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Basic Research Paper

Screening and identifying differentially expressed proteins in LST-R1 cells

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Background and Objective: Colorectal laterally spreading tumors (CLST) rarely invade deeply but always laterally spread along the colorectal mucosa; therefore, CLST could be used as a comparative model in studying the invasion and metastasis of colorectal cancer (CRC). This study identifies differentially expressed proteins between a CLST cell line LST-R1 and two colorectal carcinoma cell lines SW480 and LoVo using proteomic technology. Methods: Total proteins of LST-R1, SW480 and LoVo cells were isolated by two-dimensional electrophoresis (2-DE). Differentially expressed protein spots were analyzed with Melanie 3 software. The peptide mass fingerprints (PMFs) of differently expressed proteins were analyzed by MALDI-TOF mass spectrum. Subsequently matched proteins were searched through protein databases. Results: Using pH 4–7 IPG gels with 250 μg protein loading, the numbers of protein spots in 2-DE maps were 1285 ± 51 in LST-R1 cells, 1184 ± 47 in SW480 cells, and 1124 ± 54 in LoVo cells; with 150 μg protein loading, the numbers were 989, 935 and 893, respectively. The distribution and levels of these proteins in 2-DE maps of LST-R1, SW480 and LoVo cells were analogical which indicated CLST also expresses the protein profile of common colorectal tumors. In 2-DE maps, 96 ± 7 differential protein spots were detected between LST-R1 cells and SW480 cells with 50 ± 6 only expressed or obviously overexpressed in LST-R1 cells and 47 ± 5 in SW480 cells; 108 ± 10 differential protein spots were detected between LST-R1 cells and LoVo cells with 56 ± 8 only expressed or obviously overexpressed in LST-R1 cells and 52 ± 11 in LoVo cells. Nineteen differentially expressed proteins were identified among LST-R1, SW480 and LoVo cells. Conclusion: Nineteen differentially expressed proteins are possibly involved in laterally spreading of CLST and adhesion and invasion of CRC.

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Colorectal laterally spreading tumors (CLST) may offer information on the invasion and metastasis of colonic tumors providing novel inspiration due to the disease's unique biological characteristics. CLST morphologically presents as diffusion along the superficial layer of colonic mucosa but rarely infiltrates into the deep layer;[1,2] this differs significantly from common colonic tumors which are subject to deep infiltration and high metastasis. For this reason, CLST was decided to be the control model for our study on the invasion and metastasis of colonic tumors. LST-R1, a CLST cell line, was already established in our previous study,[1] with the in vitro experiment validating it as less invasiven than human colonic tumor cell lines SW480 and LoVo, while a series of differentially expressed genes among three cell lines were screened out with gene chip. Differentially expressed proteins were herein investigated with proteomics at the level of protein expression profile.

Materials and Methods

Cells and reagents. LST-R1 cell line was established by Southern Hospital Institute of Gastroenterology, First Military University,[3] deriving from rectal LST pathologically presented as villous adenoma with moderate to severe atypical hyperplasia, which was stably passaged in vitro; SW480 (ATCC NO. CCL-228) and LoVo (ATCC NO. CCL-229) cell lines were purchased from Institute for Biochemistry and Cell Biology (Shanghai), Chinese Academy of Sciences.

Our team purchased 0.25% trypsinase/0.02% EDTA, penicillin, streptomycin and ROMI-1640 basic medium from Gibco as well as bovine serum from Hangzhou Institute of Sijiqing Biological Products. Immobilized pH (3–10 and 4–7) gradient strips, auxiliary IEF buffer, as well as tributylphosphine (TBP) were obtained from Bio-Rad; ultra-pure urea, thiourea, iodoacetamide, dithiothreitol (DTT), glycerol, N,N-methlenebisacrylamide, tetramethylethylenediamine (TEMED), mineral oil, ammonium

