African Journal of Biotechnology Vol. 9(41), pp. 6840-6847, 11 October, 2010
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.425
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Proteome analysis of human colorectal cancer tissue using 2-D DIGE and tandem mass spectrometry for identification of disease-related proteins

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Accepted 23 August, 2010

Laser capture microdissection and two-dimensional difference gel electrophoresis were used to establish the proteomic profiles for tumor and matched adjacent tissues from 12 patients. Differential protein spots were identified by mass spectrometric analysis. The cDNA of the differential protein was transfected into colorectal cancer cells, and the biological behavior of these cells was observed. The proteomic profile in colorectal cancer tissues was significantly different from that in normal adjacent tissues. There was a 1.5-fold difference and 60 differential protein spots between cancer and adjacent tissues. Ten differential protein spots were analyzed. Among them, two protein spots were down-regulated and eight protein spots were up-regulated in the primary tumor tissues. After identification by mass spectrometry, the two down-regulated proteins were carbonic anhydrase II and protein disulfide isomerase, and these eight up-regulated proteins included APC-stimulated guanine nucleotide exchange factor, phosphoglycerate kinase 1, fumarate hydratase, aldolase A, activator protein 2B, glutathione S-transferase A3, Arginase and zinc finger protein 64 homolog. After been transfected with carbonic anhydrase II, the invasive ability, mobility and drug resistance of colon cancer lovo cells were significantly reduced. The proteomic profile was significantly different between colorectal cancer tissues and normal adjacent tissues. The down-regulation of carbonic anhydrase II and protein disulfide isomerase and up-regulation of APC-stimulated guanine nucleotide exchange facto, aldolase A, glutathione S-transferase A3 and arginase were correlated with the onset of colorectal cancer.

Key words: Colorectal cancer, proteomics.

INTRODUCTION

Colorectal cancer (CRC) is one of the common malignant tumors in the world, with an increasing morbidity, and its mortality ranks the second among all cancers (Jemal et al., 2008; Ferlay et al., 2007). The development and progression of CRC is a multistep procedure with distinct genetic events occurring at different stages (Fearon and Vogelstein, 1990). This process may require 15 to 20 years, with the transition from adenoma to adenocarcinoma taking up to 10 years (Morson, 1974). Early diagnosis is the key point for the treatment and prevention of malignant tumors. However, there is no special symptom in the early stage of malignant tumors. The majority of the malignant tumors were in the advanced stage when they were diagnosed, with extensive metastases. Therefore, new suitable tumor markers were especially important for the early diagnosis and treatment of malignant tumors (Chignard and Beretta, 2004). Colonoscopy is a reliable means for the screening and

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Abbreviations: CRC, Colorectal cancer; **2DE,** two-dimensional gel electrophoresis; **MS,** mass spectrometric; **LCM,** laser capture microdissection; **2D DIGE,** two-dimensional differential in-gel electrophoresis; **DDT,** dichlorodiphenyltrichloroethane; **SDS-PAGE,** sodium dodecyl sulfate-polyacrlamide gel electrophoresis; **LC-MS/MS,** liquid chromatography-tandem mass spectroscopy; **PDI,** protein disulfide isomerase; **GEF,** guanine nucleotide exchange factor; **GST A3,** glutathione S-transferase A3; **EDTA,** ethylenediamine tetra acetic acid; **PBS,** phosphate-buffered saline.

diagnosis of CRC. However, it is very difficult to be accepted by patients without symptoms or with slight symptoms due to its invasion. The known tumor markers such as CEA and CA199 are not suitable for the screening and early diagnosis of CRC due to their low sensitivity and specificity. Therefore, currently, tumor markers with high sensitivity and specificity were deficient for the early diagnosis of CRC clinically.

