Analysis of Differentially Expressed Proteins in Colorectal Cancer Using Hydroxyapatite Column and SDS-PAGE

Shi-Rou Lim · Boon-Hui Gooi · Manjit Singh · Lay-Harn Gam

Abstract Limitation on two dimensional (2D) gel electrophoresis technique causes some proteins to be under presented, especially the extreme acidic, basic, or membrane proteins. To overcome the limitation of 2D electrophoresis, an analysis method was developed for identification of differentially expressed proteins in normal and cancerous colonic tissues using self-pack hydroxyapatite (HA) column. Normal and cancerous colon tissues were homogenized and proteins were extracted using sodium phosphate buffer at pH 6.8. Protein concentration was determined and the proteins were loaded unto the HA column. HA column reduced the complexity of proteins mixture by fractionating the proteins according to their ionic strength. Further protein separation was accomplished by a simple and cost effective sodium dodecyl sulfate–polyacrylamide gel electrophoresis method. The protein bands were subjected to in-gel digestion and protein analysis was performed using electrospray ionization (ESI) ion trap mass spectrometer. There were 17 upregulated proteins and seven downregulated proteins detected with significant differential expression. Some of these proteins were low abundant proteins or proteins with extreme pH that were usually under presented in 2D gel analysis. We have identified brain mitochondrial carrier protein 1, T-cell surface glycoprotein CD1a, SOSS complex subunit B2, and Protein Jade 1 which were previously not detected in 2D gel analysis method.

Keywords Colon cancer · Hydroxyapatite chromatography · Proteomics · Tissue specimens · Total phosphate extraction

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Introduction

Colorectal cancer (CRC) is a worldwide disease and shows striking variation in incidence rate across countries dependent on the environmental, lifestyle, and dietary behavior adopted by people [1–3]. In Malaysia, colorectal cancer ranks the third most common cancerous disease that afflicts both men and women [3].

The completion of the Human Genome Project (HGP) in 2003 has greatly expanded the insight into the root causes of genetic disorders at the molecular level [4], and ultimately, this leads to the rapid advancement of proteomic-based cancer research.

In cancer, the malignant transformation of healthy cells usually occurs as a consequence of alterations at the genomic level; this transformation resulted in the production of defected gene products, namely proteins [5]. Hence, the integration of genomics into proteomics can provide valuable understanding to biological mechanisms underlying the disease processes [6].

The goal in cancer research is frequently to identify changes in healthy cells after being transformed into malignant cells. One of the approaches to accomplish this goal is by analysis of protein expression changes between normal and cancerous cells [7]. Profiling of the differentially expressed proteins by 2D gel electrophoresis is one of the most used methods in biomarkers discovery for screening, diagnosis as well as therapeutic targets for cancers [8].

Hydroxyapatite (HA, also called hydroxylapatite) is a naturally occurring mineral form of calcium phosphate; it can be used as a matrix for the chromatography of proteins, nucleic acids, bacteria, and viruses [9, 10]. Hydroxyapatite chromatography separates proteins based on ion exchange mechanism. It has been proven to be an effective purification method in a variety of compounds including biomolecules such as proteins [11, 12].

In this study, we aimed to identify differentially expressed proteins from normal and cancerous colon tissues that were fractionated using HA columns prior to separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). This fractionation method is beneficial in the analysis of complex proteins mixture, and it is hope that the proteins which cannot be separated by 2D gel can be identified using this approach.

Materials and Methods

Tissue Specimen Collection

Ethical approval was obtained from University Sains Malaysia. Normal and cancerous colorectal tissues were collected after received informed consents from the patients. All the patients had undergone surgical operation at Hospital Pulau Pinang. The tissues were confirmed cancerous or normal by hospital's pathologist prior to analysis. Each of the tissues were sliced into smaller pieces, weighed, and kept frozen at −70 °C.

