antigens that did not react with control sera including annexins I and IV, thymidine phosphorylase, CAI and major vault protein [72], several of which were shown to have increased expression in RCC tissue. However, no single spot was identified by more than 2/8 patient sera, suggesting that such antigens may be most useful in targeted therapies tailored for individual patients.

In a study to identify MHC class I ligands that may form the basis of cancer vaccines, expression data generated by comparing normal and tumour tissue mRNA was combined with sets of MHC-bound peptides isolated from the tumour samples and identified by MS/MS [73, 74]. Vaccine candidates were chosen based on over-expression in RCC together with limited expression in normal tissues. Known and novel tumour-associated antigens were identified including CAIX, adipophilin and IGFBP3. This approach is now being extended using stable isotope labelling to allow quantitation by MS, allowing direct identification of differentially presented peptides thereby overcoming the lack of correlation between mRNA levels and the MHC ligandome [75].

Conclusions

Proteomic technologies have advanced enormously in recent years, allowing major improvements in proteome coverage. Profiling lower abundance molecules remains a major challenge although is now becoming more feasible. Application of proteomics to RCC is an approach that is clearly in its infancy; studies addressing the biological processes associated with disease progression and the clinical problems in patient management lag far behind what is now possible from a technical viewpoint. This reflects the relatively small number of groups working in this specific area and the difficulty in accessing large numbers of samples given the relatively low incidence of this cancer. Although the small number of studies carried out in RCC to date have not identified molecules or signatures with clear-cut proven clinical utility, they do offer a flavour of the potential of proteomics-based approaches with encouraging results overall. A number of studies have generated promising results: several discriminating biomarkers and signatures now require rigorous validation in larger sample sets to assess their clinical utility and more in-depth analysis of particular molecules is required to understand their role in tumorigenesis. Further profiling studies are

now a priority, with a focus on analysis of less abundant molecules and/or particular forms of proteins for the successful identification of novel biomarkers.

References

1 Drucker BJ: Renal cell carcinoma: current status and future prospects. Cancer Treat Rev 2005;31:536–545.
2 Lam JS, Breda A, Belldegrun AS, Figlin RA: Evolving principles of surgical management and prognostic factors for outcome in renal cell carcinoma. J Clin Oncol 2006;24:5565–5575.
3 Motzer RJ, Michaelson MD, Redman BG, Hudes GR, Wilding G, Figlin RA, Ginsberg MS, Kim ST, Baum CM, DePrimo SE, Li JZ, Bello CL, Theuer CP, George DJ, Rini BI: Activity of SU11248, a multitargeted inhibitor of vascular endothelial growth factor receptor and platelet-derived growth factor receptor, in patients with metastatic renal cell carcinoma. J Clin Oncol 2006;24:16–24.
4 Motzer RJ, Rini BI, Bukowski RM, Curti BD, George DJ, Hudes GR, Redman BG, Margolin KA, Merchan JR, Wilding G, Ginsberg MS, Bacik J, Kim ST, Baum CM, Michaelson MD: Sunitinib in patients with metastatic renal cell carcinoma. JAMA 2006;295:2516–2524.
5 Motzer RJ, Hutson TE, Tomczak P, Michaelson MD, Bukowski RM, Rixe O, Oudard S, Negrier S, Szczylik C, Kim ST, Chen I, Bycott PW, Baum CM, Figlin RA: Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. N Engl J Med 2007;356:115–124.
6 Ratain MJ, Eisen T, Stadler WM, Flaherty KT, Kaye SB, Rosner GL, Gore M, Desai AA, Patnaik A, Xiong HQ, Rowinsky E, Abbruzzese JL, Xia C, Simantov R, Schwartz B, O'Dwyer PJ: Phase II placebo-controlled randomized discontinuation trial of sorafenib in patients with metastatic renal cell carcinoma. J Clin Oncol 2006;24:2505–2512.
7 Escudier B, Eisen T, Stadler WM, Szczylik C, Oudard S, Siebels M, Negrier S, Chevreau C, Solska E, Desai AA, Rolland F, Demkow T, Hutson TE, Gore M, Freeman S, Schwartz B, Shan M, Simantov R, Bukowski RM: Sorafenib in advanced clear-cell renal-cell carcinoma. N Engl J Med 2007;356:125–134.
8 Kane RC, Farrell AT, Saber H, Tang S, Williams G, Jee JM, Liang C, Booth B, Chidambaram N, Morse D, Sridhara R, Garvey P, Justice R, Pazdur R: Sorafenib for the treatment of advanced renal cell carcinoma. Clin Cancer Res 2006;12:7271–7278.
9 Yin-Goen Q, Dale J, Yang WL, Phan J, Moffitt R, Petros JA, Datta MW, Amin MB, Wang MD, Young AN: Advances in molecular classification of renal neoplasms. Histol Histopathol 2006;21:325–339.
10 Anderson NL: The roles of multiple proteomic platforms in a pipeline for new diagnostics. Mol Cell Proteomics 2005;4:1441–1444.
11 Sarto C, Marocchi A, Sanchez JC, Giannone D, Frutiger S, Golaz O, Wilkins MR, Doro G, Cappellano F, Hughes G, Hochstrasser DF, Mocarelli P: Renal cell carcinoma and normal kidney protein expression. Electrophoresis 1997;18:599–604.
12 Magni F, Sarto C, Valsecchi C, Casellato S, Bogetto SF, Bosari S, Di FA, Perego RA, Corizzato M, Doro G, Galbusera C, Rocco F, Mocarelli P, Galli KM: Expanding the proteome two-dimensional gel electrophoresis reference map of human renal cortex by peptide mass fingerprinting. Proteomics 2005;5:816–825.
13 Craven RA, Totty N, Harnden P, Selby PJ, Banks RE: Laser capture microdissection and two-dimensional polyacrylamide gel electrophoresis: evaluation of tissue preparation and sample limitations. Am J Pathol 2002;160:815–822.
14 Shi T, Dong F, Liou LS, Duan ZH, Novick AC, DiDonato JA: Differential protein profiling in renal-cell carcinoma. Mol Carcinog 2004;40:47–61.
15 Perego RA, Bianchi C, Corizzato M, Eroini B, Torsello B, Valsecchi C, Di Fonzo A, Cordani N, Favini P, Ferrero S, Pitto M, Sarto C, Magni F, Rocco F, Mocarelli P: Primary cell cultures arising from normal kidney and renal cell carcinoma retain the proteomic profile of corresponding tissues. J Proteome Res 2005;4:1503–1510.

16 Craven RA, Stanley AJ, Hanrahan S, Dods J, Unwin R, Totty N, Harnden P, Eardley I, Selby PJ, Banks RE: Proteomic analysis of primary cell lines identifies protein changes present in renal cell carcinoma. Proteomics 2006;6:2853–2864.
17 Celis JE, Gromov P, Cabezon T, Moreira JM, Ambartsumian N, Sandelin K, Rank F, Gromova I: Proteomic characterization of the interstitial fluid perfusing the breast tumor microenvironment: a novel resource for biomarker and therapeutic target discovery. Mol Cell Proteomics 2004;3:327–344.
18 Gorg A, Weiss W, Dunn MJ: Current two-dimensional electrophoresis technology for proteomics. Proteomics 2004;4:3665–3685.
19 Tonge R, Shaw J, Middleton B, Rowlinson R, Rayner S, Young J, Pognan F, Hawkins E, Currie I, Davison M: Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. Proteomics 2001;1:377–396.
20 Sheng S, Chen D, Van Eyk JE: Multidimensional liquid chromatography separation of intact proteins by chromatographic focusing and reversed phase of the human serum proteome: optimization and protein database. Mol Cell Proteomics 2006;5:26–34.
21 Jin Y, Manabe T: Direct targeting of human plasma for matrix-assisted laser desorption/ionization and analysis of plasma proteins by time of flight-mass spectrometry. Electrophoresis 2005;26:2823–2834.
22 Hortin GL: Can mass spectrometric protein profiling meet desired standards of clinical laboratory practice? Clin Chem 2005;51:3–5.
23 Karsan A, Eigl BJ, Flibotte S, Gelmon K, Switzer P, Hassell P, Harrison D, Law J, Hayes M, Stillwell M, Xiao Z, Conrads TP, Veenstra T: Analytical and preanalytical biases in serum proteomic pattern analysis for breast cancer diagnosis. Clin Chem 2005;51:1525–1528.
24 Semmes OJ, Feng Z, Adam BL, Banez LL, Bigbee WL, Campos D, Cazares LH, Chan DW, Grizzle WE, Izbicka E, Kagan J, Malik G, McLerran D, Moul JW, Partin A, Prasanna P, Rosenzweig J, Sokoll LJ, Srivastava S, Srivastava S, Thompson I, Welsh MJ, White N, Winget M, Yasui Y, Zhang Z, Zhu L: Evaluation of serum protein profiling by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry for the detection of prostate cancer. I. Assessment of platform reproducibility. Clin Chem 2005;51:102–112.
25 Rai AJ, Stemmer PM, Zhang Z, Adam BL, Morgan WT, Caffrey RE, Podust VN, Patel M, Lim LY, Shipulina NV, Chan DW, Semmes OJ, Leung HC: Analysis of Human Proteome Organization Plasma Proteome Project (HUPO PPP) reference specimens using surface enhanced laser desorption/ionization-time of flight (SELDI-TOF) mass spectrometry: multi-institution correlation of spectra and identification of biomarkers. Proteomics 2005;5:3467–3474.
26 Villanueva J, Philip J, Entenberg D, Chaparro CA, Tanwar MK, Holland EC, Tempst P: Serum peptide profiling by magnetic particle-assisted, automated sample processing and MALDI-TOF mass spectrometry. Anal Chem 2004;76:1560–1570.
27 Baumann S, Ceglarek U, Fiedler GM, Lembcke J, Leichtle A, Thiery J: Standardized approach to proteome profiling of human serum based on magnetic bead separation and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Clin Chem 2005;51:973–980.
28 Chaurand P, Sanders ME, Jensen RA, Caprioli RM: Proteomics in diagnostic pathology: profiling and imaging proteins directly in tissue sections. Am J Pathol 2004;165:1057–1068.
29 Old WM, Meyer-Arendt K, Aveline-Wolf L, Pierce KG, Mendoza A, Sevinsky JR, Resing KA, Ahn NG: Comparison of label-free methods for quantifying human proteins by shotgun proteomics. Mol Cell Proteomics 2005;4:1487–1502.
30 McDonald WH, Yates JR III: Shotgun proteomics: integrating technologies to answer biological questions. Curr Opin Mol Ther 2003;5:302–309.
31 Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlet-Jones M, He F, Jacobson A, Pappin DJ: Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. Mol Cell Proteomics 2004;3:1154–1169.
32 Nilsson P, Paavilainen L, Larsson K, Odling J, Sundberg M, Andersson AC, Kampf C, Persson A, Al-Khalili SC, Ottosson J, Bjorling E, Hober S, Wernerus H, Wester K, Ponten F, Uhlen M: Towards a human proteome atlas: high-throughput generation of mono-specific antibodies for tissue profiling. Proteomics 2005;5:4327–4337.

33 Uhlen M, Bjorling E, Agaton C, Szigyarto CA, Amini B, Andersen E, Andersson AC, Angelidou P, Asplund A, Asplund C, Berglund L, Bergstrom K, Brumer H, Cerjan D, Ekstrom M, Elobeid A, Eriksson C, Fagerberg L, Falk R, Fall J, Forsberg M, Bjorklund MG, Gumbel K, Halimi A, Hallin I, Hamsten C, Hansson M, Hedhammar M, Hercules G, Kampf C, Larsson K, Lindskog M, Lodewyckx W, Lund J, Lundeberg J, Magnusson K, Malm E, Nilsson P, Odling J, Oksvold P, Olsson I, Oster E, Ottosson J, Paavilainen L, Persson A, Rimini R, Rockberg J, Runeson M, Sivertsson A, Skollermo A, Steen J, Stenvall M, Sterky F, Stromberg S, Sundberg M, Tegel H, Tourle S, Wahlund E, Walden A, Wan J, Wernerus H, Westberg J, Wester K, Wrethagen U, Xu LL, Hober S, Ponten F: A human protein atlas for normal and cancer tissues based on antibody proteomics. Mol Cell Proteomics 2005;4:1920–1932.
34 Anderson KS, Labaer J: The sentinel within: exploiting the immune system for cancer biomarkers. J Proteome Res 2005;4:1123–1133.
35 Hillen N, Stevanovic S: Contribution of mass spectrometry-based proteomics to immunology. Expert Rev Proteomics 2006;3:653–664.
36 Lemmel C, Weik S, Eberle U, Dengjel J, Kratt T, Becker HD, Rammensee HG, Stevanovic S: Differential quantitative analysis of MHC ligands by mass spectrometry using stable isotope labeling. Nat Biotechnol 2004;22:450–454.
37 Zarling AL, Polefrone JM, Evans AM, Mikesh LM, Shabanowitz J, Lewis ST, Engelhard VH, Hunt DF: Identification of class I MHC-associated phosphopeptides as targets for cancer immunotherapy. Proc Natl Acad Sci USA 2006;103:14889–14894.
38 Sarto C, Frutiger S, Cappellano F, Sanchez JC, Doro G, Catanzaro F, Hughes GJ, Hochstrasser DF, Mocarelli P: Modified expression of plasma glutathione peroxidase and manganese superoxide dismutase in human renal cell carcinoma. Electrophoresis 1999;20:3458–3466.
39 Balabanov S, Zimmermann U, Protzel C, Scharf C, Klebingat KJ, Walther R: Tumour-related enzyme alterations in the clear cell type of human renal cell carcinoma identified by two-dimensional gel electrophoresis. Eur J Biochem 2001;268:5977–5980.
40 Dallmann K, Junker H, Balabanov S, Zimmermann U, Giebel J, Walther R: Human agmatinase is diminished in the clear cell type of renal cell carcinoma. Int J Cancer 2004;108:342–347.
41 Zimmermann U, Balabanov S, Giebel J, Teller S, Junker H, Schmoll D, Protzel C, Scharf C, Kleist B, Walther R: Increased expression and altered location of annexin IV in renal clear cell carcinoma: a possible role in tumour dissemination. Cancer Lett 2004;209:111–118.
42 Unwin RD, Craven RA, Harnden P, Hanrahan S, Totty N, Knowles MA, Eardley I, Selby PJ, Banks RE: Proteomic changes in renal cancer and co-ordinate demonstration of both the glycolytic and mitochondrial aspects of the Warburg effect. Proteomics 2003;3:1620–1632.
43 Hwa JS, Park HJ, Jung JH, Kam SC, Park HC, Kim CW, Kang KR, Hyun JS, Chung KH: Identification of proteins differentially expressed in the conventional renal cell carcinoma by proteomic analysis. J Korean Med Sci 2005;20:450–455.
44 Seliger B, Lichtenfels R, Atkins D, Bukur J, Halder T, Kersten M, Harder A, Ackermann A, Malenica B, Brenner W, Zobawa M, Lottspeich F: Identification of fatty acid binding proteins as markers associated with the initiation and/or progression of renal cell carcinoma. Proteomics 2005;5:2631–2640.
45 Perroud B, Lee J, Valkova N, Dhirapong A, Lin PY, Fiehn O, Kultz D, Weiss RH: Pathway analysis of kidney cancer using proteomics and metabolic profiling. Mol Cancer 2006;5:64.
46 Alchanati I, Nallar SC, Sun P, Gao L, Hu J, Stein A, Yakirevich E, Konforty D, Alroy I, Zhao X, Reddy SP, Resnick MB, Kalvakolanu DV: A proteomic analysis reveals the loss of expression of the cell death regulatory gene GRIM-19 in human renal cell carcinomas. Oncogene 2006;25: 7138–7147.
47 Craven RA, Hanrahan S, Totty N, Harnden P, Stanley AJ, Maher ER, Harris AL, Trimble WS, Selby PJ, Banks RE: Proteomic identification of a role for the von Hippel-Lindau tumour suppressor in changes in the expression of mitochondrial proteins and septin 2 in renal cell carcinoma. Proteomics 2006;6:3880–3893.
48 Nakamura E, Abreu-e-Lima P, Awakura Y, Inoue T, Kamoto T, Ogawa O, Kotani H, Manabe T, Zhang GJ, Kondo K, Nose V, Kaelin WG Jr: Clusterin is a secreted marker for a hypoxia-inducible factor-independent function of the von Hippel-Lindau tumor suppressor protein. Am J Pathol 2006;168:574–584.

49 Zhuang Z, Huang S, Kowalak JA, Shi Y, Lei J, Furuta M, Lee YS, Lubensky IA, Rodgers GP, Cornelius AS, Weil RJ, Teh BT, Vortmeyer AO: From tissue phenotype to proteotype: sensitive protein identification in microdissected tumor tissue. Int J Oncol 2006;28:103–110.
50 Bloom GC, Eschrich S, Zhou JX, Coppola D, Yeatman TJ: Elucidation of a protein signature discriminating six common types of adenocarcinoma. Int J Cancer 2007;120:769–775.
51 Poznanovic S, Wozny W, Schwall GP, Sastri C, Hunzinger C, Stegmann W, Schrattenholz A, Buchner A, Gangnus R, Burgemeister R, Cahill MA: Differential radioactive proteomic analysis of microdissected renal cell carcinoma tissue by 54 cm isoelectric focusing in serial immobilized pH gradient gels. J Proteome Res 2005;4:2117–2125.
52 Von Eggeling F, Junker K, Fiedle W, Wollscheid V, Dürst M, Claussen U, Ernst G: Mass spectrometry meets chip technology: a new proteomic tool in cancer research? Electrophoresis 2001;22:2898–2902.
53 Fetsch PA, Simone NL, Bryant-Greenwood PK, Marincola FM, Filie AC, Petricoin EF, Liotta LA, Abati A: Proteomic evaluation of archival cytologic material using SELDI affinity mass spectrometry: potential for diagnostic applications. Am J Clin Pathol 2002;118:870–876.
54 Junker K, Gneist J, Melle C, Driesch D, Schubert J, Claussen U, von EF: Identification of protein pattern in kidney cancer using ProteinChip arrays and bioinformatics. Int J Mol Med 2005;15:285–290.
55 Nakamura K, Yoshikawa K, Yamada Y, Saga S, Aoki S, Taki T, Tobiume M, Shimazui T, Akaza H, Honda N: Differential profiling analysis of proteins involved in anti-proliferative effect of interferon-α on renal cell carcinoma cell lines by protein biochip technology. Int J Oncol 2006;28:965–970.
56 Adam PJ, Terrett JA, Steers G, Stockwin L, Loader JA, Fletcher GC, Lu LS, Leach BI, Mason S, Stamps AC, Boyd RS, Pezzella F, Gatter KC, Harris AL: CD70 (TNFSF7) is expressed at high prevalence in renal cell carcinomas and is rapidly internalised on antibody binding. Br J Cancer 2006;95:298–306.
57 Junker K, Hindermann W, von Eggeling F, Diegmann J, Haessler K, Schubert J: CD70: a new tumor specific biomarker for renal cell carcinoma. J Urol 2005;173:2150–2153.
58 Diegmann J, Junker K, Gerstmayer B, Bosio A, Hindermann W, Rosenhahn J, von Eggeling F: Identification of CD70 as a diagnostic biomarker for clear cell renal cell carcinoma by gene expression profiling, real-time RT-PCR and immunohistochemistry. Eur J Cancer 2005;41:1794–1801.
59 Law CL, Gordon KA, Toki BE, Yamane AK, Hering MA, Cerveny CG, Petroziello JM, Ryan MC, Smith L, Simon R, Sauter G, Oflazoglu E, Doronina SO, Meyer DL, Francisco JA, Carter P, Senter PD, Copland JA, Wood CG, Wahl AF: Lymphocyte activation antigen CD70 expressed by renal cell carcinoma is a potential therapeutic target for anti-CD70 antibody-drug conjugates. Cancer Res 2006;66:2328–2337.
60 Castronovo V, Waltregny D, Kischel P, Roesli C, Elia G, Rybak JN, Neri D: A chemical proteomics approach for the identification of accessible antigens expressed in human kidney cancer. Mol Cell Proteomics 2006;5:2083–2091.
61 Chaurand P, Norris JL, Cornett DS, Mobley JA, Caprioli RM: New developments in profiling and imaging of proteins from tissue sections by MALDI mass spectrometry. J Proteome Res 2006;5:2889–2900.
62 Pieper R, Gatlin CL, McGrath AM, Makusky AJ, Mondal M, Seonarain M, Field E, Schatz CR, Estock MA, Ahmed N, Anderson NG, Steiner S: Characterization of the human urinary proteome: a method for high-resolution display of urinary proteins on two-dimensional electrophoresis gels with a yield of nearly 1,400 distinct protein spots. Proteomics 2004;4:1159–1174.
63 Won Y, Song HJ, Kang TW, Kim JJ, Han BD, Lee SW: Pattern analysis of serum proteome distinguishes renal cell carcinoma from other urologic diseases and healthy persons. Proteomics 2003;3:2310–2316.
64 Tolson J, Bogumil R, Brunst E, Beck H, Elsner R, Humeny A, Kratzin H, Deeg M, Kuczyk M, Mueller GA, Mueller CA, Flad T: Serum protein profiling by SELDI mass spectrometry: detection of multiple variants of serum amyloid α in renal cancer patients. Lab Invest 2004;84:845–856.
65 Rogers MA, Clarke P, Noble J, Munro NP, Paul A, Selby PJ, Banks RE: Proteomic profiling of urinary proteins in renal cancer by surface enhanced laser desorption ionization and neural-network

analysis: identification of key issues affecting potential clinical utility. Cancer Res 2003;63: 6971–6983.
66 Rossi L, Martin BM, Hortin GL, White RL, Foster M, Moharram R, Stroncek D, Wang E, Marincola FM, Panelli MC: Inflammatory protein profile during systemic high dose interleukin-2 administration. Proteomics 2006;6:709–720.
67 Panelli MC, White R, Foster M, Martin B, Wang E, Smith K, Marincola FM: Forecasting the cytokine storm following systemic interleukin-2 administration. J Transl Med 2004;2:17.
68 Klade CS, Voss T, Krystek E, Ahorn H, Zatloukal K, Pummer K, Adolf GR: Identification of tumor antigens in renal cell carcinoma by serological proteome analysis. Proteomics 2001;1: 890–898.
69 Lichtenfels R, Kellner R, Bukur J, Beck J, Brenner W, Ackermann A, Seliger B: Heat shock protein expression and anti-heat shock protein reactivity in renal cell carcinoma. Proteomics 2002; 2:561–570.
70 Kellner R, Lichtenfels R, Atkins D, Bukur J, Ackermann A, Beck J, Brenner W, Melchior S, Seliger B: Targeting of tumor-associated antigens in renal cell carcinoma using proteome-based analysis and their clinical significance. Proteomics 2002;2:1743–1751.
71 Lichtenfels R, Kellner R, Atkins D, Bukur J, Ackermann A, Beck J, Brenner W, Melchior S, Seliger B: Identification of metabolic enzymes in renal cell carcinoma utilizing Proteomex analyses. Biochim Biophys Acta 2003;1646:21–31.
72 Unwin RD, Harnden P, Pappin D, Rahman D, Whelan P, Craven RA, Selby PJ, Banks RE: Serological and proteomic evaluation of antibody responses in the identification of tumor antigens in renal cell carcinoma. Proteomics 2003;3:45–55.
73 Weinschenk T, Gouttefangeas C, Schirle M, Obermayr F, Walter S, Schoor O, Kurek R, Loeser W, Bichler KH, Wernet D, Stevanovic S, Rammensee HG: Integrated functional genomics approach for the design of patient-individual antitumor vaccines. Cancer Res 2002;62:5818–5827.
74 Kruger T, Schoor O, Lemmel C, Kraemer B, Reichle C, Dengjel J, Weinschenk T, Muller M, Hennenlotter J, Stenzl A, Rammensee HG, Stevanovic S: Lessons to be learned from primary renal cell carcinomas: novel tumor antigens and HLA ligands for immunotherapy. Cancer Immunol Immunother 2005;54:826–836.
75 Weinzierl AO, Lemmel C, Schoor O, Muller M, Kruger T, Wernet D, Hennenlotter J, Stenzl A, Klingel K, Rammensee HG, Stevanovic S: Distorted relation between mRNA copy number and corresponding major histocompatibility complex ligand density on the cell surface. Mol Cell Proteomics 2007;6:102–113.

Dr. Rosamonde E. Banks
Cancer Research UK Clinical Centre
St James's University Hospital
Beckett Street, Leeds LS9 7TF (UK)
Tel. +44 113 206 4927, Fax +44 113 242 9886, E-Mail r.banks@leeds.ac.uk

Thongboonkerd V (ed): Proteomics in Nephrology – Towards Clinical Applications.
Contrib Nephrol. Basel, Karger, 2008, vol 160, pp 107–126

Capillary Electrophoresis Coupled to Mass Spectrometry for Biomarker Discovery and Diagnosis of Kidney Diseases

Petra Zürbig, Harald Mischak

Mosaiques Diagnostics and Therapeutics AG, Hannover, Germany

Abstract

The main focus of urinary proteome analysis in nephrology is currently on detection and identification of polypeptides that significantly alter (in abundance, distribution, etc.) during (patho)physiological changes of the kidney structure and/or function. Capillary electrophoresis coupled online to electrospray ionization time-of-flight mass spectrometry (CE-MS) was applied to human urine to identify biomarkers for clinical diagnostics. To extract the information of the CE-MS spectra in a timely fashion, software was designed to automatically deconvolute and normalize the spectra. Furthermore, bioinformatics and statistic approaches were used to discriminate patients with different diseases and healthy individuals, respectively. Samples from patients with renal diseases display polypeptide patterns that differ significantly from those obtained from healthy individuals. Examining series of patients with the same disease allowed the establishment of polypeptide patterns typical for specific diseases. This permits the search for the disease-specific peptide markers. The combinations of several polypeptides found in urine are forming a specific pattern, which is indicative not only for the particular disease, but also for the stage of disease. These results show that proteome analysis with CE-MS of urinary polypeptides in patients with different renal diseases and urological disorders can display the current status of the kidney or the urogenital tract. This advancement offers the early diagnosis of different nephrological diseases.

The main focus of proteome analysis in nephrology is currently on detection and identification of (urinary) proteins that significantly alter (in abundance, distribution, etc.) during (patho)physiological changes of the kidney structure and/or function. To allow specific and early assessment of disease, at least some of these proteins should be biomarkers that are independent from proteinuria. These biomarkers may be directly related to the disease (e.g., IgA

immune deposits in IgA nephropathy), or may result from secondary events (e.g., generation of specific cleavage products by metalloproteases that are up-regulated during inflammatory processes in the kidney). After validation, some of these changes may potentially be new therapeutic targets or novel biomarkers for disease detection and/or prognosis.

While this book chapter is focused on urine, kidney tissue certainly contains relevant proteomic information. However, analysis of its proteome comprises several disadvantages: the kidney is composed of different cell types (all of which express different and specialized proteomes) and tissue samples must be obtained invasively, rendering proteome analysis especially of the normal human kidney (which is required as the control) ethically difficult or even impossible. Therefore, most research has focused on tissue obtained from experimental animals. The proteome of the rat kidney has been described recently by Arthur et al. [1–3]. The authors have shown differential expression of proteins in the renal cortex and medulla. Two-dimensional gel electrophoresis (2-DE) resolved 1,095 spots from the cortex and 885 spots from the medulla. By matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), 54 unique proteins were identified. Nine of them were differentially expressed in the cortex and medulla and four were expressed in only one region. Xu et al. [4] examined glomeruli obtained by laser capture dissection. Subsequently, the proteome of tissue in the 5/6 nephrectomy rat model of focal segmental glomerulosclerosis was analyzed. They identified thymosin β4 as a marker of glomerulosclerosis. Recently, the first report of such 'protein maps' from murine tissue has also been published [5].

On the other hand, urinary proteins can be analyzed directly or separated by centrifugation into distinct fractions. For example, supernatants from low-speed centrifugation contain proteins derived from filtered plasma proteins and secreted by tubular epithelial cells. This supernatant can be further centrifuged at high speed (ultracentrifugation) yielding a pellet containing exosomes, small vesicles (with diameter <80 nm) with cell membrane and cytosolic proteins. These exosomes are derived from epithelial cells lining the urinary tract with a contribution from filtered exosomes from blood cells [6, 7].

Urine Proteomics

Before the 'proteomics' era, many investigators have sought to better define the urinary proteome in a variety of clinical situations. In this respect, one of the first attempts to define proteins in the urine was published by Spahr and co-workers [8, 9]. Using liquid chromatography coupled mass spectrometry (LC-MS) they identified 124 proteins from pooled urine samples

after tryptic digestion. While this study did not attempt to define any urinary biomarkers for a disease, it clearly highlighted the plethora of information in the urinary proteome and also a possible approach towards its mining. This conclusion was underscored by Pang et al. [10], who used not only 2-DE, but also 1- and 2-dimensional LC-MS to identify potential biomarkers for inflammation. Using acetone-precipitated urine samples from healthy volunteers, Thongboonkerd et al. [11] defined the first human urinary proteome map, consisting of 67 proteins and their isoforms that could be used as a reference. In a subsequent study by Oh et al. [12], pooled urine samples from 20 healthy volunteers were used to annotate 113 proteins on a 2-DE by peptide mass fingerprinting. Additional experiments that further expanded the knowledge of the normal urinary proteome have been reported by Pieper et al. [13], Sun et al. [14], and Castagna et al. [15]. Taken together, these approaches have identified approximately 800 proteins and laid the foundation for subsequent discovery of biomarkers in the urinary proteome. In a very recent study on urine obtained from healthy individuals, Adachi et al. [16] have identified more than 1,500 proteins in the urine of healthy individuals, further underlining the complexity of the human urinary proteome. A large proportion of proteins identified in this study was represented by membrane proteins. This may be due to the presence of exosomes [7]. Recently, exosomal fetuin-A has been proposed as biomarker of acute kidney injury, based on data from a rat model [17], which were further supported by western blots on 3 patients. While these data and the concept of exosomes are very promising, these preliminary observations need to be verified and further explored.

CE-MS for Proteomic Analysis

Since the introduction of 2-DE by O'Farrell [18] in 1975, especially mass spectrometric developments have been established to enhance biomarker discovery. 2-DE-MS [19–21], surface-enhanced laser desorption/ionization (SELDI) [22, 23] or LC-MS [24–26] have been extensively used to define biomarkers for a more detailed understanding of both normal and pathological processes. Recently, capillary electrophoresis coupled mass spectrometry (CE-MS) (fig. 1) was demonstrated to be a powerful alternative to the above-mentioned commonly used proteomic technologies [27–30]. The technology was successfully applied to answer the demands of biomarker discovery of clinical significance [31–35].

CE-MS is a technology offering several advantages [36–40]: (i) it provides fast separation and high resolution [41]; (ii) it is quite robust and uses inexpensive capillaries [34]; (iii) it is compatible with most buffers and analytes (provided that the buffer is volatile, as generally required for electrospray) [42], and (iv) it provides a stable constant flow avoiding use of buffer gradients [43].

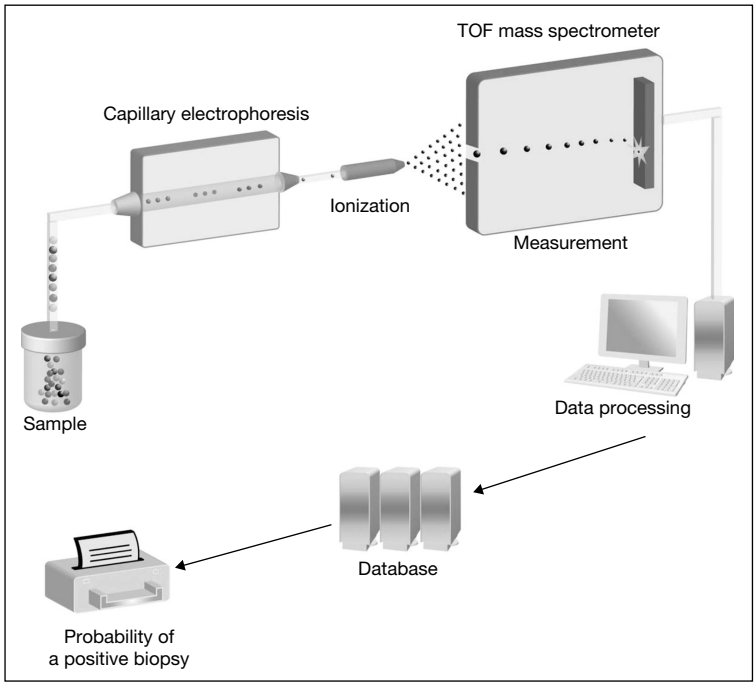


Fig. 1. Schematic drawing of CE-MS protocol (published by Sniehotta et al. [98]). Urine samples are prepared for analysis, polypeptides are separated by CE and directly sprayed into ESI-TOF-MS. Data are evaluated using specific software solutions. Each polypeptide is defined by its accurate mass and normalized CE migration time. Signal intensity serves as measure of the relative abundance. The data are stored as peak lists summarizing the information in a database.

While such buffer gradients may increase the selectivity of CE, they will on the other hand require continuous adjustment of the ionization voltage for optimal ionization (as is the case in LC-MS analysis). This would consequently reduce the robustness. As a consequence, CE certainly appears to be an excellent choice for the separation of complex biological samples, like urine. It may be owed to historical reasons – mainly the initial problems of interfacing CE with MS as well as the rather high amount of sample required for analysis in the first mass spectrometers – that CE-MS is not used more widely today. When acidic running buffers are used, as is frequently the case, CE-MS is not well suited for the analysis of proteins >20–30 kDa. A fraction of these proteins frequently precipitates in the capillary and is consequently not available for subsequent MS analysis.

Table 1. Advantages and disadvantages of different MS-based proteomic techniques for clinical applications, e.g., two-dimensional gel electrophoresis followed by mass spectrometry (2-DE-MS), liquid chromatography coupled to mass spectrometry (LC-MS), surface-enhanced laser desorption/ionization coupled to mass spectrometry (SELDI-MS), and capillary electrophoresis coupled to mass spectrometry (CE-MS)

Proteomic methods	Characteristics	Advantages	Disadvantages
2-DE-MS	Separation with 2-D electrophoresis (first dimension: isoelectric point, second dimension: molecular weight); protein identification with MS and MS/MS	Applicable to large molecules; high resolution	Not applicable to peptides <10 kDa; no automation; time consuming; quantification difficult; expensive
LC-MS	Separation with LC; analysis of peptide masses with MS	Automation; multidimensional; high sensitivity; MS/MS possibility	Time-consuming, sensitive towards interfering compounds, restricted mass range
SELDI-MS	Separation with different surface chemistries of the arrays; analysis of peptide masses with MS	Easy-to-use system; automation, low sample volume required	Restricted to selected polypeptides, low-resolution MS, interpretation of data difficult without sequence information
CE-MS	Separation with capillary electrophoresis; analysis of peptide masses with MS	Automation, high sensitivity, fast, low sample volumes required, multidimensional, low cost, MS/MS possibility	Not well suited for larger polypeptides (>20 kDa)

A major goal for clinical application is the differential display of a large number of polypeptides in a single, reproducible and time-limited step enabling comparison of different protein patterns. The advantages and limitations of the proteomic technologies, including 2-DE-MS, SELDI, LC-MS, and CE-MS with respect to this goal are summarized in table 1.

