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Proteomic Profiling of Proteins Associated With Lymph Node Metastasis in Colorectal Cancer

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ABSTRACT

Lymph node metastasis (LNM) is associated with poor prognosis in colorectal cancer (CRC). The presence or absence of lymph node metastases is a strong independent prognostic factor for CRC survival. Investigation of proteins associated with the process of lymph node metastasis (LNM) is crucial for understanding of the molecular mechanisms underlying the LNM process and for predicting the CRC prognosis. In the present study, proteins from CRC tissues and adjacent normal mucosa (NMC) were examined using two-dimensional gel electrophoresis coupled with MALDI-TOF-MS. The expression levels of Ferritin Heavy Chain (FHC) were decreased in LNM CRC as compared to those in non-LNM CRC, while the expression of Cathepsin D and Ubiquitin C-terminal hydrolase-L1 (UCH-L1) were increased in LNM CRC. The results were confirmed by Western blotting and immunohistochemical staining. Furthermore, in vitro cell invasion assay showed that the overexpression of UCH-L1 through gene transfection increased the invasive ability of HCT8 cells, suggesting that UCH-L1 is not only a biomarker for LNM in CRC, but also a functional protein that may play a significant role in cell migration. The proteins identified in the present study should further our understanding of the LNM process of CRC and may become useful markers for diagnosis and targets for therapeutic interventions. J. Cell. Biochem. 110: 1512–1519, 2010.

KEY WORDS: PROTEOMICS; LYMPH NODE METASTASIS; COLORECTAL CANCER; UCH-L1

Colorectal cancer (CRC) is one of the most common malignant tumors in the world. It causes significant morbidity and mortality, especially in industrialized nations. The morbidity of CRC in China has also risen rapidly in recent years and is the third most common cancers. In 2008, the morbidity rate of CRC grew by 4.2% in China and the estimated number of new CRC cases was up to 400,000. It has been suggested by earlier reports [Newcomb et al., 1992; Selby et al., 1992] that the survival rate decreases while progressing through the stages. A major proportion of patients in China were being diagnosed in the advanced stages and only 10% of patients were diagnosed in T1 stage. As a result, the 5-year survival rate was between 20% and 50%.

One of the most important prognostic factor for predicting the outcome of long-term survival of cancer is whether there is positive lymph nodes or distant metastases. The lymph node metastasis rate in T_3–T_4 stages was much higher than that in T_1–T_2 stages in CRC. The lymph node ratio (ratio of positive over excised lymph nodes) is a highly significant prognostic factor in stage III CRC. Studies by Derwinger et al. [2008] showed that with the increase of lymph node ratios, the 3-year disease-free survival (DFS) dropped sharply from 80% to 30%. However, previous studies has suggested that substantial variations in nodal staging is closely associated with surgical, pathological, and patients factors [Baxter et al., 2005; Chang et al., 2007]. Inadequate lymph node sampling can lead to positive lymph node being missed and thus to patients being misclassified and not being appropriately treated. Therefore, additional markers are much needed to detect lymph node metastasis (LNM) in CRC and to improve systemic treatments.

In recent years, the focus of cancer research has expanded from genomic to proteomic. Proteins are responsible for the functional execution in a diversity of cells. Many regulatory processes and disease processes occur at the protein level, and most drug targets

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are found through protein studies [Kavallaris and Marshall, 2005]. In recent studies, two-dimensional electrophoresis (2D) coupled with mass spectrometry has been successfully applied to identify tumor-associated proteins in CRC [Friedman et al., 2004; Alfonso et al., 2005]. In this study, differentially expressed proteins associated with lymph node metastasis in CRC were examined with 2D electrophoresis associated with MALDI-TOF mass spectrometry. Protein profiles in five LNM CRC tissues and five non-LNM CRC tissues were analyzed and compared using 24 cm pH 3–10 NL range immobilized pH gradient (IPG) strips. Among the proteins that were identified to be differentially expressed in CRC tissues, three proteins associated with LNM were further validated by Western blotting and immunohistochemistry staining. The identification of these proteins may lead to a better understanding of the mechanisms underlying the LNM process and provide potential diagnostic markers for lymph node metastasis in CRC.

MATERIALS AND METHODS

TISSUES

Ten cases of colorectal cancer samples and matching normal mucosa (NMC) were obtained through surgical resections at Cancer Hospital, Chinese Academy of Medical Sciences, Beijing, China. The samples were divided into two groups, non-LNM and LNM, with five cases in each. Clinical and pathologic data of the samples are shown in Table I. Fresh CRC tissues and paired normal tissues were obtained immediately after the surgery and stored in liquid nitrogen and then at −70°C until use.