Protein Extraction and Protein Concentration Determination

The tissue was defatted and gently rinsed with distilled water. It was then homogenized using a plastic homogenizer in phosphate extraction buffer, pH 6.8

(0.1 M sodium phosphate, 0.15 M NaCl, 5 M urea, 10% (v/v) glycerol, 50 mM dithiothreitol (DTT), 1% (v/v) protease inhibitor, and 1 mM benzonase) The mixture was then incubated for 5 min on ice and vortexed for 30 s followed by centrifugation at 14,000 rpm for 20 min at 18 °C. The supernatant was collected and kept at −70 °C. The protein concentration was determined by using the RC-DC Protein Assay (Bio-Rad, USA) according to the instruction provided by manufacturer. Protein standard curve was constructed by using gamma globulin.

Packing of Hydroxyapatite Column

The hydroxyapatite slurry (calcium phosphate hydroxide; Type I; Sigma®) was prepared in 1 mM sodium phosphate buffer at pH 6.8 by gently shaking for 20 min. A polypropylene cartridge (6×1.3 cm internal diameter) was positioned at 90º with a retort stand and a small piece of glass wool was inserted to the bottom of the cartridge. The cartridge was washed with distilled water. A volume of 2 mL of the HA suspension was loaded slowly into the cartridge and the HA slurry was allowed to settle down under gravitation to a height of 1 cm.

Separation of Protein Mixture by Hydroxyapatite Chromatography

The HA matrix was conditioned with 3 mL of 1 mM sodium phosphate buffer. The tissue extract was diluted to 4 mg of protein in 250 μL volume of 1 mM sodium phosphate buffer and further centrifuged at 15,000 rpm for 30 min at 4 °C. The supernatant was recovered and loaded to the hydroxyapatite column, incubated for 20 min, and subsequently washed with 2 mL of 1 mM sodium phosphate buffer. The elution of proteins was carried out in the order of increasing ionic strength of sodium phosphate elution buffer at concentrations of 200 and 700 mM and 1 M, where for each elution strength, 1.5 mL of the buffer was loaded to the column at a flow rate of 60 μL/min at room temperature. The eluate was collected as fractions of 100 μL each.

Determination of Column Trapping Efficiency

A volume of 250 μL of bovine serum albumin (BSA) at concentrations of 10, 15, 20, 25, 30, 40, and 50 mg/mL was loaded into respective HA columns and incubated for 20 min, each column was then washed using 2 mL of 1 mM sodium phosphate buffer. The wash was pooled and subjected to RC-DC protein assay to determine the non-retained BSA. The amount of retained BSA was then estimated according to the following calculation: $C = A - B$, where

A   Amount of loaded BSA (mg)
B   Amount of non-retained BSA (mg)
C   Amount of retained BSA (mg)

Determination of Protein Elution Profile

An amount of 4 mg protein from tissue extract (normal and cancerous, respectively) was reconstituted in 250 μL of 1 mM sodium phosphate buffer. It was then loaded into hydroxyapatite column and incubated for 20 min. The column was washed with 2 mL of 1 mM sodium phosphate buffer followed by elution with 200 mM, 700 mM, and 1 M buffer. A

total of 1.5 mL eluate at 100 μL per fraction was collected. The fractions were subjected to SDS-PAGE separation and the total intensity of the bands in each fraction was calculated.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Gel Images Analysis

A volume of 15 μL of the collected eluate was added with 5 μL sample buffer (0.5 M Tris–HCl pH 6.8, 10% (v/v) glycerol, 0.02% (v/v) SDS, and 0.1% (w/v) bromophenol blue). The mixtures were vortexed for 15 s and centrifuged at 3,000 rpm for 3 min at 4 °C. Electrophoresis was performed using a vertical electrophoresis slab gel apparatus at a constant voltage of 200 volts throughout the separation. The run was ended when the dye front was 2 to 3 mm away from the bottom edge of the gel. The gel was then washed three times with distilled water and stained with Bio-Safe ™ Coomassie stain (Bio-Rad). The gel images were acquired by using Versadoc system (Bio-Rad) and processed and analyzed using Quantity One software (Bio-Rad). The software was used to measure the molecular weight for each protein band relative to protein standard markers. In addition, the intensity of each band was compared between cancerous tissues and normal tissues.