CE-MS Coupling

Several technical considerations have to be taken into account to achieve stable CE-MS coupling. These aspects are extensively reviewed [42, 44–46].

Compared to HPLC, CE flow rates are much lower and the migration of analytes is determined to a large extent by the electric field strength instead of the liquid flow. This creates the physical problem of closing the electrical current for separation in addition to achieving a stable MS interface. While one of the electrodes can be easily interfaced with the capillary using the buffer at the inlet, interfacing of the other electrode at the outlet, which has to serve as the interface to the MS, is more difficult to realize.

Several solutions of this problem have been reported, such as the liquid junction approaches [47], the sheathless coupling [48] or the graphite coating [49]. However, in our hands, sheath-flow coupling has been most stable [50]. Sheath liquid circum flows the end of the capillary and closes the electrical circuit. Such a setup is quite comparable to micro-ion-spray devices, but in comparison to an LC-MS interface, has the advantage of a consistent buffer, hence no continuous change of ionization conditions is required to accommodate changes in the concentration of an organic solvent. Dilution of the sample by the sheath flow is observed to a lesser extent than expected, probably due to incomplete mixing in the Taylor cone [51]. The stability of the sheath-flow coupling is certainly beneficial for clinical applications and outweighs the lower sensitivity in comparison to the other methods of coupling.

A similar setup can be utilized for offline spotting of the CE fractions for subsequent MALDI-TOF-MS analysis [52]. In this particular case, the sheath liquid not only closes the electrical circuit, but also deposits the matrix required for analyte ionization on the target plate. The attractiveness of this approach lies in the fact that the sample is largely preserved and can be reanalyzed if necessary.

Sample Preparation

To make proteins accessible for further analysis, sample preparation is the first and the most important step. When analyzing complex biological samples, major concerns are loss of polypeptides and information as well as reproducibility. Ideally, a crude, unprocessed sample should be analyzed, which would avoid all artificial losses or biases arising from sample preparation. Easily accessible sources for analysis are body fluids, because the proteins available are already dissolved. However, body fluids are very complex mixtures of molecules with a wide range of polarity, hydrophobicity, and size over a range of several orders of magnitude. Since urine contains a large amount of different ions, lipids, carbohydrates, etc., these samples cannot be analyzed in the native form in a mass spectrometer. Although, for example, CE is relatively insensitive towards these interfering compounds and allows the direct injection of crude urine, the high salt content of this sample interferes with the CE-separation process [34]. Similar disturbances are observed

for other frequently used separation procedures, such as LC, SELDI, or 2-DE. Therefore, it appears advisable to remove salts and other low-molecular-weight compounds before analysis using e.g. size exclusion, anion-exchange [53], or reversed-phase materials [54]. In addition, larger proteins, such as albumin, tend to precipitate under the conditions employed resulting in co-precipitation of other proteins and peptides, thus reducing reproducibility and comparability [55]. Ultrafiltration or affinity columns can be used for the removal of these proteins [56]. Affinity columns tend to be less efficient and of higher cost.

Data Mining

The information content of a complex proteome analysis requires adequate tools for data analysis. The essential information that needs to be extracted includes the identity and quantity of polypeptides present. A prerequisite for the comparative evaluation of urine (or any other comparative analysis) is the ability to identify identical compounds with high probability in consecutive samples. Hence, resolution and accuracy of the parameters used for identification are of major importance. One way to increase the resolution of the MS data is to combine these with the parameters of the separation (e.g., retention or migration time, but every other unique measure may serve as additional or alternative identifying parameter). Software solutions that automatically select peaks based on parameters such as signal/noise ratio or appearance in several consecutive spectra have been reported, such as MSight [57], DeCyder MS (GE Healthcare), or MosaiquesVisu [3, 34, 55]. It is important that the software is able to perform charge deconvolution with a low error rate and combines peaks (and amplitude) that represent identical compounds at different charge states, as reported for MosaiquesVisu [3].

Furthermore, CE-MS migration time varies with the ion strength of the sample and the MS signal intensity varies depending on the efficiency of ionization, the detector gain, etc. [34]. Therefore, these parameters of the detected polypeptides have to be normalized. This normalization can be achieved using external standards or by the utilization of polypeptides that are found with high frequency in the analyzed samples and that serve as internal standards [55, 58]. Finally, a list of analyzed, unambiguously identified and standardized peptides of a given sample is obtained. Digital compilation of individual datasets to specific polypeptide pattern (see fig. 2) enables biomarker definition.

Most, if not all, proteomic studies indicated that a single biomarker does not allow reliable diagnosis, staging or prognosis of a kidney disease. This finding immediately raises the question of how to combine several biomarkers to provide a diagnostic or predictive pattern. While a definitive answer is probably still far away, a number of approaches have emerged.

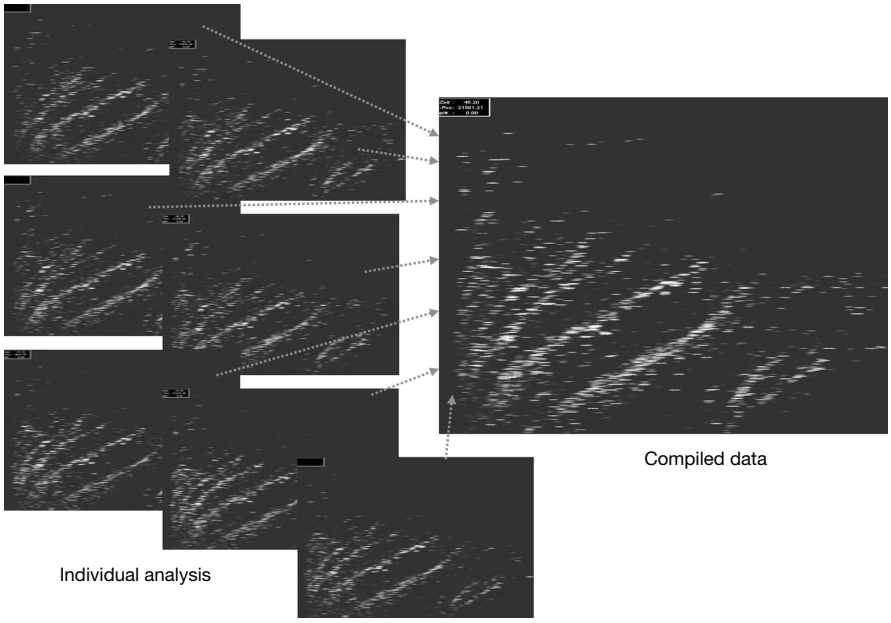


Fig. 2. Digital data compilation. Five individual CE-MS datasets of analyzed urine samples with mass (0.8–20 kDa) plotted against CE migration time (18–50 min) and MS signal intensity in the z-axis were digitally compiled to a specific polypeptide pattern.

Hierarchical decision tree-based classification methods, such as CART (Classification and Regression Trees) [59], were among the first algorithms to utilize the available information on multiple biomarkers. However, empirical observations suggested that these approaches were not too successful, because the number of incorrect predictions made by the classification algorithm increases with the complexity of the decision tree [60]. The number of datasets available to establish the decision tree is generally low, resulting in a lack of statistical significance beyond the second or third nodes of the tree.

Support vector machines (SVM) [for an example, see 61] provided a tool to overcome some of these limitations due to the theoretical principles upon which they are based. Excellent empirical performance of SVM has been reported in a number of diverse applications [60, 62, 63]. A promising probabilistic classification method that shares many of the positive characteristics of the SVM, but in addition provides the important levels of confidence with each classification prediction, is based on the gaussian process [for a comprehensive and somewhat technical text to this methodology, see 64]. The probabilistic

nature of gaussian process-based classification methods provides a means of inferring optimally weighted combinations and possible selection of biomarkers; a detailed study of this capability is currently ongoing.

No matter which of these approaches is used, two basic considerations apply: (1) the number of independent variables should be kept to a minimum, certainly less than the number of samples investigated, and (2) any such approach must be confirmed with a blinded validation set. It should be imperative to include such a blinded dataset in any report on potential biomarkers.

Urinary CE-MS Pattern for Biomarker Discovery

Analysis of changes of urinary polypeptides has been a long-standing practice in nephrology. Recent analysis of urine by CE-MS confirmed existence of disease-specific biomarkers of diseases of the kidney and the urogenital tract [60, 65–69]. The definition of disease-specific biomarkers in the urine is complicated by significant changes in the urinary proteome during the day, most likely due to exercise, variations in the diet, circadian rhythms, etc. [70]. As a consequence, the reproducibility of the assay is reduced due to these physiological changes, even if the analytical method shows high reproducibility. In addition, clear differences between first-void and midstream samples can be noted [Mischak et al., unpubl. data], further highlighting the importance of standardized protocols for urine sample collection.

Urinary Biomarkers for Kidney Diseases

Tentative and thorough optimization of CE-MS [44, 71–75] made this approach a powerful proteomic technology that has been successfully used to answer the demands for clinically relevant differential analysis. Among the clinically important and easily available body fluids, such as blood or cerebrospinal fluid, urine provides several advantages. Urine is non-invasively accessible in large quantities and may contain information on health status of the kidney [68, 69, 76–78], but also of the bladder [79–83], the prostate [55, 84, 85] and the vascularization [86]. In addition, potential problems with stability of urine samples are less an issue compared to other body fluids, such as serum or plasma [79, 87].

The polypeptide composition of urine is affected by common factors, such as nutritional state, metabolic or catabolic processes as well as by levels of different hormones. However, these variations are limited to a part of the urinary proteome; a basal part remains unaffected by these processes and facilitates urinary proteome analysis. Hence, the analysis of urine from 18 healthy volunteers using online coupled CE-MS led to the definition of a ‘normal urine

polypeptide pattern' consisting of 247 polypeptides present in more than 50% of the samples [54].

These findings were the starting point of the search for marker peptides that are specific for different diseases using CE-MS technologies. Kaiser et al. [88] described the analysis of urine samples obtained from patients with diabetes mellitus, diabetic nephropathy (DN), minimal change disease (MCD), or focal segmental glomerulosclerosis (FSGS). The obtained data suggest that a single polypeptide present in all patients with the same disease, however absent in all other diseases and healthy controls, does not exist. However, a combination of several distinct and well-defined markers provides a disease-specific pattern specific for a particular disease.

Following this methodology, differentiating polypeptide patterns could be achieved in the urine of patients suffering from renal diseases compared to healthy individuals. In addition, differentiation of different types of renal diseases, such as IgA nephropathy (IgAN), membranous nephropathy (MN), DN, MCD and FSGS has been feasible by this approach.

CE-MS data obtained from urine sample analysis sensitively reflect changes of the kidney function, enabling contemporary diagnosis of renal diseases. In this context, the work of Decramer et al. [89] can be interpreted as first proof of the capability of CE-MS-based proteomics for early diagnosis. The authors analyzed urinary polypeptides from infants with ureteropelvic junction obstruction to predict a need for surgical correction. As evident from the results shown in another chapter of this book, the authors identified and, in a prospective blinded study, validated polypeptides markers that enable diagnosis of the severity of obstruction. This resulted in the correct prediction of clinical evolution of 34/36 neonates with ureteropelvic junction obstruction several months in advance.

In addition to the definition of disease-specific polypeptide patterns, CE-MS can define polypeptide markers that are stage-specific. Mischak et al. [68] and Meier et al. [76] were able to define stage-specific biomarkers for DN patients suffering from diabetes mellitus type 1 or type 2. In both studies, the individual datasets of healthy volunteers (9 and 39, respectively), patients with diabetes type I or II without macroalbuminuria (28 and 46, respectively), those with intermittent or persistent macroalbuminuria (16 and 66, respectively) were combined to create typical polypeptide patterns. In patients with type 2 diabetes and normal albumin excretion rate, the detected polypeptide pattern differed significantly from those found in patients with higher grade albuminuria with a pattern indicative for a diabetic renal damage. Comparable results were obtained for patients with diabetes type I suggesting that the urinary proteome contains a much greater variety of polypeptides than demonstrated before.

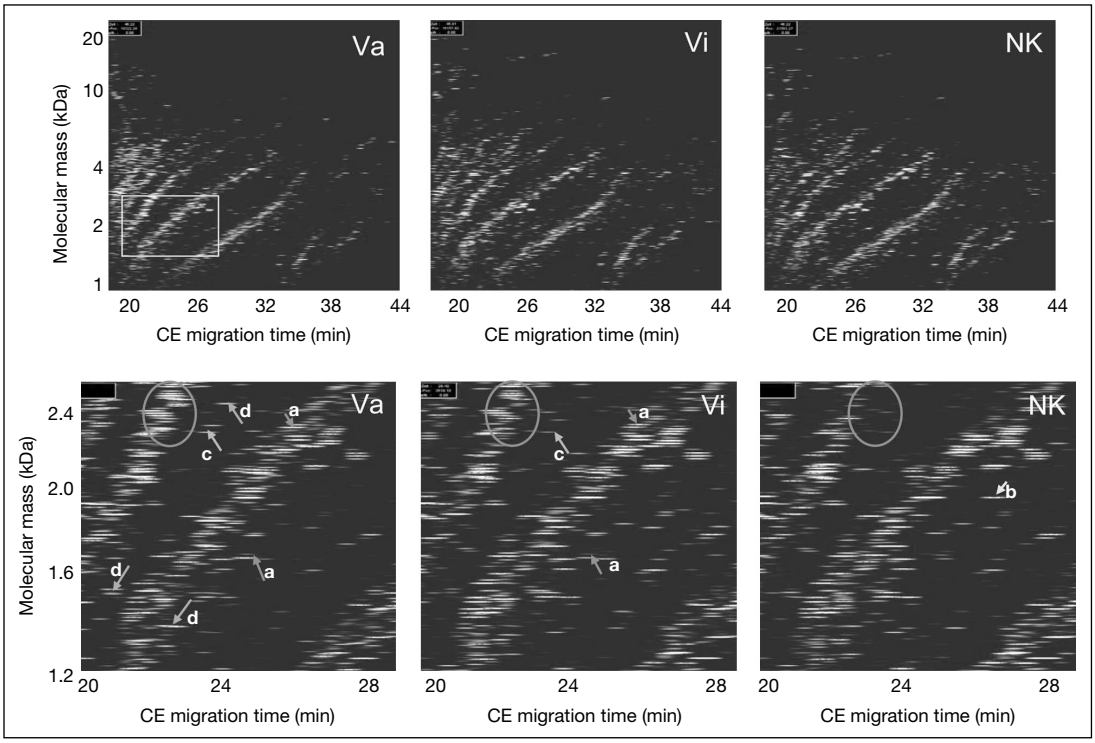


Fig. 3. Potential biomarkers for vasculitis and the activity of the disease (published by Schiffer et al. [2]). Upper panel: compiled data from active vasculitis (Va), vasculitis after therapy (Vi) and normal controls (NK). Migration time (in min) is plotted against molecular mass (in kDa). While these data appear quite similar at first sight, in the enlarged section below, several potential biomarkers become evident. Arrow (**a**) points to polypeptides that are present in chronic renal disease (in addition, an entire cluster of polypeptides specific for chronic renal disease is encircled), arrow (**b**) indicates a peptide generally present in normal controls, arrow (**c**) indicates a potential biomarker for vasculitis, and arrow (**d**) indicates potential biomarkers for active vasculitis.

In an ongoing study, we were able to define biomarkers for vasculitis [2]. Vasculitides are clinical syndromes characterized by vessel wall inflammation and resultant organ damage; one of the frequently affected organs is the kidney [90]. The diagnosis of these syndromes often presents a challenge, and especially robust markers to assess the activity of the disease do not exist. As shown in figure 3, the data we have obtained suggest that CE-MS analysis allows defining both the markers for the disease and also for the activity status, hence enabling evaluation and monitoring of therapy.

In addition to the definition of disease-specific and disease stage-specific polypeptides, monitoring patient's response to therapy using biomarkers is important for physicians. In this context, Rossing et al. [69] analyzed changes in the urinary polypeptide pattern during the treatment of DN patients with the angiotensin II receptor blocker (ARB) candesartan. In a randomized double-blinded cross-over trial, each patient received treatment with placebo, or candesartan 8, 16, or 32 mg daily for 2 months. Candesartan treatment in macroalbuminuric patients significantly changed 15 polypeptides of a 113 biomarker panel comprising 'diabetic renal damage pattern' towards the levels in normalbuminuric patients. These results suggested for the first time the potential of CE-MS to serve as a sensitive tool to monitor the effects of ARB in patients with renal diseases.

Most renal diseases ultimately progress to renal failure (end-stage renal disease). Dialysis or kidney transplantation is required after reaching end-stage renal disease. However, acute rejection of renal allografts is a common adverse situation in the kidney recipients. Hence, methods to detect acute rejection of renal allografts in a non-invasive manner avoiding the risks of renal biopsy would be beneficial. Wittke et al. [91] employed CE-MS to analyze urinary samples from 19 patients with different grades of subclinical or clinical acute rejection, 10 patients with urinary tract infection and 29 patients without evidence of rejection or infection. A distinct urinary polypeptide pattern identified 16 of the 17 cases of acute tubulointerstitial rejection, but was absent in 2 cases of vascular rejection. Potentially confounding variables, such as acute tubular lesions, tubular atrophy, tubulointerstitial fibrosis, calcineurin inhibitor toxicity, proteinuria, hematuria, allograft function and different immunosuppressive regimens did not affect the results. However, an additional polypeptide pattern that allowed differentiating between infection and acute rejection was developed. The defined polypeptide patterns were further validated in a blinded assessment of samples from transplant patients potentially exhibiting renal rejection; majority of the samples was correctly classified using these biomarkers.

Because a variety of renal diseases can be differentiated based on analysis of urine samples, it is reasonable to assume that biomarkers for renal cancer may also exist in the urine. To our knowledge, no CE-MS data for this particular diagnosis have been published. However, Rogers et al. [92] investigated renal cancer using urine samples from a total of 218 individuals with SELDI analysis. While in the first-round sensitivities and specificities of 81.8–83.3% were achieved, the values significantly declined, ranging from 41.0 to 76.6% for the second, larger set of samples 10 months later. The authors analyzed possible contributing factors including sample stability, changing laser performance, and chip variability to asses a long-term robustness of the approach.

Urinary Biomarkers for Urological Disorders

The urinary proteome reflects the status of the kidney, because its composition is directly related to the function of the organ. For example, alterations in filtration rates of the kidney are directly reflected in the ultrafiltrate and the urine. In addition, with the CE-MS analysis of urine further organs of the urinary tract can be determined, particularly malignancy. Due to the fact that urine is also in direct contact with the bladder, proteomic diagnosis of urothelial cancer was performed effectually.

Theodorescu et al. [79] described a non-invasive method for the diagnosis of urothelial carcinoma using CE-MS and bioinformatics. A bladder cancer-specific proteomic pattern was obtained from urine samples of 46 patients with urothelial carcinoma and 33 healthy volunteers. The model was further refined by the use of 366 urine samples from healthy volunteers and patients with malignant and non-malignant genitourinary diseases. In blinded assessment, a prediction model based on 22 polypeptides correctly classified all urothelial carcinoma (n = 31, sensitivity 100% [95% confidence interval (CI) 87–100]) and all healthy samples (n = 11, specificity 100% [95% CI 84–100]). In addition, the differentiation between bladder cancer from other malignant and non-malignant diseases, such as nephrolithiasis, ranged from sensitivity 86 to 100%. Upon closer examination, several of the biomarkers that were defined in this study might also enable staging of the tumor and, consequently, suggest therapeutic measures.

The analysis of urine as a diagnostic tool was also applied to prostate cancer by the use of the seminal fluid contained in male urine. In a pilot study [55], CE-MS techniques defined potential prostate cancer biomarkers in urine. 116 urine samples from patients that underwent prostate biopsy were analyzed. In this sample set, 54 patients had malignant pathology (PCa) and 62 benign prostate changes. The analysis of the data allowed definition of several polypeptides as potential biomarkers for classification of PCa patients with 92% sensitivity and 96% specificity. This pilot study suggested that CE-MS analysis of urine polypeptides may provide a tool to identify putative biomarkers for PCa.

Taking these results as a starting point, the same group refined the obtained prostate-specific pattern by the use of 116 urine samples of 54 PCa patients and 62 patients with benign pathology verified by prostate biopsy. A pattern (depicted in figure 4) of 26 potential biomarkers was validated in a blinded assessment of 81 urine samples from 58 PCa patients and 23 patients with benign prostate in a two-center study [Semjonow et al., in preparation]. The prediction model correctly classified 46 of 58 malignant prostates (89% sensitivity [95% CI 77–96]) and 17 of 23 benign samples (specificity 59% [95% CI 39–77]). These results suggest a tight connection of the prostate gland with the urinary system.

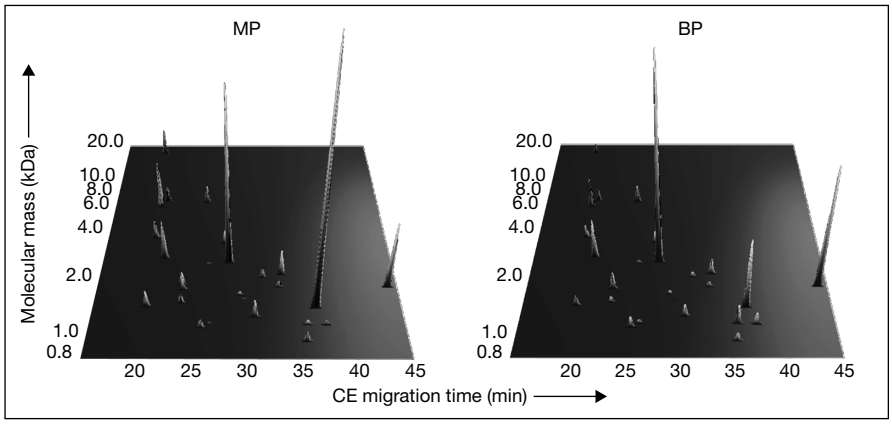


Fig. 4. 3D plot of protein patterns from patients with benign prostate (BP) and malignant cancer (MP).

Sequence Analysis of Urinary Biomarkers

Current literature indicates that CE-MS is a powerful tool allowing fast and reliable analysis of polypeptides from several types of highly complex biological samples, such as urine. Information on several hundred polypeptides from an individual sample can be obtained quickly. Although these polypeptides can serve as excellent biomarkers for diagnostic purposes, their potential physiological role remains unknown as long as their identity defined by their amino acid sequence is not determined. The identification of the defined biomarkers presents some unique challenges. The biomarkers cannot be easily isolated; the sequence analysis has to be performed from a complex mixture and potential biomarkers are frequently post-translationally modified. Potential biomarkers detected by CE-MS are likely to be small fragments of larger proteins. Thus, to identify a 2- to 10-kDa (modified) portion of a protein with a possible molecular weight >60 kDa requires extensive de novo sequencing.

For this purpose, CE can be interfaced online with MS/MS instruments. Neususs et al. [43] describe a capillary electrophoresis coupled tandem mass spectrometry (CE-MS/MS) approach for routine application in proteomic studies. Stable coupling is achieved by using a standard coaxial sheath-flow sprayer. The applied sheath flow is reduced to 1–2 μl/min in order to increase sensitivity. Detection limits are as low as 500 amol. Low femtomole amounts are

required for unequivocal identification by MS/MS experiments in the used ion trap and subsequent database search.

Alternatively, the entire CE-MS run can be spotted offline onto a MALDI target plate and, subsequently, the polypeptides of interest can be analyzed using MALDI-TOF/TOF [34, 93]. This method has the advantage that the signal of interest can be located in MS mode and optimal fragmentation conditions can be determined without repeated separation. However, sequencing with MALDI-TOF/TOF generally does not result in data of sufficient quality from urinary peptides with molecular weights above approximately 3 kDa. Several biomarker candidate peptides were identified using MALDI-MS/MS, as shown for graft-versus-host disease [94], DN [68], dialysis fluid [95] or bladder cancer [79].

Furthermore, FT-ICR MS instruments facilitate the identification of urinary polypeptides, even larger than 8 kDa [65]. The authors described CE offline coupled FT-ICR MS to identify polypeptides in the urine from patients with FSGS, MN, MCD, IgAN and DN, and to validate multiple biomarkers for the control and each of the diseases.

A comparison of the different MS/MS options was recently reported by Zürbig et al. [96]. To date, we have been able to assign 290 peptide sequences to their corresponding masses/CE migration time coordinates. The resulting contour plot is presented in figure 5b. Noticeably, the polypeptides are arranged in the same 4–5 lines that are also present in the corresponding CE-MS contour plot of the entire urine sample (fig. 5a). The members of each line are characterized by the numbers of basic amino acids (arginine; histidine; lysine) included in the peptide sequence. Specifically, the peptides in the line marked with $z = 1$ contain no basic amino acids, only the N-terminus of the peptide is positively charged at the working pH of 2. In contrast, peptides of the other lines show increasing amounts of basic amino acids ($z = 2$; $z = 3$; $z > 3$) in addition to their N-terminal ammonium group. This unique feature facilitates independent entry of different sequencing platforms for peptide sequencing of CE-MS-defined biomarkers from highly complex mixtures.

In summary, the application of CE-MS technology to proteomic analysis enables unique possibilities to solve clinical problems. The technology itself combines high sensitivity, automation and high resolution, and requires low amounts of sample. It expedites the discovery of disease-specific biomarkers and potential therapeutic targets of a variety of diseases based on molecular polypeptide patterns. The combination of excellent performance for biomarker discovery with the unique separation platform-independent biomarker sequencing makes CE-MS a powerful tool for elucidating the pathophysiological relevance of the indicative biomarkers. This may contribute to a better understanding and possibly new definitions of renal diseases and urological disorders based on molecular markers.

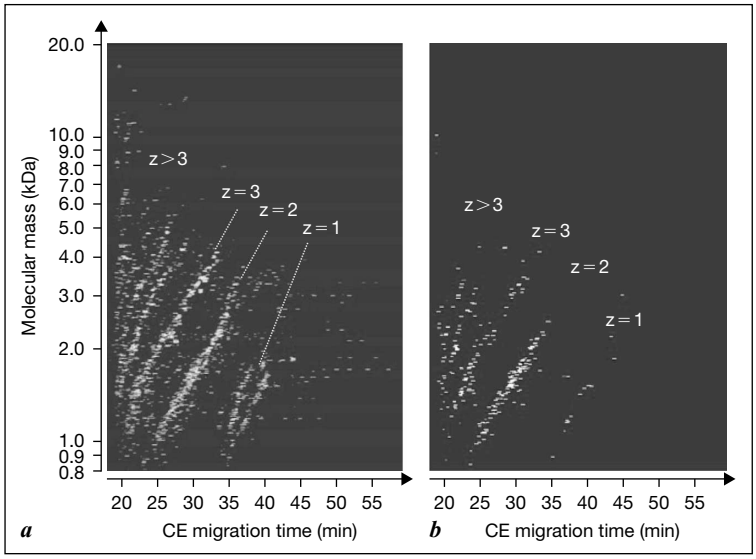


Fig. 5. Compiled CE-MS data of 204 individual datasets from healthy volunteers. ***a*** Contour plot of the entire renal proteome. The molecular weight (logarithmic application) on the y-axis is plotted against CE migration time on the x-axis. The arrangement of the analyzed peptides in distinct lines is obvious. ***b*** Contour plot of 290 identified polypeptides. The lines already observed in figure 5a could be comprehended as a result of the number of positive charges z (at pH 2).

References

1 Arthur JM, Thongboonkerd V, Scherzer JA, Cai J, Pierce WM, Klein JB: Differential expression of proteins in renal cortex and medulla: a proteomic approach. Kidney Int 2002;62:1314–1321.

2 Schiffer E, Mischak H, Novak J: High-resolution proteome/peptidome analysis of body fluids by capillary electrophoresis coupled with MS. Proteomics 2006;6:5615–5627.

3 Neuhoff N, Kaiser T, Wittke S, et al: Mass spectrometry for the detection of differentially expressed proteins: a comparison of surface-enhanced laser desorption/ionization and capillary electrophoresis/mass spectrometry. Rapid Commun Mass Spectrom 2004;18:149–156.

4 Xu BJ, Shyr Y, Liang X, et al: Proteomic patterns and prediction of glomerulosclerosis and its mechanisms. J Am Soc Nephrol 2005;16:2967–2975.

5 Kislinger T, Cox B, Kannan A, et al: Global survey of organ and organelle protein expression in mouse: combined proteomic and transcriptomic profiling. Cell 2006;125:173–186.

6 Zhou H, Yuen PS, Pisitkun T, et al: Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery. Kidney Int 2006;69:1471–1476.

7 Pisitkun T, Shen RF, Knepper MA: Identification and proteomic profiling of exosomes in human urine. Proc Natl Acad Sci USA 2004;101:13368–13373.

8 Davis MT, Spahr CS, McGinley MD, et al: Towards defining the urinary proteome using liquid chromatography-tandem mass spectrometry. II. Limitations of complex mixture analyses. Proteomics 2001;1:108–117.

9 Spahr CS, Davis MT, McGinley MD, et al: Towards defining the urinary proteome using liquid chromatography-tandem mass spectrometry. I. Profiling an unfractionated tryptic digest. Proteomics 2001;1:93–107.
10 Pang JX, Ginanni N, Dongre AR, Hefta SA, Opitek GJ: Biomarker discovery in urine by proteomics. J Proteome Res 2002;1:161–169.
11 Thongboonkerd V, McLeish KR, Arthur JM, Klein JB: Proteomic analysis of normal human urinary proteins isolated by acetone precipitation or ultracentrifugation. Kidney Int 2002;62:1461–1469.
12 Oh J, Pyo JH, Jo EH, et al: Establishment of a near-standard two-dimensional human urine proteomic map. Proteomics 2004;4:3485–3497.
13 Pieper R, Gatlin CL, McGrath AM, et al: Characterization of the human urinary proteome: a method for high-resolution display of urinary proteins on two-dimensional electrophoresis gels with a yield of nearly 1,400 distinct protein spots. Proteomics 2004;4:1159–1174.
14 Sun W, Li F, Wu S, et al: Human urine proteome analysis by three separation approaches. Proteomics 2005;5:4994–5001.
15 Castagna A, Cecconi D, Sennels L, et al: Exploring the hidden human urinary proteome via ligand library beads. J Proteome Res 2005;4:1917–1930.
16 Adachi J, Kumar C, Zhang Y, Olsen JV, Mann M: The human urinary proteome contains more than 1,500 proteins including a large proportion of membranes proteins. Genome Biol 2006;7:R80.
17 Zhou H, Pisitkun T, Aponte A, et al: Exosomal fetuin-A identified by proteomics: a novel urinary biomarker for detecting acute kidney injury. Kidney Int 2006;70:1847–1857.
18 O'Farrell PH: High-resolution two-dimensional electrophoresis of proteins. J Biol Chem 1975;250:4007–4021.
19 Smith MA, Bains SK, Betts JC, Choy EH, Zanders ED: Use of two-dimensional gel electrophoresis to measure changes in synovial fluid proteins from patients with rheumatoid arthritis treated with antibody to CD4. Clin Diagn Lab Immunol 2001;8:105–111.
20 Oh J, Pyo JH, Jo EH, et al: Establishment of a near-standard two-dimensional human urine proteomic map. Proteomics 2004;4:3485–3497.
21 Thongboonkerd V, McLeish KR, Arthur JM, Klein JB: Proteomic analysis of normal human urinary proteins isolated by acetone precipitation or ultracentrifugation. Kidney Int 2002;62:1461–1469.
22 Issaq HJ, Veenstra TD, Conrads TP, Felschow D: The SELDI-TOF-MS approach to proteomics: protein profiling and biomarker identification. Biochem Biophys Res Commun 2002;292:587–592.
23 Shiwa M, Nishimura Y, Wakatabe R, et al: Rapid discovery and identification of a tissue-specific tumor biomarker from 39 human cancer cell lines using the SELDI ProteinChip platform. Biochem Biophys Res Commun 2003;309:18–25.
24 Raida M, Schulz-Knappe P, Heine G, Forssmann WG: Liquid chromatography and electrospray mass spectrometric mapping of peptides from human plasma filtrate. J Am Soc Mass Spectrom 1999;10:45–54.
25 Hamler RL, Zhu K, Buchanan NS, et al: A two-dimensional liquid-phase separation method coupled with mass spectrometry for proteomic studies of breast cancer and biomarker identification. Proteomics 2004;4:562–577.
26 Pang JX, Ginanni N, Dongre AR, Hefta SA, Opitek GJ: Biomarker discovery in urine by proteomics. J Proteome Res 2002;1:161–169.
27 Schmitt-Kopplin P, Frommberger M: Capillary electrophoresis-mass spectrometry: 15 years of developments and applications. Electrophoresis 2003;24:3837–3867.
28 Schmitt-Kopplin P, Englmann M: Capillary electrophoresis-mass spectrometry: survey on developments and applications 2003–2004. Electrophoresis 2005;26:1209–1220.
29 Stutz H: Advances in the analysis of proteins and peptides by capillary electrophoresis with matrix-assisted laser desorption/ionization and electrospray-mass spectrometry detection. Electrophoresis 2005;26:1254–1290.
30 Neususs C, Pelzing M, Macht M: A robust approach for the analysis of peptides in the low femtomole range by capillary electrophoresis-tandem mass spectrometry. Electrophoresis 2002;23:3149–3159.
31 Oda RP, Clark R, Katzmann JA, Landers JP: Capillary electrophoresis as a clinical tool for the analysis of protein in serum and other body fluids. Electrophoresis 1997;18:1715–1723.
32 Hernandez-Borges J, Neususs C, Cifuentes A, Pelzing M: On-line capillary electrophoresis-mass spectrometry for the analysis of biomolecules. Electrophoresis 2004;25:2257–2281.

