PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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Analysis of differentially expressed proteins in cancerous and normal colonic tissues

Lay-Harn Gam, Chiuan-Herng Leow, Che Nin Man, Boon-Hui Gooi, Manjit Singh

Lay-Harn Gam, Chiuan-Herng Leow, School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia
Che Nin Man, National Poison Centre, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia
Boon-Hui Gooi, Manjit Singh, Department of Surgery, Penang General Hospital, Penang, Malaysia
Supported by the Ministry of Science, Technology and Innovation Malaysia (06-02-05-3197EA12)
Correspondence to: Dr. Lay-Harn Gam, School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 USM. Penang, Malaysia. layharn@usm.my
Telephone: +60-4-6533888-2208 Fax: +60-4-6570017
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Abstract

AIM: To identify and analyze the differentially expressed proteins in normal and cancerous tissues of four patients suffering from colon cancer.

METHODS: Colon tissues (normal and cancerous) were homogenized and the proteins were extracted using three protein extraction buffers. The extraction buffers were used in an orderly sequence of increasing extraction strength for proteins with hydrophobic properties. The protein extracts were separated using the SDS-PAGE method and the images were captured and analyzed using Quantity One software. The target protein bands were subjected to in-gel digestion with trypsin and finally analyzed using an ESI-ion trap mass spectrometer.

RESULTS: A total of 50 differentially expressed proteins in colonic cancerous and normal tissues were identified.

CONCLUSION: Many of the identified proteins have been reported to be involved in the progression of similar or other types of cancers. However, some of the identified proteins have not been reported before. In addition, a number of hypothetical proteins were also identified.

Key words: Colon cancer; Tissue specimens; Sequential protein extraction; Proteomics

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INTRODUCTION

Colorectal cancer is a worldwide public health concern and also a frequent cause of mortality and morbidity in developed, developing and industrialized countries[1]. The incidence of colon cancer increases exponentially with age. Environmental factors and host immunological characteristics could contribute to initiation and progression of the disease[2]. Colorectal cancer is among the best characterized cancers with regard to the genetic progression of the disease[3].

The ability to obtain maps of the protein content of cells provides a basis for comparison of the protein expression in normal and cancer cells. This capability becomes especially important for mapping the changes that occur during cancer progression. In the transformation process, changes in protein expression may result in expression of proteins at elevated or reduced levels[4-6]. When a cell transforms from normal to malignant, changes in protein expression ultimately are reflected in the phenotype of the cell. It is also important to map the differences between different stages of cancer and between different cancer samples at the same stage of progression in order to understand the pathways involved in these processes. Ultimately, identification of important proteins involved in the transformation process may lead to the identification of early markers for detection of specific types of cancers and their treatments[7].

In the present study, proteins from normal and cancerous colon tissues were extracted in three separate fractions. The mapping of a protein profile in each fraction was carried out individually. This approach eases the comparison of protein expression in normal and cancerous tissue as it reduces the total proteins present in each fraction. Using this method, we believe that proteins from different compartments of the cells were extracted in sequential order, where the aqueous soluble proteins were first extracted followed by non-aqueous soluble proteins consisting of proteins with moderate to extreme hydrophobicity. The proteins analyzed in this study were extracted from human tissue specimens (both cancerous and normal). In proteomics study, analysis of cancerous

tissue has advantages over analysis in established cancer cell lines, as the latter may not represent the actual expression of proteins.

MATERIALS AND METHODS

Tissue specimen collection

Normal and cancerous colonic tissues were collected from patients with colon cancer. The specimens were provided by Hospital Pulau Pinang. Normal tissue was taken from the mucosal layer of the normal colon adjacent to the tumor tissue. Both the normal and cancerous tissues were confirmed by hospital pathologists. Ulceration or infected colon cancers were exclusion criteria for the study. The tissues were collected after informed consent had been obtained from the patients. The patients had different grades of colorectal cancer as shown in Table 1. The tissues were cut into similar sizes, weighed and immediately stored at -70℃ until analyzed.

Well-differentiated adenocarcinoma refers to the less aggressive type of cancer where the cancerous tissues more closely resemble the normal tissue under histopathological examination, whereas moderately differentiated adenocarcinoma refers to moderately aggressive cancer cells.

