RESEARCH COMMUNICATION

Comparative Proteomics Analysis of Colorectal Cancer

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Abstract

Background and Objective: Protein expression in colon and rectal cancer (CRC) and paired normal tissues was examined by two-dimensional gel electrophoresis (2-DE) to identify differentially expressed proteins. Materials and Methods: Five fresh colorectal cancer and paired adjacent normal tissues were obtained and differentially expressed protein spots were determined using PDQuest software, with identification on the basis of MALDI-TOF mass spectra. Results: Compared with normal colorectal mucosa, protein abnormal expression of 65 spots varying more than 1.5 times were found in 2-DE gels from colorectal cancer samples (P<0.05); forty-two proteins were up-regulated and 23 were down-regulated; twelve protein spots were identified using mass spectrometry, of which 8 were up-regulated, includimng HSPB1and Annexin A4, while 4 were down-regulated, the results being consistent with Western blot findings. Conclusions: Two-dimensional electrophoresis reference maps for CRC tissues and adjacent normal mucosa (NMC) were established and 12 differentially expressed proteins were identified. Up-regulated HSPB1 and Annexin A4 may play many important roles in the pathogenesis of colorectal cancer.

Keywords: Colorectal cancer - two-dimensional gel electrophoresis - mass spectrometry

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Introduction

Colorectal cancer (CRC), being the world's third most common malignancy, is one of the most common digestive tract cancers (Parkin, 2001). There were over one million new cases of colorectal cancer currently around the world (Anderson et al., 2003). In our country, there has been a strong upward trend in CRC incidence with dietary changes due to economic development, social progress and improved living standards, which was the fourth leading cause of death among cancers. Approximately 30% to 40% of patients who received treatment in clinic were considered as advanced cancer patients, and the tumor recurrence rate for those patients undergoing radical resection, however, is still relatively as high as 40-70% (Nosho et al., 2007). Therefore, the research on early diagnosis of colorectal cancer has important clinical value. Nevertheless, at present, the mechanism of pathogenesis remains unclear and most recent studies have focused on differences at the molecular genetic changes, which is not enough to explain the pathogenesis of colorectal cancer (Zhao et al., 2007). As we all know, protein is the carrier of life activities and the major executors of the functions of genes. Genes achieve their effects by directing protein synthesis, and therefore, the proteomic study on mechanism of colorectal cancer will play a key role in prevention, diagnosis and treatment for colorectal cancer. In this study, the differentially expressed proteins between CRC and normal tissues were identified and differential protein expression profiles were established by proteomic approach, which provides a theoretical basis and foundation for further searching for the potential biomarkers and therapeutic targets relevant to human CRC occurrence and development.

Materials and Methods

Subjects

The colorectal cancer samples were collected from hospitalized surgical patients who were admitted in Department of Gastrointestinal Surgery, Guangdong Provincial People's Hospital from January 2010 to March 2010. Of the 32 colorectal cancer cases enrolled, 5 were Dukes' A, 15 were Dukes' B, 7 were Dukes' C and 5 were in Dukes' D. The mean age of patients was 55 years old. Five patients with Dukes' A colorectal cancer were included in colorectal cancer group and the paired normal colorectal mucosal samples were dissected from a distance of at least 10 cm from the Dukes' A colorectal cancer.

Sample preparation

Tissue samples of 100mg were taken and washed three times in cold PBS to remove residual blood plasma. Add 3ml cell lysis buffer (Carbamide7 mol/L, Thiourea 2

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mol/L, 4%CHAPS, Tris 30 mmol/L, pH8.5) and protease inhibitor to the tube, and they were cut into small pieces and sonicate on ice. After incubated at room temperature for 30 minutes, the cell lysates were centrifuged at 12,000rpm for 15 minutes at 4°C. Supernatant was used for protein purification (PlusOne 2D-Clean-up kit), and the protein concentration in samples was analyzed by 2D Quant Kit.

Immobilized pH gradient two-dimensional electrophoresis

The protein sample and lysis buffer was fully mixed, making the total sample volume to 450 μL. Hydration and isoelectric are performed automatically with a maximum current setting of 50 mA/strip at 20 °C for 60, 000 vh on an Ettan IPGphor III Isoelectric Focusing System. After the IEF gel has been run, IPG strips were equilibrated for 15 min respectively in equilibration buffer containing 10 g/LDTT and 25 g/L iodoacetamide (carbamide 6 mol/L, 75 mmol/L Tris-HCl pH8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue). The strips were then transferred to 12.5% SDS-PAGE gels, and then seal the plugs in the gel with 1% low melting point (LMP) agarose (15 W/strip). Electrophoresis was carried out at 15°C until the bromophenol blue tracking dye just reached the bottom of the gel; A mass spectrometry compatible silver staining was performed and finally the images were scanned via white light scanners.