33 Guzman NA, Park SS, Schaufelberger D, et al: New approaches in clinical chemistry: on-line analyte concentration and microreaction capillary electrophoresis for the determination of drugs, metabolic intermediates, and biopolymers in biological fluids. J Chromatogr B Biomed Sci Appl 1997;697:37–66.
34 Kolch W, Neususs C, Pelzing M, Mischak H: Capillary electrophoresis-mass spectrometry as a powerful tool in clinical diagnosis and biomarker discovery. Mass Spectrom Rev 2005;24: 959–977.
35 Kaiser T, Wittke S, Just I, et al: Capillary electrophoresis coupled to mass spectrometer for automated and robust polypeptide determination in body fluids for clinical use. Electrophoresis 2004; 25:2044–2055.
36 Dolnik V, Hutterer KM: Capillary electrophoresis of proteins 1999–2001. Electrophoresis 2001;22:4163–4178.
37 Dolnik V: Capillary electrophoresis of proteins 2003–2005. Electrophoresis 2005;27:126–141.
38 Schmitt-Kopplin P, Frommberger M: Capillary electrophoresis-mass spectrometry: 15 years of developments and applications. Electrophoresis 2003;24:3837–3867.
39 Kasicka V: Recent developments in capillary electrophoresis and capillary electrochromatography of peptides. Electrophoresis 2005;27:142–175.
40 Simpson DC, Smith RD: Combining capillary electrophoresis with mass spectrometry for applications in proteomics. Electrophoresis 2005;26:1291–1305.
41 Johannesson N, Wetterhall M, Markides KE, Bergquist J: Monomer surface modifications for rapid peptide analysis by capillary electrophoresis and capillary electrochromatography coupled to electrospray ionization-mass spectrometry. Electrophoresis 2004;25:809–816.
42 Hernandez-Borges J, Neususs C, Cifuentes A, Pelzing M: On-line capillary electrophoresis-mass spectrometry for the analysis of biomolecules. Electrophoresis 2004;25:2257–2281.
43 Neususs C, Pelzing M, Macht M: A robust approach for the analysis of peptides in the low femtomole range by capillary electrophoresis-tandem mass spectrometry. Electrophoresis 2002;23: 3149–3159.
44 Gelpi E: Interfaces for coupled liquid-phase separation/mass spectrometry techniques. An update on recent developments. J Mass Spectrom 2002;37:241–253.
45 Schmitt-Kopplin P, Frommberger M: Capillary electrophoresis-mass spectrometry: 15 years of developments and applications. Electrophoresis 2003;24:3837–3867.
46 Schmitt-Kopplin P, Englmann M: Capillary electrophoresis-mass spectrometry: survey on developments and applications 2003–2004. Electrophoresis 2005;26:1209–1220.
47 Lee ED, Muck W, Henion JD, Covey TR: On-line capillary zone electrophoresis-ion spray tandem mass spectrometry. J Chromatogr 1988;458:313–321.
48 Wahl JH, Gale DC, Smith RD: Sheathless capillary electrophoresis-electrospray ionization mass. J Chromatogr A 1994;659:217–222.
49 Nilsson S, Wetterhall M, Bergquist J, Nyholm L, Markides KE: A simple and robust conductive graphite coating for sheathless electrospray emitters used in capillary electrophoresis/mass spectrometry. Rapid Commun Mass Spectrom 2001;15:1997–2000.
50 Smith RD, Olivares JA, Nguyen NT, Udseth HR: Capillary zone electrophoresis-mass spectrometry using an electrospray ionization interface. Anal Chem 1988;60:436–441.
51 Pelzing M, Neususs C: Separation techniques hyphenated to electrospray-tandem mass spectrometry in proteomics: capillary electrophoresis versus nanoliquid chromatography. Electrophoresis 2005;26:2717–2728.
52 Johnson T, Bergquist J, Ekman R, et al: A CE-MALDI interface based on the use of prestructured sample supports. Anal Chem 2001;73:1670–1675.
53 Kaiser T, Hermann A, Kielstein JT, et al: Capillary electrophoresis coupled to mass spectrometry to establish polypeptide patterns in dialysis fluids. J Chromatogr A 2003;1013:157–171.
54 Wittke S, Fliser D, Haubitz M, et al: Determination of peptides and proteins in human urine with capillary electrophoresis-mass spectrometry, a suitable tool for the establishment of new diagnostic markers. J Chromatogr A 2003;1013:173–181.
55 Theodorescu D, Fliser D, Wittke S, et al: Pilot study of capillary electrophoresis coupled to mass spectrometry as a tool to define potential prostate cancer biomarkers in urine. Electrophoresis 2005;26:2797–2808.

56 Zolotarjova N, Martosella J, Nicol G, Bailey J, Boyes BE, Barrett WC: Differences among techniques for high-abundant protein depletion. Proteomics 2005;5:3304–3313.
57 Palagi PM, Walther D, Quadroni M, et al: MSight: An image analysis software for liquid chromatography-mass spectrometry. Proteomics 2005;5:2381–2384.
58 Weissinger EM, Wittke S, Kaiser T, et al: Proteomic patterns established with capillary electrophoresis and mass spectrometry for diagnostic purposes. Kidney Int 2004;65:2426–2434.
59 Steinberg D, Colla P: CART – Classification and Regression Trees. San Diego, Salford Systems, 1997.
60 Weissinger EM, Wittke S, Kaiser T, et al: Proteomic patterns established with capillary electrophoresis and mass spectrometry for diagnostic purposes. Kidney Int 2004;65:2426–2434.
61 Peng S, Xu Q, Ling XB, Peng X, Du W, Chen L: Molecular classification of cancer types from microarray data using the combination of genetic algorithms and support vector machines. FEBS Lett 2003;555:358–362.
62 Willingale R, Jones DJ, Lamb JH, Quinn P, Farmer PB, Ng LL: Searching for biomarkers of heart failure in the mass spectra of blood plasma. Proteomics 2006;6:5903–5914.
63 Thukral SK, Nordone PJ, Hu R, et al: Prediction of nephrotoxicant action and identification of candidate toxicity-related biomarkers. Toxicol Pathol 2005;33:343–355.
64 Girolami M, Rogers S: Variational bayesian multinomial probit regression with gaussian process priors. Neural Comput 2006;18:1790–1817.
65 Chalmers MJ, Mackay CL, Hendrickson CL, et al: Combined top-down and bottom-up mass spectrometric approach to characterization of biomarkers for renal disease. Anal Chem 2005;77:7163–7171.
66 Neuhoff N, Kaiser T, Wittke S, et al: Mass spectrometry for the detection of differentially expressed proteins: a comparison of surface-enhanced laser desorption/ionization and capillary electrophoresis/mass spectrometry. Rapid Commun Mass Spectrom 2004;18:149–156.
67 Haubitz M, Wittke S, Weissinger EM, et al: Urine protein patterns can serve as diagnostic tools in patients with IgA nephropathy. Kidney Int 2005;67:2313–2320.
68 Mischak H, Kaiser T, Walden M, et al: Proteomic analysis for the assessment of diabetic renal damage in humans. Clin Sci (Lond) 2004;107:485–495.
69 Rossing K, Mischak H, Parving HH, et al: Impact of diabetic nephropathy and angiotensin II receptor blockade on urinary polypeptide patterns. Kidney Int 2005;68:193–205.
70 Fliser D, Wittke S, Mischak H: Capillary electrophoresis coupled to mass spectrometry for clinical diagnostic purposes. Electrophoresis 2005;26:2708–2716.
71 Jensen PK, Pasa-Tolic L, Peden KK, et al: Mass spectrometric detection for capillary isoelectric focusing separations of complex protein mixtures. Electrophoresis 2000;21:1372–1380.
72 Moini M, Huang H: Application of capillary electrophoresis/electrospray ionization-mass spectrometry to subcellular proteomics of *Escherichia coli* ribosomal proteins. Electrophoresis 2004;25:1981–1987.
73 Guzman NA, Park SS, Schaufelberger D, et al: New approaches in clinical chemistry: on-line analyte concentration and microreaction capillary electrophoresis for the determination of drugs, metabolic intermediates, and biopolymers in biological fluids. J Chromatogr B Biomed Sci Appl 1997;697:37–66.
74 Oda RP, Clark R, Katzmann JA, Landers JP: Capillary electrophoresis as a clinical tool for the analysis of protein in serum and other body fluids. Electrophoresis 1997;18:1715–1723.
75 Petersen JR, Okorodudu AO, Mohammad A, Payne DA: Capillary electrophoresis and its application in the clinical laboratory. Clin Chim Acta 2003;330:1–30.
76 Meier M, Kaiser T, Herrmann A, et al: Identification of urinary protein pattern in type 1 diabetic adolescents with early diabetic nephropathy by a novel combined proteome analysis. J Diabetes Complications 2005;19:223–232.
77 Haubitz M, Wittke S, Weissinger EM, et al: Can urine polypeptide profiles be used to diagnose IgA nephropathy? Kidney Int 2005;67:2313–2320.
78 O'Riordan E, Goligorsky MS: Emerging studies of the urinary proteome: the end of the beginning? Curr Opin Nephrol Hypertens 2005;14:579–585.
79 Theodorescu D, Wittke S, Ross MM, et al: Discovery and validation of new protein biomarkers for urothelial cancer: a prospective analysis. Lancet Oncol 2006;7:230–240.
80 Dey P: Urinary markers of bladder carcinoma. Clin Chim Acta 2004;340:57–65.

81 Glas AS, Roos D, Deutekom M, Zwinderman AH, Bossuyt PM, Kurth KH: Tumor markers in the diagnosis of primary bladder cancer. A systematic review. J Urol 2003;169:1975–1982.
82 Kageyama S, Isono T, Iwaki H, et al: Identification by proteomic analysis of calreticulin as a marker for bladder cancer and evaluation of the diagnostic accuracy of its detection in urine. Clin Chem 2004;50:857–866.
83 Vlahou A, Schellhammer PF, Wright GL Jr: Application of a novel protein chip mass spectrometry technology for the identification of bladder cancer-associated biomarkers. Adv Exp Med Biol 2003;539:47–60.
84 Konety BR, Getzenberg RH: Urine based markers of urological malignancy. J Urol 2001;165: 600–611.
85 Rehman I, Azzouzi AR, Catto JW, et al: Proteomic analysis of voided urine after prostatic massage from patients with prostate cancer: a pilot study. Urology 2004;64:1238–1243.
86 Verhave JC, Hillege HL, Burgerhof JG, et al: The association between atherosclerotic risk factors and renal function in the general population. Kidney Int 2005;67:1967–1973.
87 Kolch W, Neususs C, Pelzing M, Mischak H: Capillary electrophoresis-mass spectrometry as a powerful tool in clinical diagnosis and biomarker discovery. Mass Spectrom Rev 2005;24: 959–977.
88 Kaiser T, Wittke S, Just I, et al: Capillary electrophoresis coupled to mass spectrometer for automated and robust polypeptide determination in body fluids for clinical use. Electrophoresis 2004;25:2044–2055.
89 Decramer S, Wittke S, Mischak H, et al: Predicting the clinical outcome of congenital unilateral ureteropelvic junction obstruction in newborn by urinary proteome analysis. Nat Med 2006;12: 398–400.
90 Haubitz M, Woywodt A: Circulating endothelial cells and vasculitis. Intern Med 2004;43: 660–667.
91 Wittke S, Haubitz M, Walden M, et al: Detection of acute tubulointerstitial rejection by proteomic analysis of urinary samples in renal transplant recipients. Am J Transplant 2005;5:2479–2488.
92 Rogers MA, Clarke P, Noble J, et al: Proteomic profiling of urinary proteins in renal cancer by surface enhanced laser desorption ionization and neural-network analysis: identification of key issues affecting potential clinical utility. Cancer Res 2003;63:6971–6983.
93 Rejtar T, Hu P, Juhasz P, et al: Off-line coupling of high-resolution capillary electrophoresis to MALDI-TOF and TOF/TOF MS. J Proteome Res 2002;1:171–179.
94 Kaiser T, Kamal H, Rank A, et al: Proteomics applied to the clinical follow-up of patients after allogeneic hematopoietic stem cell transplantation. Blood 2004;104:340–349.
95 Weissinger EM, Kaiser T, Meert N, et al: Proteomics: a novel tool to unravel the pathophysiology of uraemia. Nephrol Dial Transplant 2004;19:3068–3077.
96 Zürbig P, Renfrow MB, Schiffer E, et al: Biomarker discovery by CE-MS enables sequence analysis via MS/MS with platform-independent separation. Electrophoresis 2006;27:2111–2125.
97 Sniehotta M, Schiffer E, Zürbig P, Novak J, Mischak H: Capillary electrophoresis – a multifunctional application for clinical diagnosis. Electrophoresis 2007;28:1407–1417.

Harald Mischak
Mosaiques Diagnostics and Therapeutics AG
Mellendorfer Strasse 7–9, DE–30625 Hannover (Germany)
Tel. +49 511 5547 4413, Fax +49 511 5547 4431
E-Mail mischak@mosaiques-diagnostics.com

Thongboonkerd V (ed): Proteomics in Nephrology – Towards Clinical Applications.
Contrib Nephrol. Basel, Karger, 2008, vol 160, pp 127–141

Identification of Urinary Biomarkers by Proteomics in Newborns: Use in Obstructive Nephropathy

Stephane Decramer[a–c], *Petra Zürbig*[d], *Stefan Wittke*[d], *Harald Mischak*[d], *Jean-Loup Bascands*[a,b], *Joost P. Schanstra*[a,b]

[a]Inserm, U858; [b]Université Toulouse III Paul Sabatier, IFR31, Institut Louis Bugnard, and [c]CHU de Toulouse, Nephropediatric Unit, Hôpital des Enfants, TSA 70034, Toulouse, France; [d]Mosaiques Diagnostics and Therapeutics AG, Hannover, Germany

Abstract

An important issue in congenital unilateral ureteropelvic junction (UPJ) obstruction, a frequent pathology in newborns, is whether infants should undergo surgery. Non-invasive biomarkers to reduce or replace the current invasive clinical exploration are not available. The objective of this study was to identify urinary markers of UPJ obstruction. We compared a number of proteome technologies to study the urinary proteome in UPJ obstruction and selected online capillary electrophoresis coupled to mass-spectrometry for the selection of non-invasive prognostic biomarkers. We selected 53 urinary biomarkers that were able to distinguish between different levels of UPJ obstruction. In a prospective study using these 53 biomarkers, we predicted with 97% accuracy, and several months in advance, the clinical outcome of 36 UPJ-obstruction patients. Some of the discriminating biomarkers were identified. A newly identified marker, proSAAS (proprotein convertase subtilisin/kexin type 1 inhibitor), generated a new hypothesis in the physiopathology of UPJ obstruction. These results show that analysis of urinary polypeptides in newborns with UPJ obstruction can predict their clinical outcome.

The increased and routine use of prenatal ultrasound screening in developed countries has led to an increase in the detection of infants with congenital obstructive nephropathy. Obstructive nephropathy is thought to result from hydronephrosis induced by accumulation of urine in the renal pelvis or calyces [1]. The most frequently found cause of congenital obstructive nephropathy is ureteropelvic junction (UPJ) obstruction [2], although little is known about its

etiology [2, 3]. Debate is ongoing on how to treat newborns with UPJ obstruction. Until the late 1980s, neonates with UPJ obstruction were rapidly operated after birth to avoid the potential degradation of renal function. However, in a number of infants with UPJ obstruction, hydronephrosis spontaneously resolves without apparent signs of loss of renal function or renal maldevelopment. This has led to modification of the treatment of newborns with UPJ obstruction and currently most clinical teams have adopted close conservative surveillance of newborns with UPJ obstruction and surgical intervention only if renal deterioration is detected [4]. Although this attitude prevents unnecessary surgical intervention in UPJ obstruction, at least three important concerns accompany this attitude: (1) Clinical measures currently used to determine the degree of injury are far from perfect [4, 5]. (2) These imperfect clinical measures are invasive and need to be repeated several times during the first years of life in newborns with UPJ obstruction to determine the degree of obstruction. (3) Very little is known about the effect of prolonged partial obstruction on kidney function later in life. This book chapter addresses the first efforts to identify non-invasive urinary biomarkers of UPJ obstruction that might reduce/replace the invasive measures in UPJ obstruction.

Throughout this chapter we will use the following abbreviations for the different patient UPJ-obstruction groups (for details see below in the 'Patients' section): 'No_OP', non-operated UPJ obstruction; 'OP_Poss', possible operation, and 'OP', patients with severe UPJ obstruction that are scheduled to be operated rapidly after birth. For the last group, urine samples were taken before surgery. OP_Poss patients depict the neonates needing repetitive and invasive medical surveillance to determine the necessity of relief surgery.

The Search of Urinary Biomarkers of UPJ Obstruction Based on Animal Studies: The Rational Approach

The animal model (rat or mouse) of unilateral ureteral obstruction has been extensively used to study the role of a number of molecules in obstructive nephropathy and in the development of renal fibrosis [3, 6]. Based on some of these observations in the animal models, a number of groups have investigated if these molecules may be urinary biomarkers for UPJ obstruction [7–9]. Transforming growth factor β (TGFβ), the major profibrotic cytokine [10] in the kidney, was shown to be associated with the molecular, histological and functional kidney changes in obstructive nephropathy [11–14]. Two different groups have shown that indeed urinary TGFβ concentrations are significantly increased during UPJ obstruction in patients needing pyeloplasty (the OP group) [7, 9]. However, the value of TGFβ as a urinary marker in milder forms

of UPJ obstruction remains to be determined. Urinary monocyte chemoattractant peptide 1 (MCP-1) and epidermal growth factor (EGF) concentrations were also studied in UPJ obstruction in humans and revealed higher and lower concentrations in UPJ individuals, respectively [8]. Again, as in the TGFβ studies, samples were exclusively obtained from UPJ obstruction patients scheduled for pyeloplasty. Thus, although these molecules seem to be biomarkers for severe UPJ obstruction (the patients belonging to the OP group), their diagnostic and prognostic values in the low (No_OP group) and moderate (OP_Poss group) levels of UPJ obstruction remain to be determined.

Urinary Proteome Analysis: The Non-Rational, Large-Scale Approach

Based on the observation that until now 'rational' urinary UPJ-obstruction biomarker identification had only limited success, we initiated a 'non-rational' large-scale search for urinary biomarkers of UPJ obstruction by urinary proteome analysis.

As will be mentioned in other chapters of this volume of 'Proteomics in Nephrology', urine is an excellent source of biomarkers in kidney disease since it has been estimated that ~70% of the urinary proteins originate from the kidney and the urinary tract in healthy individuals [15], which might be even higher in individuals with kidney disease. A number of groups has studied and optimized urine collection [16–18]. Briefly, first morning urine, and freezing and thawing of samples are to be avoided and mid-stream urine gives the most reproducible results. In general, protease inhibitors were not used for gel-free techniques such as online capillary electrophoresis coupled to mass spectrometry (CE-MS) or surface-enhanced laser desorption/ionization mass spectrometry (SELDI-MS) but were readily employed in gel-based, i.e. two-dimensional polyacrylamide gel electrophoresis (2-D PAGE [19]). However, more recently the use of these inhibitors in 2-D PAGE was abandoned [17]. In our study, we collected urine from newborns by using urine collection bags during morning consultation in the hospital over a 30- to 45-min period. This avoids collection of first morning urine and allows obtaining samples from the same period of the day. Samples were directly frozen without addition of protease inhibitors at –20°C and were conserved 1–3 months before being thawed on ice and aliquoted into 1.5-ml samples and stored at –80°C. This way of sample handling, as analyzed by CE-MS, allows obtaining reproducible data of two samples from the same newborn taken within a short period (1 week interval; fig. 1).

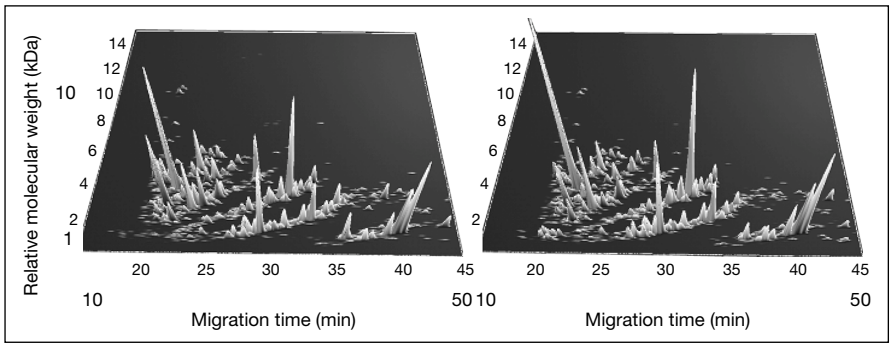


Fig. 1. Reproducibility of the CE-MS analysis. Additional data of the reproducibility of the CE-MS analysis can be found in Kolch et al. [27]. CE-MS analysis shows that 99% of the 200 most abundant polypeptides are present in two different urinary samples from a healthy newborn at a 1-week interval (age: 1 and 2 weeks respectively).

Due to the complexity of the proteome, all approaches rely on a pre-fractionation step, which is followed by ionization and subsequent mass spectrometry. Before initiating the search of urinary biomarkers in UPJ obstruction on a well-defined cohort, we first tested the ability of three specific proteomic technologies: 2-D PAGE followed by mass spectrometry, SELDI-MS and CE-MS to distinguish between the urinary proteomes of 5 healthy newborns and 5 newborns with different degrees of UPJ obstruction.

2-D PAGE pre-fractionates proteins in two steps according to two intrinsic characteristics: isoelectric point and molecular weight. After 2-D PAGE, a characteristic protein pattern (a number of spots) is obtained from a biological sample. Differentially expressed proteins can subsequently be excised from the gels, digested by a specific protease and subjected to mass spectrometry analysis for the identification [20]. We tested 2-D PAGE for the identification of biomarkers for UPJ obstruction in newborns and found that it was not suitable for the following reasons: (i) in general, we collect between 5 and 15 ml of newborn urine, yielding between 100 and 200 μg of protein after acetone precipitation, which is too low for routine 2-D PAGE urine analysis; (ii) more importantly, a large variety in protein yield after protein precipitation was observed, and (iii) finally, the inability of the 2-D PAGE analysis to study the urinary polypeptide content (2-D PAGE is generally limited to the analysis of 10–200 kDa proteins). We have observed intense Coomassie staining of the migration front suggesting the presence of a large number of peptides in urine (fig. 2) which are not exploitable by 2-D PAGE.

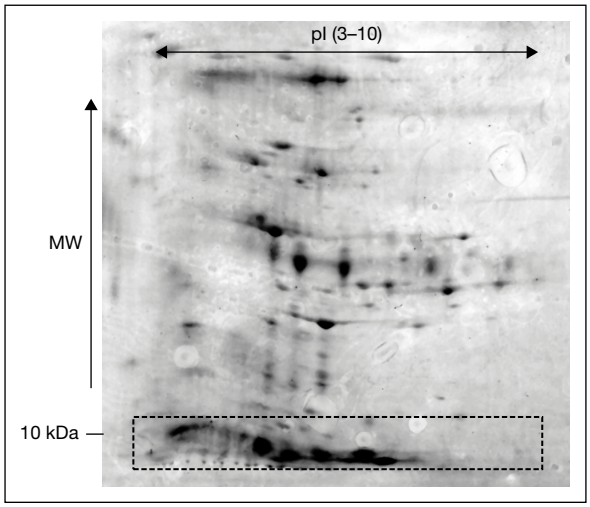


Fig. 2. 2-D PAGE shows that urine contains significant amounts of low-molecular-weight compounds (area indicated by the dotted box). Coomassie stained 2-D PAGE of acetone-precipitated urinary proteins and peptides (total 140 μg) of a healthy newborn. Linear pH 3–10 IPG strips were used.

SELDI-MS reduces the complexity of the sample by selective adsorption of proteins to different active surfaces. After removal of unbound sample by washing steps, matrix is added followed by laser desorption/ionization allowing ionization of the bound molecules and analysis by mass spectrometry [21]. Our preliminary experiments of the 10 newborn samples by SELDI-MS using a H50 (hydrophobic) surface yielded unsatisfactory results, i.e. differences in profiles from the individual samples, but no clear differences between the 5 healthy controls and the 5 UPJ-obstruction samples (data not shown). Nevertheless, others have used the SELDI-MS approach with success for the selection of urinary biomarkers, albeit only in training sets, of children with steroid-resistant and steroid-sensitive nephrotic syndrome [22, 23], in adults with active or inactive lupus nephritis [24] or in the selection of urinary biomarkers of acute rejection after kidney transplantation [25, 26].

CE-MS pre-fractionates the proteome based on migration of proteins and peptides in an electrical field by capillary electrophoresis (CE). It provides fast and high-resolution separation of the low-molecular-weight proteome but it is less adapted to larger proteins. CE seems thus well suited for the analysis of the urinary proteome consisting mostly of low molecular proteins. Online coupling

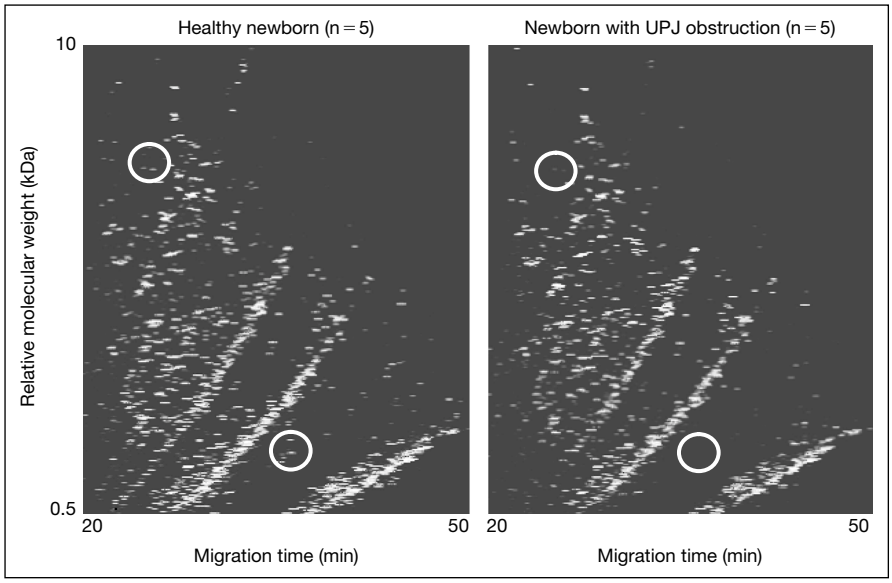


Fig. 3. CE-MS allows distinguishing between the urinary proteomes of 5 healthy newborns and 5 newborn with different degrees of UPJ obstruction. The compiled CE-MS profile of 5 individuals of each group is shown. Visual inspection allows easy identification of differences between both groups (encircled areas).

to a mass spectrometer of the CE via an electron spray interface allows direct mass spectrometry analysis of the CE outflow [27]. Using CE-MS, we were able to identify biomarkers differentially expressed in 5 healthy controls and 5 UPJ-obstruction samples (fig. 3). CE-MS was thus used for the selection and identification of biomarkers of UPJ obstruction.

CE-MS and Peptide Identification

After thawing, the urine samples were centrifuged for 10 min at 2,000 rpm. One milliliter of the supernatant was applied onto a Pharmacia C2-column to remove salts, urea, electrolytes and other interfering matrix components and to enrich the present polypeptides. The eluate was lyophilized and resuspended in 50 μl HPLC-grade water shortly before analysis by CE-MS [28, 29]. Analyses were performed as described previously [28, 29] using a P/ACE MDQ CE (Beckmann-Coulter) equipped with a bare fused silica capillary that was online coupled to an ESI-TOF mass spectrometer (Micro-TOF, Bruker-Daltonic). The

CE-MS coupling was established using an ESI-MS sprayer kit from Agilent Technologies. Spectra were accumulated every 3 s over a mass range from 350 to 3000 m/z. CE-MS runs were analyzed by MosaiquesVisu [30] and biomarkers extracted by MosaCluster [31] (see below).

The biomarkers were identified as described [32] or using a Dionex Ultimate 3000 nanoflow system connected to an LTQ Orbitrap hybrid mass spectrometer (Thermo Electron, Bremen, Germany) equipped with a nanoelectrospray ion source. Binding and chromatographic separation of the peptides took place on a 10-cm fused silica nanocolumn of 75-μm inner diameter packed with reversed-phase Biosphere C18, 5 μm resin (NanoSeparations, Nieuwkoop, The Netherlands) in a pre-column setup. The peptide mixtures were injected onto the pre-column at a flow rate of 5 μl/min and subsequently eluted with a flow of 250 nl/min using a linear gradient (60 min) from 2–50% MeCN in water (0.1% formic acid). The mass spectrometer was operated in data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey full-scan MS spectra (from m/z 300–2000) were acquired in the Orbitrap with resolution R = 60,000 at m/z 400 (target value of 500,000 charges in the linear ion trap). The most intense ions (up to 5) were sequentially isolated for fragmentation in the linear ion trap using collisionally induced dissociation and the detection took place either in the linear ion trap (parallel mode; target value 10,000) or in the Orbitrap (target value of 500,000). Orbitrap MS/MS were acquired with resolution R = 15,000 at m/z 400. General mass spectrometric conditions were: electrospray voltage, 1.6 kV; no sheath and auxiliary gas flow; ion transfer tube temperature, 225°C; collision gas pressure, 1.3 mT; normalized collision energy, 32% for MS^2. Ion selection threshold was 500 counts for MS/MS.

Patients

All patients with unilateral antenatally detected hydronephrosis, defined by a renal pelvic diameter >7 mm, were treated at the Children's Hospital Purpan, Toulouse, France. All patients underwent an ultrasonography examination within the first month of life. The degree of hydronephrosis was classified between grades I and IV according to the system developed by the Society for Fetal Urology [33, 34] and the ultrasonographic appearance of the renal parenchyma and pelvicalyceal system on longitudinal ultrasonic section [35]. A voiding cystourethrogram was systematically performed at the first visit. Patients were excluded if they had a vesicoureteric reflux, solitary kidney, bilateral hydronephrosis, ureteral dilatation or lower urinary tract abnormalities. After the first visit patients were classified in three groups (the median gestational age at detection of hydronephrosis was 25.8 weeks for all patients (range

16–33 weeks), 26 weeks in the No_OP group (range 22–33 weeks), 24.6 weeks in the OP group (range 16–33 weeks) and 27 weeks in the OP_Poss group (range 22–32 weeks).

Group No_OP

Patients with grade 1 or 2 hydronephrosis and a renal pelvic diameter between 5 and 15 mm. These patients were followed up with ultrasonography every 3 months during the first year of life, every 6 months the second year, followed by one ultrasonography once a year. In general, hydronephrosis in these patients is decreasing during the first 2 years of life. However, patients presenting increased (transitory) pelvic dilatation during this period underwent a MAG3 scan to confirm a non-obstructive washout pattern to stay in the No_OP group.

Group OP

Patients scheduled for pyeloplasty with a pelvic dilatation of at least 20 mm and grade 3 and 4 hydronephrosis. Renography was performed as soon as possible after birth, generally between weeks 3 and 6 to establish baseline differential renal function (DMSA scan) and washout pattern (MAG3 scan). Indications for surgery were: differential renal function (DRF) >10%, grade 4 hydronephrosis, obstructive washout pattern in diuretic renography with eliminated activity at 30 min <30% (or drainage half time ($T_{1/2}$) <20 min; interval necessary for half of the tracer to be eliminated after the administration of diuretic (furosemide)), sustained increase in hydronephrosis, and progressive deterioration of DRF (>5%).

Group OP_Poss

Patients with pelvic dilatation >15 mm, or grade 3 or 4 hydronephrosis, DRF <10% and a washout pattern in diuretic renography with eliminated activity at 30 min >30%.

Finally, a group of age-matched newborns were included in the study. After local ethics committee approval (CHU de Toulouse), informed consent was obtained from all participants (parents).

Searching for Biomarkers of UPJ Obstruction

After selection of, in our eyes, the most promising technique for the analysis of urinary biomarkers in UPJ obstruction in newborns, we examined the urinary proteome of a number of UPJ-obstruction patients [32]. Following CE-MS analysis of the individual samples and data deconvolution by MosaiquesVisu

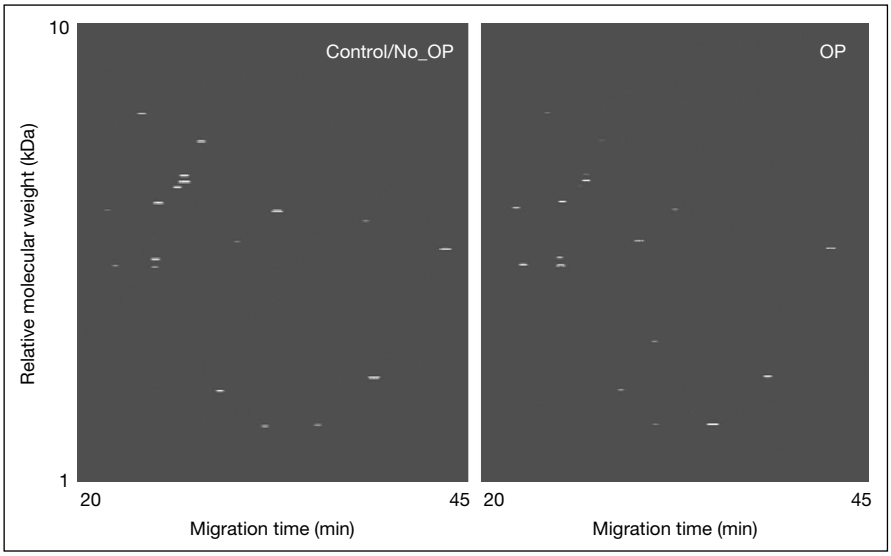


Fig. 4. The frequency of appearance of 19 biomarkers (the intensity of the marker reflects its presence in corresponding group) discriminating between the healthy newborn + No_OP group and the OP group. This figure was partly reproduced from Decramer et al. [32] with permission.

[30], group-specific profiles were generated and displayed the identical peptides within a group of individuals (healthy newborns (n = 13), No_OP newborns (n = 19) and OP newborns (n = 19)). Polypeptides in different samples were regarded identical if the deviation in molecular weight was <100 ppm and if the migration time deviation was <1 min. The software MosaCluster [31] based on support vector machines (SVM) was used for the definition of group-specific biomarkers. SVM is a method of solving classification problems rapidly and accurately by non-linear mapping of their n-dimensional input space into high-dimensional feature space. Every polypeptide used for classification represents one dimension in an n-dimensional space; patients are defined by the respective polypeptides. The program generates a model on the basis of polypeptides that are best suited to discriminate between the groups. 53 polypeptides were selected that distinguish between the healthy control, the No_OP and OP groups [31]. As an example the 19 polypeptides that discriminate between newborns of the control + No_OP group and the OP group are shown in figure 4. For our study only the frequency of appearance of the peptides was used [32] while other studies use both the frequency and the amplitude of the biomarkers for classification [31].

Prediction of the Clinical Evolution of OP_Poss Patients

The main problem in newborn with moderate UPJ obstruction (OP_Poss) is to decide between relief surgery or continued invasive and repetitive medical surveillance based on surrogate endpoints, risking unnecessary prolonged ureteral obstruction. Using a hierarchic disease model based on the 53 discriminating polypeptides between the healthy newborn, No_OP and OP group extracted as described above, we predicted the clinical outcome of patients in the OP_Poss group.