Tissue lysis and sequential extractions

The tissues were defatted and an equal weight of tissues (normal and cancerpis) was determined and subjected to analysis. The deep-frozen colon tissue specimens were disrupted by grinding in a liquid nitrogen-cooled mortar until the specimens became powder-form. The powder form-like tissues were divided into aliquots in separate eppendoft tubes. Sequential extraction of protein using three protein extraction buffers was carried out. These extraction buffers were prepared according to Molloy *et al*[8] with modifications. The extraction procedures are described in the following sections.

Tris extraction buffer

The constituent of Extraction Buffer 1 (S1 buffer) is 40 mmol/L Tris. 1000 μL of S1 buffer was added to 500 mg of homogenized tissue. The mixture was vortexed for 30 seconds and centrifuged at 20℃ with the speed of 12000 r/min for 8 min. The supernatant was collected in aliquots (50 μL) and kept at -70℃. The pellet was thoroughly washed with S1 buffer before being subjected to the second step in the sequential extraction.

Solution two extraction buffer

The extraction Buffer 2 (S2 buffer) contained 8 mol/L Urea, 50 mmol/L DTT, 40 g/L CHAPS, 2 mL/L Carrier ampholytes (pH 3-10) and 2 mg/L Bromophenol Blue. The recovered pellet was washed twice again with the T1 solution, and was then vortexed, centrifuged and the supernatant was discarded. After washing, a volume of 1000 μL S2 solution was added to the pellet. The mixture was then vortex for 30 s and then centrifuged at 20℃ with the speed of 12000 r/min for 8 min. The supernatant was collected in aliquots (50 μL) and kept at -70℃. The pellet was thoroughly washed with the same extraction buffer before being subjected to the third step in the sequential extraction.

Table 1 Clinical and pathological data of patient tissue specimens

Patient #	Age	Gender	Race	Cancer stage	Cancer grade
MMN	77	Female	Malay	Stage Ⅳ	Well differentiated adenocarcinoma
LKH	66	Male	Chinese	Stage Ⅱ	Moderately differentiated adenocarcinoma
CCH	55	Male	Chinese	Stage Ⅳ	Moderately differentiated adenocarcinoma
NSC	55	Female	Chinese	Stage Ⅲ	Moderately differentiated adenocarcinoma

Solution three extraction Buffer

The extraction Buffer 3 (S3 buffer) contained 5 M Urea, 2 mol/L Thiourea, 50 mmol/L DTT, 20g/L CHAPS, 20 mL/L Triton, 2 mL/L Carrier ampholytes (pH 3-10), 40 mmol/L Tris and 2 mg/L Bromophenol Blue. The recovered pellet from S2 extraction was washed twice with S2 buffer, it was then vortexed, centrifuged and the supernatant was discarded. After washing, 1000 μL of S3 buffer was added to the pellet. The mixture was vortexed for 30 s and then centrifuged at 20℃ with the speed of 12000 r/min for 8 min. The supernatant was collected in aliquots (50 μL) and kept at -70℃. The pellet was then discarded.

Sample preparation and electrophoresis

The preparation of protein samples for SDS-PAGE separation was carried out by adding 200 mL/L of sample buffer (0.5 mol/L Tris-HCl, 100 mL/L glycerol, 20 mg/L SDS, 100 mg/L Coomassie blue) to each fraction (5 μL) and vortexed for 30 s. The samples (normal and cancerous) and the protein molecular weights markers were then loaded into individual wells of SDS-polyacrylamide gel using a syringe. Electrophoresis was performed using a vertical electrophoresis slab gel apparatus at a constant voltage of 200 volts throughout the electrophoresis process. Electrophoresis was terminated when the dye front was 2 to 3 mm away from the bottom edge of the gel for both experiments. The gel was stained with Coomassie blue.

