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Proteome Analysis and Tissue Microarray for Profiling Protein Markers Associated with Lymph Node Metastasis in Colorectal Cancer

Haiping Pei,[†,#] Hong Zhu,[‡,#] Shan Zeng,[§,#] Yixiong Li,[*,†] Huixiang Yang,[*,||] Liangfang Shen,[‡] Jia Chen,[‡] Liang Zeng,[⊥] Jianghong Fan,[○] Xiaogang Li,[†] Yuewen Gong,[○] and Hong Shen[*,§]

Department of General Surgery, Department of Oncology, Department of Gastroenterology, and Medical Research Center, Xiangya Hospital, Central South University, Changsha, Hunan, China 410008, Department of Pathology, Tumor Hospital of Hunan Province, Changsha, Hunan, China 410006, and Faculty of Pharmacy, University of Manitoba, Winnipeg, Manitoba, Canada, R3T 2N2

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Background: Understanding the proteins associated with lymph node metastasis (LNM) in colorectal cancer (CRC) will benefit us in the prediction of CRC prognosis and provide us new potential targets in the intervention of CRC. The aim of this study is to investigate the LNM-associated proteins and to evaluate the clinicopathological characteristics of these target proteins' expression in CRC. Methods: Fresh tumor and paired normal mucosa from five cases for each group of non-LNM CRC and LNM CRC were analyzed by two-dimensional electrophoresis coupled with MALDI-TOF−MS, followed by Western blotting confirmation. In 40 paraffin-embedded CRC samples, each for non-LNM CRC and LNM CRC, four differentially expressed proteins identified by proteomics analysis were detected by tissue microarray with immunohistochemistry staining to access the clinicopathological characteristics of these proteins in LNM of CRC. Results: Twenty-five proteins were found to be differentially expressed between normal mucosa and CRC tissue. Increased expression levels of heat shock protein-27 (HSP-27), glutathione *S*-transferase (GST), and Annexin II, but a decreased expression level of liver-fatty acid binding protein (L-FABP), existed in LNM CRC as compared with non-LNM CRC ($p < 0.01$ or $p < 0.05$, respectively). Conclusion: The techniques of proteomic analysis combined with tissue microarray provide us a dramatic tool for screening of LNM-associated proteins in cancer research. The increased expression of HSP-27, GST, and Annexin II, but decreased expression of L-FABP, suggests a significantly elevated incidence of LNM in CRC.

Keywords: colorectal cancer • lymph node metastasis • proteome analysis • tissue microarray

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide in both men and women, especially in aging populations.[1] The prognosis of patients with CRC is clearly related to the presence or absence of lymph node involvement and metastasis. However, the mechanisms related to lymph node metastasis (LNM) in CRC are not fully clarified because LNM involves multiple steps and requires an accumulation of altered expression of many different proteins. Identification of specific protein signatures associated with LNM in CRC may provide novel biomarkers that allow more accurate prognostic information and may help to identify new molecular therapeutic targets, provide clues for understanding the molecular mechanisms governing CRC progression, as well as benefit the patients with an early detection of LNM.

Biomarker searching and tumor profiling in CRC have been carried out in recent years using DNA microarray,[2,3] but alterations in the proteome may reflect cellular changes more accurately since proteins are the actual mediators of intracellular processes as opposed to mRNAs. An identification of LNM-associated proteins in CRC has yet to produce reliable candidate markers. A lack of the study on proteomic profiles of LNM in CRC hinders our efforts to understand and cure this cancer. The recent application of two-dimensional electrophoresis (2-DE) coupled with mass spectrometry (MS) to the study of CRC allows the characterization of global alterations in protein expression in CRC development.[4,5] These high-throughput methods have been widely used for separating and quantifying many types of proteins from the same extracts. It has been understood that there are a series of different proteins

* To whom corespondence should be addressed at Xiangya Hospital Central South University, Changsha, Hunan, China 410008. For H.S.: e-mail, hongshen2000@yahoo.com; tel, 86-731-432-7298; fax, 86-731-4327322. For H.Y.: e-mail, yang_hx430@163.com; tel, 86-731-432-8398; fax, 86-731-4327322. For Y.L.: e-mail, liyixiong6@hotmail.com; tel, 86-731-4327021; fax, 86-731-4327322.

† Department of General Surgery, Xiangya Hospital.
‡ Department of Oncology, Xiangya Hospital.
§ Medical Research Center, Xiangya Hospital.
# These authors contributed equally to this study.
|| Department of Gastroenterology, Xiangya Hospital
⊥ Tumor Hospital of Hunan Province.
○ University of Manitoba.