Using this approach in a prospective blinded study of 36 OP_Poss patients, 25 patients were predicted to evolve towards the OP group, while 11 were predicted to evolve towards the No_OP group (fig. 5a). Nine months after this CE-MS-based prediction (which is in general the time necessary to determine if patients need surgery or have spontaneous recovery of the UPJ obstruction) the clinical evolution of these 36 patients was compared with the urinary polypeptide-based prediction. For 34 out of the 36 patients, the clinical evolution correlated with CE-MS prediction (fig. 5b): 13 patients were spontaneously resolved their UPJ obstruction while 23 were operated or scheduled to be operated [32]. Next, the prediction was re-evaluated 15 months after the CE-MS prediction and it turned out that the clinical situation of 26 patients had deteriorated, justifying pyeloplasty. This increases the success of prediction to 35 out of 36 patients. This late surgical correction of UPJ obstruction is not an exception as was recently shown by a retrospective study (16-year) on 343 children with antenatal diagnosis of hydronephrosis that led to postnatal diagnosis of UPJ obstruction. In this study, the patients (179 out of 343) that needed pyeloplasty fell into two age groups; 50% of these infants were operated before the age of 2 while the remaining ones were operated between 2 and 4 years of age [36].

Identification of CE-MS-Selected UPJ Biomarkers

The selected biomarkers thus allow the early prediction of the clinical fate of patients with intermediate UPJ obstruction. To obtain further insight into the pathophysiology of UPJ obstruction, we aimed to identify the biomarkers that were defined using the CE-MS data. We will not re-discuss the biomarkers that were identified in our previous study [32], but will focus on a newly identified marker. While the two previous markers already reported were sequenced by spotting an entire CE run on matrix-assisted laser desorption/ionization (MALDI) target plates followed by tandem mass spectrometry analysis, the current peptide was identified on an independent high-sensitivity platform based on the precise molecular weight and charge of the peptide [37]. Using a nanoflow

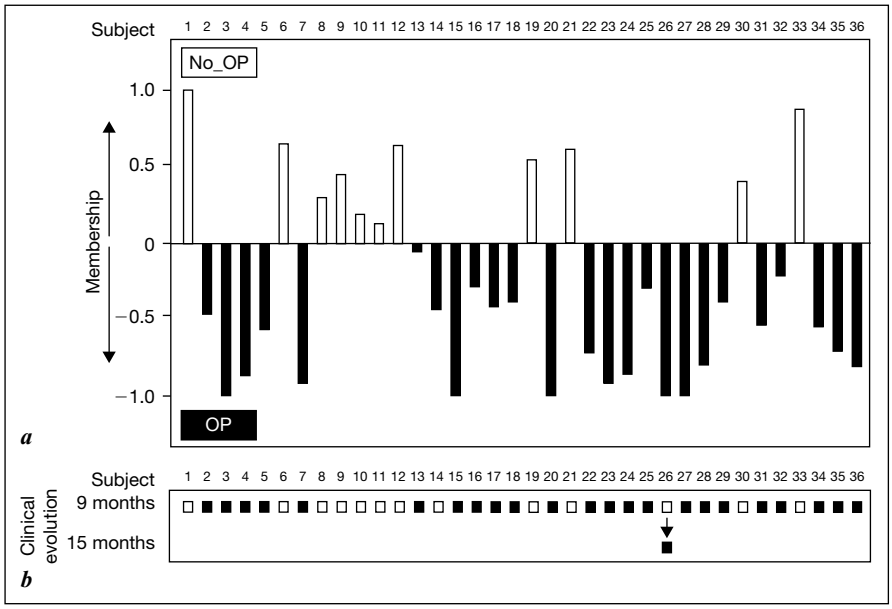


Fig. 5. ***a*** Urinary protein profiles of patients from the OP_Poss group were classified using a hierarchic disease model based on the discriminating polypeptides between the healthy newborn, No_OP and OP group. Each OP_Poss patient was scored with this model using support vector machines. This results in membership values between –1 and 1. A negative value suggests evolution towards the OP profile and a positive value suggests evolution towards the No_OP profile. Empty bars indicate a membership to the No_OP profile and black filled bars indicate a membership to the OP profile. ***b*** Clinical outcome of the OP_Poss patients 9 and 15 months after sample analysis. Empty squares indicate that the patient had evolved towards the No_OP group (spontaneous resolution of the obstruction) and filled squares indicate that these patients were operated (OP). This resulted in 34 out of 36 good predictions (94%) at 9 months and 97% of good predictions (35/36) at 15 months. This figure was partly reproduced and adapted from Decramer et al. [32] with permission.

system connected to an LTQ Orbitrap hybrid mass spectrometer, we identified a fragment of proSAAS (proprotein convertase subtilisin/kexin type 1 inhibitor, fig. 6) that was present in 77% of the individuals of the healthy newborn population and in only 18% of the UPJ-obstruction cohort (No_OP and OP). proSAAS was first identified in mouse brain [38]. Some of its N-terminal fragments inhibit the activity of the endopeptidase prohormone convertase 1 (PC1) [38]. The proSAAS fragment (221-239), poorly expressed in UPJ patients, partially carries one of the proSAAS-derived peptides that inhibits PC1 activity [39]. UPJ patients with low proSAAS can thus have increased PC1 activity.

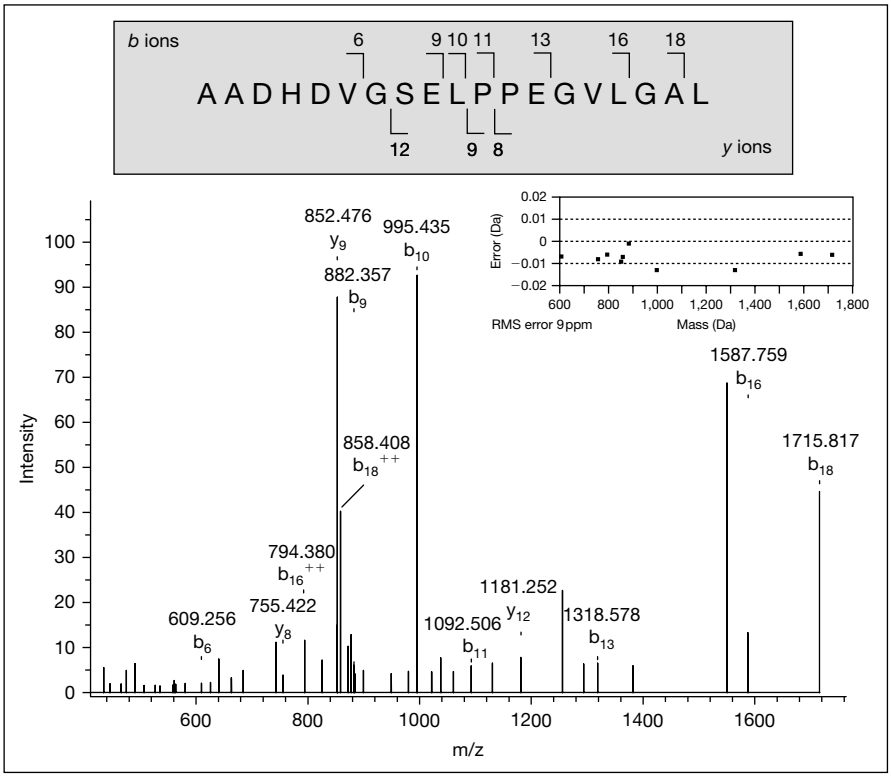


Fig. 6. MS/MS spectrum of a peptide and the deducted sequence (grey box) identified as a fragment of human proSAAS with low expression in UPJ patients compared to healthy controls. Frequency of expression in the healthy newborn population was 0.77 versus 0.18 in the UPJ obstruction cohort (No_OP and OP). The inset represents the MS/MS low mass differences (9 ppm) of the fragment ions.

PC1 was shown efficiently convert prorenin into renin [40] which might link the low expression of proSAAS to UPJ obstruction. Indeed it is well known that the renin-angiotensin system is activated in UPJ obstruction in men, as well as in animal models of UPJ obstruction [14]. We speculate that the lower proSAAS levels in UPJ obstruction lower PC1 inhibition and thus increase processing of renin from prorenin leading to increased activation of the renin-angiotensin system. However, the expression levels of both proSAAS and PC1 in the kidney are not known although PC1 is expressed in the adrenal medulla [41]. Whether lower proSAAS levels are a consequence or a (partial) cause of UPJ obstruction remains to be determined.

Conclusions

We have shown that urinary proteome analysis can predict the clinical outcome of newborns with UPJ obstruction in prospective cohort of 36 patients. The next step will be to confirm these results in a larger cohort in a multicentric study. This multicenter trial will not only help to rapidly increase patient number but will also exclude a monocentric bias. Confirmation of the results in this multicentric trial may help to replace the current imperfect and invasive standards for diagnosis, the renography. Furthermore, the continued identification of these predictive biomarkers by the ever more sensitive mass spectrometers will certainly increase our knowledge of UPJ obstruction as shown above for the proSAAS peptide and might even allow identifying the origin of UPJ obstruction which is still unknown to date.

Acknowledgements

This work was supported by grants from the Clinical Research Hospital Program from the French Ministry of Health (PHRC 2004) and from the 'Fondation pour la Recherche Médicale' called 'Development and therapy for kidney diseases'. Regulatory and ethic submission was sponsored by University Hospital of Toulouse. The work of J.P. Schanstra was supported by Inserm and the 'Direction Régional de la Recherche' (CHU de Toulouse) under the Interface program.

References

1 Chevalier RL: Pathophysiology of obstructive nephropathy in the newborn. Semin Nephrol 1998;18:585–593.
2 Roth KS, Koo HP, Spottswood SE, Chan JC: Obstructive uropathy: an important cause of chronic renal failure in children. Clin Pediatr (Phila) 2002;41:309–314.
3 Chevalier RL: Pathogenesis of renal injury in obstructive uropathy. Curr Opin Pediatr 2006; 18:153–160.
4 Csaicsich D, Greenbaum LA, Aufricht C: Upper urinary tract: when is obstruction obstruction? Curr Opin Urol 2004;14:213–217.
5 Chevalier RL, Peters CA: Congenital urinary tract obstruction. Proceedings of the State-of-the-Art Strategic Planning Workshop-National Institutes of Health, Bethesda, Md, USA, March 11–12, 2002. Pediatr Nephrol 2003;18:576–606.
6 Bascands JL, Schanstra JP: Obstructive nephropathy: insights from genetically engineered animals. Kidney Int 2005;68:925–937.
7 Furness PD 3rd, Maizels M, Han SW, Cohn RA, Cheng EY: Elevated bladder urine concentration of transforming growth factor-β_1 correlates with upper urinary tract obstruction in children. J Urol 1999;162:1033–1036.
8 Grandaliano G, Gesualdo L, Bartoli F, Ranieri E, Monno R, Leggio A, Paradies G, Caldarulo E, Infante B, Schena FP: MCP-1 and EGF renal expression and urine excretion in human congenital obstructive nephropathy. Kidney Int 2000;58:182–192.
9 El-Sherbiny MT, Mousa OM, Shokeir AA, Ghoneim MA: Role of urinary transforming growth factor-β_1 concentration in the diagnosis of upper urinary tract obstruction in children. J Urol 2002;168:1798–1800.

10 Liu Y: Renal fibrosis: new insights into the pathogenesis and therapeutics. Kidney Int 2006;69: 213–217.
11 Kaneto H, Morrissey J, Klahr S: Increased expression of TGF-β_1 mRNA in the obstructed kidney of rats with unilateral ureteral ligation. Kidney Int 1993;44:313–321.
12 Seremetis GM, Maizels M: TGF-β mRNA expression in the renal pelvis after experimental and clinical ureteropelvic junction obstruction. J Urol 1996;156:261–266.
13 Wright EJ, McCaffrey TA, Robertson AP, Vaughan ED Jr, Felsen D: Chronic unilateral ureteral obstruction is associated with interstitial fibrosis and tubular expression of transforming growth factor-β. Lab Invest 1996;74:528–537.
14 Chevalier RL: Obstructive nephropathy: towards biomarker discovery and gene therapy. Nat Clin Pract Nephrol 2006;2:157–168.
15 Thongboonkerd V, Malasit P: Renal and urinary proteomics: current applications and challenges. Proteomics 2005;5:1033–1042.
16 Schaub S, Wilkins J, Weiler T, Sangster K, Rush D, Nickerson P: Urine protein profiling with surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry. Kidney Int 2004; 65:323–332.
17 Thongboonkerd V, Chutipongtanate S, Kanlaya R: Systematic evaluation of sample preparation methods for gel-based human urinary proteomics: quantity, quality, and variability. J Proteome Res 2006;5:183–191.
18 Traum AZ, Wells MP, Aivado M, Libermann TA, Ramoni MF, Schachter AD: SELDI-TOF MS of quadruplicate urine and serum samples to evaluate changes related to storage conditions. Proteomics 2006;6:1676–1680.
19 Thongboonkerd V, McLeish KR, Arthur JM, Klein JB: Proteomic analysis of normal human urinary proteins isolated by acetone precipitation or ultracentrifugation. Kidney Int 2002;62: 1461–1469.
20 Henzel WJ, Watanabe C, Stults JT: Protein identification: the origins of peptide mass fingerprinting. J Am Soc Mass Spectrom 2003;14:931–942.
21 Issaq HJ, Conrads TP, Prieto DA, Tirumalai R, Veenstra TD: SELDI-TOF MS for diagnostic proteomics. Anal Chem 2003;75:148A–155A.
22 Woroniecki RP, Orlova TN, Mendelev N, Shatat IF, Hailpern SM, Kaskel FJ, Goligorsky MS, O'Riordan E: Urinary proteome of steroid-sensitive and steroid-resistant idiopathic nephrotic syndrome of childhood. Am J Nephrol 2006;26:258–267.
23 Khurana M, Traum AZ, Aivado M, Wells MP, Guerrero M, Grall F, Libermann TA, Schachter AD: Urine proteomic profiling of pediatric nephrotic syndrome. Pediatr Nephrol 2006;21:1257–1265.
24 Mosley K, Tam FW, Edwards RJ, Crozier J, Pusey CD, Lightstone L: Urinary proteomic profiles distinguish between active and inactive lupus nephritis. Rheumatology (Oxford) 2006;45: 1497–1504.
25 Clarke W, Silverman BC, Zhang Z, Chan DW, Klein AS, Molmenti EP: Characterization of renal allograft rejection by urinary proteomic analysis. Ann Surg 2003;237:660–665.
26 O'Riordan E, Orlova TN, Mei JJ, Butt K, Chander PM, Rahman S, Mya M, Hu R, Momin J, Eng EW, Hampel DJ, Hartman B, Kretzler M, Delaney V, Goligorsky MS: Bioinformatic analysis of the urine proteome of acute allograft rejection. J Am Soc Nephrol 2004;15:3240–3248.
27 Kolch W, Neususs C, Pelzing M, Mischak H: Capillary electrophoresis-mass spectrometry as a powerful tool in clinical diagnosis and biomarker discovery. Mass Spectrom Rev 2005;24: 959–977.
28 Weissinger EM, Wittke S, Kaiser T, Haller H, Bartel S, Krebs R, Golovko I, Rupprecht HD, Haubitz M, Hecker H, Mischak H, Fliser D: Proteomic patterns established with capillary electrophoresis and mass spectrometry for diagnostic purposes. Kidney Int 2004;65:2426–2434.
29 Wittke S, Mischak H, Walden M, Kolch W, Radler T, Wiedemann K: Discovery of biomarkers in human urine and cerebrospinal fluid by capillary electrophoresis coupled to mass spectrometry: towards new diagnostic and therapeutic approaches. Electrophoresis 2005;26:1476–1487.
30 Neuhoff N, Kaiser T, Wittke S, Krebs R, Pitt A, Burchard A, Sundmacher A, Schlegelberger B, Kolch W, Mischak H: Mass spectrometry for the detection of differentially expressed proteins: a comparison of surface-enhanced laser desorption/ionization and capillary electrophoresis/mass spectrometry. Rapid Commun Mass Spectrom 2004;18:149–156.

31 Theodorescu D, Wittke S, Ross MM, Walden M, Conaway M, Just I, Mischak H, Frierson HF: Discovery and validation of new protein biomarkers for urothelial cancer: a prospective analysis. Lancet Oncol 2006;7:230–240.
32 Decramer S, Wittke S, Mischak H, Zurbig P, Walden M, Bouissou F, Bascands JL, Schanstra JP: Predicting the clinical outcome of congenital unilateral ureteropelvic junction obstruction in newborn by urinary proteome analysis. Nat Med 2006;12:398–400.
33 Fernbach SK, Maizels M, Conway JJ: Ultrasound grading of hydronephrosis: introduction to the system used by the Society for Fetal Urology. Pediatr Radiol 1993;23:478–480.
34 Grignon A, Filion R, Filiatrault D, Robitaille P, Homsy Y, Boutin H, Leblond R: Urinary tract dilatation in utero: classification and clinical applications. Radiology 1986;160:645–647.
35 Maizels M, Mitchell B, Kass E, Fernbach SK, Conway JJ: Outcome of nonspecific hydronephrosis in the infant: a report from the Registry of the Society for Fetal Urology. J Urol 1994;152: 2324–2327.
36 Chertin B, Pollack A, Koulikov D, Rabinowitz R, Hain D, Hadas-Halpren I, Farkas A: Conservative treatment of ureteropelvic junction obstruction in children with antenatal diagnosis of hydronephrosis: lessons learned after 16 years of follow-up. Eur Urol 2006;49:734–739.
37 Zurbig P, Renfrow MB, Schiffer E, Novak J, Walden M, Wittke S, Just I, Pelzing M, Neususs C, Theodorescu D, Root KE, Ross MM, Mischak H: Biomarker discovery by CE-MS enables sequence analysis via MS/MS with platform-independent separation. Electrophoresis 2006;27: 2111–2125.
38 Fricker LD, McKinzie AA, Sun J, Curran E, Qian Y, Yan L, Patterson SD, Courchesne PL, Richards B, Levin N, Mzhavia N, Devi LA, Douglass J: Identification and characterization of proSAAS, a granin-like neuroendocrine peptide precursor that inhibits prohormone processing. J Neurosci 2000;20:639–648.
39 Basak A, Koch P, Dupelle M, Fricker LD, Devi LA, Chretien M, Seidah NG: Inhibitory specificity and potency of proSAAS-derived peptides toward proprotein convertase 1. J Biol Chem 2001; 276:32720–32728.
40 Benjannet S, Reudelhuber T, Mercure C, Rondeau N, Chretien M, Seidah NG: Proprotein conversion is determined by a multiplicity of factors including convertase processing, substrate specificity, and intracellular environment. Cell type-specific processing of human prorenin by the convertase PC1. J Biol Chem 1992;267:11417–11423.
41 Reudelhuber TL, Ramla D, Chiu L, Mercure C, Seidah NG: Proteolytic processing of human prorenin in renal and non-renal tissues. Kidney Int 1994;46:1522–1524.

Joost P. Schanstra
Inserm U388, IFR 31, Institut Louis Bugnard, BP 84225
FR–31432 Toulouse Cedex 4 (France)
Tel. +33 5 6132 2211, Fax + 33 5 6217 2554, E-Mail schans@toulouse.inserm.fr

Thongboonkerd V (ed): Proteomics in Nephrology – Towards Clinical Applications.
Contrib Nephrol. Basel, Karger, 2008, vol 160, pp 142–158

Proteomics and Kidney Stone Disease

Visith Thongboonkerd

Medical Proteomics Unit & Medical Molecular Biology Unit,
Office for Research and Development, Faculty of Medicine Siriraj Hospital,
Mahidol University, Bangkok, Thailand

Abstract

Kidney stone disease (nephrolithiasis) is an ancient and common affliction. It has been recognized for a long time with evidence of stone found in ~7,000-year-old mummies and remains a common problem worldwide, indicating ineffective prevention in the past. Precise pathogenic and molecular mechanisms of kidney stone formation are still poorly understood and should be further elucidated. Also, identification of novel therapeutic targets for better therapeutic outcome and successful prevention of the occurrence and recurrence of the stone are crucially required. One of the most promising tools for current and future biomedical research is proteomics, which has been extensively and widely applied to the nephrology field during the past 5 years. Its high-throughput capability holds a great promise also to kidney stone research. This chapter provides a brief overview of proteomic methodologies recently used for the investigation of nephrolithiasis and recent proteomic studies of nephrolithiasis are summarized.

Kidney stone disease (nephrolithiasis) remains a public health problem around the world [1]. Each stone is a mixture of crystalline and organic components. While the former is the major composition, organic compounds represent 2–5% (dry weight) of the stone and approximately two thirds of the organic matrix consists of proteins. Of all types of renal calculi, calcium oxalate (CaOx) is the most common crystalline composition found in the calculi [2].

To date, precise pathogenic mechanisms of kidney stone formation remain poorly understood. One longstanding hypothesis has suggested that the stone is initially formed inside renal tubular lumens [3]. The urine of stone formers is commonly supersaturated with calcium and oxalate ions [4], favoring CaOx crystal nucleation, growth and aggregation [5]. The nucleated crystals, predominantly monohydrate form (COM), can then retain in the kidney of these patients by

adhering to renal tubular epithelial surfaces [6, 7]. Together with the environment of supersaturated calcium and oxalate ions, the stone can thus be formed. In contrast, nucleated crystals do not retain in the normal kidney because they are eliminated from the normal kidney before they can adhere tightly to tubular epithelial cell surfaces [8–10]. CaOx in the normal urine are crystallized mostly in the dihydrate form (COD), which has the least adsorptive capability [11], thereby unfavorable for adhesion to renal tubular epithelial cells. Moreover, there are urinary substances namely 'stone inhibitors' in the normal renal tubular fluid and urine that can inhibit intratubular crystal growth, aggregation, and/or adhesion to renal epithelial cells [12]. These substances have been identified as proteins, lipids, glycosaminoglycans, and inorganic compounds. Declined levels and altered functions of these molecules in renal tubular fluid and urine can thus lead to the development of renal stones [13–15].

The other well-known hypothesis, which was first described by Alexander Randall [16], suggested that crystals initially deposited in renal interstitium (outside the renal tubular lumens) at the tip of renal papillae. These deposited crystals then form so-called 'Randall's plaques', which contain apatite crystals and are usually found in CaOx stone formers [17]. Human studies on renal biopsies have implicated that apatite crystallization occurs initially at basement membranes of the thin segment of Henle's loop. These crystals can grow and then invade to vasa recta, interstitial tissue and renal papillae [18, 19]. Subsequently, the Randall's plaques can erode into the urinary space, which is supersaturated with calcium and oxalate ions. These processes can promote heterogeneous nucleation and formation of CaOx renal calculi [18, 19].

Based on these two main hypotheses, it is still unclear whether intratubular or interstitial deposition of crystals is the main initial pathogenic mechanism of kidney stone disease. Several lines of evidence have suggested that it is unlikely to explain all forms of renal calculi by only a single mechanism or pathway. Multiple mechanisms might perhaps occur in individual patients.

Apart from idiopathic stones, CaOx stone formation is also associated with intestinal bypass that promotes hyperoxaluria. Histopathological examination reveals no plaque at the interstitium but some apatite crystals plugged inside the lumens of terminal collecting ducts that are associated with epithelial cell damage, interstitial inflammation and fibrosis [18].

Another group of the stone formers have calcium phosphate as the major crystalline composition (>50%), in which one quarter contains brushite ($CaHPO_4 \cdot 2H_2O$) that represents an early phase of calcium phosphate stone formation [20, 21]. Patients with brushite stones also have absorptive hypercalciuria type I and distal renal tubular acidosis as the associated disorders [22]. The degree of brushite supersaturation hence depends directly on urinary calcium level [23]. Brushite stone formers have histopathological changes that combine

the interstitial plaques of CaOx stone formers with the intratubular apatite plugs found in bypass stone formers. In other words, their histopathology is an amalgam of CaOx and bypass stone disease [20]. A nidus of brushite can elicit heterogeneous nucleation or epitaxial growth of CaOx. Thus, brushite has been implicated in the formation of both hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] and CaOx stones [21]. On the other hand, many of the present brushite stone formers initially had CaOx stones [20].

Even with the aforementioned knowledge, molecular mechanisms of stone formation remain poorly understood and should be further elucidated. One of the most promising tools for current and future biomedical research is proteomics, which has been extensively and widely applied to the nephrology field during the past 5 years [24–29]. Its high-throughput capability also holds a great promise to kidney stone research. This chapter provides a brief overview of proteomic methodologies recently used for the investigation of nephrolithiasis. Additionally, recent proteomic studies of nephrolithiasis are summarized.

Brief Overview of Proteomic Methodologies Recently Used for the Investigation of Nephrolithiasis

Two-Dimensional Polyacrylamide Gel Electrophoresis (2-D PAGE)

The most commonly employed proteomic technique in recent kidney stone research is gel-based method (i.e. using 2-D PAGE). The first dimension of 2-D PAGE separates proteins by differential pH or charges, whereas the second dimensional separation is based on differential molecular masses [30]. Resolved proteins in a 2-D gel can then be visualized by various stains or radiolabeling. Recently, the concept of two-dimensional difference gel electrophoresis (2-D DIGE) has been introduced to reduce gel-to-gel variability [31, 32]. Briefly, each of two samples (or sample pools) is differentially labeled with fluorescent dyes (Cy3 and Cy5). The two differentially labeled samples are then mixed and resolved simultaneously within the same 2-D gel. An internal standard labeled with a third dye (i.e. Cy2) can be also incorporated, resulting in more accurately quantitative analysis. The spots of interest can be identified mostly by peptide mass fingerprinting following matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), by other MS methods and also by immunoblotting for the known proteins.

2-D PAGE is a simple technique to perform and is available in most of proteomics laboratories. However, its procedures are time-consuming, particularly when a large number of biological samples are analyzed and spot analysis is

tedious (a totally hand-free 2-D analysis software does not really exist!). Moreover, 2-D PAGE is not suitable for proteins or polypeptides with molecular masses <10 kDa and has a limited use for an analysis of highly hydrophobic proteins.

Liquid Chromatography Coupled to Tandem Mass Spectrometry (LC-MS/MS)

LC-MS/MS has become a widely used method for gel-free proteomic analysis [33–35]. Recently, multidimensional protein identification technology (MudPIT) or 2D-LC-MS/MS has been introduced to enhance the high-throughput capability of LC-based approach [36]. MudPIT involves proteolytic digestion of the protein mixture to obtain a set of fragmented peptides that are then separated by strong cation exchange (SCX) chromatography. Peptides present in fractions from this SCX chromatography step are separated further by reversed-phase (RP) LC and then sequenced by MS/MS. Several thousands of peptides can be sequenced using this approach in a relatively short period. Analysis of the digested peptides is called 'bottom-up' approach, whereas analysis of the undigested polypeptides or protein mixture is also possible and called 'top-down' approach. In addition, LC-based technology allows opportunity to combine various kinds of LC columns to a wide spectrum of mass spectrometers.

Comparing to the 2-D PAGE approach, the LC-based method is more effective for the analysis of small proteins and peptides, as well as for membrane and highly hydrophobic proteins. However, the major limitation of the LC-based method is that quantitative analysis of two or more different samples is not an easy task. Fortunately, recent development of isotope-coded affinity tags (ICAT™) [35, 37, 38] and isobaric tags for relative and absolute quantitation (iTRAQ™) [39–42] has enabled simultaneous quantitative analysis of 2–4 samples. Even with the availability of ICAT and iTRAQ technologies, LC-based quantitative proteomics still has limitations, particularly when a much larger number of samples are subjected to comparison.

Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF-MS)

SELDI-TOF-MS (or ProteinChip™ technology) is an easy-to-use system, especially for proteome profiling of human body fluids. SELDI-TOF-MS combines MALDI-TOF-MS with surface retentate chromatography [43, 44]. Only a small volume (a few to 10 μl) of samples is required for a single analysis and this method can be readily automated, making the high-throughput analysis feasible [43, 44]. The sample is first applied onto a chip surface specifically designed for retaining particular groups of proteins. After incubation, unbound proteins are removed and the bound proteins are analyzed by TOF mass spectrometer.

This approach reduces the complexity of proteins in the sample being analyzed by selecting only a subset of proteins with particular functionality or property [43, 44].

The limitations of SELDI-TOF-MS include the restriction of information – only a particular set of proteins, which were initially bound to the chip surface, can be analyzed. Additionally, the problems of mass accuracy and precision of the measurement may also limit its utility.

Recent Proteomic Studies of Nephrolithiasis

The proteomic investigation of nephrolithiasis has been performed for quite some time. However, the number of such studies is much smaller when compared to other subdisciplines of 'renal and urinary proteomics'. During the past two decades, the proteomic investigation of nephrolithiasis has focused mainly onto four major aims: (i) to identify and characterize stone matrix proteins; (ii) to examine urinary proteome profiles for biomarker discovery, diagnostics and prognostics; (iii) to identify urinary proteins that bind to crystals for better understanding of crystal-proteins interactions, and (iv) to search for novel urinary modulators of crystal nucleation, growth, aggregation and adhesion.

Proteomic Identification and Characterization of Stone Matrix Proteins

Among all proteomic applications to kidney stone research, proteomic identification and characterization of stone matrix proteins is the major application in previous studies. Because the development of a stone may take years to decades, thorough analysis of the stone matrix proteins would lead to a better understanding of history of such developmental process (e.g. types and roles of proteins involved in the stone formation). However, one should realize the fact that renal tubular fluid and urine contains several proteins, and some of the proteins identified from the stone matrix might play no active roles in the processes of stone formation but were just simply entrapped into the stone mass.

This type of the study began even before the term 'proteome' and 'proteomics' were coined. In 1990, Jones and Resnick [45] extracted proteins from different types of renal calculi and resolved the solubilized proteins with 2-D PAGE. The results showed characteristic map of each stone type. Interestingly, the majority of all visualized proteins were low-molecular-weight (MW) proteins [45]. A subsequent study by Binette and Binette [46] also employed a similar approach to identify proteins from the crushed stone. After protein extraction by electrodialysis, recovered proteins were concentrated by filtration or lyophilization and resolved by 2-D PAGE. Some prominent protein spots were then identified by N-terminal amino acid sequencing. The results showed

that the stone matrix composed mainly of proteins with highly abundant glutamic and aspartic acids in their sequences, and with frequent occurrence of γ-carboxyglutamic acid [47]. Moreover, the findings also implicated that individual stones shared only few proteins [47].

Several years later, Kaneko et al. [48–50] reported a series of studies identifying the stone matrix proteins, using either 2-D PAGE or SDS-PAGE to separate proteins and employing LC-MS/MS for protein identification. They successfully identified prothrombin, osteopontin and protein Z from a kidney stone removed from a hyperuricemic patient (stone analysis using infrared spectroscopy and micro-area X-ray diffractometry revealed COM as the major crystalline composition) [48, 49]. Subsequent study on another hyperuricemic patient with recurrent calculi (micro-area X-ray diffractometry revealed COM as the major crystalline composition on the first and COD as the major inorganic compound in the second stone) identified uromodulin, albumin, osteopontin, protein Z, defensins, lysozyme and calgranulin A in both calculi [50].

Recently, Mushtaq et al. [51] characterized proteins in inner core and outer matrix of CaOx stones. Proteins extracted from both regions were resolved by SDS-PAGE. The results showed that the inner core contained mainly low-MW proteins (mostly with molecular masses of 12–14 kDa), whereas a 66-kDa band that were later identified as osteopontin was presented in both regions. Subsequent analyses of low-MW proteins in the inner core using RP-HPLC coupled to tandem MS (MS/MS) identified myeloperoxidase chain A, α-defensin and calgranulin. Validation with ELISA, Western blot analysis and slot-blot immunoassay confirmed the presence of these low-MW proteins only in the inner core, not in the outer matrix. The authors also proposed a hypothesis that persistent hyperoxaluria caused tubular epithelial cell injury, resulting in the release of these anti-inflammatory proteins. These proteins could have been first adsorbed on CaOx crystals and then became a part of nucleation process, leading to the inner matrix formation.

Proteomics was also applied to identify the stone matrix proteins in dogs. Forterre et al. [52] employed SELDI-TOF-MS to compare the proteome profiles of the matrix proteins derived from different types of stones, including CaOx, struvite and uric acid. The results demonstrated that the SELDI proteome profiles of these different types of stones obviously differed. However, the differential displayed SELDI peaks were not identified.

Urinary Proteome Profiling for Biomarker Discovery, Diagnostics and Prognostics

Urinary proteome profiling may lead to the discovery of urinary biomarkers for diagnostics and/or prognostics, especially for the stone recurrence.

Grover and Resnick [53] adopted 2-D PAGE to examine the urinary proteome profile of 20 stone formers with histories of idiopathic CaOx renal calculi compared to that of 20 healthy individuals. The results showed that there were 9 protein spots, whose urinary levels were greater in the stone formers. There were no differences between genders and follow-up analysis of patients with recurrent non-opaque stones revealed the same urinary proteome pattern as of the initial study. The authors suggested that these results might exclude the possibility of abrasion to be the cause of the increased levels of these proteins. One of these protein spots was identified as α_1-acid glycoprotein by comigration of known purified proteins and by comparisons with previous publications. Unfortunately, eight other protein spots were unidentified and no mass spectrometric analysis was performed in this study.

SELDI-TOF-MS is one of the powerful proteomic tools used for proteome profiling [54, 55]. It was applied by Cadieux et al. [56] for urinary proteome profiling of 25 male patients who had urological stones and underwent extracorporeal shock-wave lithotripsy, compared with 25 healthy males. The results showed that the ratio of SELDI that peaked at 67 and 24 kDa (p67:p24) was <1.0 in all normal urine samples, whereas 18 of 25 diseased urine samples had a p67:p24 ratio of >1.0. Subsequent immunological analysis suggested that p67 was indeed albumin. SELDI-TOF-MS was also applied to urinary proteome profiling in 23 dogs with urolithiasis compared to 12 healthy controls [52]. The analysis of SELDI mass spectra demonstrated that urinary proteins with molecular masses of 41.75 and 65.69 kDa had greater levels in CaOx stone formers, whereas those with masses of 10.17 and 10.45 kDa had significantly greater levels in dogs with struvite stones, and those with masses of 9.42, 15.30 and 16.03 kDa had greater levels in both struvite and uric acid stone formers, when compared to normal controls.