In-gel digestion

The SDS-Polyacrylamide gel was washed thoroughly in 100 mmol/L NH_4HCO_3 and the differentially expressed protein bands from either normal or cancerous colonic tissues were then excised from the gel. In-gel digestion was performed using trypsin according to Gam and Aishah[9] with slight modification. The gel pieces were first excised and shrunk by dehydration in acetonitrile. The solvent was then discarded and the gel pieces were dried in a vacuum centrifuge. A volume of 10 mmol/L dithiotreitol (DTT) in 100 mmol/L NH_4HCO_3 that was sufficient to cover the gel pieces was added and the protein was reduced

for 1 h at 56℃. After cooling to room temperature, the DTT solution was replaced with the same volume of 55 mmol/L iodoacetic acid in 100 mmol/L NH_4HCO_3. After 45 min incubation at ambient temperature in the dark with occasional vortexing, the gel pieces were washed with 50-100 μL of 100 mmol/L NH_4HCO_3 for 10 min, dehydrated with acetonitrile, rehydrated in 100 mM NH_4HCO_3 and dehydrated in the same volume of acetonitrile. The liquid phase was removed and the gel pieces were dried in a vacuum centrifuge. The gel pieces were swollen in digestion buffer containing 50 mmol/L NH_4HCO_3, 5 mmol/L $CaCl_2$, and 12.5 ng/μL of trypsin in an ice-cold bath. After 45 min, the supernatant was removed and replaced with 10 μL of the same buffer but without trypsin to keep the gel pieces wet during enzymatic cleavage at 37℃ overnight. Peptides were extracted from the gel matrix by adding 15 μL of 20 mmol/L NH_4HCO_3, vortexed and incubated at room temperature for 10 min and the supernatant was recovered after a brief spin. This was followed by adding (1 to 2 times the volume of gel pieces) 50 mL/L formic acid in acetonitrile:water mixture (70:30), vortex and incubated for 20 min at room temperature. It was then spun down and the supernatant was recovered. These steps were repeated 3 times. Pooled extracts were dried in a vacuum centrifuge and stored at -70℃. Each of the pooled extracts (peptides) was added with 30 μL of deionized water. Then, they were centrifuged at 500 r/min for 5 min at 15℃. The extracts were then subjected to LC/MS/MS analysis.

Mass spectrometric analysis

A volume of 5 μL of the sample was injected into a RPC-column (C_{18} 300 A, 5 μm, 1 mm × 150 mm) connected to a Hewlett Packard HPLC. A capillary pump was used to pump the mobile phase at 20 μL/min flow rate, the linear gradient used was 5% B to 95% B in 65 min. Mobile phase A was 500 μL/L formic acid in deionized water and B was 500 μL/L formic acid in ACN. The HPLC was interfaced to an ion trap mass spectrometer. The dry gas temperature was 300℃, dry gas flow rate was of 8.0 L/min, nebulizer pressure of 30.0 psi. The above parameters were used subsequently for acquiring MS data. The peptides were ionized using electrospray soft ionization technique (ESI).

The experimental method was made up of one experimental protocol consisting of 2 scan events. The first scan event was a full scan MS and the second was the data dependent MS/MS scan which is dependent on the results of the first scan event. The most intense ion in a MS scan will be automatically isolated and excited to MS/MS scan. The parameters set for data dependent scan (MS/MS scan) were default collision energy (voltage) = 1.15 V, charge state = 2, minimum threshold = 3000 counts, and the isolation width = 2 m/z.

Mascot protein identification

The protein was identified using Mascot Protein Database Search engine. The Peptide Mass Tolerance was set as ± 2 u and ± 0.8 u was set for the Fragment Mass Tolerance. Only 1 missed cleavage was allowed. This software is available at *www.matrix-science.com*. Matches were computed using a probability-based Mowse score defined as -10 × log (*P*), where *P* is the probability that the observed match was a random event.