As a technique with high-flux and high resolution, proteomics has been widely applied in proteome analysis of tumors (Cho, 2007a). The onset and development of the tissues and cells can be detected at the entire protein level through analyzing the differential expression of proteins. The combination of 2-DE and mass spectrometry can be used to identify differential proteins between tumor cells and normal original cells, and these differential proteins imply a large quantity of biological information. Some of the special proteins are special markers of tumors (Kim and Kim, 2007). The most consistently successful proteomic method is the combination of two-dimensional gel electrophoresis (2DE) for protein separation and visualization and mass spectrometric (MS) identification of proteins using peptide mass fingerprints and tandem MS peptide sequencing. Laser capture microdissection (LCM) is used to precisely harvest tumor tissues from the sample, avoiding the interference of other tissues. Therefore, it can better reflect the change of the proteins in the tumor tissues (Ornstein et al., 2000; Simone et al., 2000). Two-dimensional differential in-gel electrophoresis (2D DIGE) is a kind of method to precisely analyze the differential protein abundance of the samples, in which the protein sample was labeled before 2-D electrophoresis. The 2-D DIGE methodology was initially described by Unlu et al (1997). In this method, the compared protein samples are labeled with different fluorescent dyes and then analyzed together in the same 2-D electrophoresis gel. It is possible to separate up to three different labeled samples within the same 2D gel. The comparison between normal and cancer tissues is a common proteomic approach, with obvious benefits for the identification of differentially expressed markers. In an early study, paired patient samples from normal and neoplastic tissues were used for characterization of differences in protein profiles during CRC progression. Cancer tissues were staged according to Duke's classification. A significant alteration of 18 proteins was identified by quantitative studies during tumor formation (Stulik et al., 2001). More proteins that appear to change consistently among patients with CRC were identified through several other comparative proteomic studies of human colorectal cancers and adjacent normal tissue identified (Friedman et al., 2004; Alfonso et al., 2005; Kim et al., 2009).

In our study, the differential proteins between colorectal cancer tissues and normal adjacent tissues were analyzed by using LCM, 2D DIGE and liquid chromatography tandem mass spectrometry, and the CRC-related proteins were separated and identified.

Effects of these proteins on the biological behavior of colorectal cancer cells were explored. This approach may help to identify proteins that could be useful as new tool in the diagnosis or prognosis of this type of patients and perhaps to develop new therapeutic strategies to better aid therapy on the neoplastic target while minimizing the impact on normal tissues.

MATERIALS AND METHODS

Sampling

Tissue samples were obtained from patients (6 men and 6 women; median age: 53 years; age range: 38 -71 years) who had undergone surgical resection for primary sporadic colorectal adenocarcinomas at the Department of General Surgery, General Hospital of Beijing Military Command, Beijing, China. None of the patients received any drug therapy for at least 3 months before surgery. All patients were thoroughly informed about the study and gave written consent for the investigation in accordance with the ethical guidelines of the Local Ethical Committee.

Matched sets of colorectal cancer and normal colon mucosa (10 cm from the edge of tumorous lesions) used for 2-D DIGE analyses were obtained within 30 min after surgical resection. The tissue samples were checked and sliced by a pathologist. All samples were diagnosed by histopathology as colorectal cancer.

Samples of small colorectal cancers were collected from the distal colon and rectum and were staged according to American Joint Committee on Cancer classification. Six tumors were located in the distal colon, and six tumors were located in the rectum. All twelve tumors were adenocarcinoma classified as moderately differentiated. Every tumor was at stage I (T1-2, N0 and M0). Cancer tissue (from the edge of the tumor) and adjacent normal mucosa (at least 10 cm from the tumor) were excised from each surgical specimen.

Chemicals

DTT, urea, thiourea, CHAPS, SDS, IPG buffer, pharmalyte, IPG gel strip (18 cm, 3 – 10 NL), acrylamide, N, N-methylenebis-acrylamide and PMSF were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). 2-D gels were cast and run in a Bio-Rad Multi Cell. CyDye DIGE was purchased from GE Healthcare (USA). HPLC- grade ACN, formic acid and TFA were purchased from Sigma (St Louis, USA). All buffers were prepared with Milli-Q water (Millipore, Bedford, USA).