SAMPLE PREPARATION

Frozen tissues were washed three times with chilled phosphate-buffered saline (PBS). A total of 100 mg of tissue was homogenized in liquid nitrogen and lysed in 1 ml lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% NP-40, 20 mM DTT, 0.1 μg/ml PMFS, 2 μg/ml aprotinin, 1 mM EDTA, 1 mg/ml DNase I, 0.25 mg/ml RNaseA) at room temperature for 2 h, and then centrifuged at 12,000 rpm for 30 min at 4°C. The protein concentration was determined by 2D quant kitTM (GE Healthcare).

TWO-DIMENSIONAL ELECTROPHORESIS

Isoelectric focusing electrophoresis (IEF) was performed using 24 cm IPG dry strips (p*I* range, 3–10 NL; Amersham Biosciences). Briefly, a total of 800 μg proteins were diluted to 450 μl with rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.2% DTT and 0.5% IPG buffer) and rehydrated at room temperature for 12 h. The proteins were focused successively for 1 h at 100 V, 1 h at 500 V, 1 h at 1,000 V, 1 h at 5,000 V and 9 h at 8,000 V to give a total of 72 kVh on IPGphor III (Amersham Biosciences). After protein focusing, IPG strips were equilibrated in solution I [6 M urea, 50 mM Tris–HCl (pH 8.8), 30% glycerol, 2% SDS, 1% DTT] for 15 min, and then for an additional 15 min in solution II [6 M urea, 50 mM Tris–HCl (pH 8.8), 30% glycerol, 2% SDS, 2.5% iodoacetamide]. The second-dimensional SDS–PAGE was carried out with 12% SDS–PAGE gels on an Ettan DALT II system (Amersham Biosciences).

TABLE I. Description of Human Colorectal Cancer Samples Used in 2D Analysis

Group	Patient age	Sex	TNM stage	LNM rate
LNM CRC	51	M	T3N1M0	11% (2/18)
	47	M	T3N1M0	8% (2/24)
	39	M	T3N2M0	57% (4/7)
	61	M	T3N1M0	40% (4/10)
	62	M	T3N1M0	33% (3/9)
Non-LNM CRC	37	F	T3N0M0	0
	44	M	T3N0M0	0
	68	M	T3N0M0	0
	71	M	T3N0M0	0
	70	F	T3N0M0	0

GEL STAINING AND IMAGE ANALYSIS

All gels were stained according to a modified Neuhoff's Cooloidal Coomassie blue G-250 staining method. Gels were fixed in 40% ethanol–10% acetic acid in water for 30 min followed by washing three times in water for 10 min each. Gels were stained with Coomassie blue for 12 h.

To evaluate the spot pattern of each gel, MagicScan software on an imagescanner (Amersham Biosciences) was used to scan the 2D gels and the software of PDQuest system (Bio-Rad Laboratories) was employed for the image analysis. The matchings between different 2D gels were analyzed and the intensity of each spot was quantified by the calculation of the spot volume after normalization of the gel image. Proteins were considered as differentially expressed as follows: (1) The spot can be observed on the 2D images at least in six samples. (2) Intensities of the corresponding spots showed a difference of ≥2-fold variation in tumor tissues in comparison with normal mucosa tissues, or a difference of ≥2-fold variation in non-LNM CRC tissues in comparison with LNM CRC tissues.

IN-GEL DIGESTION AND PROTEIN IDENTIFICATION

The differentially expressed protein spots were destained in 50 mM NH_4HCO_3/acetonitrile (60/40), and dried by vacuum centrifugation. The gel pieces were then digested with 10 ng/μl modified porcine trypsin (Promega) in 50 mM NH_4HCO_3 buffer at 37°C for 16 h. Peptides were vacuum dried and redissolved in 50% acetonitrile/5% trifluoroacetic acid (TFA), dried in a speed vac, resuspended with a matrix consisting of 50% CAN and 2.5% TFA and desalted with a C18 ZipTip column (Millipore, Billerica, MA). The eluted peptides were analyzed by Bruker reflex III MALDI-TOF equipped with the SCOUT source. All MALDI spectra were externally and internally calibrated using two standard peptide mixtures. For PMF identification, the Mascot search engine (http://www.matrixscience.com) was employed against the NCBInr protein database with a mass tolerance of ±50 ppm.

WESTERN BLOTTING ANALYSIS

Briefly, 50 μg of tissue lysates were separated on 12% SDS–PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk in TBST for 4 h at room temperature, and probed with primary antibody overnight at 4°C with rabbit monoclonal antibodies against human FHC (1:1,000; Abcam), Cathepsin D (1:2,000; Abcam); UCH-L1 (1:2,000; Abcam), and β-actin (1:5,000; Sigma) followed by

incubation with peroxidase-conjugated secondary antibodies at 1:5,000 dilution for 1 h (Zhongshan Bio). The signal was visualized with ECL (Applygen Technologies).