In-Gel Digestion

Each of the protein bands from normal and cancerous colonic tissues was excised from the gels, sliced into small pieces, washed with deionized water, and subjected to in-gel digestion procedure according to [13]. In summary, the protein in the gel pieces was reduced, alkylated, and digested with trypsin. The peptides were eluted from the gel pieces and dried under the continuous flow of N_2 gas at 37 °C.

Tandem Mass Spectrometric Analysis

The dried peptides sample from in-gel digestion was reconstituted in 30 μL of H_2O/ACN, 85:15, and 0.1% formic acid and gently vortexed before centrifugation at 3,000 rpm for 5 min at 20°C. The supernatant was transferred into a 100-μL polypropylene vial. The HPLC separation was made up of an enrichment column (0.3×15mm, 5 μm, C18) and a reverse phase column (0.5×35 mm, 5 μm, C18). The two columns were connected through a switching valve device. A volume of 10 μL of sample was injected to the enrichment column that was pumped using a binary pump at 50 μL/min isocratic flow, the mobile phase used was 0.01% formic in 97:3 of H_2O/ACN. Subsequently, the trapped peptide sample were eluted from the enrichment column and subjected to chromatographic separation on the reverse phase-column by a capillary pump flowing at 4 μL/min at a linear gradient of 5% B to 95% B in 65 min. Where mobile phase A was 0.05% formic acid in deionized water and B was 0.05% formic acid in acetonitrile. This HPLC set up was interfaced to an ion trap Mass spectrometer detector. All the MS data were acquired using the data-dependent scans which consisted of mass spectrum (MS) and tandem mass spectrum (MS/MS) scans. The MS scanned all the positively charged ions, and the two most intense ions in a scan that exceeded the set threshold would be isolated and excited to MS/MS scan that produces series of product ions that contain the information of the amino acid sequence of the peptide. The MS parameters used were: dry gas flow rate of 60 μL/min, nebulizer pressure of 15 psi and dry gas temperature of 300°C. The parameters set for data-dependent scan (i.e., MS/MS scan) were; default collision energy (voltage) of 1.15 V, charge state of two, minimum threshold of 3,000 counts and isolation width of $2 m/z$. Molecular ion

extraction chromatogram was performed on each peptide to determine the level of their expression in each tissue.

Mascot Protein Identification

The MS/MS mass spectrometry data were subjected to Mascot Protein Database Search engine and the software was available at www.matrix-science.com for protein identification. The peptide mass tolerance was set as ±2 and ±0.8 u was set for the fragment mass tolerance. Up to one missed cleavage was allowed in the analysis. Primary sequence matches were computed using a probability-based Mowse score which defined as $-10 \times \log(P)$, where P is the probability that the observed match was a random event.

Results and Discussion

The conventional approach for proteomic study was through separation of proteins by 2D electrophoresis followed by analyzing the proteins using mass spectrometric analysis [14–16]. Nevertheless, 2D electrophoresis posses limitations on protein loading capacity [17] and also it is unable to separate protein with extreme acidic and basic pH, which restrict the detection of these proteins [18, 19]. Furthermore, the detection of low abundant proteins may be masked by the high spot intensities of high abundant proteins. In this study, the protein analysis was accomplished by a pre-electrophoretic protein separation method by means of hydroxyapatite column and followed by electrophoretic separation using SDS-PAGE method. SDS-PAGE is a better method for separation of protein regardless of the protein characteristic such as acidic or basic. Nevertheless, the drawback of separation of proteins by SDS-PAGE is its low resolution, where in the separation of complex mixture of proteins, co-migrating proteins in a similar band is common. The pre-electrophoretic protein separation using hydroxyapatite column was carried out to fraction the proteins according to their ionic strength and therefore reduced the number of proteins in each fraction. Although this devise did reduce the number of proteins, some of the proteins bands in SDS-PAGE still contained more than one type of proteins. This limitation was compensated for the high resolution of mass spectrometry analysis, where each distinguished protein in a band can be identified. Subsequently, quantification of peptides ions from the identical protein, which were extracted from cancerous and normal tissues, respectively, was carried out to determine of the expression levels of the proteins. Using this approach, we have identified a few differentially expressed proteins which were not previously identified by 2D gel separation on colorectal cancer tissues [20].