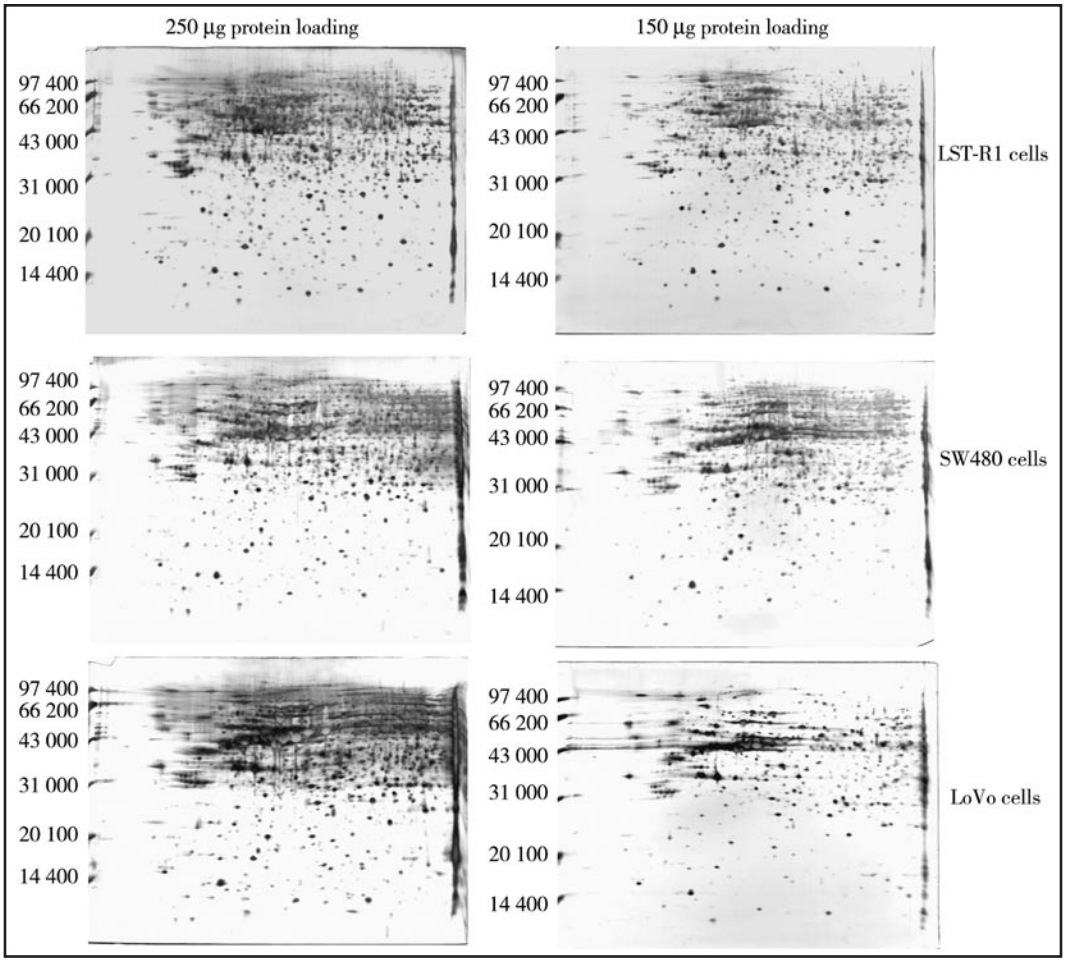

Figure 1. The 2-DE maps of 17 cm pH 4–7 IPG gels.

persulfate (APS) and bromophenol blue were obtained from Sigma; acrylamide and phenylmethylsulfonyl fluoride (PMSF) were obtained from BEBCO; Tris-(Hydroxymethyl)-aminomethane (Tris) was obtained from Genview; CHAPS was obtained from Amresco; the remaining reagents were all above analytical purity. Instruments herein used mainly consisted of the PROTEAN IEF Cell System (Bio-Rad), PROTEAN II xi System (Bio-Rad), a Voyage-DE STR MALDI-TOF mass spectrometer (ABI), as well as a domestic thermostat water bath and ultrasonic apparatus.