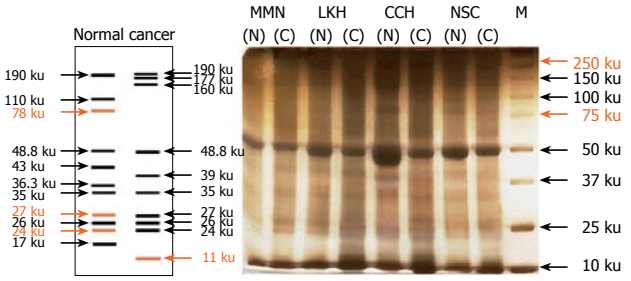

Figure 1 Proteins profiles of normal and cancer colon tissues from four different patients. The proteins were extracted using in S1 buffer. N: Normal colon tissues; C: Cancer colon tissues; M: Marker; Lanes 1 and 2: MMN samples; Lanes 3 and 4: LKH samples; Lanes 5 and 6: CCH samples and Lanes 7 and 8: NSC samples. Diagram 1 shows the consistent protein bands found in the normal and the cancer tissues of the figure.

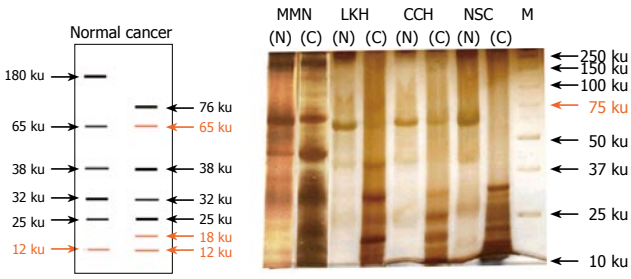

Figure 2 Proteins profiles of normal and cancer colon tissues from four different patients. The proteins were extracted using in S2 buffer. N: Normal colon tissues; C: Cancer colon tissues; M: Marker; Lanes 1 and 2: MMN samples; Lanes 3 and 4: LKH samples; Lanes 5 and 6: CCH samples and Lanes 7 and 8: NSC samples. Diagram 2 shows the consistent protein bands found in the normal and the cancer tissues of the figure.

RESULTS

Figure 1 shows the protein profiles of protein extracted using S1 buffer from normal and cancerous colon tissues of four different patients. Diagram 1 shows the consistent protein bands for both normal and cancerous tissues. The thick lines represent the protein bands that were expressed in all four tissues and the faint lines represent the protein bands that were expressed in three of the four patients. The distinct bands that were only detected in one particular tissue are ignored, as it may not represent the proteins that are involved in cancer formation. In colon cancer tissues, the protein bands that were expressed in all the four patients were at 190 ku, 177 ku, 160 ku, 48.8 ku, 39 ku, 35 ku, 27 ku, 26 ku and 24 ku. However, a protein band at 11 ku was detected in three of the four patients. In normal colon tissues, protein bands at 190 ku, 110 ku, 48.8 ku, 43 ku, 36.3 ku, 35 ku, 26 ku and 17 ku were found in all the patients' tissues while protein bands at 78 ku, 27 ku and 24 ku were found expressed only in three patients. When comparing cancerous and normal tissue, it is clear that some of the proteins were differently expressed between the two types of tissues.

Figure 2 shows the profiles of proteins extracted using S2 buffer from both normal and cancerous colonic tissues.

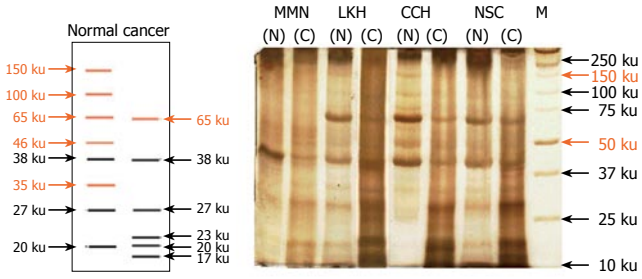

Figure 3 Proteins profiles of normal and cancer colon tissues from four different patients. The proteins were extracted using in S3 buffer. N: Normal colon tissues; C: Cancer colon tissues; M: Marker; Lanes 1 and 2: MMN samples; Lanes 3 and 4: LKH samples; Lanes 5 and 6: CCH samples and Lanes 7 and 8: NSC samples. Diagram 3 shows the consistent protein bands found in the normal and the cancer tissues of the figure.