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involved in the process of LNM in CRC. With the advantage of being non-prejudicial, proteomic analysis can give new unexpected insights into the mechanisms of carcinogensis and LNM in CRC. Tissue microarray represents a unique tool to analyze a certain protein expression of many specimens simultaneously.[6]

Employing the technologies of 2-DE/MS and tissue microarray in combination, the aim of this study is to investigate the LNM-associated proteins and to evaluate the clinicopathological characteristics of CRC with these target proteins' expression, so as to better understand the mechanisms underlying CRC prognosis.

Patients and Methods

Patients and Tissue Preparation. For 2-DE/MS proteome analysis and Western blotting, 10 cases of moderately differentiated colorectal adenocarcinoma (6 males and 4 females, aging 23−75 years, mean ± SD = 51.2 ± 12.6 years) who underwent radical resection from June 2004 to June 2005 were randomly collected from the Department of General Surgery, Xiangya Hospital, Central South University, China. The CRC samples of moderately differentiated adenocarcinoma with TNM staging from I to IV were divided into two groups of non-LNM and LNM with 5 cases in each group. The LNM rates in the group of LNM were 50% (10/20), 36.4% (8/22), 46.7% (14/30), 25% (5/20), and 13.3% (2/15), respectively. There was no LNM found in the subjects of the non-LNM group. The study was approved by the Research Ethics Committee of Central South University, China. Informed consent was obtained from all of the patients. Fresh colorectal tumor tissues and paired normal tissues located at least 15 cm away from the tumor were obtained immediately after the surgery, washed twice with chilled PBS, and stored in liquid nitrogen and then to −80 °C until use. The protein from a total of 50 mg of frozen tissue was extracted by homogenization in lysis buffer (4% CHAPS, 2 M thiourea, 7 M urea, 2% NP-40, 1% Triton X-100, 100 mM DTT, 5 mM PMSF, 0.5 mM EDTA, 2% Pharmalyte, 1 mg/mL DNase I, 0.25 mg/mL RNase A, and 40 mM Tris-HCl, pH 8.5). Following incubation at room temperature for 2 h, the lysate was centrifuged at 15 000 rpm for 30 min at 4 °C. The supernatant was precipitated with the TCA sediment method to recover the total protein, and the interfering components were removed using 2-D Clean-Up Kit (GE Healthcare, Piscataway, NJ).

With regard to the tissue microarray and clinicopathological analysis, 80 cases of CRC samples (46 males and 34 females, aging 25−76 years, mean ± SD = 52.2 ± 10.2 years, TNM staging from I to IV) were randomly selected from the Department of Pathology, Tumor Hospital of Hunan Province, China. The method of completely random design was employed for patients selecting and grouping in this retrospective study. In total, 396 cases of moderately differentiated colorectal adenocarcinoma CRC cases received radical resection from October 2002 to October 2005 in Tumor Hospital of Hunan Province; these were divided into two groups of non-LNM CRC ($n = 139$) and LNM CRC ($n = 257$) based on the occurrence of LNM. Within each group, the 10% formalin-fixed and paraffin-embedded CRC specimens were randomly numbered as 1−139 and 1−257, respectively. Randomization was performed using a random number table, and a random number was chosen from this table specifically for each case. The first 40 subjects with odd numbers in individual group, with the respectively paired normal tissue of clear margin at least 15 cm away from the tumor, were respectively assigned to non-LNM CRC and LNM CRC groups. All the patients recruited in this study received neither chemotherapy nor radiotherapy before the surgery. All tissues were examined by the same group of two senior pathologists experienced in CRC diagnosis. All specimens were handled and made anonymous according to the ethical and legal standards.

IPG-2-DE and Image Analysis. A total of 400 μg of total protein was loaded onto a 240 mm linear IPG strip (pH3−10, Amersham Biosciences, Piscataway, NJ) for first-dimensional isoelectric focusing (IEF). Protein separation in the second dimension SDS-PAGE (Bio-Rad, Hercules, CA) was carried out following the instructions of the manufacturer. After electrophoresis, the gels were stained with silver nitrate and scanned by an Imagescanner (Amersham Biosciences). The software of PD-Quest 7.3.1 (Bio-Rad) was employed for the image analysis, including background abstraction, spot intensity calibration, spot detection, and matching. The intensity of each spot was quantified by a calculation of spot volume after normalization of the gel image. Each sample was performed in triplicate, and paired Student's t test was used to evaluate the average protein abundance change corresponding to each target spot across the gels.