Methods. Preparation of samples. Cells were cultured in 10% FCS containing a RPMI-1640 culture medium. Cells of exponential phase were scraped and centrifuged for collection at 80% confluency. Cell lysis buffer (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 65 mmol/L DTT) was added into the cells at the ratio of 10:1 (cell lysis buffer:cells) andcells were also supplemented with 1 mmol/L PMSF. Lysates were vortexed, frozen and thawed repeatedly in liquid nitrogen, and were further reacted with 50 μg/mL DNase I as well as 25 μg/mL RNase A at ambient temperature for 15 min and centrifuged at 15,000 rpm at 4°C for 1 h. Supernates were collected and frozen at -80°C for further use and the concentration of proteins was measured with the Bradford method.

First dimensional immobilized pH gradient—isoelectric focusing (IPG-IEF). The procedure was modified from Görg.[4] Protein samples were obtained, added with reconstitution solution (7 mol/L urea, 2 mol/L thiourea, 2% CHAPS, 50 mmol/L DTT) into the final volume of 300 μL, and with 2 nmol/L TBP and 0.2% IEF buffer temporarily. These samples were then completely dissolved at ambient temperature, and were transferred into gel slots with the gel downside onto 17 cm IPG dry strips avoiding bubble, covered with 3 mL mineral oil 1 h later, and finally reconstituted at 20°C for 12 h. Reconstituted strips were transferred into IEF focusing plates and added with the appropriate amount of mineral oil for IEF at the condition of 250 V 1/2 h→500 V 1 h→1,000 V 1 h→5,000 V 3 h→10,000 V 70,000 V h→500 V maintaining, at the temperature of 20°C and with the maximum current of 50 μA.

Strip balance. Strips were sequentially balanced in balance solution I (5 mol/L urea, 2% SDS, 50 mmol/L tris-HCl, 20% glycerol, 2% DTT) and II (5 mol/L urea, 2% SDS, 50 mmol/L tris-HCl, 20% glycerol, 2.5% iodoacetamide) in 5 mL for 10 min each.

Two-dimensional vertical SDS-PAGE. Balanced IPG strips were transferred onto the gels. One end of the strip was set to the low molecular weight protein criterion and blocked with

0.5% agarose. The parameters of electrophoresis were as follows: 12 mA for each strip for 1/2 h and 24 mA/strip until the leading edge of bromophenol blue reached the gel bottom.

Silver staining. Silver staining was performed as follows: fixation, immersion, rinse, silver staining, colorization and termination.

Visualization of images. Images were captured with a PowerLook 1100 scanner and analyzed with Melanine 3 analysis software.

Peptide mass fingerprint with MALDI-TOF mass spectrum, in situ enzymolysis of proteins. Pointing, decolorization, deoxidization, alkylation, enzymolysis and extraction were sequentially performed.

MALDI-TOF peptide mass fingerprint. Peptide fingerprint was measured with Voyage-DE STR MALDI-TOF mass spectrometer with α-cyano-4-hydroxyl-cinnamic acid as substrate.

Database search of protein identification. Peptide Search (www.mann.embl-heidelberg.de/services/Peptide), MS-Fit (http: //prospector. ucsf. edu/ucsfhtml4.0/msfit.htm) and MOWSE (www.dl.ac.uk/SEQNET/mowse.htm/) were used for identification of the protein.

Immunohistocytochemistry. SP immunohistocytochemistry was used to detect the expression of dUTPase, a differentially expressed protein in three cell lines. After culture for 48 h, the cells were fixed in 4% paraformaldehyde at ambient temperature for 30 min, reacted with 3% H_2O_2 for 10 min, immersed in 0.3% Trtion-X100 at ambient temperature for 10 min, blocked with goat serum at ambient temperature for 10 min, incubated with dUTPase polyclonal antibody at 4°C overnight, and sequentially reacted with biotin-labeled goat-anti-rabbit secondary antibody and horseradish peroxidase-labeled tertiary antibody at ambient temperature for 10 min. Brown staining with DAB was taken as positive.

