

• COLORECTAL CANCER •

Comparative study of proteome between primary cancer and hepatic metastatic tumor in colorectal cancer

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Abstract

AIM: To identify the differential proteins associated with colorectal cancer genesis and hepatic metastasis.

METHODS: Hydrophobic protein samples were extracted from normal colorectal mucosa, primary cancer lesion and hepatic metastatic foci of colorectal cancer. With two-dimensional electrophoresis and image analysis, differentially expressed protein spots were detected, and the proteins were identified by matrix assisted laser desorption/ionization-time of flight-mass spectrometry and peptide mass fingerprint analysis.

RESULTS: Significant alterations of the proteins in number and expression levels were discovered in primary cancer and hepatic metastatic foci, the expression of a number of proteins was lost in 25-40 ku, but protein spots was increased in 14-21ku, compared with normal mucosa. Nine differentially expressed protein spots were identified. Three proteins expressed in normal mucosa, but lost in primary cancer and hepatic metastasis, were recognized as calmodulin, ribonuclease 6 precursor and mannosidase- α . Proapolipoprotein was expressed progressively from normal mucosa to primary cancer and hepatic metastasis. The differentially expressed protein of beta-globin was found in normal mucosa and hepatic metastatic tumor, but lost in primary cancer lesion. Cdc 42, a GTP-binding protein, was identified in hepatic metastasis. The protein spots of C4 from primary cancer, M7 and M9 from hepatic metastasis had less homology with the proteins in database.

CONCLUSION: Variations of hydrophobic protein expression in colorectal cancer initiation and hepatic metastasis are significant and can be observed with two-dimensional electrophoresis. The expression of calmodulin, ribonuclease 6 precursor and mannosidase- α is lost but the expression of proapolipoprotein is enhanced which is associated with colorectal cancer genesis and hepatic metastasis. Cdc 42 and beta-globin are expressed abnormally in hepatic metastasis. Protein C4, M7 and M9 may be associated with colorectal cancer genesis and hepatic metastasis.

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INTRODUCTION

Colorectal cancer genesis and metastasis are complex processes involving multiple changes in gene and protein expression^[1-5]. The liver is a common site of metastasis from colorectal cancer^[6-8]. Hepatic metastasis caused severe and fatal effects on patients who underwent radical excision for large intestine primary carcinoma^[9-12]. The success of metastatic hepatic cancer treatment is strongly dependent on early diagnosis and understanding of the molecular mechanisms and biological behaviors of colorectal cancer, especially its infiltration and metastasis. To unravel these alterations, genome and proteome approaches for the identification of qualitative and quantitative changes in gene and protein compositions provide theoretic and technical support^[13-16]. Our study was focused on the identification of differential expression proteins between primary colorectal cancer foci and hepatic metastasis with proteome approach. Hydrophobic proteins including membrane proteins play important roles in cellular signal transduction. Identification of the proteins is helpful to understand the molecular biological mechanisms of colorectal carcinogenesis and hepatic metastasis and to select tumor markers for colorectal cancer.

MATERIALS AND METHODS

Tissue sample collection

Samples of normal colorectal mucosa, primary cancer lesion and hepatic metastasis were collected from 12 colorectal cancer patients aged 36-68 years including 6 males and 6 females. The samples were stored in liquid nitrogen. Pathology examination was performed for all the specimens and the histological types consisted of moderately and poorly differentiated adenocarcinoma, signet-ring cell carcinoma and undifferentiated carcinoma, 4 cases in each type.

Protein sample preparation

A set of samples were taken from the same patient, 0.9 g of each, including normal colorectal mucosa, primary cancer and hepatic metastatic tumor. The samples were washed with PBS and then immediately ground by a liquid nitrogen cooled mortar, and homogenized in protease inhibitor buffer (cocktail formula: phenylmethylsulfonyl fluoride 40 μ g/mL, ethylenediamine tetraacetic acid 1 mmol/L, peptide inhibitin 0.7 μ g/mL, leupeptin 0.5 μ g/mL). Protein extraction was performed with Molloy procedure. Lysis buffer I (Tris 40 mmol/L, pH8.8) was added, stirred and mixed by an ultrasonic disintegrator. The mixture was centrifuged at 105 000 g for 1 h. Yellow lipids were discarded from the supernatant and the middle layer liquid was transferred and dried with a freezing dryer. The pellet was solubilized in lysis buffer II (Urea 8 mol/L, Tris 10 mmol/L, CHAPS 40 g/L, DTT 65 mmol/L) and centrifuged. The supernatant was dried and stored as *idem*. The pellet was solubilized in lysis buffer III (urea 5 mol/L, thiocarbamate 2 mol/L, SB3-10 20 g/L, 0.2 g, CHAPS 20 g/L, TBP 2 mmol/L), then the procedure was repeated once more. The proteins extracted with fractional procedure were stored at -20 °C.