In-Gel Trypsin Digestion of Target Protein. Fifty differential spots between the groups of normal colorectal mucosa and CRC were excised from the gels and digested in-gel as previously described.[7] Briefly, the gel spots were destained in 100 mM $Na_2S_2O_3$ and 30 mM $K_3Fe(CN)_6$ (v/v = 1:1), incubated at 57 °C for 1 h in the reduction buffer (100 mM NH_4HCO_3 and 10 mM DTT), and subsequently alkylated in the alkylation buffer (100 mM NH_4HCO_3 and 55 mM iodoacetamide) in the dark for 30 min at room temperature. The vacuum centrifugation-dried gel pieces were digested for 24 h at 37 °C in the solution containing 50 mM NH_4HCO_3, 5 mM $CaCl_2$, and 0.1 g/L TPCK-trypsin (Sigma-Aldrich, St. Louis, MO).

MALDI-TOF−MS. The tryptic peptide mixture was extracted three times with 50% ACN and 0.1% TFA and dried in a vacuum centrifugation, dissolved in 0.1% TFA, and desalted with a C18 ZipTip column (Millipore, Billerica, MA). The eluted peptides were analyzed using a Voyager-DETM STR Biospectrometry Workstation System 4307 (Applied Biosystems, Foster City, CA) in positive ion-reflector mode. The parameters were set up as follows: extraction voltage. 20 kV; grid voltage, 64.5%; mirror voltage ratio, 1.12; N_2 laser wavelength, 337 nm; pulse width, 3ns; acquisition mass range, 1000−3000 Da; delay, 100 ns; and vacuum degree, 4×10^{-7} Torr. Mass spectra were recorded using 50−200 shots, depending on the signal-to-noise ratio obtained from each sample. All mass spectra were externally calibrated with a standard peptide mixture (MH+: Angiotensin II. 1046.5420 Da; Angiotensin I. 1296.6853 Da; ACTH clip 18−39. 2465.199 Da). The auto digestion peaks of trypsin were served for the internal calibration. A list of the corrected mass peaks was the peptide mass fingerprinting (PMF).

Protein Identification and Database Analysis. Protein identification using PMF was performed by MASCOT Distiller (http://www.matrixscience.com/, Matrix Science Ltd, U.K.) against the NCBI nonredundant protein database (http://www.ncbi.nlm.nih.gov/). The searching parameters were set up as follows: the taxonomy was *Homo sapiens*, the enzyme was trypsin, the number of missed cleavage sites was allowed up to 1, the fixed modification was carbamidomethylation of cysteine, the variable modification was oxidation of methionine, the peptide tolerance was 50 ppm, the mass value was MH^+,

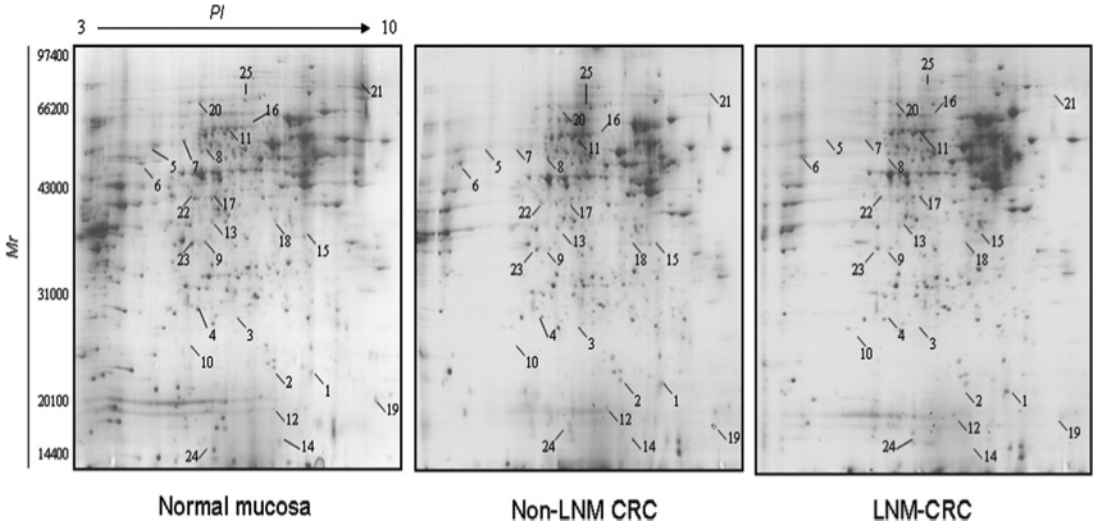

Figure 1. 2-DE maps of normal mucosa, non-LNM CRC, and LNM CRC tissues. The numbered spots represent the differentially expressed proteins between the groups of normal mucosa and CRC.

searching range within the experimental p*I* value ± 0.5 pH unit and experimental mass range (M_r) ± 20%. The criteria for positive identification of proteins were set as follows: (1) the MS match consisted of a minimum of five peptides; (2) the matched peptides covered at least 20% of the whole protein sequence; (3) the MASCOT score of a protein matching was higher than 63 ($p < 0.05$).