Gel image analysis

PDQuest Software was utilized for spot detection and matching, and spots with a P value of less than 0.05 and an average change greater than 1.5-fold were considered as statistically significant regulated spots.

In-gel digestion

Each slice was cut into1 mm3 gel particles and washed twice with sterile ultrapure water and then equal volumes of 30 mM K3Fe (CN) 6 and 100 mM $Na_2S_2O_3$ was mixed for gel destaining. 2 μL (25 ng/μL) of trypsin was added to each point and incubated overnight. Put 2 μL enzyme lysates onto Anchrochip, and then the samples were moved into MALDI TOF/TOF Mass Spectrometer (Ultraflex III) to undergo mass analysis.

Database search

Mascot database search engine was employed, and Swissprot database was selected searching for human sequence. One missed cut and a mass deviation of 100 ppm was usually allowed in the database searches, with scores over the significant threshold ($p<0.05$) were considered credible.

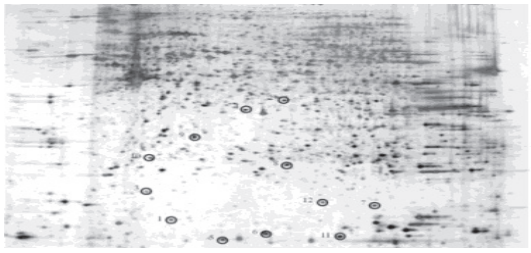

Figure 1. Distribution of Differentially Expressed Proteins

Western blot

A 50 μg of protein from each sample was fractionated by 12.5% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to PVDF membranes. Non-specific antigen was blocked with 5% nonfat dry milk, and add primary antibody and incubate overnight at 4°C; the membrane is washed three times in Tris buffered saline (TBS) containing 0.1% Tween20, and the washed membrane was then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody was added to incubate for 1 h. The dry plates were then exposed to x-ray film (Kodark, 2000) after washing three times with TBST.

Results

Differentially expressed protein

The image precisely scanned by white light scanners and image data analyzed using PDQuest software all revealed that total protein distribution pattern in normal and cancerous colon and rectal tissue was extremely similar (Figure 1). Compared with normal colorectal mucosa, 65 proteins with abnormal expression varied up to more than 1.5 times were found in 2-DE gels from colorectal cancer samples ($P<0.05$); forty-two proteins were up-regulated and 23 proteins were down-regulated in tumor tissues; twelve protein spots were identified using mass spectrometry, among which 8 proteins were

Table 1. Mass Spectrometricprotein Identification Results

Protein index	Theoretical Mr/pI	MS/MS score	Protein Coverage(%)	Protein level	Accession NO.	Protein Description
1	25133/6.0	51	9	↑	P30041	Peroxiredoxin-6
2	86847/6.67	121	3	↓	P00751	Complement factor B
3	36132/5.65	35	7	↑	P02649	Apolipoprotein E
4	68660/5.35	106	7	↓	P38606	V-type proton ATPase catalytic subunit A
5	22826/5.98	141	13	↑	P04792	Heat shock protein beta-1
6	28017/7.67	142	5	↑	P30048	Thioredoxin-dependent peroxide reductase
7	33851/5.57	135	8	↓	P25311	Zinc-alpha-2-glycoprotein
8	25537/8.26	110	12	↑	P22352	Glutathione peroxidase 3
9	41710/5.29	116	13	↑	P60709	Actin, cytoplasmic 1
10	35860/5.84	437	20	↑	P09525	Annexin A4
11	17138/5.83	124	14	↑	P15531	Nucleoside diphosphate kinase A
12	37631/6.03	187	7	↓	P01876	Ig alpha-1 chain C region

Figure 2. Western Blot Results of HSPB1 and Annexins A4. A and C: colorectal cancer tissues; B and D: normal tissue

up-regulated and 4 proteins were down-regulated.

MS protein identification

Twelve differentially expressed protein spots were preliminarily identified after in-gel digestion, mass spectrometry and database search. The results were listed in Table 1.

Western blot

The results obtained indicated,in colorectal cancer tissues, the expression of HspB1, that is heat shock proteinbeta-1, and Annexins A4 was all much higher than in normal tissues, which wasconsistent with the results gained from two-dimensional electrophoresis (Figure 2).