TISSUE MICROARRAY AND IMMUNOHISTOCHEMISTRY

The CRC tissue fragments from the dense tumor areas or the paired normal tissues were sampled by pathologists. Tissue microarray was performed using ATA-27 automated arrayer (Beecher Instruments). Appropriate areas were identified on H&E paraffin standard sections, and three to five punches of 1 mm in diameter were assessed for each sample. Immunohistochemistry was carried out on the 5 μm sections of paraffin-embedded specimens using a standard immunohistochemical technique. The slides were deparaffinized, rehydrated, and dripped in 3% hydrogen peroxide solution for 15 min. For antigen retrieval, the slides were heated in citrate buffer (pH 6.0) at 95°C for 15 min and slowly cooled to room temperature. The sections were incubated with anti-FHC (1:250), anti-Cathepsin D (1:500), and anti-UCH-L1 (1:500) overnight at 4°C, and then incubated with a biotinylated second antibody (goat anti-mouse IgG/goat anti-rabbit/IgG 1:100 dilution) for 30 min at 37°C. The development of the slides was carried out using diaminobenzidin solution. Following a hematoxylin counterstaining, immunostaining was scored by two independent experienced pathologists. The immunoreactivity of the proteins detected was recorded through the intensity of staining and the percentage of immunoreactive cells as follows: tissues with no staining were rated as 0, with a faint or moderate staining to strong staining in $<25\%$ of cells as 1, or strong staining in 25–50% of cells as 2, and strong staining in $>50\%$ of cells as 3.

STATISTICAL ANALYSIS

To evaluate the clinicopathological correlation between the LNM and the target proteins' expression in CRC, the data was analyzed by a χ^2 test. The differences were considered to be significant when the P value was less than 0.05.

CELL MIGRATION ASSAY

This assay was performed using a 24-well chemotaxis chamber transwell. The upper chambers of transwells was coated with 50 μg Matrigel for 1 h at 37°C and then placed into 24-well chambers. The lower chambers were filled with RPMI-1640 media containing 10% FBS. 2×10^5 cells transfected with either pcDNA 3.1 (PCDB) vector or pcDNA 3.1/UCH-L1 plasmid were seeded into one upper chamber in 100 μl RPMI-1640 medium containing 1% FBS and incubated for 20 h at 37°C. After incubation, cells which did not migrate were removed from the upper side of the filters with cotton balls and the migrating cells were stained with crystal violet (0.5% w/v crystal violet and 25% methanol) and counted at 100-fold magnification under a microscope.

RESULTS

2D ANALYSIS OF PROTEINS FROM LNM AND NON-LNM OF CRC, AND NORMAL COLORECTAL MUCOSA TISSUES

Six proteins were differentially expressed in LNM CRC and non-LNM CRC. In normal colorectal mucosa tissues, 913 ± 73 (mean $\pm$ SD) spots were detected, in LNM CRC, 1157 ± 93 (mean $\pm$ SD) spots were detected, and in non-LNM CRC, 1067 ± 73 (mean $\pm$ SD) spots were observed (Fig. 1). Thirty-three proteins expressed in both LNM and non-LNM CRC groups compared to the paired normal tissues. Among the above 33 proteins, 21 were down-regulated and 12 were up-regulated in CRC. All of these differentially expressed protein spots were excised from gels and identified by peptide mass fingerprinting (PMF). All of these spots were analyzed using *M*r, p*I*, the sequence coverage for protein identification and MASCOT scores for protein matching (Table II). In comparison to non-LNM CRC, LNM CRC had six differentially expressed protein spots, which were identified as serum amyloid p-component (SAP), UMP-CMP kinase, Ubiquitin C-terminal hydrolase L1 (UCH-L1), Cathepsin D, Ferritin Heavy Chain (FHC), and serum albumin, respectively.

WESTERN BLOTTING ANALYSIS OF DIFFERENTIALLY EXPRESSED PROTEINS

Considering their possible functions in carcinogenesis, three proteins were selected for Western blotting analysis to verify the results of 2D and MS. Tissues from three cases of LNM CRC tissue, three cases of non-LNM CRC, and their paired normal colorectal mucosa were analyzed by Western blot. The results confirmed our findings that the expressions of Cathepsin D, and UCH-L1 were significantly higher in LNM CRC tissue than that in non-LNM CRC group. For FHC, the expression levels were significantly decreased in LNM CRC tissues (Fig. 2). All the three proteins showed positive expressions in normal mucosa.