Western Blotting. In total, 50 μg of total protein was run on a 10% sodium dodecyl sulfate-polyacrylamide (SDS-polyacrylamide) gel under reducing conditions; the transferred membranes were incubated overnight at 4 °C with mouse monoclonal antibodies against human heat shock protein-27 (HSP-27), glutathione *S*-transferase (GST), Annexin II, β-actin (Santa Cruz Biotechnology. Inc., Santa Cruz, CA), and liver-fatty acid binding protein (L-FABP) (Cell Sciences, Canton, MA) at 1:1000 dilutions, respectively. They were subsequently incubated with HRP-conjugated Sheep anti-mouse IgG (Amersham Biosciences) at 1:1000 dilutions. Bands were visualized by employing the enhanced chemiluminescence (ECL) method.[8]

Tissue Microarray and Immunohistochemistry. The CRC tissue fragments were sampled by pathologists from the dense tumor areas or the respectively paired normal tissue. Tissue microarray was performed using an ATA-27 automated arrayer (Beecher Instruments, Inc., Sun Prairie, WI). Three punches of 600 μm in diameter were assessed for each sample in appropriate areas selected on H&E paraffin standard sections. The 5-μm tissue microarray slides were deparaffinized with xylene and ethanol for further H&E or peroxidase (DAB) immunohistochemistry staining employing DAKO EnVision System (Dako Diagnostics, Zug, Switzerland). Antigen retrieval was carried out using 6.5 mM citrate buffer (pH 6.0) pressure-cooking, and endogenous peroxidase activity was blocked with 2.5% hydrogen peroxide in methanol for 30 min at room temperature.[9] The slides were incubated with the primary antibody against respective target protein at a dilution of 1:1000 overnight at 4 °C. Peroxidase-labeled polymer and substrate-chromogen were then employed to visualize the staining of the interested protein.[10] Following a hematoxylin counterstaining, immunostaining was scored by two independent experienced pathologists. The results of the two reviewers were compared. and any discrepant scores were re-examined by both pathologists to achieve a consensus score. Given the homogenicity of the target proteins' staining, tumor specimens were scored in a semiquantitative manner using the following scale: 0 = less than 10% positive staining cells; 1 = 10−25%; 2 = 25−50%; 3 = more than 50% (negative/weak/moderate/intense staining). Cases with scores of 2+ or 3+ were designated as "positive", whereas cases with scores of 0 or 1+ were designated as "negative".[11] The lymphatic vessels density in primary CRC tissues were stained and detected by enzyme histochemical method as described previously.[12] Meanwhile, the depth of lymphatic permeation and the existence of tumor cell emboli in lymphatic vessels were observed under the light microscope.

Statistical Analysis. The data was analyzed using χ^2 test with StatView software (version 5.0, SAS Institute, Inc. Cary, NC) to evaluate the clinicopathological correlation between the LNM and the target proteins' expression in CRC. Differences were considered to be significant when the p value was less than 0.05.

Results

Proteome Differential Expression among LNM, non-LNM of CRC, and Normal Colorectal Mucosa Tissue. Compared with the control of normal tissue, the groups of LNM and non-LNM had average matching rates of 89.7% and 86.1%, and differentially expressed protein spots of 72.0 ± 12.3 and 64.0 ± 9.5, respectively. The number of differential protein expression spots was 15.0 ± 4.5 between LNM and non-LNM groups (Figure 1). There were 50 differential spots that simultaneously existed in both LNM and non-LNM CRC groups compared with the paired normal tissue, including 10 differential spots in LNM CRC as compared with non-LNM CRC. All of these spots were excised from gels and analyzed using M_r, p*I*, the sequence coverage for protein identification, and MASCOT scores for protein matching (score > 63, $p < 0.05$). Compared with the normal tissue, 25 spots were identified to be differentially expressed in CRC tissue (Table 1). Compared with non-LNM CRC, LNM CRC had four differentially expressed protein spots, which were identified as HSP-27, GST, Annexin II, and L-FABP, respectively. Figure 2 showed the PMF of protein spots 2, 10, 18, and 24 representing HSP-27, GST, Annexin II, and L-FABP, respectively.