Table 2 Up-regulated, down-regulated proteins extracted in S1, S2, S3 buffers from colon tissues

SWISS-PROT accession number	Down-regulated proteins (Normal tissues)	SWISS-PROT accession number	Up-regulated proteins (Cancer tissues)
P12718	Actin, gamma-enteric smooth muscle	P04262	Type Ⅱ, keratin subunit protein
P17661	Desmin	P35900	Keratin 20
P08670	Vimetin	P35908	Keratin, 67k type Ⅱ epidermal
Q9DFD0	Hypothetical protein	P13647	Keratin k5
Q7Z5W1	Hypothetical protein	Q86Y46	Keratin 6 irs 3
CAD19027	Hypothetical protein	P04264*	Keratin 1, type Ⅱ cytoskeletal
BAC87457	Hypothetical protein	P35527	Cytoskeletal 9
CAC08866	Hypothetical protein	P04264	Keratin, type Ⅱ cytoskeletal Ⅰ
CAC08835	Hypothetical protein	P35527	Keratin 9, type Ⅰ, cytoskeletal
Q8WTU6	Hypothetical protein	P08779	Keratin, type 1, cytoskeletal 16
Q01995	Transgelin	P48669	Keratin, type Ⅱ, cytoskeletal 6F
Q91706	Skeletal muscle beta-tropomyosin	P02538	Keratin 6A, type Ⅱ
P12277	Creatine kinase	P19013	Keratin, type Ⅱ, cytoskeletal 4
P00915	Actin-gamma	P08729	Keratin, type Ⅱ, cytoskeletal 7
Q96QE3	ATP[GTP]-binding protein	P12035	Keratin, type Ⅱ, cytoskeletal 3
		P05783	Keratin, type Ⅰ, cytoskeletal 18
		P13645	Keratin, type Ⅰ, cytoskeleton 10
		Q8NF17	Hypothetical protein
		CAE99935	Hypothetical protein
		P02675	Fibrinogen beta chain
		P00915	Carbonate dehydratase EC 4.2.1.1
		Q9PTK9	Kinesin-like protein 2
		P60174	Triosephosphate isomerase EC. 5.3.1.1
		Q6PJ43	ACTGI protein
		Q13733	Sodium/potassium-transporting ATPase alpha-4 chain EC 3.6.3.9

Diagram 2 represents the consistent protein bands that were expressed in the tissues analyzed. For the cancerous tissues, the protein bands at 76 ku, 38 ku, 32 ku and 25 ku were consistently expressed in all the cancerous tissues analyzed while protein bands at 65 ku, 18 ku and 12 ku were only found in the tissues of three of the four patients. For the normal tissues, the bands at 180 ku, 65 ku, 38 ku, 32 ku and 25 ku were found in the tissues of all four patients while only one band at 12 ku was detected in the tissues of three patients. Most of the proteins extracted from cancerous tissues using S2 buffer were found at the low molecular weight region, in the range of 35 ku to 10 ku. In contrast, relatively few protein bands from normal tissues were detected in this region and those that were detected were present at low protein intensity.

Figure 3 shows the protein profiles of proteins extracted using S3 buffer from normal and cancer colon tissues of the four patients. Diagram 3 represents the consistent protein bands expressed in the four tissues analyzed. Protein bands at 38 ku, 27 ku, 23 ku 20 ku and 17 ku from cancerous tissues were found in all four patients whereas only the band at 65 ku was found in three of the four patients. Normal tissues of the corresponding patients displayed bands at 38 ku, 27 ku and 20 ku whereas tissues from three patients were found to express consistent bands at 150 ku, 100 ku, 65 ku, 46 ku and 35 ku. The protein band at 65 ku was detected at the highest intensity compared to the other bands and it was uniquely expressed in all the patients except for patient #MMN, which was the only patient diagnosed with well differentiated adenocarcinoma cancer. Thus, its expression is probably related to the cancer grade. Nevertheless, this may just be an ambiguous assumption as only one patient with well differentiated cancer grade was analyzed. When comparing the protein profiles of protein extracted from tissues using S3 buffer, it is obvious that the expression of

Table 3 Unique proteins extracted in S1, S2, S3 buffers from colon tissues

SWISS-PROT accession number	Unique proteins (Normal tissues)	SWISS-PROT accession number	Unique proteins (Cancer tissues)
P32119	Peroxiredoxin 2 precursor	Q9BX84	LTRPC6 channel kinase 6
Q03001	Bullous pemphigoid antigen 1 precursor	Q9AVW8	DNA-directed RNA polymerase II largest chain EC 2.7.7.6
P01009	Alpha-1-antitrypsin precursor	P29312	14-3-3 protein zeta
P20848	Alpha-1-antitrypsin related protein presursor	P31946	14-3-3 protein beta/alpha
1QMBA	Alpha-1-antitrypsin mutant YES, chain A	S38532	Protein 14-3-3 eta chain
		S31975	14-3-3 protein epsilon

low molecular weight proteins (below 37 ku) in cancerous tissues was greater than that of the normal tissues.