LCM/2D-DIGE analysis

2-D DIGE was carried out according to the instructions of Ettan DIGE which was provided by GE Healthcare. Tumor and patient-matched normal colon mucosa from 12 patients were stained with nuclear fast red and subjected to LCM using an Arcturus PixCell IIe microscope (Arcturus, Conifer, CO). Protein from captured cells was extracted and quantified. The 12 patients were then randomly divided into four groups, with 3 patients in each group. 2-D DIGE was carried out according to Table 1. An internal standard pool generated by combining equal amounts of extracts from all 3 pairs of tumor and normal mucosa tissues (totally 6 samples) was labeled with Cy2 fluorescent dye. It is able to minimize gel-to-gel variation by allowing the inclusion of an internal standard within each gel. All six samples evenly distributed between CyDye DIGE fluorescence Cy3 and Cy5 to minimize the variation between fluorescence. Fifty micrograms of each tumor and paired normal mucosa protein extracts were minimally labeled with Cy3 or

Table 1. Experiment design of different fluorescent dye labeling for internal standard (Cy2) and sample 1, 2 and 3 (Cy3 or Cy5).

Gel	Different fluorescent dye labeling		
	Cy2	Cy3	Cy5
1	Internal standard	Normal 1	Cancer 2
2	Internal standard	Cancer 1	Normal 3
3	Internal standard	Normal 2	Cancer 3

Cy5 fluorescent dyes (400 pmol fluorescence/ 50 µg of protein extracts). Labeling reaction wasperformed at 4℃ for 30 min and quenched with 10 µl of lysine for 10 min on ice in dark. Equal amounts (50 µg) of quenched Cy3 or Cy5 labeled samples from each patient, together with the aliquoted 50 µg of Cy2-labeled internal standard pool (described above), were focused using IPG strips (Ready Strip, Bio-Rad, pH 3-10,18 cm) in the protein IEF Cell (Bio-Rad), with the addition of DeStreak Reagent (GE Healthcare). The IPG strips were equilibrated with equilibration buffers containing 2% DTT and 2.5% iodoacetamide for 10 min each sequentially. For the first electrophoresis, an electric potential of 50 V was applied for 12 h, 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 10000 V for 1 h and 1000 V for 4 h. The second 12% SDS-PAGE was then carried out for all 3 gels simultaneously using Ettan DALT electrophoresis system (Bio-Rad). After electrophoresis, the gel was placed on light-resistant container with SDS electro- phoresis buffer at room temperature, and the gel was scanned immediately.

DIGE image analysis

Labeled proteins in each gel were visualized using a Typhoon 9410™ (GE Healthcare) fluorescence scanner at 488/600 nm for Cy2, 532/580 nm for Cy3 and 633/520 nm for Cy5 dyes. Images were analyzed with the DeCyder™ software platform v 6.5 (GE Healthcare, USA). Gel image pairs were processed by the DeCyder™-DIA (Differential In-gel Analysis) software module to co-detect and differentially quantify the protein spots in the images, taking the sample as a reference to normalize the data, so the rest of the normalized spot maps could be compared among them. At the second stage, the DeCyder™-BVA (Biological Variation Analysis) software module was applied. BVA performs a gel-to-gel matching of the internal standard spot maps from each gel. Comparison between the different experimental groups and the control group was tested by student's t-test.

Mass spectrometry analysis and database searching

A replicate gel was made with 1 mg samples, and stained by Coomassie Brilliant Blue and then matched with the DIGE gel maps. The matched proteins spots were excised from gels and cleaved with trypsin by in-gel digestion. The peptides were subjected to liquid chromatography-tandem mass spectroscopy (LC-MS/MS) using an LTQ ion trap mass spectrometer (Thermo Scientific, Waltham, MA). Protein identities were determined from LC-MS/MS data using the sequest algorithm as implemented by the BioWorks Browser v 3.2 (Thermo Scientific) and searching against the National Center for Biotechnology Information (NCBI) database.

Cell transfection

The expression vector pcDNA3.1- carbonic anhydrase II was successfully constructed and then transfected into human colon adenocarcinoma Lovo cells with the lipidosome transfection method.

Biological behavior observation

Cell invasion analysis

Cell invasion basement membrane experiment was carried out in Transwell chambers (Costar Corporation). The number of cells in the field of vision was calculated. The invasive ability of cancer cells was expressed with the relative cell number of the invasive cells.

Cell mobility analysis

After polyester acid filter membrane (8 µm in pore size) was adhesive to the Transwell chamber, 5 µg of Fibronectin (Promega) was spread on the up- and down-surfaces of the filter membrane. After being digested with EDTA/PBS, about 2.5×10^5 cells were added into the Transwell chamber. The number of cells in the field of vision was calculated. The mobility of cancer cells was expressed with the relative cell number.