Table 1. The 25 Characterized Differentially Expressed Proteins in CRC Tissue Compared to the Paired Normal Colorectal Tissue[a]

no.	GenBank	protein description	MASCOT score	theortical M_r (kDa)/pI	calculated M_r (kDa)/pI	sequence covered
1	TAGL_HUMAN	Transgelin	88	22.52/8.88	25.52/8.00	63%
*2	E980237	HSP−27	95	22.43/7.83	26.52/7.15	67%
3	TPIS_HUMAN	Triosephosphate isomerase	113	26.81/6.51	28.96/6.10	50%
4	CAA00975	APOA1 PROTEIN	161	28.06/5.27	29.74/5.09	73%
5	A26561	Tubulin beta chain	108	49.73/4.75	50.22/4.92	52%
6	A37047	Calreticulin precursor	102	48..11/4.29	48.13/4.72	49%
7	A25074	Vimentin	157	53.62/5.06	52.77/5.03	76%
8	K2C8	Cytokeratin 8	169	53.51/5.52	51.97/5.33	68%
9	1FZEC	fibrinogen fragment d, chain C	80	34.46/5.68	36.25/5.25	69%
*10	A37378	Glutathione S−transferase	80	23.34/5.43	27.52/5.07	62%
11	KRHU2	Keratin 1	66	65.45/6.03	62.95/6.04	30%
12	1AVOB	11S regulator chain B	81	16.34/7.14	17.34/6.84	34%
13	A42077	Annexin IV	70	36.06/5.84	39.25/5.37	29%
14	AAD20503	Immunoglobulin heavy chain	65	13.00/7.88	15.35/7.45	55%
15	ALDOA_HUMAN	Fructose−bisphosphate aldolase	81	39.06/8.39	38.62/7.95	32%
16	ENOA_HUMAN	Alpha enolase	96	47.01/6.99	62.90/6.59	27%
17	AAH01166	BC001166 NID	82	43.35/6.04	42.58/5.44	30%
*18	ANXA2_HUMAN	Annexin II	125	38.45/7.56	39.64/7.15	43%
19	JE0350	Anterior gradient−2	73	19.97/9.03	20.51/8.41	42%
20	AAA64922	HSU22961 NID	99	52.05/5.69	65.97/5.23	27%
21	Q1HBA5_HUMAN	transferrin precursor	198	79.28/6.81	79.31/6.97	48%
22	Q9UMN3_HUMAN	ACTB protein	92	40.19/5.55	42.19/5.64	44%
23	Q96HG5_HUMAN	Actin, beta	91	40.98/5.56	36.44/5.16	43%
*24	FZHUL	Liver fatty acid−binding protein	65	14.26/6.6	13.52/5.30	74%
25	Q53HU0-HUMAN	Chaperonin containing TCP1	76	57.94/6.46	66.34/6.22	32%

[a] The protein numbers with "*" represents the proteins differentially expressed between non-LNM CRC and LNM CRC tissue.