EXPRESSION OF UCH-L1, CATHEPSIN D, AND FHC IN NORMAL COLORECTAL MUCOSA, COLORECTAL CANCER, AND POSITIVE LYMPH NODE METASTASIS TISSUES

The expression levels of FHC, Cathepsin D, and UCH-L1 were detected in 27 normal colorectal mucosa, 65 cases of primary CRC and 26 positive lymph nodes by immunohistochemical staining. The result is shown in Figure 3. The immunostaining of FHC was predominantly localized in the cytoplasm, and 74% (20/27) normal tissues showed positive expression. Compared with non-LNM CRC, the expression of FHC was significantly decreased in LNM CRC and positive lymph node. The positive rates in non-LNM CRC, LNM CRC, and positive lymph nodes were 37%, 13%, and 23%, respectively. Cathepsin D immunostaining was typically cytoplasmic, and strong stainings were found in stromal cells in normal tissues and LNM CRC tissues. The results also showed that Cathepsin D immunostaining in epithelial cells were elevated in LNM CRC tissues and positive lymph node. The positive rates in non-LNM CRC group, LNM CRC group, and positive lymph node group were 34.8%, 63.6%, and 61.5%. For UCH-L1, immunostaining was mainly cytoplasmic, and the proteins were mainly expressed in nerves in normal tissues while 48% (13/27) normal tissues show positive immunostaining in epithelial cells. The expression levels of UCH-L1 were significantly decreased in non-LNM CRC tissue, but elevated in LNM CRC tissue and positive lymph node where nuclear localization was observed in some neoplastic cells. The positive rates of each group were 35%, 68%, and 73%.

The expression levels of all three proteins were correlated with lymph node metastasis ($P < 0.05$), and the UCH-L1 expression level was also correlated with patients' gender, age, and primary tumor stages (Table III).

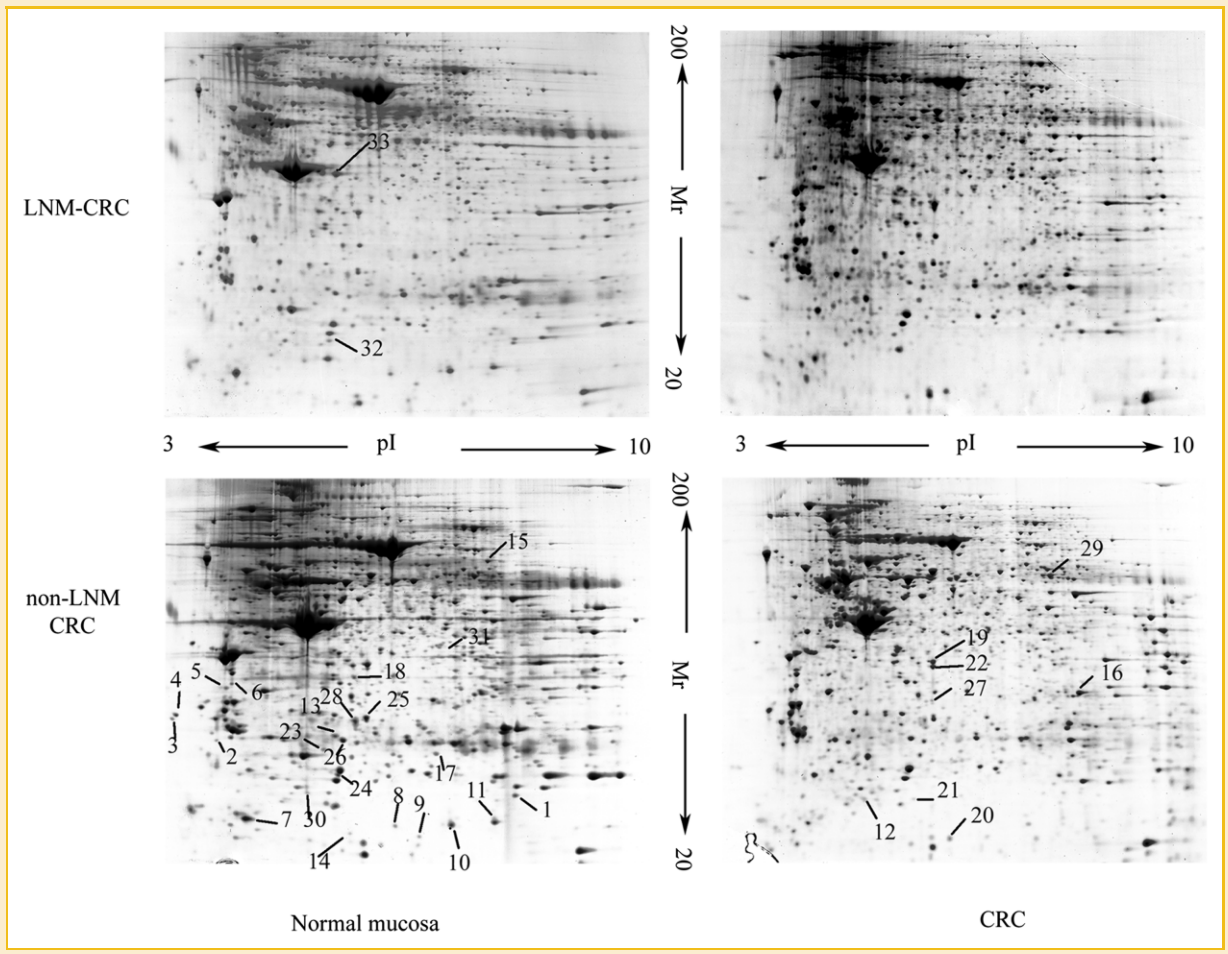

Fig. 1. Representative 2D maps of non-LNM CRC tissue, LNM CRC tissue and their paired normal mucosa tissue.