Two-dimensional gel electrophoresis and image analysis

The first dimension of isoelectric focusing (IEF) was performed

in immobilized pH gradient (IPG) gel strips with nonlinear immobilized pH 3-10 gradient. Three hundred μg of protein sample from each tissue specimen of normal mucosa, primary cancer and hepatic metastatic tumor was loaded respectively. IPG strips were placed onto the rehydration buffer (urea 8 mol/L, CHAPS 40 g/L, TBP 2 mmol/L, IPG buffer 3 g/L) and added into the sample solution without trapping air bubbles. IEF with a low voltage (30 V, followed by 60 V and 200 V) was carried out according to the programmed settings. After IEF was terminated, the IPG strips were equilibrated for 20 min in equilibration buffer. The second dimension of SDS-PAGE was performed on vertical systems. IPG strips were loaded and run on a 125 g/L acrylamide SDS-PAGE gel in electrode buffer (Tris 0.025 mol/L, glycine 0.192 mol/L, SDS 1 g/L, pH 8.3). Electrophoresis was performed with a current of 30 mA/gel for 15 min, then at maximum settings of 60 mA/gel for 4 h. The temperature of the cooling plate was set at 20 °C. After SDS-PAGE, the gels were stained with silver nitrate. Proteins were visualized by silver-staining and then scanned using Scan Prisa 640 UT. Two DE image computer analysis was carried out with Melanie 3.0 software (GeneBio, Geneva). Isoelectric points and molecular weights of individual proteins were evaluated with polypeptide SDS-PAGE-standards. Differential protein spots among normal colorectal mucosa, primary cancer lesion and hepatic metastasis were picked out by comparison of 2-DE images with Melanie 3.0 analysis.

Mass spectrometry and peptide mass fingerprint analysis

Selected differential protein spots were excised from 2-DE gels and transferred to a 96-well plate ready for trypsin digestion. The gel pieces were washed successively in water, Tris-HCl (20 mmol/L pH 8), Tris-HCl (20 mmol/L pH 8)/500 g/L acetonitrile and finally 1 000 g/L acetonitrile, and dried. The dried gel pieces were incubated in trypsin solution for 16 h at 37 °C. The resultant peptide mixture was extracted with 20 μL 5 g/L TFA/300 g/L

acetonitrile, followed by extraction with 20 μL 5 g/L TFA/600 g/L acetonitrile. α -cyano-4-hydroxycinnamic acid was used as matrix solution. Matrix assisted laser desorption/ionization-time of flight-mass spectrometry analysis were performed with MALDI-TOF mass spectrometer (Bruker, USA). The spectrum was obtained and recorded. Proteins were identified by searching NCBI and SwissProt databases using Profound peptide mass fingerprinting retrieval software (http://129.85.19.192/profound_bin/WebProFound.exe?FORM=1). Protein identities were assigned if at least four peptide masses were matched within a maximum of 100 ppm error spread across the data set and the candidate agreed with the estimated pI and molecular weight from the 2-DE gel.

Statistical analysis

Experimental data were analysed statistically with Crosstab chi-Square test and Student's *t*-test using SPSS 10.0. $P < 0.05$ was considered statistically significant.