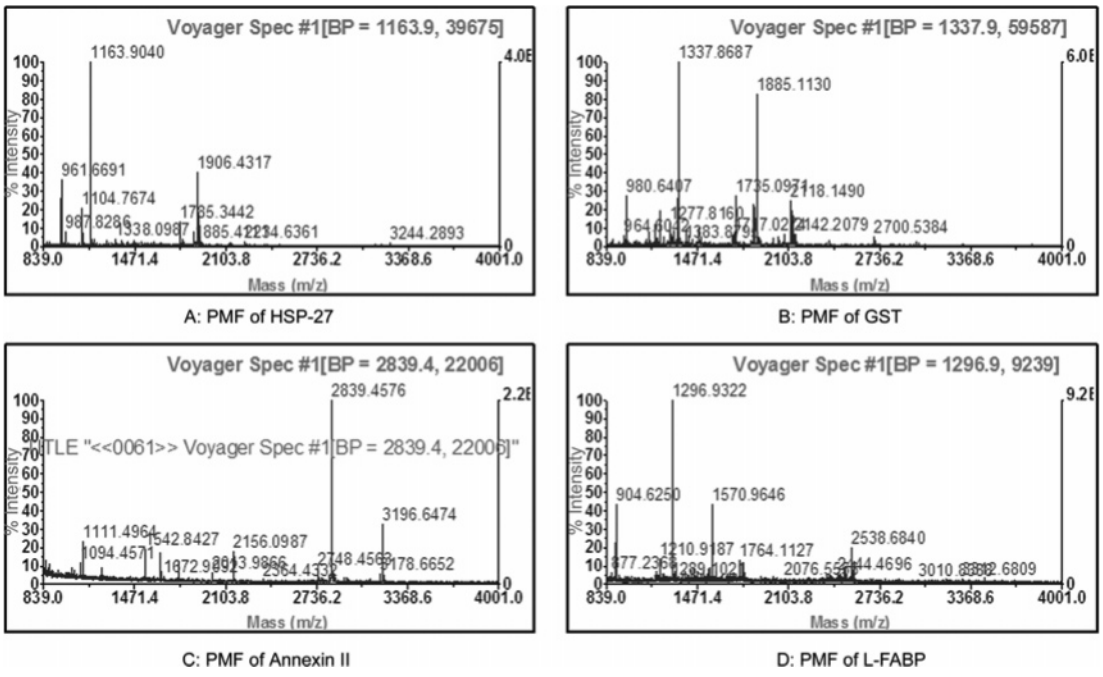

Figure 2. PMF of protein spots 2, 10, 18, and 24, respectively, representing HSP-27 (panel A), GST (panel B), Annexin II (panel C), and L-FABP (panel D), which are expressed differentially between the groups of LNM and non-LNM CRC (MASCOT scores are 95, 80, 125, and 65, respectively, $p < 0.05$). Ions score is $-10^*\text{Log}(P)$, where P is the probability that the observed match is a random event.

Confirmatory Studies of Differentially Expressed Proteins by Western Blotting. Western blotting results confirmed our MALDI-TOF−MS findings that the expression of three proteins, HSP-27, GST, and Annexin II, was significantly higher in LNM CRC tissue than that in the non-LNM CRC group, while the normal tissue had the lowest proteins' expression. However, the lowest and highest expression of L-FABP was, respectively, found in LNM-CRC and normal colorectal mucosa. A representative Western blotting result was presented in Figure 3.

Tissue Microarray, Clinicopathological, and Immunohistochemistry Analysis in LNM and Non-LNM CRC. The intratumoral lymphtic vessels were strip-like. The mean lymphatic vessels density of LNM CRC (23.42 ± 6.89) was significantly higher than that of non-LNM CRC (8.26 ± 2.16) ($p < 0.01$). The average infiltration depth of lymphatic vessels in cases of LNM CRC (3.55 ± 0.67 mm) was significantly higher than that of non-LNM CRC (1.06 ± 0.21 mm) ($p < 0.01$). The percentage of the existence of tumor cell emboli in lymphatic vessels was 87.5% (35/40) in LNM CRC and 17.5% (7/40) in non-LNM CRC, respectively.

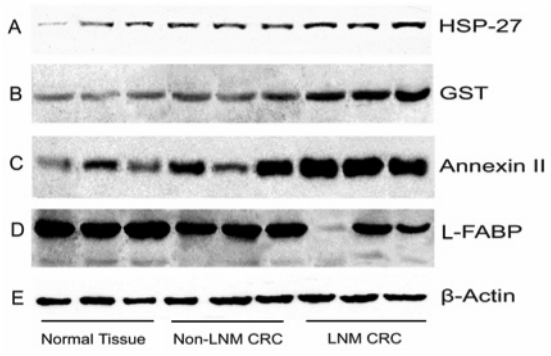

Figure 3. Protein expression of HSP-27, GST, Annexin II, and L-FABP in normal tissue, non-LNM CRC, and LNM CRC and normal tissue identified by Western blotting. There are three representative samples in each group, which demonstrate that the expression levels of HSP-27, GST, and Annexin II significantly increase in LNM CRC group, while the expression level of L-FABP significantly decreases in LNM CRC tissues. β-Actin is used as the internal loading control.

A representative immunohistochemistry staining of different proteins in individual study group was presented in Figure 4. In the groups of LNM CRC, non-LNM CRC, and normal tissue, the positive rates of HSP-27 expression were 90.0%, 50%, and 5%; the positive rates of GST expression were 90%, 57.5%, and 25%; the positive rates of Annexin II expression were 82.5%, 50%, and 15%; the positive rates of L-FABP expression were 30%, 60%, and 87.5%, respectively. The statistical analysis demonstrated that LNM CRC group had significantly higher positive expression rates of HSP-27, GST, and Annexin II, but a significantly lower positive expression rate of L-FABP than non-LNM CRC group (Table 2, $p < 0.01$ or $p < 0.05$, respectively).