EFFECT OF UCH-L1 OVEREXPRESSION ON CELL MIGRATION

The effects UCH-L1 overexpression on cell migration was examined in colorectal cancer cell line HCT8 as there is no endogenous Uchl1 expression due to a densely methylated UCHL1 promoter region (Fig. 4a). Over expression of UCH-L1 significantly increased in the invasiveness of HCT8 cells (Fig. 4b,c). This suggests that UCH-L1 may plays a important role in cell migration and in tumor-cell metastasis.

DISCUSSION

Lymph node metastasis is a multiple step process and requires accumulative effects of altered expressions of proteins. Identification of new biomarkers for lymph node metastasis will play an important role in diagnosis and treatment of LNM, and may provide new insights into its pathogenesis. Proteomic analysis makes it possible to look directly into the proteins that perform important functions. In the present study, 10 pairs of CRC tissues within stage III were selected and divided into two groups according to presence or absence of lymph node metastasis and their protein patterns were analyzed by 2D. It was noticed by Ott et al. [2001] that the heterogeneity of CRC and normal mucosa, such as blood vessels, stroma, bacteria, necrosis, lymph, and inflammatory cells, might hamper the proteomic study. However, Hanash [2003] and Mueller and Fusenig [2004] suggested that heterotypic interactions between tumor cells and their microenvironments can affect the phenotype of the tumor, and some differentially expressed proteins in stromal cells correlated with tumor progression. Accordingly, we chose to include the stroma in the analysis instead of purifying the epithelial cells as previously done by other teams.

A total of 33 proteins were identified to be associated with carcinogenesis in CRC, among which 21 were up-regulated and 12 were down-regulated in CRC tissue. Our results also showed that the three isoforms of transgelin were down-regulated in CRC tissues, which might be the result of different splicing, processing or post-translational modifications.

Western blotting was done to confirm the differential expression levels of the three proteins associated with lymph node metastasis (FHC, Cathepsin D, and UCH-L1) in LNM CRC and non-LNM CRC tissues. The results further validated the proteomic identifications. Immunohistochemistry analysis by tissue microarray revealed that the expression levels of FHC, cathepin D, and UCH-L1 were significantly associated with lymph node metastasis and some other clinicopathologic factors of CRC.

TABLE II. The Differentially Expressed Proteins Identified Between CRC Tissue and Adjacent Normal Mucosa Tissue

Spot no.	Protein AC[a]	Protein description	MASCOT score	Theoretical *Mr* (kDa)/p*I*	Sequence coverage (%)	Change fold (CRC/NMC)
1	gi|4503057	Crystalline alphaB	142	20.14/6.76	68	−6.87
2	gi|4504771	Eukaryotic translation initiation factor 6	71	26.58/4.56	39	+4.96
3	RAB3D_HUMAN	Ras-related protein Rab-3D	40	24.25/4.76	23	−5.82
4	gi|33875631	ANP32A protein	100	24.05/4.75	31	−0.216
5	gi|49456555	PCNA	97	28.69/4.57	62	+4.06
6	gi|339956	Tropomyosin alpha-1	106	32.69/4.69	32	+3.97
7	gi|29568111	Myosin regulatory light polypeptide 9	90	19.81/4.8	52	−3.57
8	gi|48255905	Transgelin	95	22.60/8.87	38	−3.34
9	gi|21389433	HSP 27	77	17.125/5.95	33	−2.67
10	gi|49168456	TAGLN	87	22.60/8.87	50	−13.25
11	gi|49168456	TAGLN	87	22.60/8.87	50	−12.23
12	gi|15082258	Chromobox protein homolog 3	43	20.80/5.23	27	+2.12
13	gi|4502133[b]	Serum amyloid p-component	110	25.37/6.1	31	↓
14	gi|453155	Keratin, type 1cytoskeletal 9	84	62.09/5.19	31	−6.52
15	gi|133922562	Phosphoglucomutase-like protein 5	176	62.19/6.81	37	−22.4
16	gi|7669492	Glyceraldehyde-3-phosphate dehydrogenase	134	36.03/8.57	44	−2.00
17	gi|4557966	GST M2-2	100	25.56/6.02	42	−2.2
18	gi|10835035	Sulpotransferase 1A3	103	34.17/5.68	44	−2.45
19	gi|4557032	L-Lactate dehydrogenase B chain	133	36.61/5.71	36	+2.24
20	NDKA_HUMAN	Nucleoside dephosphate kinase A	91	17.14/5.83	51	+2.25
21	GRP78_HUMAN	78 kDa glucose-regulated protein	58	72.29/5.07	11	−10.89
22	gi|67464392	Pkm2	130	59.70/8.22	32	+3.00
23	gi|21361091[b]	Ubiquitin carboxyl-terminal hydrolase isozyme L1	125	24.81/5.33	46	↑
24	gi|12644008[b]	UMP-CMP kinase	103	22.21/5.44	43	↓
25	gi|30581141	Proteasome activator complex subunit 1	77	28.705/5.78	38	−2.33
26	gi|662841	Heat shock protein 27	103	22.77/5.98	40	+2.11
27	gi|119597993	Annexin A2	175	32.43/5.93	60	+2.32
28	gi|4503143[b]	Cathepsin D	116	44.52/6.1	41	↑
29	gi|31416989	Pyruvate kinase isozymes M1/M2	212	57.90/7.96	42	+2.17
30	gi|56682959[b]	Ferritin heavy chain	64	21.21/5.3	28	↓
31	gi|56682959[b]	Short chain specific acyl-coA dehydrogenase	142	44.27/8.13	53	−2.07
32	gi|119626070	Albumin	88	22.00/5.66	46	−2.53
33	gi|28592[b]	Serum albumin	74	69.32/6.05	24	↓