Results

2-DE. Experiments were triplicated with the identical experimental conditions and parameter settings. With the loading quantity of 250 μg for protein, the protein spots of LST-R1, SW480 and LoVo cells in 2-DE spectrum using IPG pH 4–7 strips were 1285 ± 51, 1184 ± 47 and 1124 ± 54, respectively (Fig. 1); with the loading quantity of 150 μg, those were 989, 935 and 893 protein spots, respectively (Fig. 1).

Analysis of differential protein spots. Comprehensive analysis showed that LST-R1, SW480 and LoVo cells were similar in distribution and expression value of most proteins with 2-DE spectrum, indicating the expression of protein spectrum in common colon tumors by CLST with the majority of properties of common colon tumors. LST-R1 and SW480 cells were found different in (96 ± 7) protein spots with pH 4–7 IPG strip 2-DE spectrum wherein (50 ± 6) spots were only expressed or significantly enhanced in LST-R1 cells but (47 ± 5) spots were only expressed or significantly enhanced in SW480 cells instead; LST-R1 and LoVo cells were found different in (108 ± 10) protein spots wherein (56 ± 8) spots were only expressed or significantly enhanced in LST-R1 cells but (52 ± 11) spots were only expressed or significantly enhanced in LoVo cells instead (Fig. 2).

Peptide mass fingerprint. Protein PMFs obtained were measured with a MALDI-TOF mass spectrometer, followed by internal correction of any two degraded fragments from trypsinase for more accurate PMFs (Fig. 2).