MTT assay

Cells with and without pcDNA3.1-carbonic anhydrase II transfection served as the experimental group and the control group. The inhibition ratio of 5-Fu and Oxaliplatin on cells were observed. Inhibition ratio = [1-(OD value of the experimental group/ OD value of the control group)] × 100%.

Statistical analyses

All data were analyzed with SPSS 12.0 software and the protein expression of different tissues was compared with t test. A p value of less than 0.5 was considered statistically significant.

RESULTS

2-D DIGE

Tissues from 12 colorectal cancer patients were analyzed by 2-D DIGE. The spots were clearly distinguished, with about 1200 spots in each gel. The protein profile was significantly different between the colorectal cancer tissues and normal adjacent tissues. The DIGE image was analyzed with DeCyder™ software to detect the differential spots (Figure 1). After background elimination, gel normalization and artifacts removal, the matching rate of each gel to the MASTER gel was more than 90%. There were 1.5-folds differential expression and 60 differential protein spots between tumor tissues and normal adjacent tissues. The differential spots were seen in Figures 2 and 3. It was found that there were two differential spots with 25-60 kD and pH 6.0 - 8.0 and eight differential spots with 25-80 kD, pH 6.0 - 10.0. These spots stably appeared on the parallel gel. These differential spots were excised and analyzed by mass spectrum.