[a]Accession number as in Swissprot or Entrez Protein.
[b]The proteins differentially expressed between non-LNM and LNM CRC tissues.
The "+" and "−" indicate the up-regulated and down-regulated proteins in tumors, respectively, as compared with normal tissues.
↓ the proteins down-regulated in LNM CRC tissue as compared with normal tissues.
↑ the proteins up-regulated in LNM CRC tissues as compared with normal tissues.

Previous studies [Wilkinson et al., 1989; Schofield et al., 1995] showed that Ubiqitin C-terminal hydrolase L1 is a member of Deubiqitinating Enzymes (DUBs) and is widely expressed in neuronal tissues. It was also found that mutation of the UCH-L1 gene was associated with Parkinson's and Alzheimer's diseases [Liu et al., 2002; Betarber et al., 2005]. More recently, research data suggested that UCH-L1 was highly expressed in some malignant cancers, including pancreatic, prostate, esophageal, and colorectal carcinomas [Tezel et al., 2000; Yamazaki et al., 2002; Takase et al., 2003; Leiblich et al., 2007]. Moreover, silencing of UCH-L1 expression by the methylation of its promoter was observed in some cancer cell lines and primary tumors, such as esophageal squamous cell carcinoma [Mandelker et al., 2005], colorectal and ovarian cancers [Fukutomi et al.,2007; Okochi-Takada et al.,2006]. Mizukami et al. [2008] showed that hypomethylation might also play an important role in re-expression of the UCH-L1 gene in

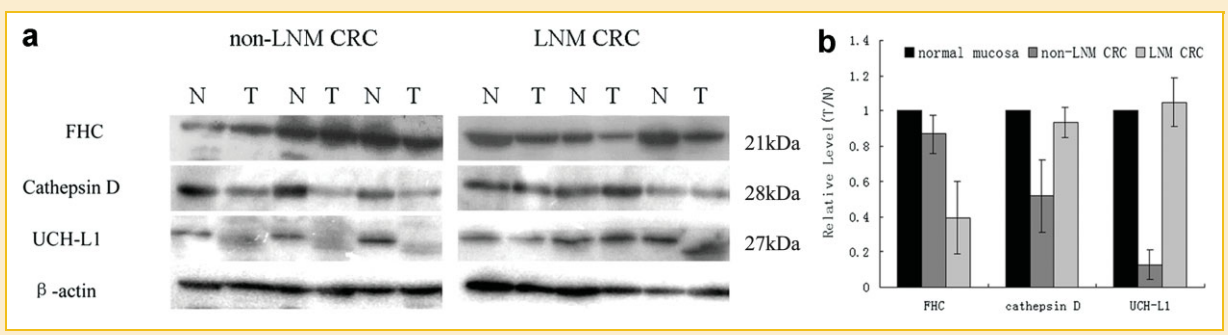

Fig. 2. Results of Western blotting analysis. a: Western blotting shows changes in the expression levels of FHC, Cathepsin D, and UCH-L1 in normal mucosa, non-LNM CRC, and LNM CRC tissue. β-Actin was used as an internal control for equal protein loading. b: Histogram of relative changes in the expression level of the three proteins in normal mucosa, non-LNM CRC, and LNM-CRC tissue as determined by densitometric analysis.