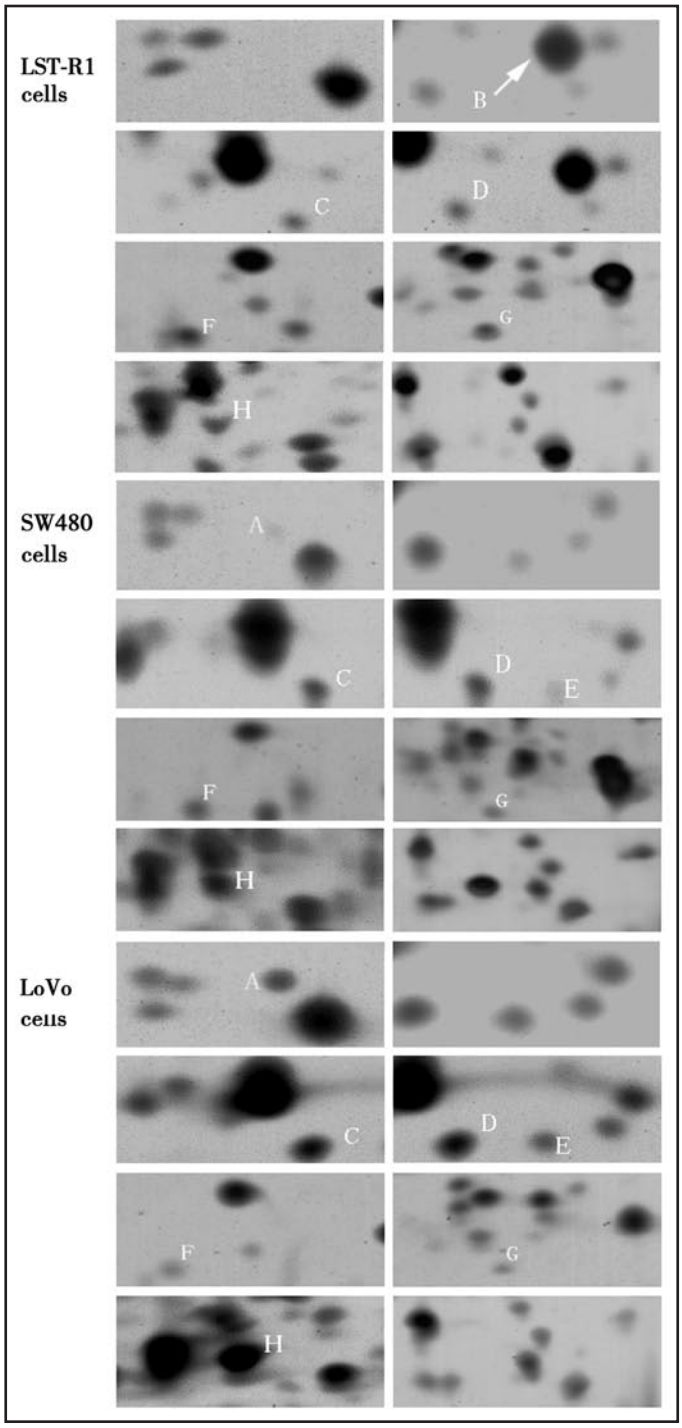

Figure 2. Magnification of the differentially expressed protein spots among LST-R1, SW480 and LoVo cells. The letters indicate differentially expressed protein spots.

Database search and identification of protein. Through a series of parameters searched in the protein database, 19 differentially expressed proteins were identified preliminarily (Table 1) among

Table 1 **The differentially expressed proteins among LST-R1, LoVo and SW480 cells**

LST-R1>SW480>LoVo	LoVo>SW480>LST-R1
Cytochrome c oxidase subunit Vib	Cyclin B1
14 ku beta-galactoside-binding lectin	dUTPatase
OK/SW-CL.19	Platelet glycoprotein IX precursor
Polyamine oxidase isoform-3	Prefoldin subunit 3
Ki nuclear autoantigen	Annexin A8
Proteasome activator subunit 3	Cyclin-dependent kinase 4 inhibitor A
Aflatoxin B1-aldehyde reductase	Chain A, Human Peroxiredoxin 5
Epsilon subunit of coatomer protein complex isoform b	Peptidylprolyl isomerase A; cyclophilin A
N-methyl-D-aspartate receptor subunit	Urokinase-type plasminogen activator receptor
Trafficking protein particle complex 6B	

which proteins auch as galectin-1, dUTPatase and uPAR were closely associated with the metastasis of tumors.

Detection of dUTPase with immunohistocytochemistry. Our study showed that dUTPase proteins were all expressed in LST-R1, SW480 and LoVo cells with nuclear dominance in SW480 and LoVo cells and cytoplasmal dominance in LST-R1 cells in the following sequence of expression intensity: LoVo > SW480 > LST (Fig. 3).

Discussion

Currently, the study on CLST was restricted to endoscopy and clinical pathology[5-9] while the association between the unique biological characteristics of CLST and its molecular biology was less reported, and mainly categorized into colonic superficial lesions other than individual lesions.[10-14] Overexpression and mutation of p53 to various extent and K-ras mutation were found in CLST,[10] although including the seemingly unclear association with less lateral diffusion and invasiveness of CLST. It was of great significance when Yamada et al.[13] found that in non-polyp-like colonorectal tumors the apoptotic index was higher in deep layers than in superficial ones, presuming such difference in apoptosis in various layers associates with CLST lateral diffusion other than vertical growth. However, the unique growth mechanism of CLST was far from this. In China, CLST was initially reported by Jiang et al.[15] of Southern Hospital Institute of Gastroenterology, First Military University in 1,920 patients using colonoscopy to detect 25 CLST patients with a positive rate of 0.8%, indicating the substantial incidence of CLST in China. In vitro culture, as well as the establishment and detection of oncogene expression were successfully conducted in the study for the first time,[3] establishing the LST-R1 cell model for LST, based on which the differential gene expression spectrum of LST-R1 cells relates to common tumor cells.[16] The protein expression spectrum was herein further investigated with proteomics.

Comprehensive analysis showed that LST-R1, SW480 and LoVo cells were similar to the distribution and expression value of most proteins with pH 4–7 IPG strip 2-DE spectrum, indicating the expression of the protein spectrum in common colon tumors by CLST with the majority of properties of common colon tumors. Furthermore, LST-R1 and SW480 cells were found to be different in (96 ± 7) protein spots wherein (50 ± 6) spots were only expressed or significantly enhanced in LST-R1 cells but (47 ± 5) spots were only expressed or significantly enhanced in SW480 cells instead; LST-R1 and LoVo cells were found different in (108 ± 10) protein spots wherein (56 ± 8) spots were only expressed or significantly enhanced in LST-R1 cells but (52 ± 11) spots were only expressed or significantly enhanced in LoVo cells instead. Presumably there existed some difference in the expression of proteins involving cell adhesion, migration and invasiveness.