Table 2 shows the list of proteins detected, with a total of 15 and 24 down-regulated and up-regulated proteins identified from normal and cancerous tissues, respectively. Table 3 shows the unique proteins that were identified form normal and cancerous tissues.

DISCUSSION

The sequential protein extraction technique consists of three types of protein extraction buffers, namely the S1 buffer, S2 buffer and S3 buffer. The S1 buffer is used mainly to extract aqueous soluble proteins, such as cytosolic proteins and nuclear proteins while the S2 and S3 buffers were used to extract proteins with intermediate to extreme hydrophobicity. The constituent of S2, which is made up of chaotropic agents and other reagents may disturb rugged protein-protein interaction found among structural, cytoskeletal proteins and aggregating proteins[10]. However, the combination of urea and thioureas in S3 was used to enhance the solubility of membrane proteins[8]. The extraction buffers were always used in the orderly sequence of S1 followed by S2 and then S3. The use of sequential protein extraction buffers was first suggested by Molloy *et al*[8] for separation of protein mixtures using 2D-gel electrophoresis. However, in our present study, the extracted proteins were separated using the SDS-PAGE technique, whereby direct comparison of protein profiles from tissues of four different patients was carried out.

In general, use of sequential extraction buffers is beneficial to this study because proteins were extracted according to their increasing hydrophobic strength, which may imply that proteins from different cellular compartments were extracted in different fractions. This method not only allows the analysis of proteins according to their localization in the cells but also reduces the number of proteins extracted in each fraction, which provides better resolution of proteins and their visualization in SDS-PAGE.

The protein profiles of the proteins extracted using the S1 and S2 buffers are significantly different, however the protein profiles of proteins extracted using S2 and S3 showed only slight differences. The significant variation between the protein profiles of S1 and S2 can be explained by the vast difference in the property of the proteins extracted, where S1 buffer extracts proteins only solubilize in aqueous solution while S2 buffer extracts the non-aqueous soluble proteins. The protein profiles displayed by proteins extracted using S2 and S3 also showed a certain degree of variation as S3 buffer was used to enhance the solubility of more stringent proteins from the tissue[11].

The differentially expressed proteins in each of the gels were then subjected to further analysis and the identity of each protein was analyzed using LC/MS/MS. Proteins were identified using the Mascot protein search engine on the basis of peptide mass matching[12] with redundancy of post-translational modification and proteolysis. The definition of "up-regulated", "down-regulated" and unique proteins in this study are solely dependent on their existence in SDS-PAGE. When analyzed using Quantity one software (BioRad), the intensity of the bands for up-regulated proteins was more intense in cancerous as compared to normal tissues while the "down-regulated" proteins were the proteins that were more intense in normal as compared to cancerous tissues. However, some of the intense protein bands in SDS-PAGE were found to contain more than one type of protein. Therefore, for the up-regulated and down-regulated protein bands, both the protein bands of the identical molecular weight from normal and cancerous tissues were excised and subjected to LC/MS/MS analysis. The proteins that were detected in both tissues were then quantified by their peptides' peak areas in selected ion chromatogram analysis. The proteins present in one tissue and not the other are reported as unique proteins.

A total of ninety-five proteins were identified from the protein bands indicated earlier for both the normal and cancerous tissues. However, some of these proteins were of serum origin, due to the embedded blood vessels in the tissues, and these proteins were excluded from the protein list. Furthermore, some of the proteins were represented by more than one band as described by heterogeneity. The heterogeneous proteins were mainly derived from structural, transport and enzyme subunits such as Actin, gamma-enteric smooth muscle, Desmin, Vimetin, Keratin, type I cytoskeletal 10, Cytoskeletal 9, and Triosephosphate isomerase EC 5.3.1.1. Phospholylation, glycosylation and limited proteolytic activities have been considered as major modification of proteins and they therefore affect the mobility of the modified proteins in SDS-PAGE[13-15].