Sodium phosphate buffer was chosen as extraction buffer because of its ability to extract a wide variety of proteins from tissue in a single extract, which include hydrophilic, hydrophobic, and membrane proteins [21]. Salts [22, 23] and glycerol in the extraction buffer were used to increase the solubility of proteins [24], while benzonase was used to reduce the viscosity of the tissue extract by digesting its DNA content [10]. On the other hand, anti protease was added to prevent proteolytic digestion of proteins by proteases present in the extract. The solid debris present in the tissue extract was removed by prolonged centrifugation. The supernatant was recovered and loaded to the hydroxyapatite column, we find that these steps were critical to prevent clogging of the HA column during protein separation.

The mechanism of adsorption and desorption between the protein and hydroxyapatite is complex [25, 26] and considered as a "mixed mode" ion exchange because it involves both

the anionic and cationic exchange [27]. The functional group of HA comprises of negatively charged phosphate ions and positively charged calcium ions [26]. The adsorption of proteins on HA is usually performed at low concentrations of phosphate buffer by ionic forces, and elution was carried out at higher concentrations of the same buffer, most often at the same pH value [10]. During the chromatographic separation, competition occurs between acidic proteins and phosphate ions for adsorption onto the C crystal sites (calcium ion) of HA matrix. While basic protein competes with sodium ions for adsorption onto P crystal sites (phosphate ion) of HA matrix [28]. We found that the optimum incubation time to allow binding of amino groups and carboxyl groups of proteins onto the PO_4^{3-} and Ca^{2+} crystal sites of hydroxyapatite was 20 min. Due to fragile feature of the hydroxyapaptide material, the column flow rate was fixed at 60 μL/min and it was done under gravitation without applying any pressure to the column. One prime concern is the compatibility of the buffer additives with the hydroxyapatite material although the composition of the extraction buffer used in this study was previously reported by Broadhurst [25] and Diamantidis et al. [29].

The column binding capacity of a column volume of 1 cm³ and a diameter of 1.3 cm was in the range of 21–23 mg of proteins, which is much higher than the loaded amount of colonic tissues extract carried out in this study. A range of hydroxyapatite solid-phase protein association period between 5 and 30 min was tested. Twenty minutes of incubation time was found to be the optimum period for retention of protein in the column. The unbound or loosely bound proteins were washed using 1 mM sodium phosphate buffer before the elution of retained proteins was taking place. The dissociation period of the interactions between hydroxyapatite solid phase and proteins varied according to the characteristic of the proteins. In this study, we loaded an amount of 4 mg of proteins in tissue extract to the self-pack hydroxyapatite column, which is within the range of column binding capacity (21–23 mg). This amount of proteins was suspended in a volume of 250 μL of 1 mM of phosphate buffer, a volume which can be fully trapped within the bed volume of the column (1 mL).

Figure 1 shows the elution profiles of tissue extracts separated by HA chromatography. The elution was carried out using phosphate buffer at different ionic strengths. In order to avoid dilution of protein collected, only the fractions that contained high concentration of proteins were collected and pooled for subsequent SDS-PAGE separation. When eluting
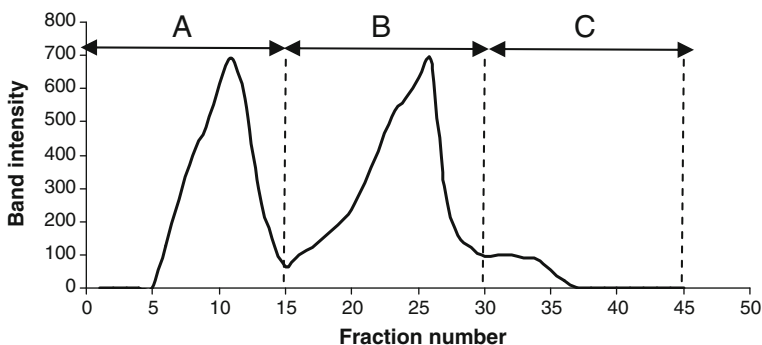