RESULTS

2-DE image analysis of protein spots in matched sets of colorectal cancer

The hydrophobic protein profiles including partial membranous proteins from colorectal normal mucosa, primary cancer and metastatic foci in liver are displayed in Figures 1A-C. Comparing the 2-DE protein images of the three tissues, we found that the number of protein spots and protein expression level were significantly changed in primary cancer and hepatic metastatic lesion. Under the same experimental conditions, 390 ± 28 protein spots and 206 ± 22 , 236 ± 19 spots were found in normal colorectal mucosa and in primary cancer and hepatic metastasis, respectively. Compared with normal colorectal mucosa, the number of protein spots in primary cancer and metastatic tumor was significantly different $t = 53.116$, $t = 33.399$,

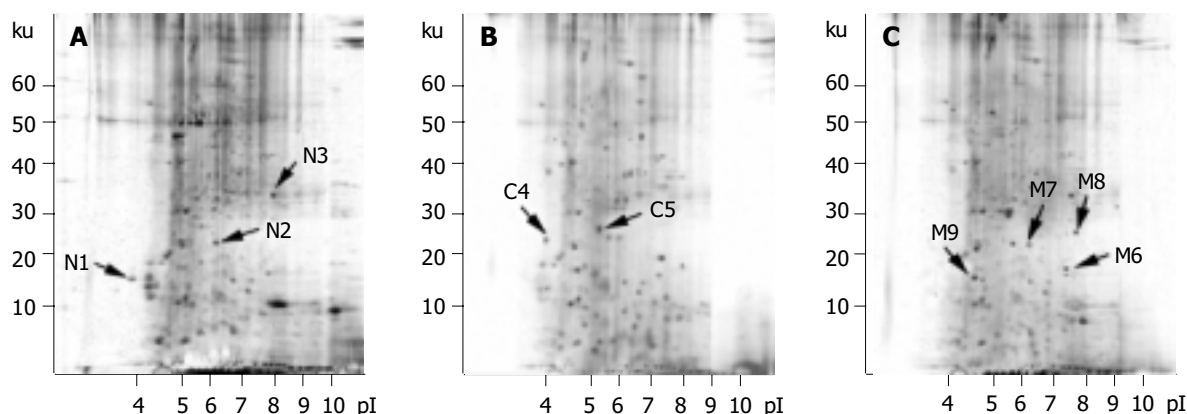


Figure 1 Silver-stained two-dimensional electrophoretic images of hydrophobic proteins from (A) Normal colon mucosa, (B) Primary colon cancer lesion, (C) Hepatic metastasis.

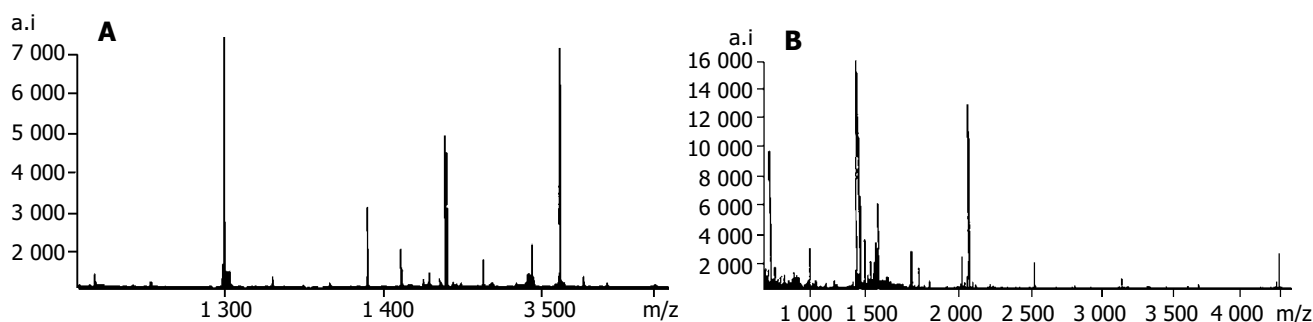


Figure 2 MALDI-TOF mass spectrometry and peptide mass fingerprint analysis of the differential protein spots (A) N2 protein spot from normal colon mucosa, (B) M6 protein spot from hepatic metastasis.

Table 1 Identification of nine differentially expressed proteins by peptide mass fingerprint and matching with proteins in databases

Spot No.	Accession (NCBI nr)	Theoretical pI	Theoretical M_r	Length (AA)	Protein name
N1	1CDL_B	4.0	16.56	147	Calmodulin complexed with calmodulin-binding peptide
N2	NP_003721	6.7	29.46	256	Ribonuclease 6 precursor
N3	XP_040720	8.4	32.76	287	Hypothetical protein XP_040720
C4		4.5	22.48	217	
C5	AAA51747	5.4	28.94	249	Proapolipoprotein
M6	AAA88054	6.8	15.96	147	Beta-globin
M7		5.5	22.57	203	
M8	XP_010554	6.8	21.25	191	Similar to cell division cycle 42
M9		4.5	14.72	127	

The difference of protein spot number between hepatic metastatic tumor and primary cancer was also significant ($t = 24.407, P < 0.01$).