Proteomic Analysis of Urinary Proteins that Bind to Crystals for Better Understanding of Crystal-Proteins Interactions

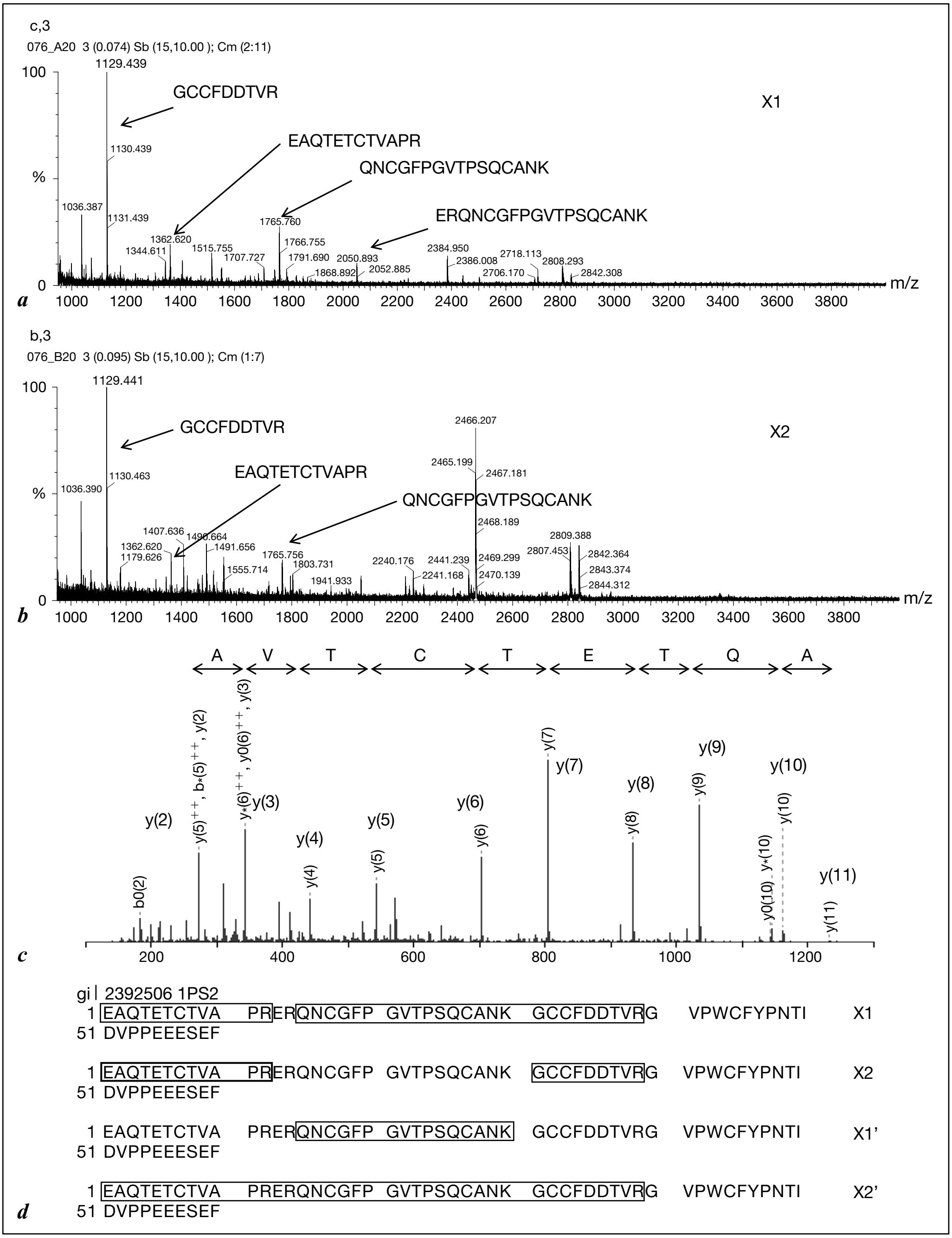
Identification of urinary proteins that bind to crystals would lead to a better understanding of crystal-proteins interactions. Recently, urinary crystal surface-binding substances (CSBS) were studied by Koide et al. [57]. The naturally existing CSBS in healthy individuals were isolated and fractionated using gel chromatography RP-LC. CSBS fractions from RP-LC were then analyzed by gas chromatography coupled to MS and by N-terminal amino acid sequencing. Functional assay demonstrated that the CSBS exhibited very strong inhibitory activity against CaOx crystal growth and aggregation. Surprisingly, no promoting activity of CSBS was detected. Although several RP-LC fractions were found to have both peptides and saccharides, no distinct macromolecules were identified or characterized in this study.

Proteomic Identification of Novel Urinary Modulators of CaOx Crystal Nucleation, Growth, Aggregation and Adhesion

Functional characterization of urinary proteins with regard of their inhibitory and promoting activities in stone formation would definitely lead to a clearer picture of their roles in crystal modulation (either inhibition or promotion of crystal nucleation, growth, aggregation and adhesion to renal tubular epithelial cells). Such of studies may also lead to defining and development of new therapeutic targets for better treatment outcome and successful prevention of occurrence and recurrence of nephrolithiasis.

Currently known stone inhibitory proteins include nephrocalcin [22], Tamm-Horsfall protein [58], uropontin [59], inter-α-trypsin inhibitor (bikunin) [60], and urinary prothrombin fragment 1 (crystal matrix protein) [61]. Interestingly, these stone inhibitors have similar physicochemical properties as they are small anionic proteins that can bind to calcium and inhibit either growth or aggregation of CaOx crystals. Only a few other proteins have been identified as inhibitors of stone formation [62–64].

Recently, our group applied a proteomics approach to identify human urinary trefoil factor 1 (TFF1) as a novel potent inhibitor of CaOx crystal growth by combining conventional biochemical methods with recent advances in MS [65]. Because most of previously known inhibitory proteins are anionic proteins with calcium-binding property, we focused our attention on anionic urinary proteins. Anionic proteins were isolated by DEAE (DE-52; Whatman Inc.) adsorption and separated by HiLoad 16/60 Superdex 75 (GE Healthcare) gel filtration. A fraction with potent inhibitory activity against CaOx crystal growth was isolated and further purified by anion exchange chromatography (Resource Q; GE Healthcare). The protein in two subfractions that retained inhibitory activity was identified by quadrupole time-of-flight (Q-TOF) MS and MS/MS analyses. Such protein was identified as TFF1 in both subfractions (fig. 1) and its identity was clearly confirmed by Western blot analysis. Concentrations and normalized levels (normalized with urine creatinine and total protein) of TFF1 in the urine of patients with idiopathic CaOx renal calculi were significantly less (2.5-fold for the concentrations and 5- to 22-fold for the normalized levels) than those in the normal controls. Functional studies of urinary TFF1 demonstrated that its inhibitory potency was similar to that of nephrocalcin and the functional domain for the inhibitory activity was at its C-terminus, which contains multiple repeated glutamic acid residues. In addition, homodimerization frequently occurs in the native form of TFF1 (at Cys58-Cys58) [66, 67], producing a potent negatively charged pocket. We therefore hypothesized that this negatively charged area is crucial for entrapment of free calcium ions in the urine, thereby reducing the growth rate of CaOx crystals [65] (fig. 2).


c,3
076_A20 3 (0.074) Sb (15,10.00); Cm (2:11)
GCCFDDTVR
EAQTETCTVAPR
QNCGFPGVTPSQCANK
ERQNCGFPGVTPSQCANK
X1
m/z
a
b,3
076_B20 3 (0.095) Sb (15,10.00); Cm (1:7)
GCCFDDTVR
EAQTETCTVAPR
QNCGFPGVTPSQCANK
X2
m/z
b
A V T C T E T Q A
c
gi| 2392506 1PS2
1 EAQTETCTVA PRERQNCGFP GVTPSQCANK GCCFDDTVRG VPWCFYPNTI X1
51 DVPPEEESEF
1 EAQTETCTVA PRERQNCGFP GVTPSQCANK GCCFDDTVRG VPWCFYPNTI X2
51 DVPPEEESEF
1 EAQTETCTVA PRERQNCGFP GVTPSQCANK GCCFDDTVRG VPWCFYPNTI X1’
51 DVPPEEESEF
1 EAQTETCTVA PRERQNCGFP GVTPSQCANK GCCFDDTVRG VPWCFYPNTI X2’
d
51 DVPPEEESEF

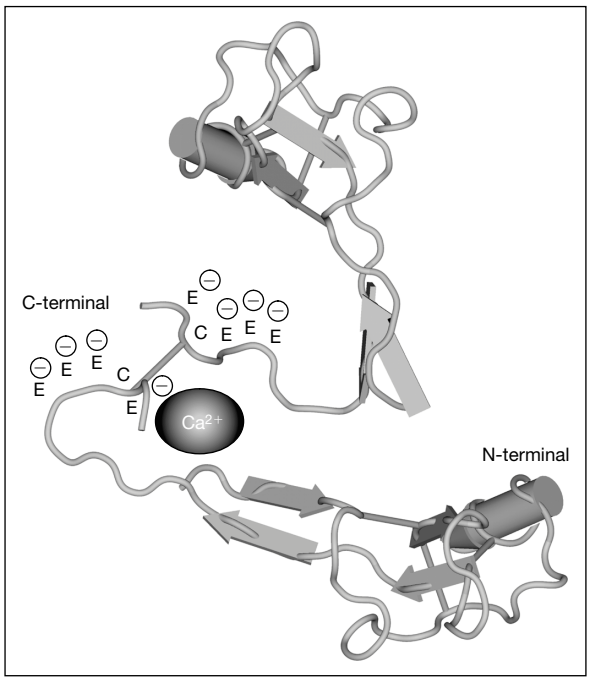
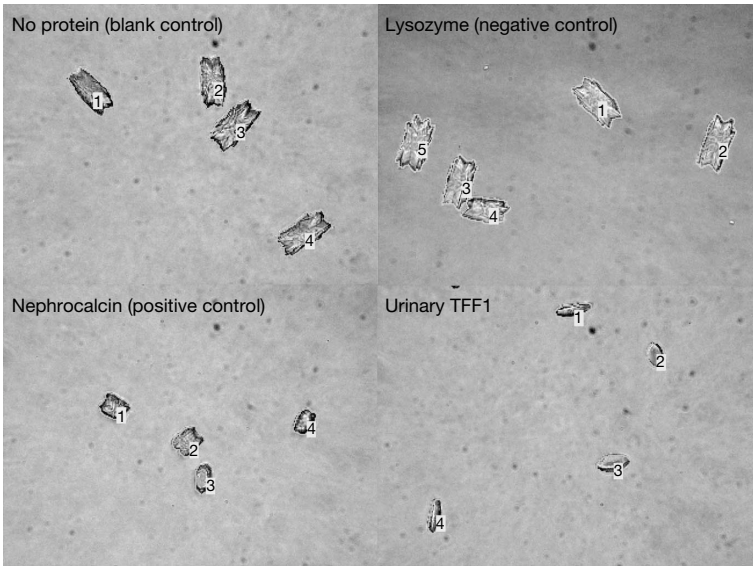


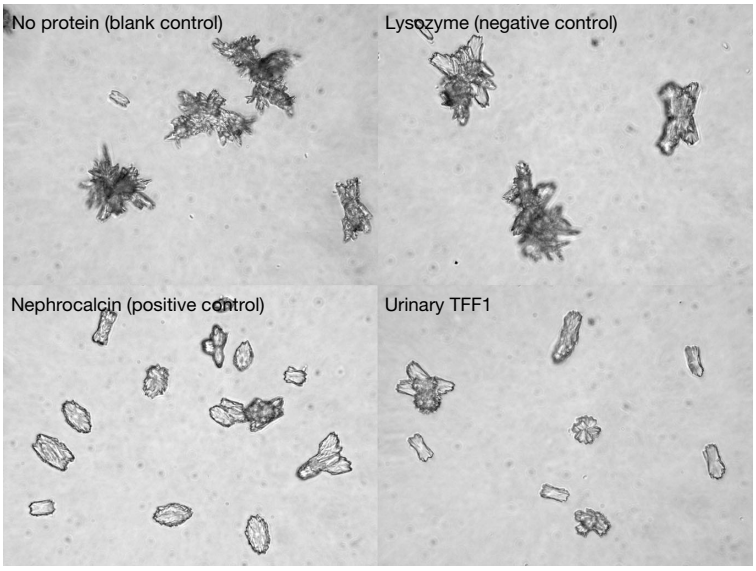
Fig. 2. The proposed model of Ca^{2+}-binding site in urinary TFF1 molecules. Based on the functional data and because the C-terminus of TFF1 contains multiple repeated glutamic residues, which are negatively charged, it has been hypothesized that this area is particularly important for mediating the CaOx crystal growth inhibitory function of TFF1. Additionally, homodimerization, which frequently occurs in the native form of TFF1 (at Cys58-Cys58) [66, 67], can also facilitate entrapment of Ca^{2+} ions in this area [modified from 65, with permission from the American Society for Clinical Investigation].

Our subsequent study [68], which offered more direct evidence, confirmed the initial result that human urinary TFF1 is a novel potent CaOx crystal growth inhibitor. Moreover, additional data indicated that urinary TFF1 can also inhibit COM crystal aggregation (fig. 3). We also found that urinary TFF1 at a supraphysiologic level can transform the COM crystals to the COD type (fig. 3),

Fig. 1. Identification of urinary TFF1 by Q-TOF-MS and MS/MS analyses. ***a***, ***b*** MS data obtained from subfractions X1 and X2, respectively. ***c*** MS/MS mass spectra were significantly matched with the peptide AQTETCTVA of human 1pS2 (gi|2392506) or TFF1. ***d*** Using the MASCOT search engine, peptide masses obtained from all subfractions (X1, X2, X1', and X2') were significantly matched with human TFF1, and the identified residues obtained from MS/MS analysis are labeled [modified from 65, with permission from the American Society for Clinical Investigation].

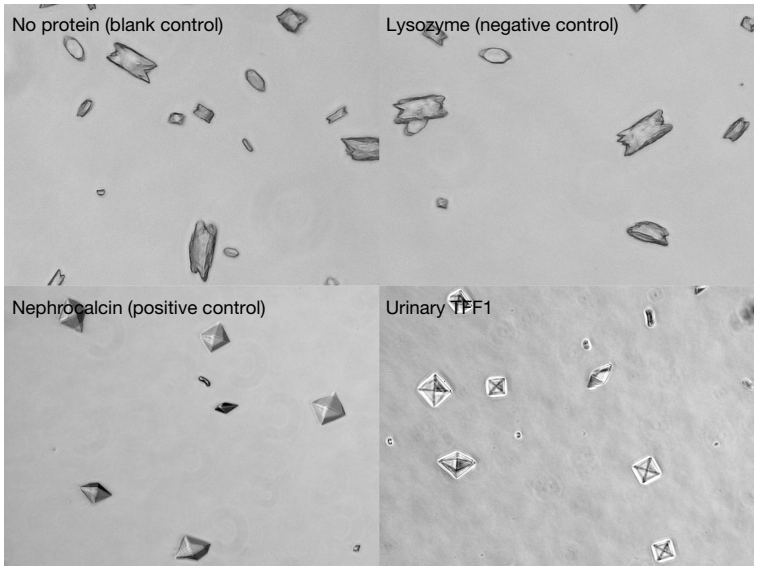


a



b

Fig. 3. Urinary TFF1 can inhibit CaOx crystal growth (***a***) and aggregation (***b***), and at its supraphysiologic level, can also transform COM (typically crystallized in monoclinic prismatic, hexagonal or dendrite shape) to COD (typically crystallized with tetragonal bipyramidal or weddellite shape) (***c***). Orig. magnif. 400× [modified from 68, with permission from the American Urological Association and Elsevier].



c

which has much less adsorptive capability [11]. This transformation is unfavorable for the stone formation [11, 69, 70]. Taken together, the data indicate that human urinary TFF1 is a novel potent inhibitor of CaOx kidney stone formation.

Summary and Outlook

Some progress has been made in proteomic applications to kidney stone research. To identify and characterize stone matrix proteins, recent proteomic studies have identified, however, only a small number of the stone matrix proteins. Therefore, an extensive proteomic study to characterize the stone matrix proteome is crucially required to better understand the involvements or roles of such proteins during stone developmental phases. Also, differential proteomics of different parts of the stone mass would also lead to an unraveling of the stone history.

Although opaque kidney stones can be simply detected by conventional radiography and ultrasonography, non-opaque stones are more difficult to detect and may be precisely diagnosed at a later stage or when patients have complications. Biomarker discovery would be therefore beneficial for the early diagnosis of non-opaque stones. Moreover, the discovery of biomarkers that can

be used as the predictive or prognostic markers is definitely required for the prediction of the stone recurrence and for prognosis. However, there are only few previous studies that focused on this goal. Most of these studies utilized SELDI-TOF-MS for proteome profiling. CE-MS [71–73], microarrays [74], and microfluidic technology on a chip [75] should be also applied in the near future to achieve this goal.

Other aspects of proteomic applications to the stone research that will draw lots of attention from proteomists, nephrologists, urologists and scientists in the coming years include the identification of proteins that bind to crystals for better understanding of crystal-proteins interactions and identification of urinary proteins that serve as novel inhibitors or promoters of the stone formation. These interesting areas seem to be achievable most likely by an initial screening using proteomic methodologies followed by subsequent functional validation using conventional biochemical methods. Finally, one of the interesting areas, which had not been previously approached by any of proteomic strategies, is the study of cellular responses during crystal adhesion to renal tubular epithelial cells. This type of the study will sooner or later be explored by proteomic techniques.

In summary, proteomics provides a wealth of useful information and holds a great promise for the investigation of nephrolithiasis. The ultimate goals are to better understand the pathogenic and molecular mechanisms of stone formation, to identify biomarkers for earlier diagnosis and successful prediction of the stone recurrence, and to define novel therapeutic targets for better therapeutic outcome and effective prevention of kidney stone disease.

Acknowledgements

This work was supported by Siriraj Grant for Research and Development, Mahidol University, Vejdusit Foundation, Thailand Research Fund, Commission on Higher Education, National Center for Genetic Engineering and Biotechnology, and National Research Council of Thailand.

References

1 Ramello A, Vitale C, Marangella M: Epidemiology of nephrolithiasis. J Nephrol 2000;13(suppl 3): S45–S50.
2 Coe FL, Parks JH, Asplin JR: The pathogenesis and treatment of kidney stones. N Engl J Med 1992;327:1141–1152.
3 Lieske JC, Deganello S: Nucleation, adhesion, and internalization of calcium-containing urinary crystals by renal cells. J Am Soc Nephrol 1999;10(suppl 14):S422–S429.
4 Parks JH, Coward M, Coe FL: Correspondence between stone composition and urine supersaturation in nephrolithiasis. Kidney Int 1997;51:894–900.

5 Mandel N: Mechanism of stone formation. Semin Nephrol 1996;16:364–374.
6 Kok DJ, Papapoulos SE, Bijvoet OL: Crystal agglomeration is a major element in calcium oxalate urinary stone formation. Kidney Int 1990;37:51–56.
7 Lieske JC, Swift H, Martin T, Patterson B, Toback FG: Renal epithelial cells rapidly bind and internalize calcium oxalate monohydrate crystals. Proc Natl Acad Sci USA 1994;91:6987–6991.
8 Lieske JC, Deganello S, Toback FG: Cell-crystal interactions and kidney stone formation. Nephron 1999;81(suppl 1):8–17.
9 Finlayson B, Reid F: The expectation of free and fixed particles in urinary stone disease. Invest Urol 1978;15:442–448.
10 Kok DJ, Khan SR: Calcium oxalate nephrolithiasis, a free or fixed particle disease. Kidney Int 1994;46:847–854.
11 Tomazic BB, Nancollas GH: The dissolution of calcium oxalate kidney stones. A kinetic study. J Urol 1982;128:205–208.
12 Ryall RL: Glycosaminoglycans, proteins, and stone formation: adult themes and child's play. Pediatr Nephrol 1996;10:656–666.
13 Coe FL, Nakagawa Y, Asplin J, Parks JH: Role of nephrocalcin in inhibition of calcium oxalate crystallization and nephrolithiasis. Miner Electrolyte Metab 1994;20:378–384.
14 Wesson JA, Johnson RJ, Mazzali M, Beshensky AM, Stietz S, Giachelli C, Liaw L, Alpers CE, Couser WG, Kleinman JG, Hughes J: Osteopontin is a critical inhibitor of calcium oxalate crystal formation and retention in renal tubules. J Am Soc Nephrol 2003;14:139–147.
15 Hess B: Tamm-Horsfall glycoprotein and calcium nephrolithiasis. Miner Electrolyte Metab 1994;20:393–398.
16 Randall A: The origin and growth of renal calculi. Ann Surg 1937;105:1009–1027.
17 Low RK, Stoller ML: Endoscopic mapping of renal papillae for Randall's plaques in patients with urinary stone disease. J Urol 1997;158:2062–2064.
18 Evan AP, Lingeman JE, Coe FL, Parks JH, Bledsoe SB, Shao Y, Sommer AJ, Paterson RF, Kuo RL, Grynpas M: Randall's plaque of patients with nephrolithiasis begins in basement membranes of thin loops of Henle. J Clin Invest 2003;111:607–616.
19 Bushinsky DA: Nephrolithiasis: site of the initial solid phase. J Clin Invest 2003;111:602–605.
20 Evan AP, Lingeman JE, Coe FL, Shao Y, Parks JH, Bledsoe SB, Phillips CL, Bonsib S, Worcester EM, Sommer AJ, Kim SC, Tinmouth WW, Grynpas M: Crystal-associated nephropathy in patients with brushite nephrolithiasis. Kidney Int 2005;67:576–591.
21 Pak CY, Poindexter JR, Peterson RD, Heller HJ: Biochemical and physicochemical presentations of patients with brushite stones. J Urol 2004;171:1046–1049.
22 Nakagawa Y, Ahmed M, Hall SL, Deganello S, Coe FL: Isolation from human calcium oxalate renal stones of nephrocalcin, a glycoprotein inhibitor of calcium oxalate crystal growth. Evidence that nephrocalcin from patients with calcium oxalate nephrolithiasis is deficient in γ-carboxyglutamic acid. J Clin Invest 1987;79:1782–1787.
23 Pak CY: Physicochemical basis for formation of renal stones of calcium phosphate origin: calculation of the degree of saturation of urine with respect to brushite. J Clin Invest 1969;48:1914–1922.
24 Thongboonkerd V, Klein JB (eds): Proteomics in Nephrology. Contrib Nephrol. Basel, Karger, 2004, vol 141, pp 1–327.
25 Thongboonkerd V: Proteomics in nephrology: current status and future directions. Am J Nephrol 2004;24:360–378.
26 Thongboonkerd V, Malasit P: Renal and urinary proteomics: current applications and challenges. Proteomics 2005;5:1033–1042.
27 Thongboonkerd V: Proteomic analysis of renal diseases: unraveling the pathophysiology and biomarker discovery. Expert Rev Proteomics 2005;2:349–366.
28 Thongboonkerd V: Recent progress in urinary proteomics. Proteomics Clin Appl 2007;1: 780–791.
29 Thongboonkerd V: Practical points in urinary proteomics. J Proteome Res 2007;6:3881–3890.
30 Klein E, Klein JB, Thongboonkerd V: Two-dimensional gel electrophoresis: a fundamental tool for expression proteomics studies. Contrib Nephrol. Basel, Karger, 2004, vol 141, pp 25–39.
31 Lilley KS, Friedman DB: All about DIGE: quantification technology for differential-display 2D-gel proteomics. Expert Rev Proteomics 2004;1:401–409.

32 Marouga R, David S, Hawkins E: The development of the DIGE system: 2D fluorescence difference gel analysis technology. Anal Bioanal Chem 2005;382:669–678.
33 McCormack AL, Schieltz DM, Goode B, Yang S, Barnes G, Drubin D, Yates JR III: Direct analysis and identification of proteins in mixtures by LC/MS/MS and database searching at the low-femtomole level. Anal Chem 1997;69:767–776.
34 Wu CC, MacCoss MJ, Howell KE, Yates JR III: A method for the comprehensive proteomic analysis of membrane proteins. Nat Biotechnol 2003;21:532–538.
35 Gygi SP, Rist B, Griffin TJ, Eng J, Aebersold R: Proteome analysis of low-abundance proteins using multidimensional chromatography and isotope-coded affinity tags. J Proteome Res 2002;1:47–54.
36 Washburn MP, Wolters D, Yates JR III: Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat Biotechnol 2001;19:242–247.
37 Goodchild A, Raftery M, Saunders NF, Guilhaus M, Cavicchioli R: Cold adaptation of the Antarctic archaeon, *Methanococcoides burtonii*, assessed by proteomics using ICAT. J Proteome Res 2005;4:473–480.
38 Yi EC, Li XJ, Cooke K, Lee H, Raught B, Page A, Aneliunas V, Hieter P, Goodlett DR, Aebersold R: Increased quantitative proteome coverage with $^{13}C/^{12}C$-based, acid-cleavable isotope-coded affinity tag reagent and modified data acquisition scheme. Proteomics 2005;5:380–387.
39 Choe LH, Aggarwal K, Franck Z, Lee KH: A comparison of the consistency of proteome quantitation using two-dimensional electrophoresis and shotgun isobaric tagging in *Escherichia coli* cells. Electrophoresis 2005;26:2437–2449.
40 Chong PK, Gan CS, Pham TK, Wright PC: Isobaric tags for relative and absolute quantitation (iTRAQ) reproducibility: implication of multiple injections. J Proteome Res 2006;5: 1232–1240.
41 Aggarwal K, Choe LH, Lee KH: Shotgun proteomics using the iTRAQ isobaric tags. Brief Funct Genomic Proteomic 2006;5:112–120.
42 Wiese S, Reidegeld KA, Meyer HE, Warscheid B: Protein labeling by iTRAQ: a new tool for quantitative mass spectrometry in proteome research. Proteomics 2007;7:340–350.
43 Fung E, Diamond D, Simonsesn AH, Weinberger SR: The use of SELDI ProteinChip array technology in renal disease research. Methods Mol Med 2003;86:295–312.
44 Petricoin EF, Liotta LA: Clinical proteomics: application at the bedside. Contrib Nephrol. Basel, Karger, 2004, vol 141, pp 93–103.
45 Jones WT, Resnick MI: The characterization of soluble matrix proteins in selected human renal calculi using two-dimensional polyacrylamide gel electrophoresis. J Urol 1990;144: 1010–1014.
46 Binette JP, Binette MB: Sequencing of proteins extracted from stones. Scanning Microsc 1994;8: 233–239.
47 Binette JP, Binette MB, Gawinowicz MA, Kendrick N: Urinary stone proteins: an update. Scanning Microsc 1996;10:509–517.
48 Kaneko K, Yamanobe T, Nakagomi K, Mawatari K, Onoda M, Fujimori S: Detection of prothrombin and osteopontin in a renal stone found in a hyperuricemic patient using 2D-PAGE and LC-MS analysis. Nucleosides Nucleotides Nucleic Acids 2004;23:1127–1129.
49 Kaneko K, Yamanobe T, Nakagomi K, Mawatari K, Onoda M, Fujimori S: Detection of protein Z in a renal calculus composed of calcium oxalate monohydrate with the use of liquid chromatography-mass spectrometry/mass spectrometry following two-dimensional polyacrylamide gel electrophoresis separation. Anal Biochem 2004;324:191–196.
50 Kaneko K, Yamanobe T, Onoda M, Mawatari K, Nakagomi K, Fujimori S: Analysis of urinary calculi obtained from a patient with idiopathic hypouricemia using micro area x-ray diffractometry and LC-MS. Urol Res 2005;33:415–421.
51 Mushtaq S, Siddiqui AA, Naqvi ZA, Rattani A, Talati J, Palmberg C, Shafqat J: Identification of myeloperoxidase, α-defensin and calgranulin in calcium oxalate renal stones. Clin Chim Acta 2007;384:41–47.
52 Forterre S, Raila J, Kohn B, Brunnberg L, Schweigert FJ: Protein profiling of organic stone matrix and urine from dogs with urolithiasis. J Anim Physiol Anim Nutr (Berl) 2006;90:192–199.

53 Grover PK, Resnick MI: Evidence for the presence of abnormal proteins in the urine of recurrent stone formers. J Urol 1995;153:1716–1721.
54 Papale M, Pedicillo MC, Thatcher BJ, Di Paolo S, Lo ML, Bufo P, Rocchetti MT, Centra M, Ranieri E, Gesualdo L: Urine profiling by SELDI-TOF/MS: monitoring of the critical steps in sample collection, handling and analysis. J Chromatogr B Analyt Technol Biomed Life Sci 2007;856:205–213.
55 Poon TC: Opportunities and limitations of SELDI-TOF-MS in biomedical research: practical advices. Expert Rev Proteomics 2007;4:51–65.
56 Cadieux PA, Beiko DT, Watterson JD, Burton JP, Howard JC, Knudsen BE, Gan BS, McCormick JK, Chambers AF, Denstedt JD, Reid G: Surface-enhanced laser desorption/ionization-time of flight-mass spectrometry (SELDI-TOF-MS): a new proteomic urinary test for patients with urolithiasis. J Clin Lab Anal 2004;18:170–175.
57 Koide T, Yoshioka T, Yamaguchi S, Hosokawa S, Utsunomiya M, Sonoda T: Urinary crystal surface binding substances on calcium oxalate crystals. Urol Res 1990;18:387–392.
58 Hess B: Tamm-Horsfall glycoprotein – inhibitor or promoter of calcium oxalate monohydrate crystallization processes? Urol Res 1992;20:83–86.
59 Shiraga H, Min W, VanDusen WJ, Clayman MD, Miner D, Terrell CH, Sherbotie JR, Foreman JW, Przysiecki C, Neilson EG: Inhibition of calcium oxalate crystal growth in vitro by uropontin: another member of the aspartic acid-rich protein superfamily. Proc Natl Acad Sci USA 1992;89:426–430.
60 Atmani F, Khan SR: Role of urinary bikunin in the inhibition of calcium oxalate crystallization. J Am Soc Nephrol 1999;10(suppl 14):S385–S388.
61 Ryall RL, Grover PK, Stapleton AM, Barrell DK, Tang Y, Moritz RL, Simpson RJ: The urinary F1 activation peptide of human prothrombin is a potent inhibitor of calcium oxalate crystallization in undiluted human urine in vitro. Clin Sci (Lond) 1995;89:533–541.
62 Han IS, Nakagawa Y, Park JW, Suh MH, Suh SI, Shin SW, Ahn SY, Choe BK: FKBP-12 exhibits an inhibitory activity on calcium oxalate crystal growth in vitro. J Korean Med Sci 2002;17: 41–48.
63 Tsujihata M, Miyake O, Yoshimura K, Kakimoto KI, Takahara S, Okuyama A: Fibronectin as a potent inhibitor of calcium oxalate urolithiasis. J Urol 2000;164:1718–1723.
64 Selvam R, Kalaiselvi P: A novel basic protein from human kidney which inhibits calcium oxalate crystal growth. BJU Int 2000;86:7–13.
65 Chutipongtanate S, Nakagawa Y, Sritippayawan S, Pittayamateekul J, Parichatikanond P, Westley BR, May FE, Malasit P, Thongboonkerd V: Identification of human urinary trefoil factor-1 as a novel calcium oxalate crystal growth inhibitor. J Clin Invest 2005;115:3613–3622.
66 Chadwick MP, Westley BR, May FE: Homodimerization and hetero-oligomerization of the single-domain trefoil protein pNR-2/pS2 through cysteine 58. Biochem J 1997;327:117–123.
67 Polshakov VI, Williams MA, Gargaro AR, Frenkiel TA, Westley BR, Chadwick MP, May FE, Feeney J: High-resolution solution structure of human pNR-2/pS2: a single trefoil motif protein. J Mol Biol 1997;267:418–432.
68 Thongboonkerd V, Chutipongtanate S, Semangoen T, Malasit P: Urinary trefoil factor-1 is a novel potent inhibitor of calcium oxalate crystal growth and aggregation and can transform calcium oxalate monohydrate to dihydrate form. J Urol 2008;179:1615–1619.
69 Jung T, Kim WS, Choi CK: Biomineralization of calcium oxalate for controlling crystal structure and morphology. Mat Sci Eng C 2004;24:31–33.
70 Tomazic BB, Nancollas GH: A study of the phase transformation of calcium oxalate trihydrate-monohydrate. Invest Urol 1979;16:329–335.
71 Fliser D, Wittke S, Mischak H: Capillary electrophoresis coupled to mass spectrometry for clinical diagnostic purposes. Electrophoresis 2005;26:2708–2716.
72 Kolch W, Neususs C, Pelzing M, Mischak H: Capillary electrophoresis-mass spectrometry as a powerful tool in clinical diagnosis and biomarker discovery. Mass Spectrom Rev 2005;24: 959–977.
73 Weissinger EM, Hertenstein B, Mischak H, Ganser A: Online coupling of capillary electrophoresis with mass spectrometry for the identification of biomarkers for clinical diagnosis. Expert Rev Proteomics 2005;2:639–647.

74 Liu BC, Zhang L, Lv LL, Wang YL, Liu DG, Zhang XL: Application of antibody array technology in the analysis of urinary cytokine profiles in patients with chronic kidney disease. Am J Nephrol 2006;26:483–490.
75 Thongboonkerd V, Songtawee N, Sritippayawan S: Urinary proteome profiling using microfluidic technology on a chip. J Proteome Res 2007;6:2011–2018.

Visith Thongboonkerd, MD, FRCPT
Medical Proteomics Unit & Medical Molecular Biology Unit
Office for Research and Development
12th Floor, Adulyadej Vikrom Building, Siriraj Hospital
2 Prannok Road, Bangkoknoi, Bangkok 10700 (Thailand)
Tel./Fax +66 2 418 4793, E-Mail thongboonkerd@dr.com

Thongboonkerd V (ed): Proteomics in Nephrology – Towards Clinical Applications.
Contrib Nephrol. Basel, Karger, 2008, vol 160, pp 159–171

Exploring the Uremic Toxins Using Proteomic Technologies

Eric Schiffer[a], *Harald Mischak*[a,b], *Raymond C. Vanholder*[c]

[a]Mosaiques Diagnostics & Therapeutics, Hanover; [b]Hanover Medical School, Hanover, Germany, and [c]Nephrology Section, Department of Internal Medicine, University Hospital, Gent, Belgium

Abstract

Kidney failure leads to the uremic syndrome that is the clinical expression of the malfunction of vital organs due to the accumulation of uremic toxins, which are normally cleared by the kidneys. Progressively more uremic retention solutes have been identified and their potential toxicity has been characterized. Polypeptides constitute a heterogeneous group of uremic molecules. Therefore, proteome analysis represents a new and promising analytical approach to identify new uremic toxins. Proteomic technologies cover applicability to a broad molecular mass range. For polypeptides >10 kDa classical proteomic techniques, such as two-dimensional gel electrophoresis followed by mass spectrometry, are able to identify uremic polypeptides. In the mass range from approximately 1 to 10 kDa, capillary electrophoresis coupled to mass spectrometry (CE-MS) emerged as a fast possibility to analyze of up to 1,400 compounds in a single step. This chapter will provide an overview about proteomic technologies as efficient tools for the detection of uremic toxins, emphasizing the features of CE-MS. Subsequently, examples of the application of proteomic techniques to define novel biomarkers for renal diseases and uremic toxins will be discussed.