Mass spectrum analysis

Eight differential protein spots with 5-folds different expression

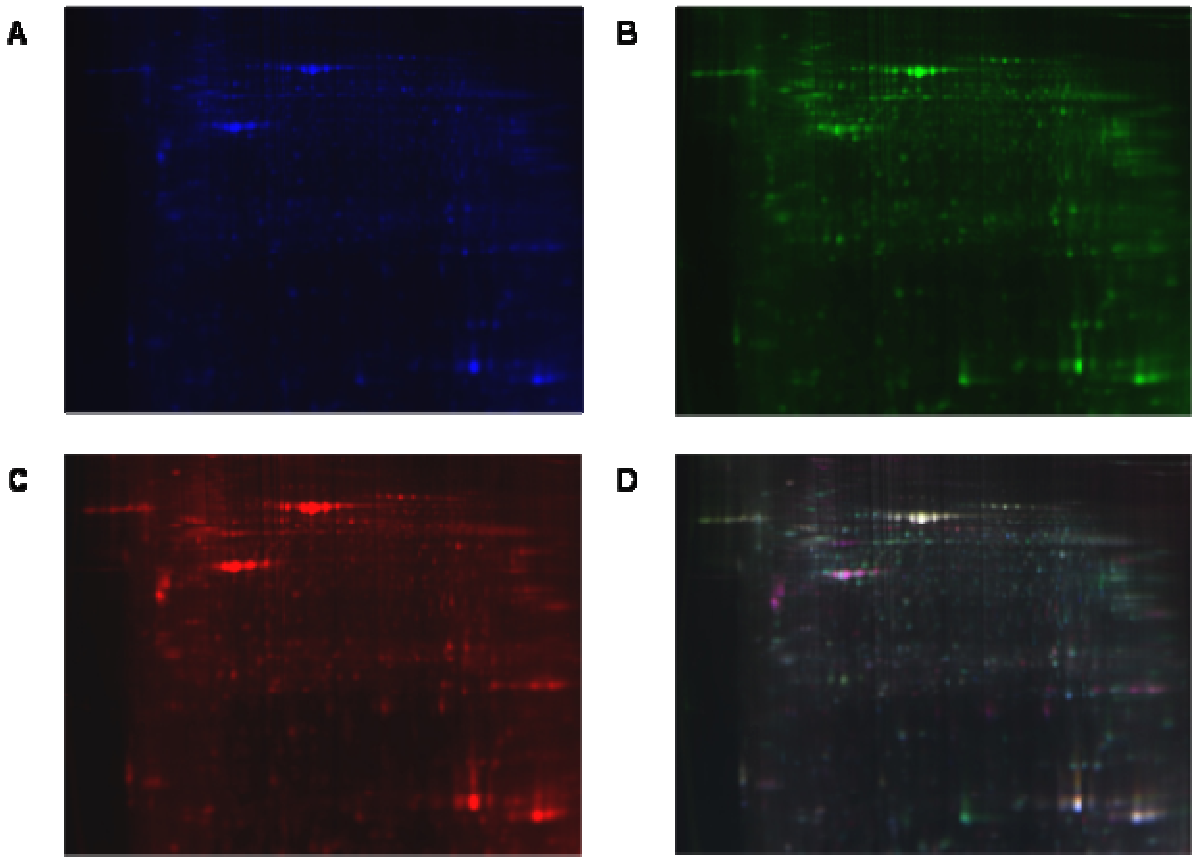

Figure 1. The scan result of one gel and the overlay map. Each gel contained the same pooled internal standard sample minimal dye labeled with CyDye DIGE Fluor Cy2 and two protein samples labeled with fluor Cy3 or Cy5 minimal dyes. (A) Internal standard Cy2 dye. (B) Normal mucosa tissues Cy3 dye. (C) Cancer tissues Cy5 dye. (D) Image analysis overlay images.

were analyzed and identified with the LC- MS/MS method. Two differential spots with 25 - 60 kD and pH 6.0 - 8.0 were up-regulated in normal adjacent tissues as compared with the tumor tissues. The result of mass spectrum showed that these two proteins were Carbonic Anhydrase II and protein disulfide isomerase (PDI). Eight differential spots with 25 - 80 kD and pH 6.0 - 10.0 were up-regulated in tumor tissues as compared with the normal adjacent tissues. They were APC-stimulated guanine nucleotide exchange factor, phosphoglycerate kinase 1, fumarate hydratase, aldolase A, activator protein 2B, glutathione S-transferase A3, arginase and zinc finger protein 64 homolog (Table 2).

Effects of carbonic anhydrase II cDNA transfection on the biological behavior of cells

The pcDNA3.1- carbonic anhydrase II was successfully constructed and transfected into lovo cells. The invasion ability and mobility of carbonic anhydrase II-transfected cells were significantly decreased as compared with the non-transfected cells. The inhibition ratio of 5-Fu and oxaliplatin was significantly increased in the carbonic anhydrase II-transfected cells as compared with the non-transfected cells ($P < 0.05$).

DISCUSSION

The differential analysis of the protein profiles between tumor tissue and normal tissue is the most extensive and effective method in tumor proteomics (Lawrie et al., 2001; Simpson and Dorow, 2001). The combination of 2-D DIGE and MS is an effective method for screening the differential proteins of colorectal cancer. DIGE ensures the sensitivity and accuracy of protein detection, which is suitable for the analysis of differential protein profiles. The newly deve- loped quantitative proteomics has high sensitivity, dynamic range and reproducibility vs the conventional 2-D DIGE (Ong and Mann, 2005; Unlü et al., 1997). Proteomic, together with other global technologies, have taken CRC research to a new era, providing a wealth of new informa- tion, sometimes at a pace too fast for proper validation and evaluation. Basic research relating to the understanding of the mechanisms of cancer