The structural proteins were mostly extracted from the cancerous tissues using the S3 buffer. The majority of the structural proteins identified belongs to the cytokeratin family. Williams *et al*[16] reported that cytokeratin expressing tumor cells contain cytokeratins 8 and 18, but not

cytokeratin 19 or vimentin. The roles of cytokeratins are commonly known to encompass both structural and signaling capabilities. In the present study, cytokeratin 18 was found in cancerous colon tissues in S3 buffer extracts. The expression of cytokeratin 18 in colonic cancerous tissues is consistent with those reported by Leong *et al*[17]. In contrast, the expression of cytokeratin 18 was found reduced in malignant tumors of prostate carcinoma compared to benign prostatic hyperplasia[4]. The expression of cytokeratins is sometimes used as an indicator of the behavioral changes of a tissue. For instance, in breast tissue, cytokeratin 8 has been reported to be the major receptor for plasminogen on breast cells[18].

Vimentin is expressed at higher levels in the low-secreting variant. It is present in many cell lines, but normally absent from differentiated cells. Thus, lower vimentin expression in the high secretors may be indicative of a more differentiated phenotype[19]. In our present study, vimentin is a down-regulated protein found in S1 buffer extracts. Williams *et al*[16] has reported that the absence of vimentin in colon cancer tissue is due to its correlation with cytokeratin 19 or other cytokeratin expressions. However, Birchmeier *et al*[20] and Seshadri *et al*[21] reported no unequivocal correlation between a gain of vimentin and poor prognosis with primary tumors.

Hypothetical proteins were extracted in all three extraction buffers from both normal and cancerous colonic tissues. Generally, hypothetical proteins are defined as the proteins that were not described at the protein level, but were predicted from cDNA sequences[22]. As shown in Table 2, a series of hypothetical proteins were identified. Afjelhi-Sadat *et al*[23] suggested that the hypothetical proteins may serve as marker or protein vaccine candidates. Particularly when the hypothetical protein is found in cancerous tissue exclusively, it can be considered as novel and of pivotal importance. Some of the hypothetical proteins were detected as heterogeneous proteins suggesting the post-translational nature of the proteins.

Peroxiredoxin 2 is a type transport protein and it was extracted in the S1 buffer as a unique protein in normal tissues. Peroxiredoxin 2 is part of the peroxidase family, which are proteins that have been known to connect with cell proliferation, differentiation and apoptosis.

Fibrinogen is a type of channel protein and its role was reported to be supporting the binding of growth factors and promoting the cellular responses of adhesion, proliferation, migration during angiogenesis and tumor cell growth[24]. Fibrinogen was detected in the S2 buffer of cancerous tissues. The presence of fibrinogen in cancer cells was demonstrated to affect the progression of tumor cell growth and metastasis on the basis that fibrinogen alters the ability of breast cancer cells to migrate[24]. In addition, fibrinogen was also found present in lung cancer following the detection of elevated plasma fibrinogen levels in advanced stages of the disease[25] and the localization of fibrinogen in tumor tissue[26,27]. There are also reports of the deposition of fibrinogen on the surfaces of tumor cells and lymphocytes[28,29]. In primary and metastatic tumor[30], the presence of fibrinogen was regarded as an adverse event leading to tumor development. Yamaguchi *et al*[31] reported that lung cancer was developed through induction of hyperfibrinogenemia.

Transgelin (SM22-alpha) is a cytoskeletal-binding protein that was isolated in S1 buffer as a down-regulated protein. Maguire *et al*[32] has reported that loss of transgelin expression is an indication of early tumor progression and the authors suggested that transgelin may serve as a diagnostic marker for breast and colon cancer. We found that the expression of transgelin in cancerous tissue is much lower than that of normal tissue. Other cytosketal-binding proteins that were detected in S1 buffer extract were Bullous pemphigoid antigen 1 precursor and Skeletal muscle beta-tropomyosin.