Kidney failure results in the functional failure of vital organs, attributable to the accumulation of compounds that under normal conditions are excreted into the urine by healthy kidneys [1]. 'Uremic toxins' are defined as 'uremic retention solutes' that modify biological and/or biochemical functions and contribute to the uremic syndrome [1]. According to classical definitions of toxicity, a uremic toxin has to be a chemical or biological agent capable of producing a response that is deleterious to the biological system [2].

Since the 1980s, progressively more uremic retention solutes have been identified and characterized with respect to their potential toxicity [1, 3]. The European Uremic Toxin Work Group (EUTox) reported an encyclopedic list containing 90

uremic compounds that had been described at that moment [3]. However, conceivably the information presented was incomplete as compared to the number of compounds that are retained in reality. Uremic toxins can be subdivided into three major groups: (1) small solutes (<500 Da) with unknown protein binding; (2) solutes with known or likely protein binding, and (3) middle molecules (>500 Da).

Polypeptides constitute a heterogeneous group of uremic retention molecules that, following this subdivision, correspond to the characteristics of 'middle molecules', which arbitrarily are defined as molecules with a molecular weight in excess of 500 Da. Under physiological conditions, glomerular filtration and luminal re-absorption in the tubular system, with the former as the rate-limiting step, are the sequential renal handling processes of polypeptides. Degradation of the reabsorbed polypeptide takes place within the tubular cell, followed by anti-luminal re-absorption by peritubular capillaries. These elimination pathways are hampered once the function of the kidney begins to fail. Therefore, many polypeptides show an increased serum concentration in uremia [3].

For a long time, the search for polypeptide uremic toxins has been biased by the preferential analysis of known solutes that might be of pathophysiological relevance. Proteome analysis represents a completely different approach whereby all present peptides can be registered and potentially identified, offering the possibility to come to the unbiased identification of markers or solutes [4, 5].

The following section will provide an overview about proteomic technologies as efficient tools for the detection of biomarkers. Afterwards, examples of the application of proteomic techniques for the definition of novel biomarkers for renal disease and uremic toxins will be discussed in detail.

Proteome Analysis

During the last several years, the number of reports on the application of proteomics to analyze complex biological fluids has been rapidly growing [6]. Particularly high sensitivity, speed, and reproducibility of mass spectrometry (MS) have boosted its application in all aspects of body fluid profiling, including detection, definition and structural characterization (i.e., peptide mapping, peptide sequencing).

The current state of the art does not allow analyzing the proteome of a complex biological sample by MS without preceding separation. At the moment, there is no indication that obviating the latter step may be possible in the near future. Therefore, high-resolution pre-MS separation is a prerequisite for proteomic profiling. In the experiments reported by O'Farrell [7] in 1975, two-dimensional gel electrophoresis (2-DE) may well be considered the basis for today's proteomics. Separation of polypeptides according to their isoelectric point (pI) and molecular

mass (Da) provided a basis for high-resolution fractionation of complex protein mixtures. Years later, the implementation of MS (fig. 1) led to a step-by-step identification of hundreds of proteins based on a proteolytic in-gel digest, gel extraction and MS analysis of the resultant peptide fragments [8–10].

However, the method of peptide mass fingerprinting is rather time-consuming and remains technically challenging, since comparability and reproducibility are difficult to achieve. Additionally, up until today the approach lacks automation [9–12]. Systematic analysis of polypeptides <10 kDa by 2-DE-MS is difficult or even impossible. Despite all of these problems, 2-DE, especially in combination with difference gel electrophoresis-labeling procedures [13], still appears to be the method of choice for comparative analysis of large proteins [6, 14].

At first glance, surface-enhanced laser desorption/ionization (SELDI), appeared to represent a solution to several limitations of 2-DE-MS. Consequently, a number of clinically relevant proteome investigations have used the approach of reducing the complexity of samples by fractionation using selective interactions of polypeptides with different immobilized matrices [15–22]. These 'active' surfaces consist of reversed-phase materials, ion-exchange materials, ligands, receptors, antibodies, or DNA, to name just a few. Theoretically, a specific small fraction of all polypeptides in a given sample binds to the surface of the SELDI chip, facilitating mass spectrometric analysis and characterization of potential biomarkers from highly complex samples. At least in part due to its benefit of an easy-to-use system, numerous reports on biomarkers for a variety of diseases merged [23–26]. Unfortunately, the reproducibility and hence reliability of the results were subsequently heavily debated [27–30]. A drawback of this approach seems to be the loss of the majority of information contained in a biological sample, consequently limiting the significance of the data. Additionally, comparability of obtained datasets is hard to achieve due to the usage of different chip surfaces, varying binding/washing conditions, low capacities of surfaces, and the very low resolution of the used mass spectrometers. At least limited binding capacity and low spectral resolution can be solved by the use of magnetic beads instead of surfaces, and of course more suitable mass spectrometer instruments [31, 32].

If sensitivity is a consideration, liquid chromatography (LC) may be an excellent choice. This powerful approach represents a high-resolution separation method with very high capacity for analytes (up to a preparative gram scale) in as far as they can be loaded onto an LC column [9, 11, 33–35]. The high resolution can be multiplied using LC in multidimensional (MudPIT) experiments [36, 37]. MudPIT approaches generate vast amounts of information, but are very time-consuming in terms of data generation and evaluation. In addition, sensitivity towards interfering compounds and precipitation of analytes on LC-column materials complicates LC separation of highly complex mixtures of analytes covering broad ranges of hydrophobicity [9, 11].

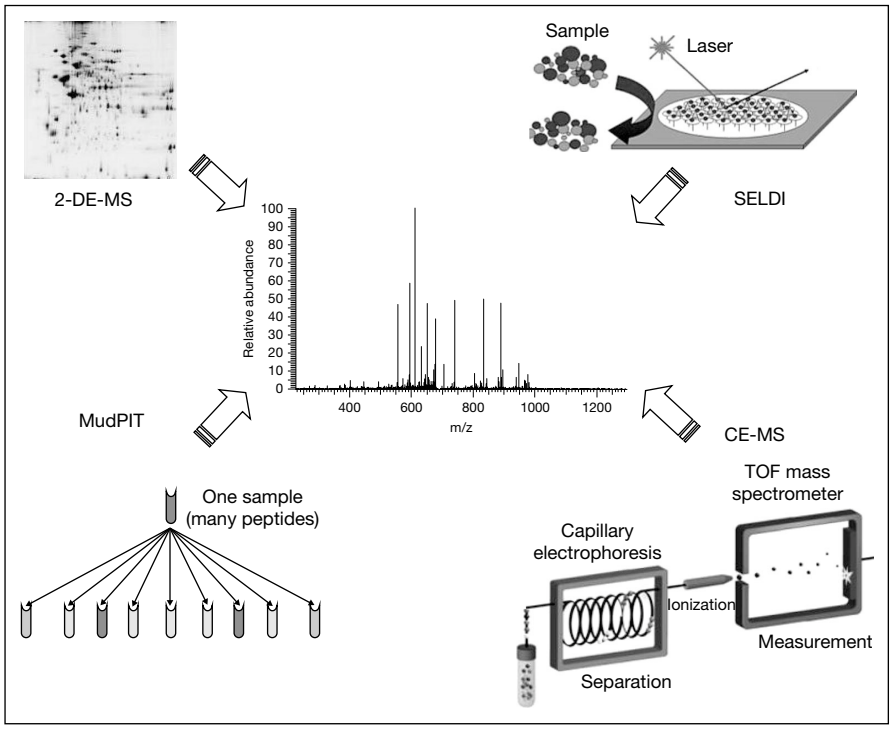


Fig. 1. Schematic representation of proteomic technologies. High-resolution pre-MS separation is a prerequisite for proteomic profiling. Two-dimensional gel electrophoresis (2-DE) separates polypeptides according to their isoelectric point (pI) and molecular mass (Da). The implementation of mass spectrometry (MS) led to a step-by-step identification of proteins by peptide mass fingerprinting (upper left panel). SELDI uses the approach of selective binding of polypeptides to different 'active' surfaces facilitating mass spectrometric analysis and characterization of potential biomarkers from complex samples (upper right panel). If sensitivity is a consideration, liquid chromatography (LC) offers a high-resolution separation method that can be multiplied using LC in multidimensional (MudPIT) experiments (lower left panel). Capillary electrophoresis coupled to mass spectrometry (CE-MS) provides fast separation and high resolution. It is quite robust and uses inexpensive capillaries and is compatible with most buffers and analytes (lower right panel). After pre-separation the mass spectrometric profiling characterizes biomarker candidates by its molecular mass (and its polypeptide sequence).

Alternatively, capillary electrophoresis coupled to mass spectrometry (CE-MS) [38–42] offers several advantages (for a technical introduction to CE-MS, see chapter 'CE-MS for Biomarker Discovery and Diagnosis of Kidney Diseases'): CE-MS provides fast separation and high resolution [43]. It is quite robust and uses inexpensive capillaries [44] and is compatible with most buffers and analytes [45]. A stable constant flow avoids need for buffer gradients resulting and the

Table 1. Comparison of 2-DE-MS, SELDI, LC-MS and CE-MS methods

Technology	Advantages	Limitations
2-DE-MS	Applicable to large molecules, high resolution	Not applicable to peptides <10 kDa, no automation, time-consuming, quantification difficult, expensive
SELDI	Easy-to-use system, high throughput, automation, low sample volume required, TOF/TOF sequencing possible	Restricted to selected polypeptides, low-resolution MS, interpretation of data difficult without sequence information
LC-MS	Automation, multidimensional, high sensitivity, MS/MS possibility	Time-consuming, sensitive towards interfering compounds, restricted mass range
CE-MS	Automation, high sensitivity, fast, low sample volumes required, multidimensional, low cost, MS/MS possibility	Not well suited for larger polypeptides (>20 kDa)

subsequent continuous changes in electrospray ionization (ESI) conditions as observed for LC-ESI-MS [46]. As a consequence, CE certainly appears to be an excellent choice for the separation of complex biological samples. Unfortunately, CE-MS is not widely used today, probably due to the initial problems of interfacing CE with MS as well as the rather high amount of sample required for analysis in the first mass spectrometers. The advantages and limitations of the proteomic technologies (2-DE-MS, SELDI, LC-MS and CE-MS) are summarized in table 1.

Proteomics can be a powerful tool for the fast and reliable analysis of polypeptides from several types of highly complex biological samples, such as urine, blood or cerebrospinal fluid. Information on several hundred polypeptides from an individual sample can be obtained quickly. Although these polypeptides can serve as excellent biomarkers for diagnostic purposes, their potential physiological role remains unknown as long as their identity defined by their amino acid sequence is not determined. The identification of the defined biomarkers presents some unique challenges. The biomarkers cannot easily be isolated; the sequence analysis has to be performed from a complex mixture and potential biomarkers are frequently prototypically processed and/or posttranslationally modified. Potential biomarkers detected by CE-MS are likely to be small fragments of larger proteins.

Thus, to identify a 2- to 10-kDa (modified) portion of a protein with a possible molecular weight >60 kDa requires extensive top-down peptide sequence analysis. Such an approach is more demanding than e.g. MudPIT approaches on tryptic digests (bottom-up approaches), where the ion mass of the peptide after tryptic digestion already serves as one good parameter for identification, since the bottom-up approaches are able to provide theoretical parent protein masses in body fluids [47]. Unfortunately, modifications that are generally

observed (e.g. oxidation, proteolytic processing, or glycosylation) prevent the direct correlation of such data to defined biomarkers.

For top-down polypeptide sequencing, CE or LC can be interfaced either offline or online with tandem MS (MS/MS) instruments. Neususs et al. [46] describe a capillary electrophoresis-tandem MS (CE-MS/MS) approach for routine application in proteomic studies. Stable coupling is achieved by using a standard coaxial sheath-flow sprayer. Low femtomole amounts are required for unequivocal identification by MS/MS experiments in the used ion trap and subsequent database search.

Alternatively, the entire separation run (CE or LC) can be spotted offline onto a MALDI (matrix-assisted laser desorption/ionization) target plate and subsequently the polypeptides of interest can be analyzed using MALDI time-of-flight tandem MS (MALDI-TOF/TOF) [44, 48]. This method has the advantage that the signal of interest can be located in MS mode and optimal fragmentation conditions can be determined without repeated separation. Several biomarker candidates can be identified following this strategy, as shown for graft-versus-host disease [49], diabetic nephropathy [50], or bladder cancer [51]. However, sequencing with MALDI-TOF/TOF generally does not result in data of sufficient quality for urinary peptides with molecular masses above approximately 3,000 Da [52]. The enormous spectral resolution ($<$1 ppm) of Fourier transform ion cyclotron resonance MS instruments tends to shift these limitations and enables top-down peptide sequencing of urinary polypeptides even larger than 8 kDa [52].

A comparison of the different CE-MS/MS options was recently reported by Zurbig et al. [53]. Compared to other high-performance separation methods coupled either online or offline to MS devices, CE-MS provides a unique advantage: at pH 2 the number of basic amino acids directly determines polypeptide migration time. This unique property facilitates the independent entry of different platforms for peptide sequencing of CE-MS-defined biomarkers from highly complex mixtures. In consequence, proteomic biomarker definition may be the basis for the generation of a urinary proteome map. In a form of a digital database, biomarker-defining parameters, statistics of the biological background of a biomarker, biomarker sequence information, and obtained MS/MS raw data can be unified.

Application of Proteomic Techniques to Uremic Toxicity

The previous sections described the need for new analytical methods in the identification of middle uremic toxins. Different proteomic methods were introduced with special respect to polypeptide analysis and their technical aspects. The following section will provide an overview about the application of different proteomic methods in the field of uremia.

Hemodialysis liquids are suitable fluids for proteomic analysis, since they have low contents of albumin and other interfering large proteins, e.g. transferrin or immunoglobulins. Compared to plasma or serum, the concentration of albumin in hemofiltrate (HF) is 1,500-fold reduced, while the concentration of the polypeptides in the range of 1–30 kDa remains nearly unaffected [54]. A first attempt towards the analysis of middle molecules for HF from patients with chronic uremia was published by Brunner et al. [55] in 1978. At that time, the absence of suitable high-throughput techniques for polypeptide identification restricted the methods for single protein identification to Western blotting, ELISA, or RIA, one at a time.

To overcome these limitations, Forssmann et al. [54, 56] suggested an LC-MS-based approach to identify polypeptides from HF. This work resulted in a 'peptide bank' containing up to 300 different chromatographic fractions generated from about 10,000 liters of human HF. Starting from this bank, bioactive peptides were isolated and identified. In the first proteomic approaches, LC-MS-guided purification of the human peptide hormone guanylin from HF was performed [57]. Several additional peptides with various biochemical functions could be isolated, e.g. endostatin and resistin as angiogenesis inhibitors or a propiomelanocortin-derived peptide with lipolytic activity [58, 59].

Further improvements of the LC-MS approach were introduced by Wagner et al. [60]. They applied an automated two-dimensional HPLC system with integrated sample preparation and MALDI-TOF-MS detection for the analysis of polypeptides $<$20 kDa.

A proteomic approach based on 2-DE and MALDI-TOF-MS to identify uremic toxins from ultrafiltrate (UF) was presented by Ward and Brinkley [61] in 2004. In brief, the sample was prepared by a 50-fold concentration step using a 1-kDa cut-off membrane, followed by dialysis for desalting. After 2-DE, protein spots of interest were excised, trypsinized and identified by peptide mass fingerprinting. Following this approach, 21 spots from the proteome map were identified, which represented six different proteins with several different post-translational modifications resulting in multiple spots of the same protein. The identified proteins were β_2-microglobulin, one of the major uremic toxins [62], as well as α_1-antitrypsin, albumin (mature and complexed), complement factor D, cystatin C and retinol-binding protein.

Lefler et al. [63] presented a combination of reversed-phase (RP) chromatography, 2-DE and MALDI-TOF/TOF MS/MS for the identification of proteins in UF. Therefore, UF was loaded onto an RP C-4 column followed by stepwise elution with 10% (fraction 1), 25% (fraction 2) and 50% acetonitrile in water (fraction 3). After lyophilization, each fraction was applied to 2-DE and protein spots were identified by peptide mass fingerprinting. To verify these database matches, additional MS/MS experiments were performed using a MALDI-TOF/TOF instrument. 47 protein spots representing 10 different proteins were identified. Albumin

was identified in 9 spots and transferrin in 8 spots. β_2-Microglobulin was also identified. The addition of RP chromatography as a third dimension of separation (compared to the approach of Ward and Brinkley) evidently resulted in a higher number of visible protein spots very likely due to the reduction matrix effects and signal suppression.

Molina et al. [64] performed a proteome analysis of human hemodialysis fluid applying SDS-PAGE in combination with LC-MS/MS. After desalting of the hemodialysis fluid, samples were concentrated using a 3 kDa cut-off filter. Subsequently, the sample was separated by SDS-PAGE, silver stained and the bands excised, in-gel digested with trypsin and analyzed by LC-MS/MS. Nanoflow RP C-18 chromatography coupled via ESI sources to either a quadrupole time-of-flight MS or an ion trap MS was used. Applying this approach, 292 different proteins from hemodialysis fluid were identified; 205 of them had not previously been identified in serum or plasma. Additional Western blot analysis of a subset of these proteins revealed their presence in normal serum. The detection limit might be the major reason why the majority of these proteins had previously not been identified. The authors concluded that this might be due to the lower dynamic range of protein concentration in HF compared to serum/plasma samples and the enrichment of the lower-molecular-weight proteins.

A further outcome of this proteomic analysis was that, similar to the results reported by Ward and Brinkley [61], proteins in hemodialysis fluid often harbor posttranslational modifications, which makes identification by MS/MS difficult when searching in databases with standard settings. Most posttranslational modifications reported by Molina et al. [64] were oxidization at methionine or tryptophan residues, pyroglutamine formation, N-terminal acetylation, N-glycosylation of peptides and proline hydroxylation. The question that arises from these findings is: Do even minor modifications change the properties of these proteins in a way that their renal metabolic processing or their biological impact is influenced? Hence, should all these differently modified polypeptides be put together under one single denominator or are they representing different uremic entities?

In conclusion, all presented bottom-up proteomic techniques showed differences of protein expression within a mass range of >10 kDa, and many of these proteins could be identified by their sequence. The results of these proteomic studies are of great relevance in the evaluation of uremic toxins. Unfortunately, all these techniques lack the identification of uremic retention molecules in the lower molecular range from 1 kDa up to 10 kDa, due to methodological restriction to the analysis of proteins with higher molecular masses (>10 kDa). Two- or multidimensional approaches are time-intensive and hence suited for the analysis of a very limited number of different samples.

CE-MS is an alternative, which allows the analysis of hundreds of samples in a timely fashion. Recently, this approach was used to investigate the effect of

different dialysis membranes (low-flux vs. high-flux) on the number of polypeptides in dialysate [65]. For sample preparation anion-exchange chromatography with DEAE-Sepharose to remove interfering salts and matrix followed by lyophilization was used. Due to the relative insensitivity of CE towards salts, this fast and simple procedure has resulted in a reproducible setup. CE-MS analysis was performed on a Beckman P/ACE MDQ CE coupled via a CE-ESI-MS sprayer kit from Agilent to an Applied Biosystems Mariner ESI-TOF-MS. The sample was injected hydrodynamically on an untreated silica capillary (inner diameter 75 μm, length 90 cm). More than 600 polypeptides could be analyzed in a single sample. Larger polypeptides (>10 kDa) were only present in the dialysates from high-flux membranes, while in dialysates from low-flux membranes the majority of polypeptides was smaller than 10 kDa (fig. 2). In a further study the potential of CE-MS followed by CE-MS/MS to identify uremic retention molecules in dialysis fluids from low- and high-flux membranes was examined [4]. To obtain further insight into the uremic toxins within a mass range of 800 up to 15,000 Da, the same CE-MS setup was used. Sample preparation was modified by using a RP C-18 column for desalting. Subsequent analysis detected 1,394 different polypeptides in samples from high-flux membrane dialysis, whereas 1,046 polypeptides were recovered in the dialysate of the same patient obtained with the low-flux membrane. For sequencing, a complete CE run was spotted onto a MALDI target plate (one spot every 15 s.) and examined subsequently in MS mode on a MALDI-TOF/TOF instrument. Polypeptides of interest were fragmented in MS/MS mode and their sequence identified with MASCOT search against the SwissProt database. With the exemplary identification of fragments of a 950.6-Da polypeptide as a fragment of salivary praline-rich protein, and a second 1,292.8-Da peptide was identified as a fragment of α-fibrinogen. It should be stressed that these two peptides were chosen randomly to demonstrate the possibilities of identification following the isolation of compounds by a proteomic approach. These findings demonstrate the potential of the CE-MS application for proteomics and the identification of yet unknown uremic retention molecules.

Summary and Outlook

In contrast to the classical search for polypeptide uremic toxins by the preferential analysis of known solutes that might be of pathophysiological importance, proteome analysis has proven to be a powerful analytic tool for the identification of previously unknown uremic middle molecules. Detected peptides can be registered and identified, offering the possibility to come to the unbiased characterization of uremic polypeptides. Proteomic technologies cover applicability to a broad molecular mass range. For polypeptide proteins, >10 kDa

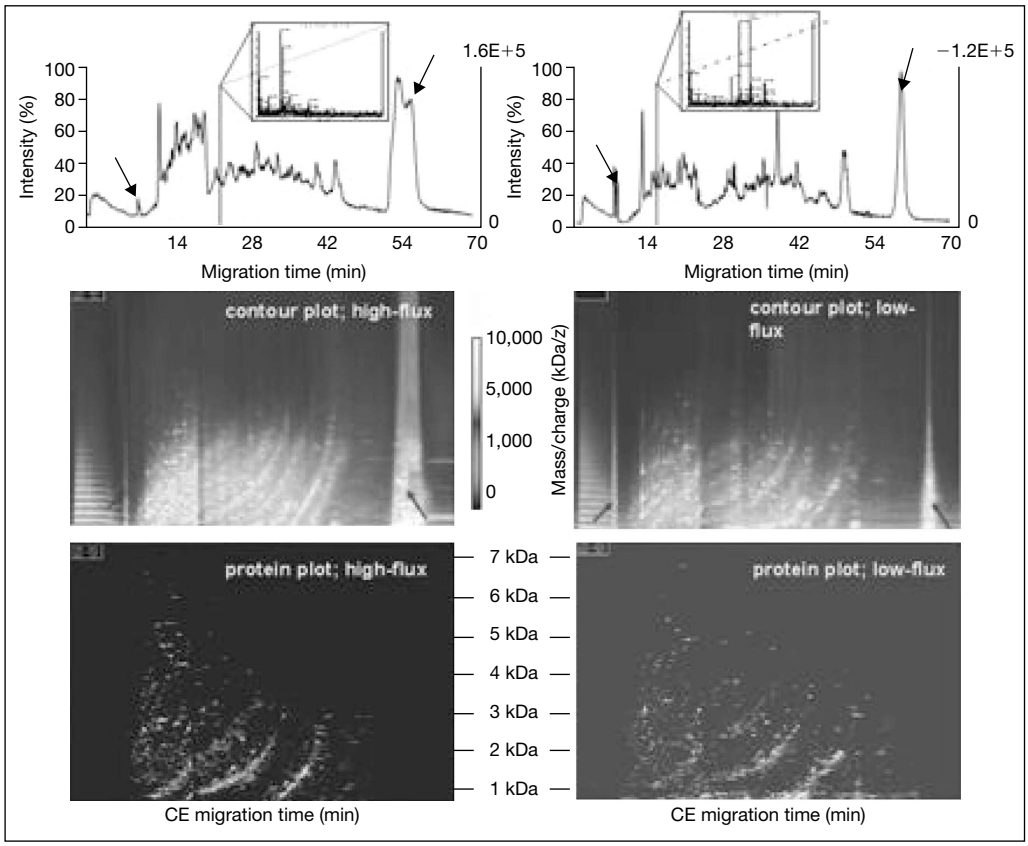


Fig. 2. Comparison of proteomic analysis of ultrafiltrate obtained from high-flux (left) or low-flux (right) polysulfone membranes as published by Weissinger et al. [4]. The total ion chromatogram (upper graphs) is obtained after CE-MS analysis. The insert shows individual spectra, obtained every 3 s, yielding the total ion chromatogram. These data are converted by application of MosaiquesVisu software to a three-dimensional contour plot (middle graph). Mass per charge is shown on the y-axis against the migration time in min on the x-axis, and the signal intensity is color coded (black to white; 0 to 10,000 MS counts). The start of the spectrum is marked by the appearance of highly charged formiates, while the end of the spectrum is marked by the appearance of organic polymers. The position of these are indicated by arrows in the raw data plots (middle panel) and in the total ion chromatogram (upper graphs). Next, the signal to noise is calculated and the noise removed, thus leaving only signals and the actual mass is calculated. The resulting individual peak list contains more than 1,000 different molecules defined by their mass and migration time in the CE (bottom graph). Both the number of individual compounds and the intensity of the signal (corresponding to concentration) are higher for the high-flux membrane.

2-DE-based proteomic techniques are able to identify dozens of new uremic polypeptides. In the mass range from approximately 1 to 10 kDa, especially CE-MS-based technology allows the fast analysis of up to 1,400 compounds in a single analysis step. Currently, proteomic approaches merely intimate its enormous potential to revolutionize uremic toxin definition and identification. However, that poses the question, how to link the obtained uremic toxins to pathophysiological states. While classically uremic toxin identification was performed from bottom-up to bottom-down, from the physiological observation to the identified molecule, proteomics may reverse this process. This may promise new insights into the context of uremia and in our understanding of pathological states.

References

1 Vanholder R, De Smet R: Pathophysiologic effects of uremic retention solutes. J Am Soc Nephrol 1999;10:1815–1823.
2 Vanholder R, Argiles A, Baurmeister U, Brunet P, et al: Uremic toxicity: present state of the art. Int J Artif Organs 2001;24:695–725.
3 Vanholder R, De Smet R, Glorieux G, Argiles, et al: Review on uremic toxins: classification, concentration, and interindividual variability. Kidney Int 2003;63:1934–1943.
4 Weissinger EM, Kaiser T, Meert N, De Smet R, et al: Proteomics: a novel tool to unravel the pathophysiology of uraemia. Nephrol Dial Transplant 2004;19:3068–3077.
5 Weissinger EM, Nguyen-Khoa T, Fumeron C, Saltiel C, et al: Effects of oral vitamin C supplementation in hemodialysis patients: a proteomic assessment. Proteomics 2006;6:993–1000.
6 Thongboonkerd V: Proteomics in nephrology: current status and future directions. Am J Nephrol 2004;24:360–378.
7 O'Farrell PH: High-resolution two-dimensional electrophoresis of proteins. J Biol Chem 1975;250: 4007–4021.
8 Patterson SD, Aebersold R: Mass spectrometric approaches for the identification of gel-separated proteins. Electrophoresis 1995;16:1791–1814.
9 Aebersold R, Goodlett DR: Mass spectrometry in proteomics. Chem Rev 2001;101:269–295.
10 Yanagida M: Functional proteomics; current achievements. J Chromatogr B Analyt Technol Biomed Life Sci 2002;771:89–106.
11 Issaq HJ: The role of separation science in proteomics research. Electrophoresis 2001;22: 3629–3638.
12 Morrison RS, Kinoshita Y, Johnson MD, Uo T, et al: Proteomic analysis in the neurosciences. Mol Cell Proteomics 2002;1:553–560.
13 Patton WF: Detection technologies in proteome analysis. J Chromatogr B Analyt Technol Biomed Life Sci 2002;771:3–31.
14 Thongboonkerd V, Malasit P: Renal and urinary proteomics: current applications and challenges. Proteomics 2005;5:1033–1042.
15 Shiwa M, Nishimura Y, Wakatabe R, Fukawa A, et al: Rapid discovery and identification of a tissue-specific tumor biomarker from 39 human cancer cell lines using the SELDI ProteinChip platform. Biochem Biophys Res Commun 2003;309:18–25.
16 Issaq HJ, Veenstra TD, Conrads TP, Felschow D: The SELDI-TOF MS approach to proteomics: protein profiling and biomarker identification. Biochem Biophys Res Commun 2002;292: 587–592.
17 Forde CE, Gonzales AD, Smessaert JM, Murphy GA, et al: A rapid method to capture and screen for transcription factors by SELDI mass spectrometry. Biochem Biophys Res Commun 2002;290: 1328–1335.
18 Yip TT, Lomas L: SELDI ProteinChip array in oncoproteomic research. Technol Cancer Res Treat 2002;1:273–280.

19 Merchant M, Weinberger SR: Recent advancements in surface-enhanced laser desorption/ionization-time of flight-mass spectrometry. Electrophoresis 2000;21:1164–1177.
20 Von Eggeling F, Junker K, Fiedle W, Wollscheid V, et al: Mass spectrometry meets chip technology: a new proteomic tool in cancer research? Electrophoresis 2001;22:2898–2902.
21 Issaq HJ, Veenstra TD, Conrads TP, Felschow D: The SELDI-TOF MS approach to proteomics: protein profiling and biomarker identification. Biochem Biophys Res Commun 2002;292:587–592.
22 Weinberger SR, Viner RI, Ho P: Tagless extraction-retentate chromatography: a new global protein digestion strategy for monitoring differential protein expression. Electrophoresis 2002;23: 3182–3192.
23 Tang N, Tornatore P, Weinberger SR: Current developments in SELDI affinity technology. Mass Spectrom Rev 2004;23:34–44.
24 Petricoin EF, Ardekani AM, Hitt BA, Levine PJ, et al: Use of proteomic patterns in serum to identify ovarian cancer. Lancet 2002;359:572–577.
25 Rosenblatt KP, Bryant-Greenwood P, Killian JK, Mehta A, et al: Serum proteomics in cancer diagnosis and management. Annu Rev Med 2004;55:97–112.
26 Schaub S, Wilkins J, Weiler T, Sangster K, et al: Urine protein profiling with surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry. Kidney Int 2004;65:323–332.
27 Kolch W, Mischak H, Chalmers MJ, Pitt A, Marshall AG: Clinical proteomics: a question of technology. Rapid Commun Mass Spectrom 2004;18:2365.
28 Baggerly KA, Morris JS, Edmonson SR, Coombes KR: Signal in noise: evaluating reported reproducibility of serum proteomic tests for ovarian cancer. J Natl Cancer Inst 2005;97:307–309.
29 Baggerly KA, Morris JS, Coombes KR: Reproducibility of SELDI-TOF protein patterns in serum: comparing datasets from different experiments Bioinformatics 2002;20:777–785.
30 Check E: Running before we can walk. Nature 2004;429:496–497.
31 Cheng AJ, Chen LC, Chien KY, Chen YJ, et al: Oral cancer plasma tumor marker identified with bead-based affinity-fractionated proteomic technology. Clin Chem 2005;51;2236–2244.
32 Tolson JP, Flad T, Gnau V, Dihazi H, et al: Differential detection of S100A8 in transitional cell carcinoma of the bladder by pairwise tissue proteomic and immunohistochemical analysis. Proteomics 2006;6:697–708.
33 Aebersold Rl, Mann M: Mass spectrometry-based proteomics. Nature 2003;422:198–207.
34 Wittke S, Kaiser T, Mischak H: Differential polypeptide display: the search for the elusive target. J Chromatogr B Analyt Technol Biomed Life Sci 2004;803:17–26.
35 Issaq HJ, Conrads TP, Janini GM, Veenstra TD: Methods for fractionation, separation and profiling of proteins and peptides. Electrophoresis 2002;23:3048–3061.
36 Chen EI, Hewel J, Felding-Habermann B, Yates JR III: Large-scale protein profiling by combination of protein fractionation and multidimensional protein identification technology (MudPIT). Mol Cell Proteomics 2006;5:53–56.
37 Cagney G, Park S, Chung C, Tong B, et al: Human tissue profiling with multidimensional protein identification technology. J Proteome Res 2005;4:1757–1767.
38 Dolnik V, Hutterer KM: Capillary electrophoresis of proteins 1999–2001. Electrophoresis 2001; 22: 4163–4178.
39 Schmitt-Kopplin P, Frommberger M: Capillary electrophoresis-mass spectrometry: 15 years of developments and applications. Electrophoresis 2003;24:3837–3867.
40 Kasicka V: Recent developments in capillary electrophoresis and capillary electrochromatography of peptides. Electrophoresis 2006;27:142–175.
41 Simpson DC, Smith RD: Combining capillary electrophoresis with mass spectrometry for applications in proteomics. Electrophoresis 2005;26:1291–1305.
42 Dolnik V: Capillary electrophoresis of proteins 2003–2005. Electrophoresis 2006;27:126–141.
43 Johannesson N, Wetterhall M, Markides KE, Bergquist J: Monomer surface modifications for rapid peptide analysis by capillary electrophoresis and capillary electrochromatography coupled to electrospray ionization-mass spectrometry. Electrophoresis 2004;25:809–816.
44 Kolch W, Neususs C, Pelzing M, Mischak H: Capillary electrophoresis-mass spectrometry as a powerful tool in clinical diagnosis and biomarker discovery. Mass Spectrom Rev 2005;24:959–977.
45 Hernandez-Borges J, Neususs C, Cifuentes A, Pelzing M: On-line capillary electrophoresis-mass spectrometry for the analysis of biomolecules. Electrophoresis 2004;25:2257–2281.