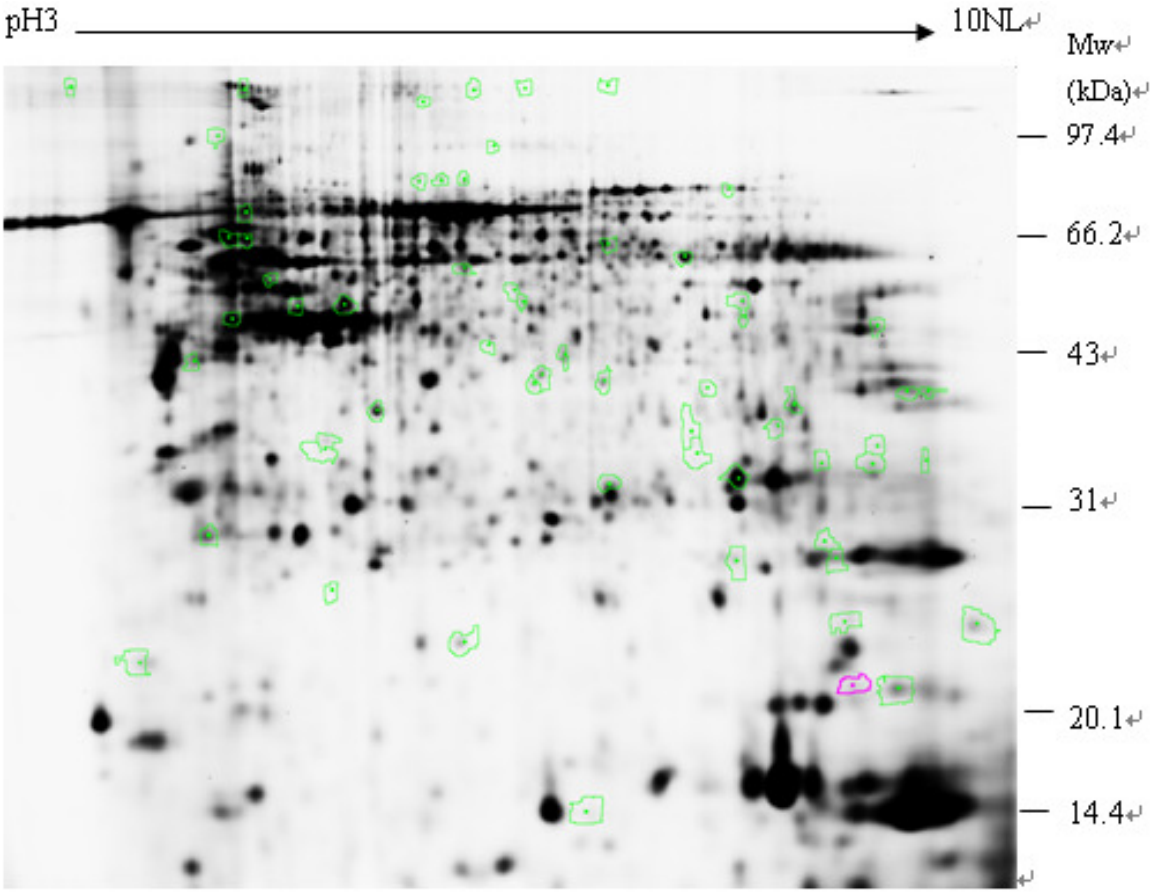

Figure 2. 1.5-fold difference and 60 differential protein spots between colon cancer tissues and normal adjacent tissues.

development has particularly benefited from these new approaches. But the promise of rapid development of novel clinical applications has not materialized yet. There is no doubt that there is room for improvement in all aspects of proteomic research (Georgia et al., 2009).

As a difference with the previous studies, we used the approach of protein separation by 2D DIGE coupled with protein identification by LC-MS/MS analysis and database search. This analysis identified strongly discriminated 2 down-regulated proteins and 8 up-regulated protein spots in colorectal cancer tissues. The identified proteins may be involved in the process of tumorigenesis and invasion.

There were 2 unique proteins including CAII and PDI that were down-regulated in CRC tissues versus normal colon mucosa, which might be related to the onset and development of colorectal cancer. CAII is the key enzyme for adjusting acid-base homeostasis in human bodies. Our study showed that CAII could decrease the invasive ability and mobility of tumor. The low expression of CAII could promote the proliferation and metastasis of cancer cells. Therefore, CAII could be used for screening colon cancer, and could also be used for the prevention and treatment of the recurrence and metastasis of colorectal cancer (Okoyama et al., 1997). PDI which is located in endocyto-plasmic reticulum is an important protein fold catalyzer. It catalyzes the formation of protein disulfide linkage and the rearrangement of mispairing disulfide linkage, and also has molecular chaperones activity for inhibiting the aggregation of misfolding proteins. Disulfide bond formation is probably involved in the biogenesis of approximately one third of human proteins. A central player in this essential process is protein disulfide isomerase (PDI). PDI was the first protein-folding catalyst reported (Hatahet et al., 2009). The abnormal expression of PDI was also found in the CRC caco-2 cells (Stierum et al., 2003). The restricted protein synthesis due to the low PDI expression may be correlated with the onset and development of colorectal cancer, the mechanism of which still requires further studies.