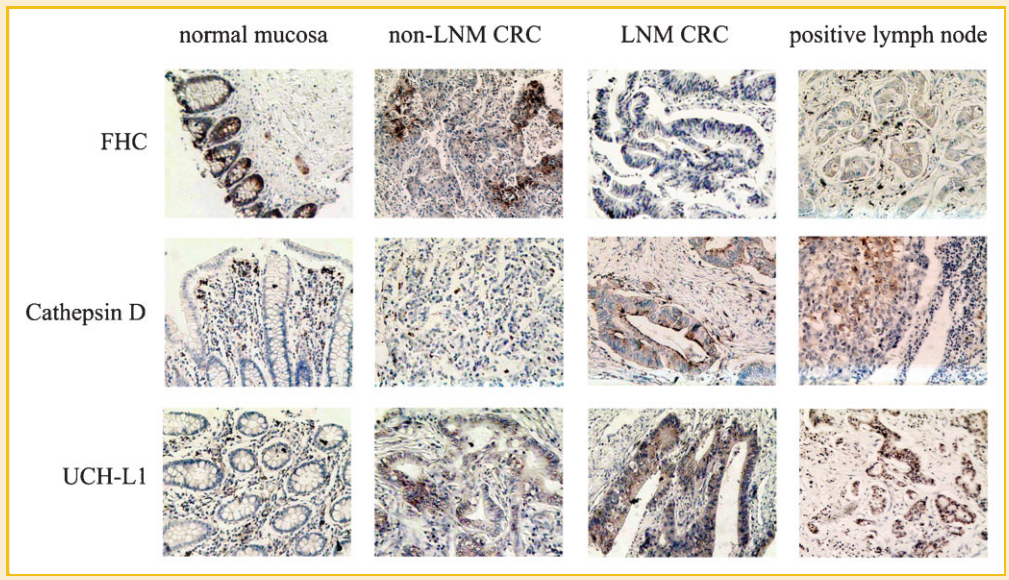

Fig. 3. Immunohistochemical staining of FHC, Cathepsin D, and UCH-L1 in normal mucosa, non-LNM CRC, LNM-CRC, and positive lymph node (magnification 100×).

gallbladder cancer and colorectal cancer, and UCH-L1 was less frequently methylated in metastatic colorectal cancer. It was also reported that UCH-L1 is markedly expressed in primary lung cancer and lung cancer cell lines [Hibi et al., 1999]. The overexpression of UCH-L1 in cancer cells enhances their invasive potential and tumor metastasis probably due to Akt activation [Kim et al., 2009]. Our data suggest that UCH-L1 expressions in epithelial cells in LNM CRC and positive lymph node were elevated compared to that in non-LNM CRC, and epithelial cells in normal tissue also showed positive expression. Thus, UCH-L1 could be used as a biomarker for lymph node metastasis in CRC and it also could be an important player in cell migration.

Furthermore, we studied the relationship between the over-expression of UCH-L1 and cell migration in vitro. The results showed that high expression level of UCH-L1 significantly increased the migration ability of HCT8 cells in vitro. Recent results suggested that UCH-L1 could up-regulate β-catenin/TCF signaling which is closely associated with cancer metastasis [Bheda et al., 2009]. FHC plays an important role in the storage and release of iron and is pivotal to coordinating iron metabolism. Iron accumulation leads to the formation of reactive oxygen species, which in turn causes damages to proteins, lipids, and DNA [Ames et al., 1993]. When supplemented with iron in animals, the rate of esophageal adenocarcinoma was 10-folds higher than that in nonsupplemented animals [Chen et al., 1999]. This protein was demonstrated to be up-regulated in esophageal adenocarcinoma [Boult et al., 2008], papillary thyroid cancer [Giusti et al., 2008], hepatocarcinogenesis [Wu et al., 1997], testicular seminoma [Cohen et al., 1984], and breast cancer [Weinstein et al., 1982]. Conversely, cancers with the highest metastatic potential showed the lowest levels of ferritin expression in rat transitional cell carcinoma of the urothelium [Vet et al., 1997]. Early results [Birchmeier et al., 1993; Bilello et al., 2003; Brookes et al., 2006] also confirmed that elevated levels of intracellular iron can repress E-cadherin expression. Our results showed that the expression level of iron storage protein FHC in LNM CRC was much lower than that in non-LNM CRC, which may cause iron accumulation in tumor cells. The data suggested that FHC might be a biomarker for lymph node metastasis in colorectal cancer.

Cathepsin D is an aspartyl proteinase that functions in the normal degradation of intracellular and endocytosed proteins. Overexpression of Cathepsin D has been suggested to increase the metastatic potential of breast tumor and been associated with poor prognosis in patients with primary breast cancer [Ravdin, 1993; Westley and May, 1996; Foekens et al., 1999; Scorilas et al., 1999]. It was reported that Cathepsin D is expressed in both epithelial neoplastic and stromal cells in CRC. Cathepsin D expression in stromal cells may be an important indicator of poor prognosis in colorectal adenocarcinomas [Kanber et al., 2002], and act as a predictor of lymph node metastasis in submucosal colorectal cancer [Oh-e et al.,

TABLE III. Relationships Between FHC, Cathepsin D, and UCH-L1 Expression in Primary CRC and Clinical Pathologic Factors