46 Neususs C, Pelzing M, Macht M: A robust approach for the analysis of peptides in the low femtomole range by capillary electrophoresis-tandem mass spectrometry. Electrophoresis 2002;23:3149–3159.
47 Adachi J, Kumar C, Zhang Y, Olsen JV, Mann M: The human urinary proteome contains more than 1,500 proteins including a large proportion of membranes proteins. Genome Biol 2006;7:R80.
48 Rejtar T, Hu P, Juhasz P, Campbell JM, et al: Off-line coupling of high-resolution capillary electrophoresis to MALDI-TOF and TOF/TOF MS. J Proteome Res 2002;1:171–179.
49 Kaiser T, Kamal H, Rank A, Kolb HJ, et al: Proteomics applied to the clinical follow-up of patients after allogeneic hematopoietic stem cell transplantation. Blood 2004;104:340–349.
50 Mischak H, Kaiser T, Walden M, Hillmann M, et al: Proteomic analysis for the assessment of diabetic renal damage in humans. Clin Sci (Lond) 2004;107:485–495.
51 Theodorescu D, Wittke S, Ross MM, Walden M, et al: Discovery and validation of new protein biomarkers for urothelial cancer: a prospective analysis. Lancet Oncol 2006;7:230–240.
52 Chalmers MJ, Mackay CL, Hendrickson CL, Wittke S, et al: Combined top-down and bottom-up mass spectrometric approach to characterization of biomarkers for renal disease. Anal Chem 2005; 77:7163–7171.
53 Zurbig P, Renfrow MB, Schiffer E, Novak J, et al: Biomarker discovery by CE-MS enables sequence analysis via MS/MS with platform-independent separation. Electrophoresis 2006;27:2111–2125.
54 Schepky AG, Bensch KW, Schulz-Knappe P, Forssmann WG: Human hemofiltrate as a source of circulating bioactive peptides: determination of amino acids, peptides and proteins. Biomed Chromatogr 1994;8:90–94.
55 Brunner H, Mann H, Essers U, Schultheis R, et al: Preparative isolation of middle molecular weight fractions from the hemofiltrate of patients with chronic uremia. Artif Organs 1978;2:375–377.
56 Schulz-Knappe P, Schrader M, Standker L, Richter R, et al: Peptide bank generated by large-scale preparation of circulating human peptides. J Chromatogr A 1997;776;125–132.
57 Schrader M, Jurgens M, Hess R, Schulz-Knappe P, et al: Matrix-assisted laser desorption/ionisation mass spectrometry guided purification of human guanylin from blood ultrafiltrate. J Chromatogr A 1997;776:139–145.
58 John H, Radtke K, Standker L, Forssmann WG: Identification and characterization of novel endogenous proteolytic forms of the human angiogenesis inhibitors restin and endostatin. Biochim Biophys Acta 2005;1747:161–170.
59 Fricke K, Schulz A, John H, Forssmann WG, Maronde E: Isolation and characterization of a novel proopiomelanocortin-derived peptide from hemofiltrate of chronic renal failure patients. Endocrinology 2005;146:2060–2068.
60 Wagner K, Miliotis T, Marko-Varga G, Bischoff R, Unger KK: An automated on-line multidimensional HPLC system for protein and peptide mapping with integrated sample preparation. Anal Chem 2002;74:809–820.
61 Ward RA, Brinkley KA: A proteomic analysis of proteins removed by ultrafiltration during extracorporeal renal replacement therapy; in Thongboonkerd V, Klein JB (eds): Proteomics in Nephrology. Contrib Nephrol. Basel, Karger, 2004, vol 141, pp 280–291.
62 Gejyo F, Yamada T, Odani S, Nakagawa Y, et al: A new form of amyloid protein associated with chronic hemodialysis was identified as β_2-microglobulin. Biochem Biophys Res Commun 1985; 129: 701–706.
63 Lefler DM, Pafford RG, Black NA, Raymond JR, Arthur JM: Identification of proteins in slow continuous ultrafiltrate by reversed-phase chromatography and proteomics. J Proteome Res 2004; 3:1254–1260.
64 Molina H, Bunkenborg J, Reddy GH, Muthusamy B, Scheel PJ, Pandey A: A proteomic analysis of human hemodialysis fluid. Mol Cell Proteomics Mol Cell Proteomics 2005;4:637–650.
65 Kaiser T, Hermann A, Kielstein JT, Wittke S, et al: Capillary electrophoresis coupled to mass spectrometry to establish polypeptide patterns in dialysis fluids. J Chromatogr A 2003;1013:157–171.

Eric Schiffer
Mosaiques Diagnostics & Therapeutics AG
Mellendorfer Strasse 7–9, D-30625 Hanover (Germany)
Tel. +49 511 5547 4417, Fax +49 511 5547 4431, E-Mail schiffer@mosaiques-diagnostics.com

Thongboonkerd V (ed): Proteomics in Nephrology – Towards Clinical Applications.
Contrib Nephrol. Basel, Karger, 2008, vol 160, pp 172–185

Proteomic Approaches for the Study of Cell Signaling in the Renal Collecting Duct

Ewout J. Hoorn, Trairak Pisitkun, Ming-Jiun Yu, Mark A. Knepper

Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Md., USA

Abstract

In the current era of large-scale biology, proteomics has evolved as a powerful, new technique that aims to identify, quantify, and analyze a large number of proteins in a functional context. Therefore, proteomics can be used to study cellular pathways and identify disease biomarkers. In this review, we first outline the principles of two important proteomics techniques that either use difference gel electrophoresis (DIGE) or liquid chromatography (LC) for protein separation, followed by tandem mass spectrometry (MS/MS). The advantages and limitations of each technique are discussed, emphasizing the ability of DIGE to perform quantitative proteomics and the high-throughput and high-sensitivity characteristics of LC-MS/MS. We have employed both techniques to unravel the molecular machinery of vasopressin signaling, which governs water homeostasis by recruiting aquaporin-2 (AQP2) water channels after activation of the vasopressin-2 receptor by vasopressin. Several aspects of vasopressin signaling in the inner medullary collecting duct (IMCD) were investigated, including the short- and long-term regulation of AQP2, phosphoproteomics, signaling during vasopressin escape, and the proteomes of AQP2-bearing vesicles and the IMCD plasma membranes. We also emphasize that proteomics of body fluids will be the strategy to identify disease biomarkers, and therefore conclude the review by highlighting the perspectives of biomarker discovery in urinary exosomes.

Introduction

Proteomics aims to identify, quantify, and analyze a large number of proteins in a functional context [1]. Therefore, proteomics can be used to study cellular pathways and identify disease biomarkers. Proteomics focuses on generating new hypotheses instead of testing existing hypotheses. This stimulates the discovery of new proteins or the discovery of new roles for known

proteins, such as their involvement in certain signaling cascades, interactions with other proteins, or associations with human disease. Unavoidably, large-scale protein analysis also bears the risk of identifying false positives or proteins that turn out to be innocent bystanders or housekeepers, addressing the need for rigorous post-hoc testing. Technically speaking, the birth of proteomics resulted from the coupling of protein separation techniques with mass spectrometry. Two mass spectrometry techniques, MALDI (matrix-assisted laser desorption/ionization) and electrospray ionization (ESI), were acknowledged by the award of the Nobel Prizes in chemistry to Tanaka and Fenn, respectively. Currently, many different types of proteomics platforms can be distinguished based on the protein separation technique that is being used. Protein separation can be achieved by gel-based approaches using one- or two-dimensional electrophoresis (1-DE or 2-DE), by high-performance liquid chromatography (HPLC or LC), or by affinity media such as surface enhanced laser desorption ionization (SELDI) [2, 3]. Difference gel electrophoresis (DIGE) is a technical modification of 2-DE in which the experimental and control samples are derivatized with different fluorophores and are run in the same gel [4]. Note that there are other techniques that could be listed such as capillary electrophoresis [5]. The separations are followed by a mass spectrometry step for identification of the proteins of interest. Our studies have employed two approaches for protein quantification and identification, viz. DIGE followed by MALDI-TOF (time-of-flight) MS and LC-MS/MS. In this review, we will first outline the principles of these two proteomics techniques and then illustrate how they increase our understanding of vasopressin signaling in the inner medullary collecting duct (IMCD), which is a major focus of our laboratory [6].

Principles of Proteomics Based on DIGE

Figure 1 summarizes the principles of DIGE- and LC-based proteomics, while table 1 lists the strengths, limitations, and solutions to overcome some of the limitations of these two proteomics techniques.

The most important step in preparing the sample for DIGE is the covalent labeling of proteins with three different fluorescent dyes (for case, control, and internal standard), which should cause little shift in isoelectric point and an identical shift in molecular weight [4, 7]. During 2-DE, proteins are separated based on their isoelectric points (first dimension) and their molecular weights (second dimension) (fig. 1a) [2, 4, 7]. It may be useful to select a subproteome prior to 2-DE by using subcellular fractionation, sequential extraction, laser microdissection, or other separation techniques [8]. 2-D gels constitute a map of intact proteins which allows the analysis of differences in relative abundance

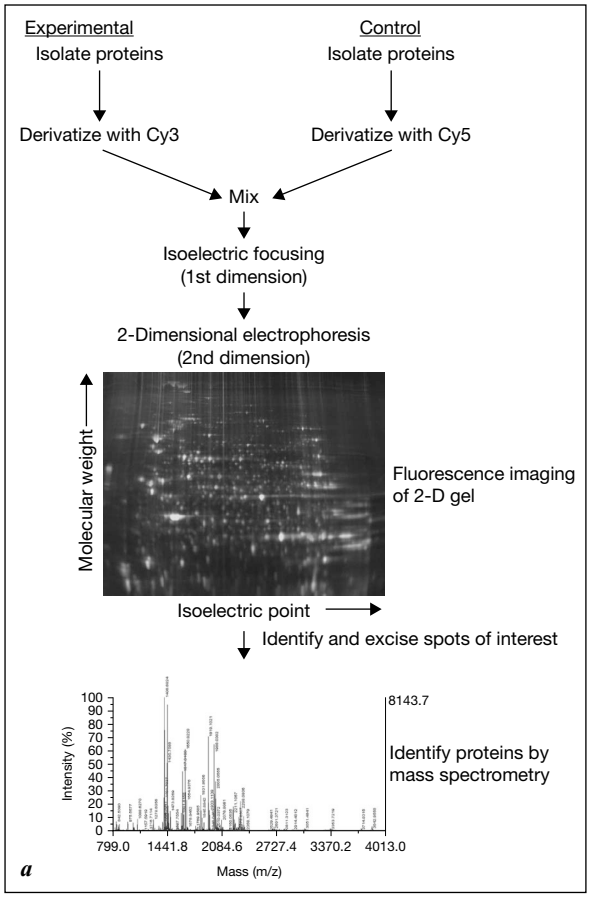


Fig. 1. Principles and flowcharts of DIGE-based (***a***; reprinted from Hoorn et al. [4] with permission and LC-based (***b***) proteomics. In LC-based proteomics, the bottom-up approach requires an initial digestion step with proteases to break proteins into peptides. Survey mass analysis yields mass-to-charge (m/z) spectra of parent peptide ions (MS1). MS1 peaks are selected for collision-induced dissociation (the light grey peak at 860.1 in this example) allowing protein identification based on peptide fragmentation spectra (MS2). LC-ESI = Liquid chromatography coupled to electrospray ionization.

and pre- and posttranslational modifications. The ability to accurately analyze differential expression is the most important strength of DIGE-based proteomics, making it one of the few platforms to routinely perform quantitative proteomics [4]. Notwithstanding, several challenges with regard to statistics in DIGE-based proteomics remain, including which type of statistical test to

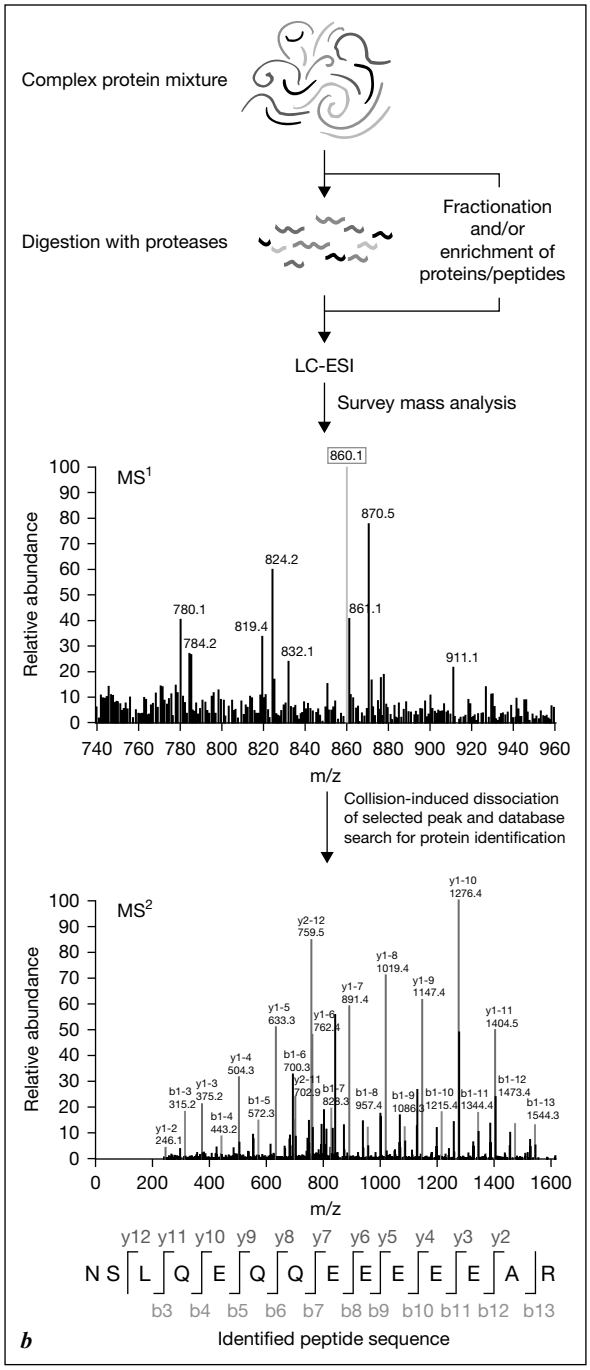

Complex protein mixture
Digestion with proteases
Fractionation and/or enrichment of proteins/peptides
LC-ESI
Survey mass analysis
MS1
Relative abundance
m/z
860.1
870.5
824.2
780.1
861.1
819.4
784.2
832.1
911.1
Collision-induced dissociation of selected peak and database search for protein identification
MS2
y1-10 1276.4
y2-12 759.5
y1-8 1019.4
y1-9 1147.4
y1-7 891.4
y1-5 633.3
y1-6 762.4
y1-11 1404.5
y1-4 504.3
b1-6 700.3
b1-12 1473.4
y1-3 375.2
b1-3 315.2
y2-11 702.9
b1-7 828.3
b1-8 957.4
b1-9 1086.3
b1-10 1215.4
b1-11 1344.4
b1-13 1544.3
b1-5 572.3
b1-4 443.2
y1-2 246.1
y12 y11 y10 y9 y8 y7 y6 y5 y4 y3 y2
N S L Q E Q Q E E E E E A R
b3 b4 b5 b6 b7 b8 b9 b10 b11 b12 b13
b
Identified peptide sequence

Table 1. Strengths, limitations, and solutions for DIGE- and LC-based proteomics

	DIGE-based proteomics	LC-based proteomics
Strengths	• Quantitative proteomics • Visual output • Low experimental variation • Information on MW and pI	• High throughput • High sensitivity
Limitations	• Low identification rate of hydrophobic and low-abundance proteins • Difficult analysis of proteins with overlapping spots	• Not intrinsically quantitative • No visualization of protein isoforms including splice variants and posttranslational modifications
Solutions to overcome limitations	• Modified 2-DE (using 16-BAC and SDS) • Estimation of overall false discovery rate • Combination with bioinformatics pathways analysis	• Combination of 2-DE with LC-MS/MS • Specialized techniques for labeling and detecting specific posttranslational modifications

choose when analyzing multiple differential expression profiles, how to correct for multiple testing, and how to normalize for dye-specific biases [9]. Prudence is therefore warranted when interpreting the results of 2-D gel analyses, and it is recommended that proteins of interest are confirmed by other methods such as immunoblotting [4].

After the statistical analysis of 2-D gels, proteins of interest are cut from the gels, treated with a protease (usually trypsin) and prepared for mass spectrometry. In DIGE-based proteomics, usually a MALDI-TOF mass spectrometer is used. MALDI refers to the process in which laser pulses ionize and volatilize peptides that have been embedded in a matrix. The time-of-flight (TOF) of peptides from ionization until arrival at the detector plate is related to the mass of the ion (heavier ions travel longer). Subsequently, a procedure called 'peptide mass fingerprinting' (PMF) is used to identify the protein by measuring the molecular masses of the trypsin products and matching these with databases of theoretical sizes of trypsinized fragments from known protein sequences. The introduction of tandem mass spectrometry or MS/MS has further strengthened proteomics in the sense that peptide ions can now be fragmented in a second step by using a technique called collision-induced dissociation, allowing the partial sequencing of these peptides [1, 2, 4].

Principles of Proteomics Based on LC

Proteomics based on LC typically uses a 'bottom up' approach (fig. 1b), which means protein identification depends on the initial treatment with a protease (usually trypsin) to digest proteins into relatively small peptides [10]. The next step is protein separation, which is achieved by exploiting different physicochemical properties than in DIGE, viz. hydrophobicity, surface charge, and affinity to particular compounds. The small peptides are subsequently fragmented by collision-induced dissociation, which allows a reliable identification based on peptide fragmentation spectra. LC-based proteomics has two important advantages compared to DIGE-based proteomics (table 1). The first advantage is that LC can be coupled online to mass spectrometry, establishing a truly high-throughput system. The second advantage is that the sensitivity of LC-based proteomics is higher than that of DIGE-based proteomics, allowing the identification of low-abundance proteins and increasing the yield of the overall protein identification.

Until recently, a major limitation of LC-MS/MS was the ability to perform quantitative proteomics. Namely, the design of LC-MS/MS complicates parallel processing of multiple samples to obtain a statistical output to differential protein expression, as in DIGE-based proteomics. However, several quantification strategies are evolving, and can be classified into non-labeling and labeling methods [10]. Non-labeling methods resolve the two samples in separate LC-MS/MS runs and quantify appropriate peptides by integrating the area under the pseudochromatograms constructed from the MS^1 peak heights for the appropriate peptide ions [11]. Labeling methods include ICAT (isotope-coded affinity tags) [12], iTRAQ (isobaric tags for relative and absolute quantification) [13], and end-labeling approaches using O^{18}-labeled water [14] or in vacuo isotope-coded alkylation technique (IVICAT) [15].

Proteomics Studies of Vasopressin Signaling in the Renal Collecting Duct

Water balance regulation depends on the release of vasopressin by the pituitary and its end-organ effect on the renal collecting duct system. The activation of the vasopressin 2 receptor (V2R, a protein of 371 residues with 7 transmembrane domains) by vasopressin is the most important step in water balance regulation, because it increases water permeability of the renal collecting duct by inserting aquaporin-2 (AQP2) water channels in the apical membrane of the collecting duct principal cell. Binding of vasopressin to the V2R activates the Gs adenylyl cyclase system, stimulating cAMP and protein kinase A, which

triggers phosphorylation of many proteins including AQP2. In addition, V2R receptor occupation stimulates a rise in intracellular calcium that plays a critical role in the water permeability increase by stimulating calmodulin-dependent phosphorylation of the regulatory light chain of non-muscle myosin-II in collecting duct cells [16, 17]. In general, two forms of regulation of AQP2 are recognized, short- and long-term regulation. Short-term regulation occurs through the trafficking of AQP2-containing vesicles to the apical plasma membrane. Long-term regulation of AQP2 results from a variety of mechanisms, including transcriptional and translational regulation.

Figure 2 is a schematic representation of vasopressin signaling and AQP2 trafficking in the IMCD and shows the sequence of events from the activation of V2R by vasopressin until the insertion of AQP2 water channels in the apical plasma membrane. Figure 2 also summarizes the new insights in vasopressin signaling that were generated by our recent proteomics studies. Several aspects of vasopressin signaling were investigated, including the long-term regulation of AQP2 (fig. 2, Legend A) [18, 19], IMCD signaling during vasopressin escape (Legend B) [20], calcium-calmodulin signaling (Legend C) [17], phosphoproteomics of IMCD signaling (Legend D) [21], proteomics of AQP2-bearing vesicles (Legend E) [22] and, finally, proteomics of IMCD plasma membranes (Legend F) [23].

Two studies on the long-term regulation of AQP2 investigated the effects of the V2R-selective vasopressin analog dDAVP on protein expression in IMCD (fig. 2, Legend A) [18, 19]. To identify vasopressin-responsive proteins, both studies were performed in Brattleboro rats, which lack endogenous vasopressin because of a mutation in the neurophysin-vasopressin gene. The first study used DIGE-based proteomics [18], whereas the second used ICAT in combination with LC-MS/MS [19]. The study using DIGE-based proteomics identified 43 proteins that were found to be regulated in response to vasopressin infusion, including 18 that were increased in abundance, 22 that were decreased, and 3 that were shifted in the gel, presumably because of posttranslational modification [18]. The identified proteins pointed to several intracellular processes regulated by vasopressin, including determinants of nitric oxide levels in the cell (nitric oxide synthase 2, arginase 2, and NADPH oxidase or NOX4), the production of cAMP (adenylyl cyclase VI), receptor internalization (GPCR kinase 4), endoplasmic reticulum function (GRP78 and protein disulfide isomerase), and calcium-mediated processes (annexins II and V) [18].

The study using ICAT and LC-MS/MS identified 33 proteins that were found to be regulated in response to vasopressin infusion, including 22 proteins that were increased in abundance and 11 proteins that were decreased in abundance [19]. A number of the identified proteins seemed of relevance to vasopressin signaling. Syntaxin-7, which belongs to the family of so-called

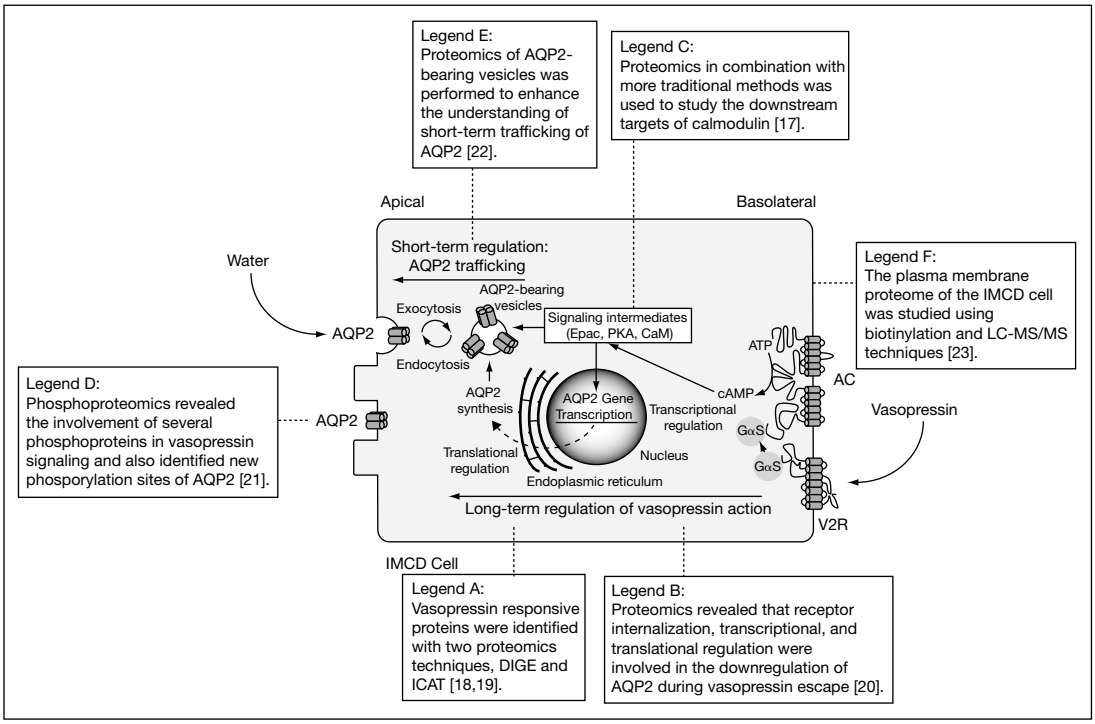


Fig. 2. Summary of proteomics studies on vasopressin signaling in the inner medullary collecting duct. See Legends A–F in the figure and text for more details. AC = Adenylate cyclase; CaM = calmodulin; DIGE = difference gel electrophoresis; ICAT = isotope coded affinity tags; PKA = protein kinase A; V2R = vasopressin 2 receptor.

t-SNARE proteins, plays a critical role in vesicle fusion, and could therefore be involved in endocytosis of AQP2 (fig. 2). The identification of Rap1 was of interest, because it is the downstream target for Epac, which in turn is activated by cAMP. Therefore, cAMP-induced calcium mobilization, which stimulates AQP2 trafficking, could be mediated by Epac and Rap1. Finally, cathepsin D, a renin-like proteolytic enzyme regulated by the transcription factor p53 was identified [24]. p53 was recently shown to be involved in the regulation of AQP2 during the vasopressin escape phenomenon [20].

During vasopressin escape, patients and experimental animals undergo a brisk water diuresis despite high levels of circulating vasopressin. Vasopressin escape therefore forms an important defense mechanism against the development of severe hyponatremia. DIGE-based proteomics was used to identify

IMCD proteins that trigger and maintain vasopressin escape (fig. 2, Legend B) [20]. For this purpose, we used an animal model of vasopressin escape, in which animals continually received dDAVP via osmotic minipump, while experimental animals also received a water load. A set of 22 (mostly high-abundance) proteins were identified and subjected to a bioinformatics pathways analysis. The pathways analysis indicated that 8 of the 22 proteins were part of a larger protein regulatory network consisting of 33 proteins, including low-abundance regulatory proteins and transcription factors. These identified proteins pointed to several candidate regulators of vasopressin escape, including proteins that could regulate the *AQP2* gene (c-myc, c-fos, c-jun and SRC-1), proteins that may be involved in receptor internalization (c-src and RACK1), and proteins involved in protein folding in the endoplasmic reticulum (GRP78, protein disulfide isomerase and heat-shock protein 70).

Because phosphorylation is a key event in cell signal transduction, it is likely to be involved in both the short- and long-term regulation of water transport. Therefore, we were also interested in pursuing phosphoproteomics. In a study by Chou et al. [17], phosphoproteomics, in combination with more traditional methods such as immunoblotting and ^{32}P labeling, was used to study the downstream targets of calmodulin (fig. 2, Legend C). We previously showed that the trafficking of AQP2 to the apical plasma membrane is dependent on intracellular calcium mobilization and calmodulin activation [16]. 2-DE proteomics identified two isoforms of the protein myosin light chain (MLC) as well as their phosphorylated forms (by using a fluorescent dye that recognizes phosphoproteins). Subsequently, the phosphorylation of MLC by myosin light chain kinase (MLCK) was shown to be a downstream effect of vasopressin-activated calcium-calmodulin signaling. Thus, MLC and MLCK appear important proteins in the vasopressin signaling cascade downstream from calmodulin.

In a more recent study by Hoffert et al. [21], the study of IMCD phosphorylation and its role in vasopressin signaling was expanded to the complete IMCD proteome (fig. 2, Legend D). A large-scale phosphoproteomics analysis was carried out, which was facilitated by using a combination of phosphopeptide enrichment by immobilized metal affinity chromatography (IMAC) and phosphorylation site identification by LC-MS^n neutral loss scanning. This approach identified 30 phosphoproteins with potential relevance to vasopressin signaling and AQP2 trafficking. In addition, previously unidentified phosphorylation sites of important membrane proteins were found, including eight sites among AQP2, aquaporin-4, and the urea transporter isoforms A1 and A3. The final step consisted of a quantitative phosphoproteomics approach using label-free quantification, which showed a significantly changed phosphorylation state of five proteins, including AQP2. A follow-up study on one of the

new AQP2 phosphorylation sites, serine-261, confirmed its regulation by vasopressin [25].

Another aim of our proteomics studies was to better understand the short-term regulation of AQP2, because little is known about the specific intracellular protein trafficking pathways and the nature of the intracellular compartments in which AQP2 resides. Therefore, Barile et al. [22] performed an LC-MS/MS analysis of AQP2-bearing vesicles (fig. 2, Legend E). To do so, AQP2 vesicles were first immunoisolated by using biotinylated chicken anti-AQP2 attached to magnetic beads. Subsequently, proteins were separated using 1-DE, which was sliced and trypsinized prior to LC-MS/MS analysis. Using compartmental marker proteins as references, the results showed that AQP2-containing vesicles are heterogeneous and that intracellular AQP2 resides chiefly in endosomes, the trans-Golgi network, and the rough endoplasmic reticulum. Importantly, the repertoire of compartmental marker proteins revealed in this study included many Rab, ARF, and SNARE family proteins.

The identification of membrane proteins with proteomics remains a challenge. Membrane proteins represented approximately 2% of the total number of identifications using DIGE [19], whereas this percentage increased to approximately 7% when using ICAT and LC-MS/MS [20]. However, these yields still contrast with the average percentage of membrane proteins in cells (~30%) and the physiological importance of membrane proteins. Therefore, in a recent proteomics study, Yu et al. [23] attempted to enrich membrane proteins from the apical and basolateral plasma membranes by using surface biotinylation (fig. 2, Legend F). Subsequently, LC-MS/MS analysis was performed, which identified a total of 62 integral and glycosylphosphatidyl inositol (GPI)-linked and 159 peripheral membrane proteins (fig. 3). Among the integral membrane proteins identified were activin A receptor and TauT (a sodium- and chloride-dependent taurine transporter), both of which appear relevant to water balance regulation [26, 27]. Among the peripheral membrane proteins identified were proteins that could act as transducing molecules, which couple the vasopressin stimulus to AQP2 trafficking, including cAMP-dependent kinase catalytic subunit, Ca^{2+}/calmodulin-dependent nitric-oxide synthase 1, and calcyclin. A number of the identified scaffold proteins were bassoon, piccolo, and septin 9, which are involved in cytoskeleton and membrane organization as well as synaptic vesicle trafficking [23]. They are of potential interest because vasopressin-stimulated AQP2 trafficking also involves cytoskeletal reorganization [28].

Finally, in an effort to integrate all of the current knowledge of the mechanisms of vasopressin-regulated water transport in the IMCD, three WWW-based proteomic databases have been constructed: (1) the IMCD Proteome Database [19]; (2) the Collecting Duct Phosphoprotein Database (CDPD) [21], and (3) the

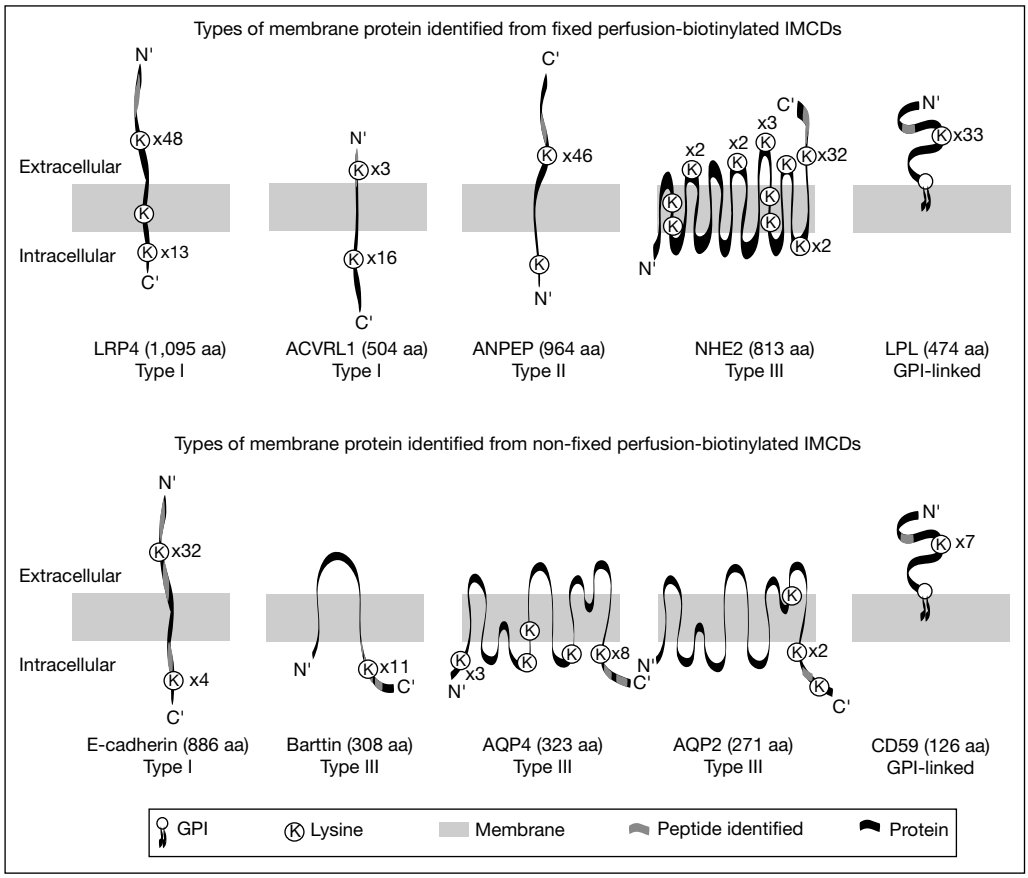


Fig. 3. Exploring the plasma membrane proteome: examples of membrane proteins in the inner medullary collecting duct identified by proteomics. Type I and II membrane proteins have one single transmembrane span with N' or C' terminus facing the extracellular space, respectively. Type III membrane proteins contain multiple membrane-spanning topology. Some membrane proteins are anchored to the membrane via a GPI anchor. ANPEP = Aminopeptidase N; GPI = glycosylphosphatidyl inositol; IMCD = inner medullary collecting duct; LPL = lipoprotein lipase; LRP4 = low-density lipoprotein receptor-related protein 4 [reprinted from 23, with kind permission].

Inner Medullary Collecting Duct Membrane Protein (IMP) Database [23]. These databases provide an important tool for studying the systems biology of the IMCD, and can be publicly accessed at http://dir.nhlbi.nih.gov/labs/lkem/rm/proteomics_db.asp.

From Renal Mechanisms to Clinical Disorders: Clinical Applications of Proteomics

Proteomics based on DIGE and LC offers a revolutionary novel approach to study cellular processes. However, clinically more applicable may be proteomics of body fluids. In nephrology, urinary proteomics is a promising method to identify urinary biomarkers for renal disease [29]. In general, urinary proteins may originate from the glomerular filtrate, renal tubular secretion, cell shedding, proteolytic cleavage of glycoproteins (e.g., Tamm-Horsfall), or exosomes [29]. Urinary exosomes may be of special interest, because exosome formation appears to be an active and potentially regulated process by which renal transporters and intracellular proteins are excreted into the urine [30]. In addition, proteomic profiling of urinary exosomes demonstrated them to contain several disease-associated proteins [30]. Currently, early urinary proteomics studies are being conducted, studying various diseases, including genetic renal electrolyte disorders, minimal change disease, membranous nephropathy, focal segmental glomerular sclerosis, diabetic nephropathy, acute renal allograft rejection, urolithiasis, acute renal failure, renal and bladder cancer, and autosomal dominant polycystic kidney disease [10, 29, 31]. Especially in diseases where early identification, differential diagnosis or therapeutic monitoring is important, urinary proteomics may be applicable as a non-invasive and cost-effective technology. For example, if unique fingerprints in the urinary proteome exist for different types of (essential) hypertension, this may eventually help to personalize therapy and classify this heterogeneous disorder. The application of urinary proteomics to hypertension was recently illustrated in a study by Olivieri et al. [32]. In patients with primary aldosteronism and hypertension, they demonstrated a specific pattern of the urinary excretion of prostasin, which is a membrane-bound serine protease regulating the epithelial sodium channel (ENaC) in the renal collecting duct [32]. Thus, urinary prostasin was postulated to be a marker for ENaC activation in humans, making it an attractive candidate marker for primary aldosteronism and other forms of hypertension [32].