There were eight unique proteins that were up-regulated in CRC tissues versus normal colon mucosa. APC- stimulated guanine nucleotide exchange factor acts as the guanine nucleotide exchange factor (GEF) for RhoA and RAC1 GTPases. Binding of APC may activate RAC1 GEF activity. The APC-ARHGEF4 complex seems to be involved in cell migration as well as in E-cadherin-

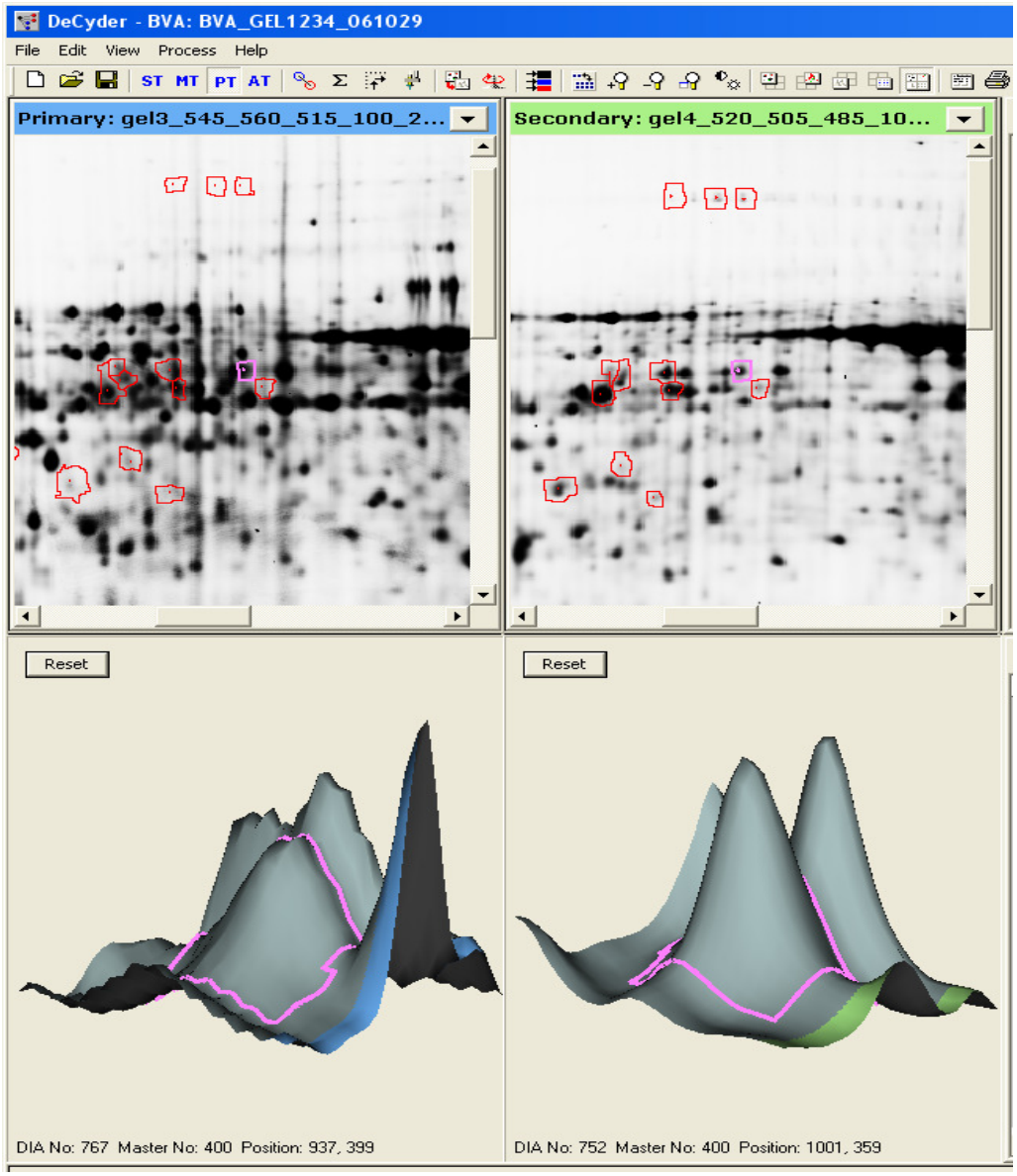

Figure 3. Analysis of protein disulfide isomerase with DeCyder software (Left: tumor tissue; Right: NORMAL intestinal mucosa tissue).

mediated cell-cell adhesion (Sjöblom et al., 2006). Mutation of the tumor suppressor is a key early evented in the development of most colorectal tumors. APC promotes degradation of betacatenin and thereby negatively regulates Wnt signaling, whereas mutated APCs present in colorectal tumor cells are defective in this activity. APC also stimulates the activity of the guanine nucleotide exchange factor Asef and regulates cell morphology and migration. Truncated mutant APCs constitutively activate Asef and induce aberrant migration of colorectal tumor cells. Asef plays a critical role in tumor angiogenesis and may be a promising target for cancer chemotherapy (Kawasaki et al., 2010).