Among the aforementioned differential spots, 40 differential protein spots were chosen for MALDI-TOF mass spectral analysis to obtain a series of PMFs. A search through the protein data bank was subsequently conducted in combination with such protein properties as isoelectric point and molecular weight from 2-DE spectrum in order to identify 19 proteins involving cell proliferation, apoptosis and adhesion, including GAL1, dUTPase, uPAR and coat protein of interests. The expression of dUTPase was shown to increase in the LST-R1, and sw480 to LoVo cells, consistent with the research of Fleischmann et al.[17] which reported significantly lower expression of primary colon tumors without lymphatic metastasis than with lymphatic metastasis, indicating the involvement of dUTPase in the progression of colon tumor invasion and metastasis. Immunohistocytochemical analysis showed dUTPase proteins were all expressed in LST-R1, SW480 and LoVo cells with nuclear dominance in SW480 and LoVo cells and cytoplasmal dominance in LST-R1 cells in the following sequence of expression intensity: LoVo > SW480 > LST. Whether the difference between expression intensity and locality was associated the unique growth pattern of CLST is yet to be investigated. As a multifunctional protein, urokinase-type plasminogen receptor (uPAR) played a key role in tumor invasion, metastasis and angiogenesis. Highly expressed uPAR was reported to be positively correlated with distant metastasis of primary colon tumors but negatively correlated with the survival rate of patients with colon tumors, indicating the critical role of uPAR in the progression, invasion and metastasis of colon tumors.[18] Expression of dUTPase was herein in the increasing trend from LST-R1, sw480 to LoVo cells, consistent with the previous report.[18] As the downstream protein of ARF1 interactive protein network,[19] the expression gradient of coat protein in the three cells was identical to that in ARF1. ARF1