		FHC			Cathepsin D			UCH-L1		
	n	N	P	*P*	N	P	*P*	0–1	2–3	*P*
Gender										
Male	41	31	10	0.262[a]	22	19	0.714[a]	17	24	0.009[a*]
Female	24	15	9		14	10		18	6	
Age (years)										
≥50	37	25	12	0.514[a]	23	14	0.206[a]	15	22	0.013[a*]
<50	28	21	7		13	15		20	8	
Primay tumor (T) stage										
T_1–T_2	9	6	3	1.000[a]	6	3	0.710[a]	8	1	0.056[a]
T_3–T_4	56	40	16		30	26		27	29	
Lymph node (N) metastasis										
N_0	43	27	16	0.048[a*]	28	15	0.027[a*]	28	15	0.011[a*]
N_x	22	19	3		8	14		7	15	
Distant metastasis (M)										
M_0	59	41	18	0.662[a]	33	26	1.000[a]	31	28	0.817[a]
M_1	6	5	1		3	3		4	2	

[a]Pearson χ^2-test.
*P-value <0.05.

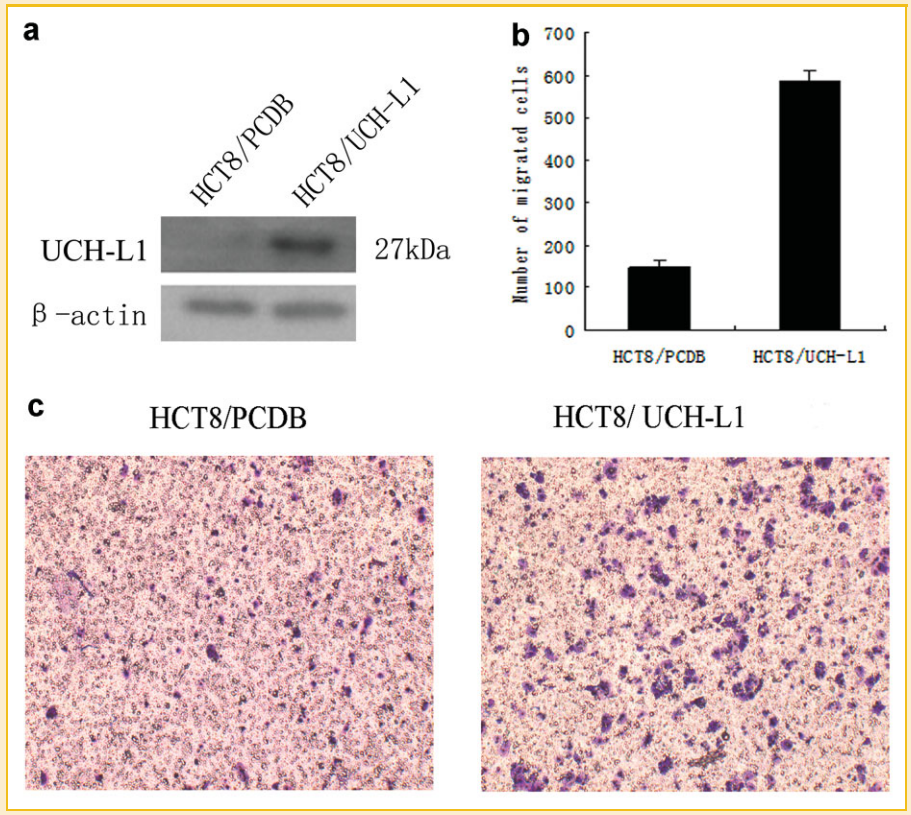

Fig. 4. UCH-L1 modulates tumor-cell invasion. a: HCT8 cells were transiently transfected with PCDB vector, pcDNA 3.1/UCH-L1. Western blotting showing the expression level of UCH-L1 in transfected and control HCT8 cells. b: After 24 h, same amount of cells (2×10^5 cells) were seeded on the upper chamber of transwell coated with Matrigel. Tumor cells penetrating the precoated polycarbonate membrane were photographed. c: The number of invasive tumor cells per field in transfected HCT8 cells (magnification 100×).

2001]. Our results were consistent with those reports. The expression levels of Cathepsin D in normal epithelial cells were negative or very low, while elevated expression of Cathepsin D in tumor cells in CRC was closely related to lymph node metastasis. However, the detailed intracellular mechanisms of the association of Cathepsin D and lymph node metastasis need to be further investigated.

In summary, we screened the differential expression patterns of proteins between non-LNM CRC and LNM CRC using comparative proteomic technology. Three proteins associated with lymph node metastasis in CRC were identified and further confirmed by Western blotting and immunonhistochemical staining. The elevated expression of Cathepsin D, UCH-L1, and decreased expression of FHC in tumor cells indicated a higher incidence of lymph node metastasis in colorectal cancer. Moreover, our results might provide some useful information for the development of clinical biomarkers for diagnosis or targets for therapeutic intervention and drug development to lymph node metastasis in CRC.

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