Discussion

Colorectal cancer is one of the malignant tumors, which pose a serious hazard to human health. In recent years, along with lifestyle and diet changes, the incidence of colorectal carcinomas showed a gradual increase and occurs at younger age. The statistics conducted in Beijing and Shanghai in 2005 had been noted that colorectal cancer is the second most common cancer, and was the third most common cause of cancer death. Therefore, the prevention, diagnosis and treatment of colorectal carcinoma have been attracted worldwide attention. Now it is considered that the incidence of colorectal cancer is a multi-step, multi-stage, multiple genes involved genetic disorder. Nevertheless, its oncogenic mechanism remains unclear. Proteomics is the large-scale study of proteins, concerning the quantitative analysis of dynamic changes in protein on the overall level, which affiliate the scanning of specific markers for early diagnosis and prognosis as well as effective therapeutic target of early-stage cancer (Jimenez et al., 2010).

The small heat shock proteins (sHSPs) are a diverse group of stress-inducible proteins with a molecular mass of 15-30 kDa, which characterized by a common structural feature of the α2-crystallin domain. The region is of approximately 85-100 amino acid residues in the highly conserved C-terminal region of the protein (Oya-Ito et al., 2011). Small heat shock proteins (sHSPs) are products of heat shock response and are abundant and ubiquitous in almost all organisms. HspB1, that is Heat shock protein beta-1, also donated Hsp25 (mouse) and Hsp27 (human), is the most widely distributed and well studied sHsp (Ferns et al., 2006). Thus it may play a vital role in maintaining normal protein structure, and cell response to stress tolerance (Doshi et al., 2010). High expressed HspB1 play a vital role in promoting neuronal survival and regeneration following peripheral nerve injury, survival of injured sensory and motor neurons (Carmichael et al., 2002; O'Reilly et al., 2010). The up-regulated HspB1 can suppresses the cell death induced by PolyQ (Friedman et al., 2009) and interferes in the construction of cytoskeleton neurofilament network (Kalaydjleva et al., 2000); mutations in HSPB1 can intermediate filament protein assembly, reduce the actin binding, trigger actin polymerization, thereby affecting axonal transport, leading to axonal degeneration (Hartl et al., 2002; Jakubowicz-Gil et al., 2008). In addition, it is confirmed that HspB1 exhibits high expression in several cancers including stomach, breast, ovary and prostate, and is a sign of poor prognosis (Sirchia et al., 2008; Sharma et al., 2009; Morri et al., 2010) High levels of Hsp27 constitutive expression have also been detected in colorectal cancer tissue, indicating it may play an important role at the early stage of the colorectal tumorigenesis and has also been associated with a variety of tumor types.

Annexins are a family of Ca^{2+}/phospholipid-binding proteins that function as organizers of membrane domains and membrane- transport as well as of ion fluxes across membranes; Meanwhile, the changes in expression and cellular localization of Annexins had been observed in a variety of cell types that undergo tumor progression and development in a variety of tumors, linking to tumor development and progression in various diseases (Gerke and Moss 2002; Lim and Pervaiz 2007; Baskic et al., 2010). Annexins A4 is a member of the annexin superfamily, which can promote membrane fusion and exocytosis, and inhibit phospholipase activity after binding with Ca^{2+}, involving in cell signaling, anti-apoptotic and other important physiological processes (Miao et al., 2009). It is reported in literature that the expression of AnnexinA4 in pancreatic cancer tissues was significantly higher than that in normal and pancreatitis tissues, indicating AnnexinA4 was related to the development and occurrence of pancreatic cancer (Shen et al., 2004). Annexin A4 is overexpressed in renal cell carcinoma, and immunohistochemical analysis showed altered location Annexin A4 in tumor cells, which is found in the cell membrane and in the cytoplasm, revealing AnnexinA4 may be involved in development and progression (Zimmermann et al., 2004). Altogether our data suggests that Annexin A4 is overexpressed at early-stage colorectal cancer, which indicates that it may play a role in the oncogenesis of colorectal cancer. Those relevant researches suggested that a change of Annexin A4 expression may have impact on cellular behavior such as migration, invasion, proliferation rate, etc, which may cause abnormal cell proliferation and regulation in the tumor. Therefore, Annexin A4 may eventually serve as diagnostic markers or therapeutic targets for malignant tumors. Nevertheless, the specific mechanism on tumor metastasis remains unclear.

In conclusion, colorectal cancer is a genetic disorder, and its incidence is a multi-step, multi-stage, multiple genes involved long time process. Furthermore, many expression and function changes in protein occur in this process. In this article, 12 differentially expressed spots were identified by two-dimensional electrophoresis and MALDI-TOF mass spectrometry, and two of the meaningful proteins were verified. The differentially expressed proteins were systemically analyzed and their

functions and interrelationships were detected, which is of great clinical significance and development value on diagnosis, treatment and prevention of tumor.

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