References

1 Aebersold R, Mann M: Mass spectrometry based proteomics. Nature 2003;422:198–207.
2 Knepper M: Proteomics and the kidney. J Am Soc Nephrol 2002;13:1398–1408.
3 Xiao Z, Prieto D, Conrads TP, Veenstra TD, Issaq HJ: Proteomic patterns: their potential for disease diagnosis. Moll Cell Endocrinol 2005;230:95–106.
4 Hoorn EJ, Hoffert JD, Knepper MA: The application of DIGE-based proteomics to renal physiology. Nephron Physiol 2006;104:61–72.
5 Fliser D, Wittke S, Mischak H: Capillary electrophoresis coupled to mass spectrometry for clinical diagnostic purposes. Electrophoresis 2005;26:2708–2716.

6 Nielsen S, Frokiaer J, Marples D, Kwon TH, Agre P, Knepper MA: Aquaporins in the kidney: from molecules to medicine. Physiol Rev 2002;82:205–244.
7 Lilley KS, Friedman DB: All about DIGE: quantification technology for differential-display 2D-gel proteomics. Expert Rev Proteomics 2004;1:401–409.
8 Stasyk T, Huber LA: Zooming in: fractionation strategies in proteomics. Proteomics 2004;4: 3704–3716.
9 Fodor IK, Nelson DO, Alegria-Hartman M, Robbins K, Langlois RG, Turteltaub KW, Corzett TH, McCutchen-Maloney SL: Statistical challenges in the analysis of two-dimensional difference gel electrophoresis experiments using DeCyder™. Bioinformatics 2005;21:3733–3740.
10 Pisitkun T, Johnstone R, Knepper MA: Discovery of urinary biomarkers. Mol Cell Proteomics 2006;5:1760–1771.
11 Wang G, Wu WW, Zeng W, Chou CL, Shen RF: Label-free protein quantification using LC-coupled ion trap or FT mass spectrometry: reproducibility, linearity, and application with complex proteomes. J Proteome Res 2006;5:1214–1223.
12 Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R: Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotechnol 1999;17:994–999.
13 Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlet-Jones M, He F, Jacobson A, Pappin DJ: Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. Mol Cell Proteomics 2004;3:1154–1169.
14 Yao X, Freas A, Ramirez J, Demirev PA, Fenselau C: Proteolytic ^{18}O labeling for comparative proteomics: model studies with two serotypes of adenovirus. Anal Chem 2001;73:2836–2842.
15 Simons BL, Wang G, Shen RF, Knepper MA: In vacuo isotope coded alkylation technique (IVICAT); an N-terminal stable isotopic label for quantitative liquid chromatography/mass spectrometry proteomics. Rapid Commun Mass Spectrom 2006;20:2463–2477.
16 Chou CL, Yip KP, Michea L, Kador K, Ferraris JD, Wade JB, Knepper MA: Regulation of aquaporin-2 trafficking by vasopressin in the renal collecting duct. Roles of ryanodine-sensitive Ca^{2+} stores and calmodulin. J Biol Chem 2000;24;275:36839–36846.
17 Chou CL, Christensen BM, Frische S, Vorum H, Desai RA, Hoffert JD, de Lanerolle P, Nielsen S, Knepper MA: Non-muscle myosin II and myosin light chain kinase are downstream targets for vasopressin signaling in the renal collecting duct. J Biol Chem 2004;279:49026–49035.
18 Van Balkom BW, Hoffert JD, Chou CL, Knepper MA: Proteomic analysis of long-term vasopressin action in the inner medullary collecting duct of the Brattleboro rat. Am J Physiol Renal Physiol 2004;286:F216–F224.
19 Pisitkun T, Bieniek J, Tchapyjnikov D, Wang G, Wu WW, Shen RF, Knepper MA: High-throughput identification of IMCD proteins using LC-MS/MS. Physiol Genomics 2006;25:263–276.
20 Hoorn EJ, Hoffert JD, Knepper MA: Combined proteomics and pathways analysis of collecting duct reveals a protein regulatory network activated in vasopressin escape. J Am Soc Nephrol 2005; 16:2852–2863.
21 Hoffert JD, Pisitkun T, Wang G, Shen RF, Knepper MA: Quantitative phosphoproteomics of vasopressin-sensitive renal cells: regulation of aquaporin-2 phosphorylation at two sites. Proc Natl Acad Sci USA 2006;103:7159–7164.
22 Barile M, Pisitkun T, Yu MJ, Chou CL, Verbalis MJ, Shen RF, Knepper MA: Large-scale protein identification in intracellular aquaporin-2 vesicles from renal inner medullary collecting duct. Mol Cell Proteomics 2005;4:1095–1106.
23 Yu MJ, Pisitkun T, Wang G, Shen RF, Knepper MA: LC-MS/MS analysis of apical and basolateral plasma membranes of rat renal collecting duct cells. Mol Cell Proteomics 2006;5:2131–2145.
24 Sun Y, Wicha M, Leopold WR: Regulation of metastasis-related gene expression by p53: a potential clinical implication. Mol Carcinog 1999;24:25–28.
25 Hoffert JD, Nielsen J, Yu MJ, Pisitkun T, Schleicher SM, Nielsen S, Knepper MA: Dynamics of aquaporin-2 serine-261 phosphorylation in response to short-term vasopressin treatment in collecting duct. Am J Physiol Renal Physiol 2007;292:F691–F700.
26 Kobayashi T, Tanaka H, Kuwana H, Inoshita S, Teraoka H, Sasaki S, Terada Y: Wnt4-transformed mouse embryonic stem cells differentiate into renal tubular cells. Biochem Biophys Res Commun 2005;336:585–595.

27 Huang DY, Boini KM, Lang PA, Grahammer F, Duszenko M, Heller-Stilb B, Warskulat U, Haussinger D, Lang F, Vallon V: Impaired ability to increase water excretion in mice lacking the taurine transporter gene TAUT. Pflügers Arch 2006;451:668–677.
28 Valenti G, Procino G, Tamma G, Carmosino M, Svelto M: Minireview: aquaporin 2 trafficking. Endocrinology 2005;146:5063–5070.
29 Hoorn EJ, Pisitkun T, Zietse R, Gross P, Frokiaer J, Wang NS, Gonzales PA, Star RA, Knepper MA: Prospects for urinary proteomics: exosomes as a source of urinary biomarkers. Nephrology (Carlton) 2005;10:283–290.
30 Pisitkun T, Shen RF, Knepper MA: Identification and proteomic profiling of exosomes in human urine. Proc Natl Acad Sci USA 2004;101:13368–13373.
31 Zhou H, Pisitkun T, Aponte A, Yuen PS, Hoffert JD, Yasuda H, Hu X, Chawla L, Shen RF, Knepper MA, Star RA: Exosomal fetuin-A identified by proteomics: a novel urinary biomarker for detecting acute kidney injury. Kidney Int 2006;70:1847–1857.
32 Olivieri O, Castagna A, Guarini P, Chiecchi L, Sabaini G, Pizzolo F, Corrocher R, Righetti PG: Urinary prostasin: a candidate marker of epithelial sodium channel activation in humans. Hypertension 2005;46:683–688.

Dr. Mark A. Knepper, MD, PhD
NIH, NHLBI, NIH, 10 Center Drive, Building 10, Room 6N260
Bethesda, MD 20892 (USA)
Tel. +1 301 496 3064, Fax +1 301 402 1443, E-Mail knep@helix.nih.gov

Thongboonkerd V (ed): Proteomics in Nephrology – Towards Clinical Applications.
Contrib Nephrol. Basel, Karger, 2008, vol 160, pp 186–197

Overview of Kidney and Urine Proteome Databases

Yutaka Yoshida[a], *Masahito Miyamoto*[a,b], *Xu Bo*[a], *Eishin Yaoita*[a], *Tadashi Yamamoto*[a]

[a]Department of Structural Pathology, Institute of Nephrology, Graduate School of Medical and Dental Sciences, Niigata University, Niigata, and [b]Division of Nephrology and Hypertension, Department of Internal Medicine, St. Marianna University School of Medicine, Kawasaki, Japan

Abstract

With the completion or almost completion of genome sequences of many organisms in combination with the tremendous development of mass spectrometric analysis of proteins, several comprehensive proteomic studies, targeting whole organisms, body fluids, organs, tissues, cells, cellular organelles, or functional protein complexes, have produced valuable resources that can be shared and retrieved. In the present review, we provide current concept of construction of protein databases with special emphasis on high-throughput identification of protein using mass spectrometry, annotations, computational tools, and search engines to retrieve information of the identified proteins. We then update the current status of available protein databases of kidney and urine proteomes.

Introduction

The availability of genome sequences of organisms and the tremendous development in technology of mass spectrometry (MS) have paved way to identification of a large number of proteins in complex mixtures [1–3]. Accordingly, many comprehensive proteomic studies have emerged targeting whole organisms, body fluids, organs, tissues, cells, cellular organelles, or complex protein machineries executing specific biological functions. Among them, some studies have provided valuable resources that could be shared and retrieved by many researchers in various biological fields. As the public domain databases of genes, mRNAs, and proteins such as Entrez Gene, GeneCards, Uni-Prot, Swiss-Prot/

TrEMBL, RefSeq, IPI, and OMIM are now essential and necessary bioinformatics tools for most of investigators, databases of particular proteomes are obviously useful to gain information of a protein under investigation and gain insight into a proteome of interest, and an increasing number of databases are now available on the web [4].

In the present review we first describe the current concept of the proteome database with special emphasis on MS instrumentations for protein identification to achieve in-depth and confident protein identification, annotations and computational annotation tools of identified protein, search engines to retrieve information required, and dealing with MS dataset used for protein identification. We then take an overview of databases of kidney and urine proteome currently available.

Database Construction: Protein Identification

Two-Dimensional Gel Electrophoresis-Based Proteome Analysis

Construction of a database of a given proteome requires comprehensive protein identifications. Proteomic analysis for protein identification can be generally categorized into two-dimensional gel electrophoresis (2-DE)-based and liquid chromatography (LC)-based methodologies. The current 2-DE-based approach combines high-resolution 2-DE using immobilized pH gradient gels (IPGs) with identification of selected protein spots by MS [5]. The 2-DE is the classical method, and has been routinely used as a mature and well-established method for quantitative expression profiling of large sets of complex protein mixture. The advantage of 2-DE analysis over the conventional LC-based proteomic analysis is its capability of providing intuitive images of proteomes in a highly quantitative manner and visualization of protein isoforms and their derivatives produced by alternative splicing, proteolytic cleavage, or a variety of posttranslational modifications. This is in contrast to the LC-based method which performs analysis on peptides and where intrinsic structural features of intact proteins such as molecular weight (MW) and isoelectric point (pI) are lost. Depending on the gel size and pH gradient used, the best 2-DE can separate more than 5,000 proteins simultaneously (~2,000 routinely) with the detectability threshold of <1 ng of protein spot. Despite the superiority of 2-DE for analysis of complex protein mixtures, this technique is limited to the high abundant proteins if a crude protein mixture is used, mainly due to its low capacity of protein load (~150 μg to several milligrams). Another problem pertaining to 2-DE is the relative incompatibility of some proteins including large, hydrophobic proteins and proteins of extreme pIs with the first dimension or isoelectric focusing (IEF) step; these proteins simply do not behave well in this

type of analysis and are not well separated on 2-DE gels. While the 2-DE separation could be best for analysis of abundant proteins, the major problem is its low sensitivity to detect low abundant proteins. The usage of narrow overlapping pH gradient IPGs and prefractionation of a crude protein mixture to reduce its complexity is necessary for more comprehensive profiling of protein expression in a targeted proteome [6]. Protein identification of selected protein spots on 2-DE gels are performed by the peptide mass fingerprinting using MALDI-TOF-MS and/or more reliably by the sequence tag method using LC-MS/MS. The results of protein identification of protein spots separated on 2-DE gels are generally reliable, partly because the structural information of intact proteins to be identified is provided. The task of identification of protein spots on 2-DE, however, is time-consuming and laborious, and identification of many proteins required for construction of a comprehensive database of proteome is practically difficult to accomplish.

LC-MS-Based Proteome Analysis

LC-MS-based methodologies are alternative and complementary technologies that have emerged recently. The most widely used method to separate protein or peptide mixtures is HPLC. The diversity of stationary phase and separation modes gives HPLC considerable resolving power, and combination of HPLC separation modes is one of the most effective tools in analytical proteomics. Yates and colleagues [7] have developed a two-dimensional HPLC separation technique termed MudPIT (Multidimensional Protein Identification Technology) to analyze complex peptide mixtures. In MudPIT, peptides are first separated in a strong cation exchange (SCX) column, and fractions from SCX column by stepwise elution are then separated respectively by reversed-phase (RP)-HPLC column, which is linked in series with SCX column. The peptides separated on RP column are directly eluted into the tandem MS instrument through ESI interphase. This approach greatly increases the number of peptides that were identified in a single run. The improvement of separation technique of peptides using HPLC, IEF in IPG, or free-flow IEF in combination with the marked advance in tandem MS technology such as linear ion trap-Fourier transform or orbitrap, which has an extremely high mass accuracy and a very high-resolution power, leads to identification of many proteins with high confidence and in-depth profiling of proteomes [8]. Combination of prefractionation of proteins using free-flow IEF and/or SDS-PAGE with the tandem MS has also been proven to be very effective in identification of low abundant proteins as well as increase in the number of identified proteins [9].

The increase in the number of identified proteins and effective detection of low abundant proteins by LC-MS-based approaches provide us with much more comprehensive profiling of protein expression in a targeted proteome. However,

experimental strategies for protein identification employed by LC-based proteomic analysis are based solely on interpreted sequences of proteolytically digested peptides and introduce the complication of loss of connectivity between peptides and their protein precursors. The multiple unique peptides map to more than one protein sequences introduces an uncertainty between the possibility that the shared peptides can be mapped to more than one protein sequence owing to bioinformatics redundancy and the possibility that more than one precursor is actually present in the original protein mixture (physical redundancy) [10]. In addition, large-scale experiments aimed at identifying an enormous number of proteins may include false-positive matching of peptide sequence, especially in protein identification with single peptide match, and should estimate a false-positive rate by a searching randomized or inversed database [11].

Recent advancements in protein/peptide separation and tandem MS enable identification of an enormous number of proteins in a high-throughput, high-confidence manner. With the availability of these instrumentations, a comprehensive database of proteomes does not appear be a difficult task. However, it should be noted that complete profiling of a given proteome is difficult and still challenging even with the currently best technology.

Database Construction: Annotating the Proteome

Fundamental Annotations

After completing protein identifications of a given proteome with defined confidence, annotations of identified proteins are necessary for characterizing the proteome and constructing database. Protein name and its aliases, gene name or gene symbol, accession number of representative protein databases in public domain, and the number of unique peptides matched to the identified protein as well as score provided from the search engine used for protein identification, as indexes to indicate the reliability of identification, are the least required annotations to construct a proteome database. Among them, the accession number of public domain protein databases including Swiss-Prot, Refseq, IPI, or UniProt is critical to gain knowledge of a protein of interest. Swiss-Prot (http://www.expasy.org) and RefSeq (http://www.ncbi.nlm.nih.gov/RefSeq/) are the most curated and integrated databases, which provide comprehensive, integrated, and non-redundant information of a large number of proteins with several annotations concerning: correct sequences, splice variants, sites of polymorphisms, potential sites of posttranslational modifications, systemic protein names as well as all known synonyms, gene name, citation information, function(s), enzyme-specific information, biologically relevant domains and sites,

MW theoretically or experimentally determined, subcellular location(s), tissue-specific expression, developmentally specific expression, secondary structure, quaternary structure, similarity to other proteins, diseases associated with deficiencies in the protein, and so forth. IPI (http://www.ebi.ac.uk/IPI/) merges the experimentally determined protein sequences held in the UniProt sequence database with the protein predictions of Ensemble and both protein predictions and experimentally derived datasets provided by RefSeq to provide a minimally redundant yet maximally complete set of human, mouse, rat and zebrafish proteins consisting of one sequence per transcript. All annotated splice variants are included in IPI as separate entries unless their protein sequences are identical. UniProt (http://www.ebi.ac.uk/uniprot/) was created by reconciling, curating, and merging protein sequence information from the Swiss-Prot, TrEMBL and PIR sequence databases to provide extensively curated, non-redundant, detailed and comprehensive information of protein of a particular species. One of the many strengths of the UniProt is the extensive cross-referencing made to other more specialized databases, and may be regarded as a central hub of knowledge extending out to many additional sources to expand the information [12]. These protein databases enable users to acquire a lot of knowledge about a particular protein from the databases themselves and by tracking in cross-references, which are linked to other specialized databases.

Descriptions of Physiological Functions of Identified Proteins

As mentioned above, most of the information intrinsic to a particular protein can be obtained by referring to related records in public domain protein databases. Additional annotations related to molecular functions, biological processes, cellular localization, metabolic and signal transduction pathways are especially helpful for gaining insight into the biological significance of a protein and also to characterize the proteome. The Gene Ontology (GO) Consortium (http://www.geneontology.org/index.shtml) has developed structured controlled vocabularies (ontologies) to describe gene products in terms of three categories, namely molecular functions, biological processes, and cellular components (cellular localizations) in a species-independent manner. The use of GO terms in describing gene products in databases facilitates consistent description of gene products in different databases. PANTHER (http://www.pantherdb.org/) is software freely accessible on the web and provides a platform of assigning families, functional classifications in the terms of molecular function, biological process, and pathways to gene products [13]. A high-throughput batch search using a large number of queries is also possible in addition to search with a single query. The PANTHER classification is based on experimental evidence and evolutionary relationships to predict function. Its ontologies are controlled as structured vocabularies of molecular function and biological

process terms are similar to those of GO, but greatly abbreviated and simplified to facilitate high-throughput analyses. The tools for mapping of their ontology terms to GO terms, or vice versa, are also available. Recent development of the PANTHER classification is the improvement in pathway classification resulting in an increased number of available pathways. The databases of biological pathways are also available in STKE [14], KEGG [15], MetaCyc [16], FREX [17] and Reactome [18]. KEGG and MetaCyc have collected mostly metabolic pathways, while STKE and Reactome contain well-curated, publicly available data on signaling pathways. STKE provides the most comprehensive description of signaling pathways.

Bioinformatics Tools for Annotation

Annotating of identified proteins is a daunting work: the larger number of identified proteins leads to a more difficult, laborious, and time-consuming task of annotations. Bioinformatics software that can automatically annotate the identified proteins is thus very useful. XOME (Mitsui Knowledge Industry, Tokyo, Japan) is software that creates a peak list from MS and MS/MS spectra produced by a variety of tandem MS instruments. It also automatically performs an integral identification using commonly used search engines such as MASCOT, SEQUEST, and X'TANDEM, and additionally executes quantitative calculations for analyses using stable isotope labeling methods such as ICAT, SILAC, and a label-free method such as emPAI for estimation of the absolute amount of a particular protein. XOME also automatically annotates the identified protein with GO terms. GoMiner (http://discover.nci.nih.gov/gominer/) is freely accessible software originally designed for creating lists of 'interesting' genes (down- and up-regulated genes) from a microarray experiment for biological interpretation in the context of GO terms [19]. GoMiner is also applicable to proteomic dataset and provides results of analysis on the basis of GO annotations [20]. INGENUITY (Ingenuity Systems, Redwood City, Calif., USA) is web-based software that is designed for pathway analysis of proteomic dataset based on knowledge created by experts curated experimentally to prove relationships between proteins.

Searching Proteome Database

Browsing of all entries of identified proteins or entries categorized under biological functions, cellular localization or pathways is fundamental to searching proteome databases. Searching the database by selected index word(s) is also essential for users to find the information attributable to a particular protein or proteins, and most of databases are equipped with a search engine which allows users to search by a variety of index words. It is recommended that the search form is as simple as possible like 'Google' and an advanced search by

multiple index words should be kept in a separate linked page [4]. Besides the fundamental index words including protein name, gene symbol and identifier (accession number of protein or gene databases), text query using keywords or phrases is also helpful to reach the information required. Searching by pI or MW range is also useful to find the proteins of concern.

MS Dataset

The number of matched peptides, sequence coverage and scores, which are provided by search engines and used for protein identification, are useful parameters to indicate confidence of identification. The sequences, interpreted MS/MS spectra, observed masses, and retention times and charge states of the matched peptides are valuable information for further proteomic analysis by MS such as quantitative analysis of the targeted protein(s). These MS data used for protein identification are recommended to be deposited in the database as supplemental data.

Kidney Proteome Database

Glomerulus Proteome

We have previously analyzed glomerulus proteome of the normal human kidney by conventional 2-DE and constructed an XML-based database. The glomeruli were highly purified by the standard sieving method from renal cortices without apparent pathologic manifestations obtained from 4 patients after surgical nephrectomy due to renal tumor. 2-DE gave a well-separated electrophoretic pattern in which 1,713 valid spots were observed. Of the observed spots, 1,559 spots were commonly found in 2-DE gels derived from all 4 subjects, and then identified by peptide mass fingerprinting using MALDI-TOF-MS and/or by the sequence tag method using LC-ion trap tandem MS. Having done so, we succeeded in identification of 347 protein spots representing 212 unique proteins [21]. The database on the web (http://www.hkupp.org/) provides a 2-DE image created by compiling those obtained from 4 subjects to which tabulated fundamental information of identified proteins, including functional classification based on GO terms, are linked. Users can easily obtain the information by clicking the protein spot of interest, and perform search by protein name, pI and MW ranges, accession number of the public domain protein database (Swiss-Prot or RefSeq), gene name, and accession number of the nucleotide database (Entrez Nucleotide). The gel image is scalable for easily matching between the protein spot on the 2-DE gel image produced by users and the corresponding spot on the synthetic 2-DE gel image on the database.

We have recently completed in-depth profiling of glomerulus proteome of the normal human kidney by using 1-D prefractionation (SDS-PAGE) and 2-D prefractionation (free-flow IEF coupled with SDS-PAGE) of proteins in combination with RP-LC ion trap tandem MS. Proteomic analysis by combination of protein prefractionation and LC-MS/MS identification is a powerful approach for comprehensive analysis of proteome. The 1-D prefractionation approach resulted in 3,426 protein identifications, whereas the 2-D prefractionation approach resulted in 6,339 protein identifications, of which 3,077 proteins were overlapped. The identified proteins were compiled to a 1-D protein array consisting of 15 pixels defined by MW and to a 2-D protein array consisting of 75 pixels defined by MW and pI range [22]. The detailed annotations of identified proteins and construction of the database on the web is now accessible on the web (http://www.hkupp.org/).

Collecting Duct Proteome

Knepper and colleagues [23–25] have conducted qualitative and quantitative analyses of proteome of the inner medullary collecting duct cells and its changes in response to vasopressin in rats. They identified 848 proteins by two-dimensional difference gel electrophoresis (2-D DIGE) [23] and LC-MS/MS analysis [24, 25], and have also created such a database (http://cddb.nhlbi.nih.gov/cddb/) [26]. The identified proteins are divided into 25 categories based on their molecular functions and searchable by protein name, its abbreviation and alternate name through filter of a public domain database such as OMIM, GeneCards, rat genome database, and mouse genome informatics. Additionally, each entry of the identified proteins is linked to a PubMed search, Entrez Gene, OMIN, GeneCards, Rat Genome database, and Mouse genome database to retrieve the detailed information of a protein.

Urine Proteome Database

Urinary proteomics is becoming an important field by virtue of its non-invasiveness, especially when studies are conducted in human subjects. An enormous number of proteins and peptides can be detected in human urine. They are derived from a variety of sources including glomerular filtration of blood plasma, cell and plasma membrane shedding, proteolytic cleavage of cell surface proteins, and secretion of exosomes [27–30]. Although a lot of studies on urine proteome in the normal and diseased states has been conducted using 2-DE, multidimensional LC-MS/MS, capillary electrophoresis coupled with MS (CE-MS), and other methods, databases of urine proteome are very limited possibly due to variability of urine proteome depending on age, gender,

physiological and pathophysiological conditions, and sampling and storage conditions. We here describe the two urine proteome databases now available on the web.

Urine Proteome

Adachi et al. [31] have recently reported a comprehensive proteomic analysis of normal urine obtained from 10 healthy volunteers. They employed prefractionation of urinary proteins by SDS-PAGE and RP-HPLC in combination with protein identification by high-performance, high-resolution LC-MS/MS instruments such as LTQ-FT and LTQ-Orbitrap with two consecutive stages of mass spectrometric fragmentation (MS^3). They identified 1,543 proteins and essentially eliminated false-positive identification. Analysis of urine proteome based on the identified proteins revealed that nearly half of the proteins were membrane proteins according to GO annotations. Furthermore, extracellular, lysosomal, and plasma membrane proteins were enriched in the urine proteome. The database is deposited on the website as one of the Max-Planck Unified (MAPU) proteome databases (http://www.mapuproteome.com/urine/). Annotations of identified protein are relatively simple, consisting of accession numbers of IPI, Swiss-Prot and NCBI protein databases, gene symbol, MW, protein name, and number of the matched peptides. The respective entry can be searched by IPI accession number, protein name, description, and peptide sequence. In addition, the BLAST search engine using protein sequence as an inquiry is available on the website.

Urinary Exosome Proteome

Urine contains relatively high-density solid materials consisting mainly of shedded epithelial cells or casts, small fragments of membranes derived by shedding of microvilli or by apoptosis. These solid materials can be removed by centrifugation at low to moderate speed. Pisitkun et al. [32] found the existence of urinary exosomes derived from virtually every epithelial cell type facing the urine, including glomerular podocytes, renal tubular epithelial cells, and transitional epithelial cells lining the urinary tract. The exosomes are low-density vesicles and can be recovered in purified forms by differential centrifugation. A comprehensive analysis of exosome proteome from healthy volunteers using LC-MS/MS identified numerous protein components of multivesicular bodies and of the endosomal pathway, in addition to cytoplasmic proteins. These findings suggest that the exosome is the potential source of biomarker discovery [30]. They have so far identified 304 proteins in the exosome preparation and created a database available on the web (http://dir.nhlbi.nih.gov/papers/lkem/exosome/). Annotations of the exosome proteome include protein name, accession number of the RefSeq protein database, gene name, subcellular localization

and the number of matched peptides. Currently, users cannot use the search engine on the web, and only browsing of the identified proteins is allowed.

Summary and Outlook

The completion or almost completion of genome sequences of many organisms and the tremendous development of MS towards analysis of proteins paved way to identification of a large number of proteins in complex mixtures. As the public domain databases of genes, mRNAs, and proteins are now essential for most of investigators in various biological fields, construction of databases of particular proteomes are obviously useful to obtain essential information of a protein under investigation and to gain insight into a proteome of interest. A large number of proteomic studies aiming at comprehensive analysis of whole organisms, body fluids, organs, tissues, cells, cellular organelles, functional protein complexes or posttranslational modifications, such as phosphorylation and glycosylation, have been conducted to produce valuable resources that can be retrieved and shared among several researchers. Under these circumstances, construction of the database of a particular proteome will surely contribute to development of biological research. A certain number of proteome databases of kidney and urine have been recently available on the web, and can provide useful information for further in-depth analyses. Although extensive studies have been conducted aiming at comprehensive analysis of kidney and urine proteomes or their subproteomes, the number of identified proteins is still limited (not covering the whole proteome) obviously due to the heterogeneity, variability, and the wide dynamic range of these proteomes, especially when the studies are conducted using human samples. Complete profiling of a given proteome is difficult and still challenging, and awaits further development of technologies for protein/peptide separation and identification.

References

1 Aebersold R, Mann M: Mass spectrometry-based proteomics. Nature 2003;422:198–207.
2 Sadygov RG, Cociorva D, Yates JR III: Large-scale database searching using tandem mass spectra: looking up the answer in the back of the book. Nat Methods 2004;1:195–202.
3 Peng J, Gygi SP: Proteomics: the move to mixtures. J Mass Spectrom 2001;36:1083–1091.
4 Nucleic Acids Res, Database Issue, 2007;35.
5 Görg A, Weiss W, Dunn MJ: Current two-dimensional electrophoresis technology for proteomics. Proteomics 2004;4:3665–3685.
6 Okano T, Kondo T, Kakisaka T, Fujii K, Yamada N, Kato H, Nishimura T, Gemma A, Kudoh S, Hiroshi S: Plasma proteomics of lung cancer by a linkage of multi-dimensional liquid chromatography and two-dimensional difference gel electrophoresis. Proteomics 2006;6:3938–3948.

7 Washburn MP, Wolters D, Yates JR: Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat Biotechnol 2001;19:242–247.
8 Zhang Y, Zhang Y, Adachi J, Olsen JV, Shi R, de Souza G, Pasini E, Foster LJ, Macek B, Zoungman A, Kumar C, Wisniewski JR, Jun W, Mann M: MAPU: Max-Planck unified database of organellar, cellular, tissue and body fluid proteomes. Nucleic Acids Res 2007;35:D771–D779.
9 Tang HY, Ali-Khan N, Echan LA, Levenkova N, Rux JJ, Speicher DW: A novel four-dimensional strategy combining protein and peptide separation methods enables detection of low-abundance proteins in human plasma and serum proteomes. Proteomics 2005;5:3329–3342.
10 Publication guideline for the analysis and documentation of peptide and protein identifications. Mol Cell Proteomics 2006; available on the journal website (http://www.mcponline.org).
11 Qian WJ, Liu T, Monroe ME, Strittmatter EF, Jacobs JM, Kangas LJ, Petritis K, Camp DG II, Smith RD: Probability-based evaluation of peptide and protein identifications from tandem mass spectrometry and SEQUST analysis: the human proteome. J Proteome Res 2005;4:53–62.
12 Orchard S, Hermjakob H, Apweiler R: Annotating the human proteome. Mol Cell Proteomics 2005;4:435–440.
13 Mi H, Guo N, Kejariwal A, Thomas PD: PANTHER version 6: protein sequence and function evolution data with expanded representation of biological pathways. Nucleic Acids Res 2007;35: D247–D252.
14 Gough NR: Science's signal transduction knowledge environment: the connections maps database. Ann NY Acad Sci 2002;971:585–587.
15 Kanehisa M, Goto S, Hattori M, Aoki-Kinoshita KF, Itoh M, Kawashima S, Katayama T, Araki M, Hirakawa M: From genomics to chemical genomics: new developments in KEGG. Nucleic Acids Res 2006;34:D354–D357.
16 Capsi R, Roerster H, Fulcher CA, Hopkinson R, Ingraham J, Kaipa P, Krummenacker M, Paley S, Pick J, Rhee SY, Tissier C, Zhang P, Karp PD: A multiorganism database of metabolic pathways and enzymes. Nucleic Acids Res 2006;34:D511–D516.
17 Fukuda KI, Yamagata Y, Takagi T: FREX: a query interface for biological processes with hierarchical and recursive structure. In Silico Biol 2004;4:63–79.
18 Joshi-Tope G, Gillespie M, Vastrik I, D'Eustachio P, Schimidt E, de Bono B, Jassal B, Gopinath GR, Wu GR, Matthews L, Lewis S, Birney E, Stain L: Reactome: a knowledgebase of biological pathways. Nucleic Acids Res 2005;33:D428–D432.
19 Zeeberg BR, Feng W, Wang G, Wang MD, Fojo AT, Sunshine M, Narasimhan S, Kane DW, Reinhold WC, Lababidi S, Bussey KJ, Riss J, Barrett JC, Weinstein JN: GoMiner: a resource for biological interpretation of genomic and proteomic data. Genome Biol 2003;4:R28.
20 Lim MS, Elenitoba-Johonson KSJ: Mass spectrometry-based proteomic studies of human anaplastic large cell lymphoma. Mol Cell Proteomics 2006;5:1787–1798.
21 Yoshida Y, Miyazaki K, Kamiie J, Sato M, Okuizumi S, Kenmochi A, Kamijo K, Nabetani T, Tsugita A, Xu B, Zhang Y, Yaoita E, Osawa T, Yamamoto T: Two-dimensional electrophoretic profiling of normal human kidney glomerulus proteome and construction of an extensible markup language (XML)-based database. Proteomics 2005;5:1083–1096.
22 Miyamoto M, Yoshida Y, Taguchi I, Tasaki M, Osawa T, Kimura K, Yaoita E, Yamamoto T: Comprehensive proteomic analysis of normal human kidney glomerulus with two-dimensional protein prefractionation in combination with nanoflow LC-MS/MS. J Am Soc Nephrol 2006;17: 434A.
23 Hoffert JD, van Balkom BW, Chou RA, Knepper MA: Application of difference gel electrophoresis to the identification of inner medullary collecting duct proteins. Am J Physiol Renal Physiol 2004;286:F170–F179.
24 Pisitkun T, Bieniek J, Tchapyjnikov D, Wang G, Wu WW, Shen RF, Knepper MA: High throughput identification of IMCD proteins using LC-MS/MS. Physiol Genomics 2006;25: 263–276.
25 Yu MJ, Pisitkun T, Wang G, Shen RF, Knepper MA: LC-MS/MS analysis of apical and basolateral plasma membranes of rat renal collecting duct cells. Mol Cell Proteomics 2006;5:2131–2145.
26 Legato J, Knepper MA, Star RA, Mejia R: Database for renal collecting duct regulatory and transporter proteins. Physiol Genomics 2003;13:179–181.
27 O'Riordan E, Goligorsky MS: Emerging studies of the urinary proteome: the end of the beginning. Curr Opin Nephrol Hypertens 2005;14:579–585.

28 Thongboonkerd V, Malasit P: Renal and urinary proteomics: current applications and challenges. Proteomics 2005;5:1033–1042.
29 Schiffer E, Mischak H, Novak J: High resolution proteome/peptidome analysis of body fluids by capillary electrophoresis coupled with MS. Proteomics 2006;6:5615–5627.
30 Pisitkun T, Johnstone R, Knepper MA: Discovery of urinary biomarkers. Mol Cell Proteomics 2006;5:1760–1771.
31 Adachi J, Kumar C, Zhang Y, Olsen JV, Mann M: The human urinary proteome contains more than 1,500 proteins, including a large proportion of membrane proteins. Genome Biol 2006;7:R80.
32 Pisitkun T, Shen RF, Knepper MA: Identification and proteomic profiling of exosomes in human urine. Proc Natl Acad Sci USA 2004;101:13368–13373.

Yutaka Yoshida, PhD
Department of Structural Pathology, Institute of Nephrology
Graduate School of Medical and Dental Sciences, Niigata University
Niigata 951-8510 (Japan)
Tel. +81 25 227 2152, Fax +81 25 227 0768, E-Mail yyoshi@med.niigata-u.ac.jp

Author Index

Subject Index