Discussion

In the present study, 25 proteins are identified to be differentially expressed between CRC tissues and normal colorectal mucosa. To some extent, this result is consistent with the data reported by other groups, who listed several proteins involved in intracellular fatty acid binding and chromatin binding (L-FABP), toxin catabolism and water deprivation (GST), protein synthesis and folding (heat shock proteins), cell communication and signal transduction (Annexin IV), and cellular reorganization and cytoskeleton (cytokeratins, vimentin, beta actin) in the proteomic profiles of CRC cell line Caco-2 and CRC tissue. However, there are still some different proteins between our data and other researchers' reports.[4,13,14] We consider that 2-DE and MALDI-TOF−MS-based PMF analysis for human tissue is more complex than that for cell line. Meanwhile, 2-DE gels have extremely numerous biological information which is hard to be fully revealed by a single laboratory. A systemic collection and analysis for the sorted complementary data from various research groups will benefit the scientists in making global proteomic profiles of CRC. Moreover, the difference of races and region distributions, as well as the different methods of tissue collection and management, may contribute to the distinction among various laboratories. The methods employed in this study of tissue washing and surface scraping from tissue are important for collecting pure tumor cell population free of contaminating serum proteins, red blood cells, connective tissues, and necrotic tissue materials.[15]

Fifty spots of differentially expressed proteins between normal mucosa and CRC, as well as 10 spots of differentially expressed proteins between non-LNM CRC and LNM CRC, are excised from 2-DE gels to perform subsequent analysis in this study. However, only 50% and 40% of these spots are finally identified, respectively. This identification rate can be caused by a low-to-medium protein abundance of these unidentified spots, as well as the limited sensibility of the mass spectrometer. It is also possible that these unidentified spots may consist of some undiscovered proteins, which cannot be verified by current protein database search. Moreover, current 2-DE system has a limited apparent molecular M_r, where molecules smaller than 10K are generally lost. This has prompted much interest in non-2-DE approaches for studying global protein profiles.[16] As new proteomics techniques with better protein separation and extraction, higher-sensitivity, and specificity are developed, more sorted data will help us to fully understand the protein profiles of CRC. Moreover, advances in the fields of proteomics may someday provide a clinically useful serum test, which could have dramatic implications in improving compliance with LNM screening and decreasing morbidity and mortality of CRC.

The results of Western blotting confirmed our proteomic identification that three proteins including HSP-27, GST, and Annexin II have elevated expression levels, while L-FABP has a decreased expression level in the cases of LNM CRC as compared with non-LNM CRC. The clinicopathological analysis by tissue microarray reveals that the expression levels of these four proteins are significantly associated with the incidence of LNM in CRC and are possibly correlated with the prognosis of CRC. HSP-27 has important biological functions of cellular signal transduction regulating cell proliferation, differentiation, and apoptosis. In human CaCo2 colorectal cancer cell lines, HSP-27 inhibits apoptosis induced by a variety of different stimuli.[17]

Glutathione *S*-transferase-pi overexpression is supposed to be closely associated with K-ras mutation via AP-1 activation during human colon carcinogenesis.[18] The protein expression of both HSP-27 and GST has been found to be related to the carcinogenesis and metastasis of the gastric carcinomas.[19,20] An increase of HSP-27 expression level appears to be associated with parameters of unfavorable prognosis and shorter overall survival of gastric carcinomas,[20] while some evidence indicates that GST measurement may be useful as a tumor marker in gastric, liver, and colorectal cancer. Moreover, a combined determination of GST and other markers increases the detection sensitivity for cancers of human digestive tract.[21] The present study suggests the possible poor prognostic characteristics of HSP-27 and GST positive expression in CRC, because the CRC tissue with increased expression levels of HSP-27 and GST are suspected to have a high incidence of LNM. Although the mechanisms of these proteins' involvement in LNM of CRC have not yet been understood, our findings will attract more interest and efforts to further investigate the roles of HSP-27 and GST in the prognosis of CRC.

The differentiation of human colon adenocarcinoma cell upregulates the expression of Annexin II and alters the subcellular relocation of this protein.[22] Annexin II has been noted to be overexpressed in advanced colorectal carcinoma and considered as an independent factor of poor prognosis in patients with colorectal carcinoma.[23] L-FABP is specially expressed in hepatocytes and enterocytes. It plays a central role in the solubilization and the intracellular compartmentalization of