Peptide mass fingerprinting of differential protein spots from 2-DE gels

Nine differential protein spots of the 2-DE gels were analysed using mass spectrometry. Three protein spots, N1, N2, N3, were taken from normal colorectal mucosa. C4 and C5 spots were from primary cancer lesions and four spots, M6, M7, M8, M9, from hepatic metastatic cancer. Molecular weight and isoelectric points of the nine protein spots were determined according to the standard molecular markers and peptide mass fingerprint analysis, and the data are shown in Table 1 and Figure 2.

Identification of differential expression proteins of colorectal cancer and hepatic metastasis

The peptide mass fingerprints obtained from the nine differential protein spots were compared to fingerprints obtained by theoretical cleavage of protein sequences in databases and the protein identities were assigned. Protein spots of N1, N2 and N3 from normal colorectal mucosa represented calmodulin, ribonuclease 6 precursor and hypothetical protein XP_040720, respectively. The expression of three proteins was lost in primary cancer and hepatic metastatic foci. Protein C5, matching to proapolipoprotein, expressed progressively from normal mucosa to primary cancer and hepatic metastatic tumor. Protein spot M6 was observed in normal mucosa and in hepatic metastatic cancer, but lost in primary carcinomas, being recognized as beta-globin. Expressed in hepatic metastasis, M8 was identified with cell division 42 (GTP-binding protein), but not found in normal mucosa and primary cancer lesion. Differential protein spots of C4 from primary carcinomas, M7 and M9 from hepatic metastasis were not identified by peptide mass fingerprint analysis because their peptide mass fingerprints had less homology with the known proteins in databases.

DISCUSSION

The initiation and hepatic metastasis of colorectal cancer involved multiple gene and protein alterations^[17-19]. Understanding the molecular basis of the disease is of great significance for its early detection and treatment. In this study, the proteome approach was applied to the identification of differential proteins between primary colorectal cancer lesion and its hepatic metastasis. We used 2-DE to isolate and analyze the set of hydrophobic proteins from normal colorectal mucosa, primary

cancer and hepatic metastatic tumor. It was of clinical importance to identify the differential expression proteins that had potentiality of being tumor markers and anticancer targets.

By comparison with 2-DE images, significant differences of protein expression were found in normal mucosa, primary cancer and hepatic metastasis, and the number and distribution of protein spots changed noticeably in the range of pH 4.0-9.0. Compared with normal mucosa, a number of protein spots with a molecular weight of 25-40 ku were lost in primary cancer and hepatic metastasis, but proteins with a molecular weight of 14-21 ku were observed in the same pH range. What the differential proteins were and what functions they performed in colorectal carcinogenesis and in hepatic metastasis attracted our attention. We identified nine protein spots and studied their roles in the course of initiation and hepatic metastasis of colorectal cancer.

Proteins of calmodulin (N1), ribonuclease 6 precursor (N2) and hypothetical protein XP_040720 (N3) were expressed in normal colorectal mucosa, but lost in primary cancer lesion and in hepatic metastasis. It indicated the loss of proteins was associated with colorectal carcinogenesis and hepatic metastasis. Calmodulin could regulate the concentration of calcium ions in cells and had important effects on normal cellular functions. Ca^{2+} regulation was necessary for cell differentiation and apoptosis. Combination of calcium ions and the receptors could influence cell signal transduction that controls cell differentiation and division. Low concentration of calcium ions made cell division easily, and the high concentration was advantageous to cell differentiation. Calcium ions promoted cancer cells into apoptosis. Thus, calcium was regarded as a chemoprophylaxis agent for colorectal cancer^[20,21]. We propose loss of calmodulin expression is connected with initiation and hepatic metastasis of colorectal cancer.