Aldolase A expression was significantly and stably up-regulated in each sample. Aldolase plays an important role in glucose metabolism. Aldolase has a molecular weight of 160 kDa and has three isozymes, namely: aldolase A, B and C. The enzyme is probably present in all cells; it occurs in particularly large quantities in the muscles, liver and brain. Aldolase becomes elevated in serum with malignant tumors, and isozyme A is predominant in serum (Cho, 2007b), which has been confirmed by Western-blot and immunohistochemistry (Tomonaga et al., 2004). Aldolase A could serve as a biomarker for the diagnosis of colorectal cancer. However, its sensitivity and specificity need further studies.

Some proteins such as glutathione S-transferase A3 (GST A3) may be related to drug resistance of tumor cells. GST can promote drug metabolism and reduce the cytotoxic effect of anti-tumor drug through binding lipophilic

Table 2. List of differently expressed proteins in CRC, determined by use of 2DE and MS.

Spot number	M W [a]	PI [b]	NCBI ID	Name	Trend of expression [c]
1	29070	6.94	GI:4557395	carbonic anhydrase II [Homo sapiens]	T < N
2	57043	6.10	GI:860986	protein disulfide isomerase [Homo sapiens]	T < N
3	44615.4	8.30	GI : 4505763	phosphoglycerate kinase 1 [Homo sapiens]	T > N
4	50385.6	7.23	GI:13111881	Fumarate hydratase [Homo sapiens]	T > N
5	39289	8.39	GI:28614	Aldolase A [Homo sapiens]	T > N
6	40874	9.33	GI:178705	activator protein 2B [Homo sapiens]	T > N
7	76159	6.69	GI:52782759	APC-stimulated guanine nucleotide exchange factor (Asef)	T > N
8	25302.5	9.21	GI:10443247	glutathione S-transferase A3 [Homo sapiens]	T > N
9	25357	8.67	GI:1197498	Arginase[Homo sapiens]	T > N
10	25933	8.19	GI:123233576	zinc finger protein 64 homolog (mouse) [Homo sapiens]	T > N

[a] Theoretical molecular weight of the matching protein in kDa; [b] Theoretical isoelectric point of the matching protein; [c] T, tumorous tissue; N, nontumorous tissue.

cytotoxic drug and increasing its water-solubility. GSTA3 is also a high-performance catalytic isomerase during the biosynthesis of steroid hormone (Johansson and Mannervik, 2001), the mechanism of which still needs further study.

Some proteins may be related to the metastasis of colorectal cancer. Arginase is a kind of hydrolase in liver, which plays a catalytic effect in the final process of ornithine cycle and forms urea and ornithine eventually. Porembska et al. (2002) considered that the CRC patients with high serum arginase activity had high recurrence and metastasis. In our study, human arginase was significantly up-regulated in the colorectal cancer tissues. Human arginase may become a prognostic marker for the recurrence and metastasis of CRC.

In summary, in the present study, we obtained some differential proteins through analyzing the proteome of colorectal cancer. These proteins may play a certain role in the tumorigenesis and metastasis. Some proteins may serve as the early prognostic biomarkers for colorectal cancer, which still needs large-sample studies. With the development of colorectal cancer proteomics, more significant differential proteins will be found and the mechanism of CRC will be further illuminated, and new significant biomarkers will be found.

ACKNOWLEDGEMENTS

We thank Mr. Ying Jiang for instructions of using the DIGE system (Beijing Proteome Research Center, Beijing, China) and also appreciate Mr. Qianglin Duan for English language revision of the manuscript. This study was supported by grants from National Natural Science Foundation of China (No. 30471700) and the "Eleventh Five Year Plan" Science and Technique Foundation of PLA, China (No.06G027).

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