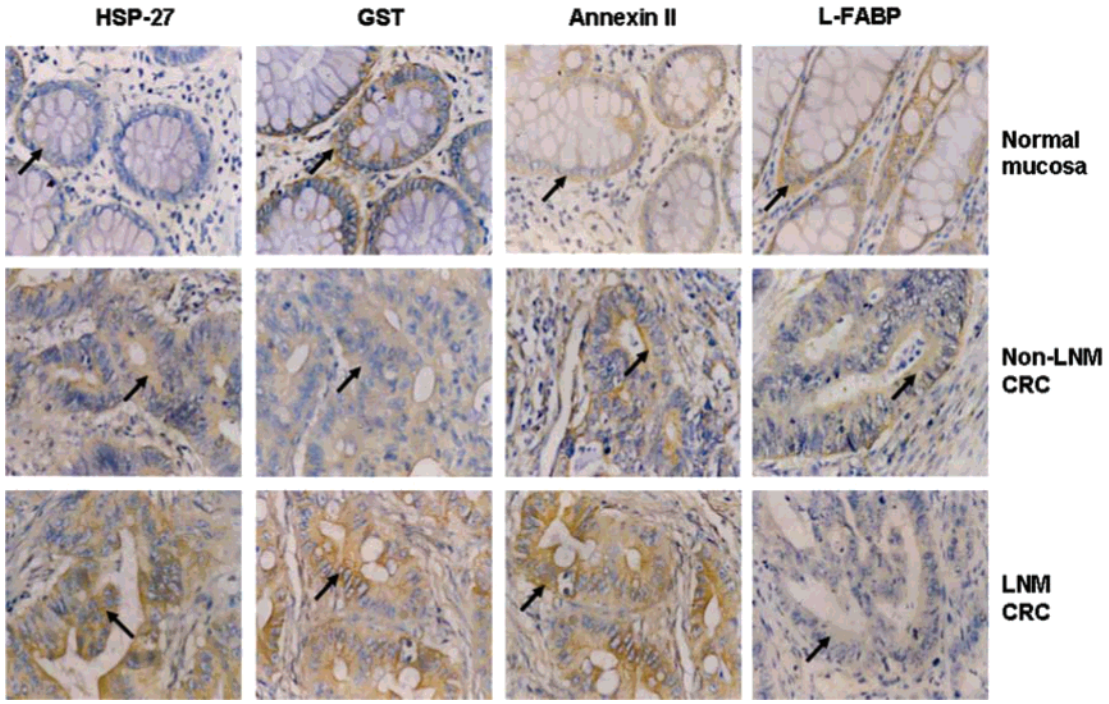

Figure 4. Immunohistochemical detection of HSP-27, GST, Annexin II, and L-FABP in normal mucosa, non-LNM CRC, and LNM CRC. The arrows indicate the target protein staining in brown-yellow color in colorectal glandular epithelium.

Table 2. The Protein Expression of HSP-27, GST, Annexin II, and L-FABP in Normal Mucosa, Non-LNM CRC, and LNM CRC[a]

		HSP−27			GST			Annexin II			L-FABP		
group	case	N	P	rate	N	P	rate	N	P	rate	N	P	rate
Normal	80	76	4	5%	60	20	25%	68	12	15%	10	70	87.5%
Non−LNM	40	20	20	50%	17	23	57.5%	20	20	50%	16	24	60%
LNM	40	4	36	90%	4	36	90%	7	33	82.5%	28	12	30%
χ value			15.238			10.912			9.448			7.273	
p value			<0.01			<0.01			<0.01			<0.05	

[a] $\chi 2$ and p values represent the statistic analysis results between non-LNM CRC and LNM CRC groups. "N" means negative protein expression, "P" means positive protein expression.

fatty acids, thus, involved in the modulation of cell division, cell growth, and differentiation and the protection of cells against the cytotoxic effects of fatty acids by preventing high intracellular fatty acid concentrations.[24] It is reported that patients bearing L-FABP-positive CRC showed better prognosis than those bearing L-FABP-negative CRC.[25,26] L-FABP may be a sensitive marker for differentiating cancer cells, while histologically undifferentiated cancers in the colon and rectum grow rapidly and aggressively.[24,27] The results of our study can directly explain the previously described prognostic roles of Annexin II and L-FABP, which is found by other groups. An increased expression of Annexin II or a decreased expression of L-FABP is associated with LNM in CRC, thus, leading to poor prognosis of CRC. However, the detailed intracellular mechanisms of the association between the LNM of CRC and these proteins' expression of Annexin II and L-FABP remain unknown.

It is interesting that the primary LNM CRC tissues, which exhibit upregulated expression of HSP-27, GST, Annexin II, and downregulated expression of L-FABP, have poorer clinicopathological characteristics than non-LNM CRC. The high density, infiltration depth, and tumor cell emboli of intratumoral lymphatic vessels in LNM CRC will favor the incidence of LNM in CRC patients. This may be a part of the biological ground of the roles of HSP-27, GST, Annexin II, and L-FABP in LNM of CRC. However, the detailed molecular mechanisms of the association between these four proteins and LNM in CRC remain unclear and need to be further investigated.

In conclusion, the techniques of proteomic analysis combined with tissue microarray provide a dramatic tool of screening of LNM-associated proteins in colorectal cancer research. The increased expression of HSP-27, GST, and Annexin II, but decreased expression of L-FABP suggests a significantly elevated incidence of LNM in CRC.

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