Ribonuclease 6 precursor protein belongs to the Rh/T2/S-glycoprotein class of extracellular ribonucleases and the gene is present in a single copy in the human genome and has been mapped to 6q27. This has been found to be a region of the human genome prone to rearrangements associated with several human malignancies^[22]. The family of the proteins possesses the function of ribozyme and self-splicing. They catalyze breaking of RNA, synthesis of polypeptide bonds and nucleotides. The protein can disintegrate DNA fragments, regulate cell biological behaviors and cell division. Loss of ribonuclease 6 precursor expression would facilitate carcinogenesis and infiltration. Ribozyme has been found useful in anticancer therapy^[23]. The loss of ribonuclease 6 precursor expression in primary colorectal cancer and hepatic metastasis could provide an experimental interpretation of ribozyme treatment.

XP_040720, a hypothetical protein is now defined as a

member of mannosidase- α class 1A. The protein is located in Golgi complex and participates in N-glycoprotein synthesis and oligosaccharide processing. It could play an important role in synthesis of membranous proteins and receptor proteins^[24]. But we do not know what functions the protein performs in colorectal carcinogenesis and metastasis. It could be regarded as a differentiation-related protein in normal mucosa cells, and loss of the protein expression is a dedifferentiation phenotype in the primary cancer lesion and hepatic metastasis, which is still lack of evidence.

Loss of the three protein expressions was considered to be connected with colorectal cancer initiation and hepatic metastasis. However proapolipoprotein expression was found stepwisely increased from normal mucosa to primary cancer and hepatic metastasis, and enhanced expression of the protein was in association with colorectal cancer. Proapolipoprotein was hydrolyzed by the signal peptidase and propeptidase, through which apolipoprotein was generated^[25]. Apolipoprotein is a carrier of lipids and regulates many cellular functions. It was found that apolipoprotein had an antiapoptosis effect and was related with carcinogenesis and progression. Enhanced expression of apolipoprotein has been reported in hepatoma^[26]. Our study provided an evidence of apolipoprotein in colorectal cancer.

A differentially expressed protein in our study was beta-globin. Its expression was found in normal mucosa and hepatic metastasis, but lost in primary cancer lesion. We suggested beta-globin was an associated protein with hepatic metastasis. The gene family of beta-globin consists of five functional genes and is located on chromosome 11. The family members expressed in order of the genes as they were arranged on the chromosome during various developmental stages. Beta-globin gene was activated and expressed continuously in late stage of pregnancy^[27,28]. Study on regulation of beta-globin showed that the variations of transcription frequency and cycle of phasic and specific expression of beta-globin family genes in developmental regulation were more important than changes of the gene transcription speed and expression quantity^[29]. Understanding the mechanisms of beta-globin regulation is helpful to researches on cell growth, carcinogenesis and progression. The cycle variation of beta-globin expression from the normal mucosa to primary cancer and hepatic metastasis implied genetic recombination and regulation changes in hepatic metastasis of colorectal cancer. It was reported that beta-globin expression was induced by treatment of chemotherapy agents in some cancer cells. This might indicate that the expression of beta-globin increased drug resistance of cancer cells and facilitated hepatic metastasis.

It has been found that Cdc 42, a differential protein expressed in hepatic metastasis, is a Rho-related member of the Ras superfamily, and acts as a GTP-binding protein^[30,31]. The protein performs the function of a molecular switch to control a diversity of cellular processes, and regulates cytoskeleton actin recombination, cell polarity and cell movement. Enhanced expression of Cdc 42 might facilitate cell division and accelerate cancer cell growth and proliferation, as well as interrupt signal transduction of apoptosis^[32]. Invasive behavior of cancer cells was reinforced by enhanced Cdc 42 expression through regulating cellular skeleton, cell adhesiveness and neovascularization. Therefore, Cdc 42 has been regarded as an associated protein in hepatic metastasis of colorectal cancer.

C4 expressed in primary cancer, M7 and M9 in hepatic metastasis, had low homology with the proteins known in database, and the three proteins were not identified. They manifested the possibility to be new proteins associated with colorectal cancer. Sequencing of the proteins and study of their functions are needed to help understand the mechanisms

of colorectal carcinogenesis and hepatic metastasis.

In summary, we identified 9 differentially expressed proteins that were associated with colorectal cancer genesis and hepatic metastasis. Relations of these proteins with colorectal cancer were not or seldom reported before. The differential proteins will help understand the mechanism of colorectal cancer genesis and hepatic metastasis. The results prove that proteome study represents a very useful and promising tool in discovering new tumor markers and anticancer targets of colorectal cancer.

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