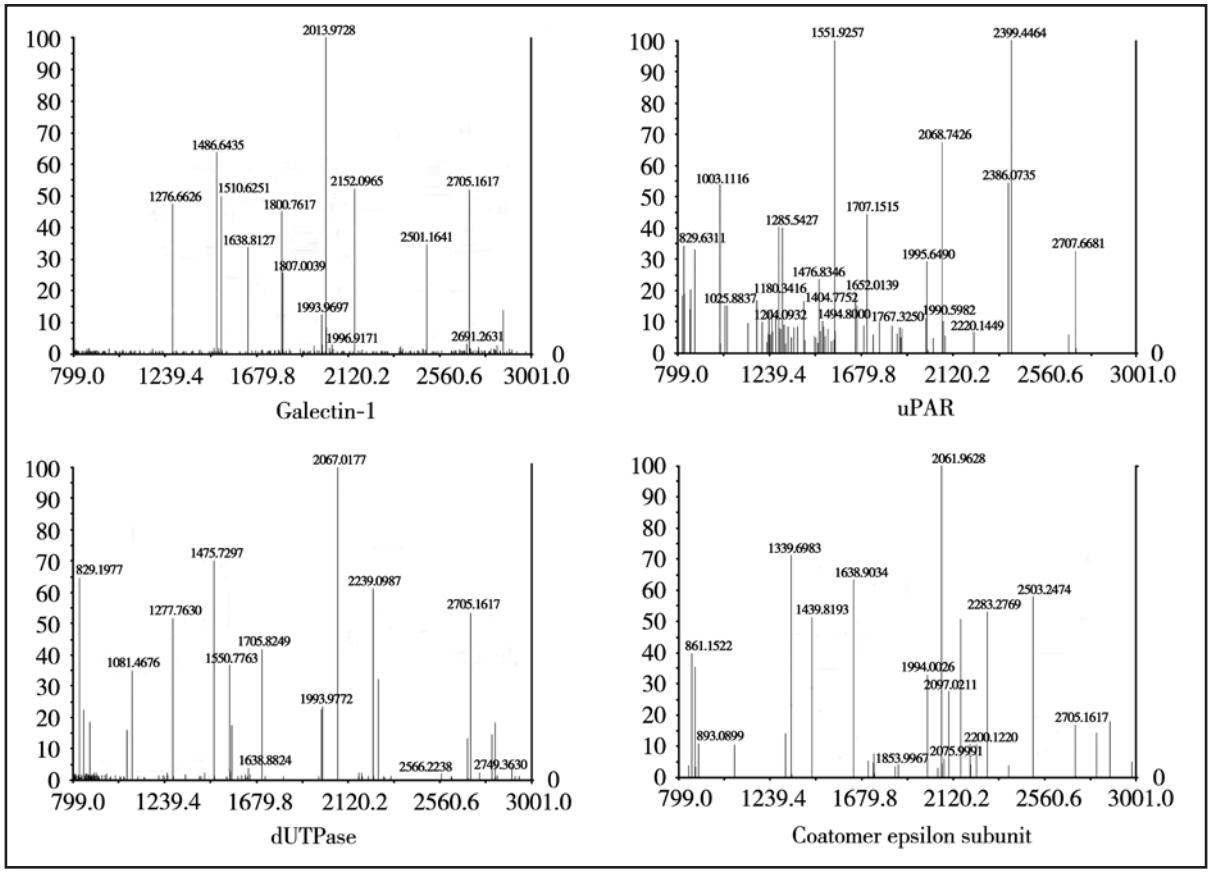

Figure 3. The peptide mass fingerprints (PMFs) of four proteins.

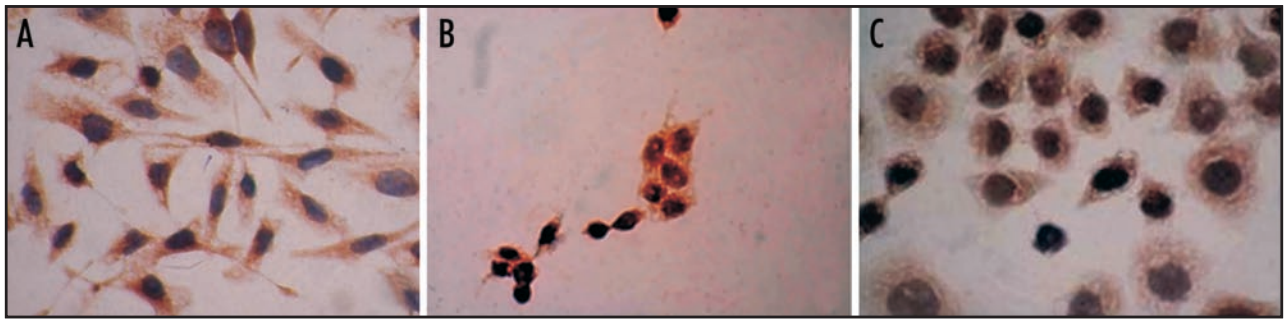

Figure 4. Expression of dUTPase in LST-R1, SW480 and LoVo cells (SP x400). (A) dUTPase is expressed in cytoplasm of LST-R1 cells. (B) dUTPase is expressed in cytoplasm and nuclei of SW480 cells, mainly in nuclei. (C) dUTPase is expressed in cytoplasm and nuclei of LoVo cells, mainly in nuclei.

was found to be associated with lateral growth of CLST.[20] Such protein network was likely to play a certain role the progression of CLST. Another differentially expressed protein, galectin-1, a homogenous dimer of 14.5 ku in molecular weight was reported to function in cell adhesion, proliferation regulation and immune reaction involving invasion and metastasis of multiple tumors,[21] including those of colon tumor.[22]

Therefore in our study, protein expression spectra of CLST LST-R1 cells and common colon tumor cells were analyzed with proteomics to preliminarily identify 19 differentially expressed proteins. Based on the literature available, some proteins were likely to be closely associated with invasion and metastasis, and further functional analysis of these differential proteins would explore not only the potential molecular mechanism of CLST lateral growth but also the invasion and metastasis of colon tumors.

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