Some of the enzymes that play important roles in metabolic pathways and are essential for energy production were detected as up-regulated protein in S1 buffer. These enzymes include triosephosphate isomerase and LTRPC6 channel kinase 6. LTRPC6 channel kinase 6 is an essential ion channel and serine/threonine-protein kinase is crucial for magnesium homeostasis. It plays an important role in epithelial magnesium transport and in the active magnesium absorption in the gut and kidney.

Sodium/potassium-transporting ATPase alpha-4 chain was detected as the up-regulated protein in S3 buffer. It is an integral membrane protein that is located in the cell membrane[33]. It catalyzes the hydrolysis of ATP coupled with the exchange of sodium and potassium ions across the plasma membrane.

Carbonate dehydratase is also the up-regulated protein detected in S1 and S2 buffer, it is a zinc-containing enzyme that catalyses the reversible hydration of carbon dioxide, which is significant in the transport of CO_2 from the tissues to the lungs. It was reported that over expression of the zinc enzyme carbonic dehydratase is observed in certain human cancers[34]. Its expression was reported to be elevated in the renal cancer cells as compared to the surrounding normal kidney tissue[35].

DNA-directed RNA polymerase Ⅱ largest chain is the DNA-RNA binding protein that was detected as unique protein in S1 buffer extract of cancer tissues. It was also reported in human brain tumors[36].

Proteins 14-3-3 are the protein-binding proteins that were isolated in the S1 buffer as unique proteins from colon cancer tissue. Protein 14-3-3 plays a role in the regulation of signal transduction protein pathways implicated in the control of cell proliferation, differentiation and survival[37]. This protein was reported to play multiple roles in maintaining cell survival[38,39]. Friedman *et al*[40] has reported that protein 14-3-3 in three different isoforms was expressed up to 1.7 fold in the colon tumors as compared to normal colon tissue.

Alpha-1-antitrypsin is an endopeptidase inhibitor protein that was isolated as a unique protein in S1 buffer from normal tissue only. In individuals who lack this inhibitor protein, the protease destroys the membrane system, leaving the colon and rectum vulnerable to colorectal cancer development[41]. The main role of alpha-1-antitrypsin is in defense against elastase damage that occurs in the lung under normal physiology conditions[42]. Alpha-1-antitrypsin was detected in patients with lung cancer[43] and was used as a cancer marker for cervical

cancer[44]. Contrary to our finding, Friedman *et al*[40] reported the detection of alpha-1-antitrypsin in colon cancer.

ATP (GTP)-binding protein is ATP-binding protein that was detected as down-regulated protein in S2 buffer. It is a mitochondrial membrane protein.

Kinesin is a motor activity protein that was detected as an up-regulated protein in S2 buffer extract. It is a microtubule-associated force-producing protein that may play a role in organelle transport[45]. This protein was also reported in breast cancer tissue[46]. Kinesin-like protein 2 is an immunogenic breast cancer antigen[47]. In addition, kinesin-like protein 2 was also reported in pancreatic cancer[48].

ACTG1 is another type of motor activity protein that was detected as an up-regulated protein. It is a protein involved in the formation of filaments, which is the major component of the cytoskeleton[49]. Its existence in colorectal cancer was also reported by Vadlamudi and Shin[50]. Alteration of cytoskeletal proteins may have an important role in cancer initiation or progression. Mutations affecting four major cytoskeletal components have now been identified in human neoplastic cells. Chou *et al*[51] postulated that mutated cytoskeletal genes may be members of a class of oncogenes, which are fundamentally different from both the nuclear-acting and growth factor/receptor/protein kinase-related types of oncogenes.

In this study, a number of the differentially expressed proteins extracted from the normal and cancerous colonic tissues were identified. Amongst the proteins identified, some of the proteins have their roles identified in similar or other types of cancers. There are also a series of hypothetical proteins with unknown functions. Some of these proteins have their primary location at the cell membrane, which may serve as potential antigens for drug-targeted therapy or as candidates for vaccines against colon cancer. These proteins are under further investigation in our laboratory.

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