Fig. 1 Elution profiles of colonic tissues extracts separated by hydroxyapatite column at flow rate of 60 μL/min; 4 mg of proteins in 250 μL was loaded to the column and the fractions were collected at 100 μL/fraction and separated on SDS-PAGE. Each fraction was represented by the total bands intensity as quantified using Quantity one software. **a** Elution with 200 mM sodium phosphate buffer. **b** Elution with 700 mM sodium phosphate buffer. **c** Elution with 1 M sodium phosphate buffer

with 200 mM sodium phosphate buffer, fractions 5–15 were pooled, while for the 700 mM and 1 M sodium phosphate buffers, fractions 18–28 and fractions 31–37 were pooled, respectively. In each of the elution, 1.5 mL of elution buffer was loaded to the column. We find that this volume was sufficient to completely elute most of the proteins at respective ionic strength before proceeds to higher buffer strength. Nevertheless, carryover effect of high abundant proteins was still observable, such as the detection of serum albumin in the eluate of 200 and 700 mM elution buffers. The packing of each hydroxyapatite column was consistent in term of column volume, diameter, and column packing procedure. The columns were not recycled to avoid carryover effect.

Upon SDS-PAGE separation, each of the elution fractions was found contained less number of protein bands compared to the total extract without hydroxyapatite column separation. Figure 2 shows the protein profiles at each different ionic strengths. It is clear that the pre-fractionation procedure reduced the complexity of protein mixture when compared with the un-fractionated tissue extract. The different in protein profiles indicated that different types of protein were eluted by elution buffers of different ionic strengths.

Proteins that can be separated on hydroxyapatite chromatography are in the molecular weight range of 10,000 Da to several millions [30]. Protein molecules of small dimensions are eluted from a column by a lower phosphate buffer concentration than are molecules of high molecular weight [10]. This statement was supported in Fig. 3, where higher molecular weights of proteins were eluted when the elution concentration increases from 200 mM to 1 M sodium phosphate. Larger proteins are presumably creating stronger binding strength between the crystal binding sites of hydroxyapatite and hence require higher elution concentration of phosphate buffer. Figure 3 shows the comparison of elution profiles of 5 patients. The intensity of the identical protein bands between cancerous and normal tissues of the same patients were quantified in order to determine the expression levels of the proteins. Cross comparison between all the five patients was carried out and only the proteins that were consistent in differential expressed between cancerous and normal tissue of all five patients were termed upregulated protein (higher in cancer tissue) or downregulated protein (higher in normal tissue) dependent on their expression level between cancerous and normal tissues. The consistent elution patterns in all the five patients were an indication of the reproducibility of the separation method. Nevertheless,
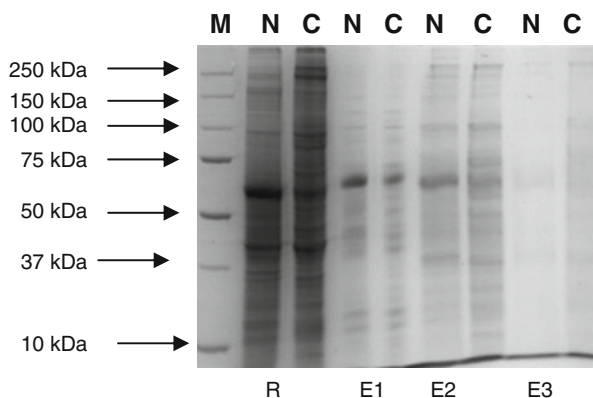

Fig. 2 Protein elution profiles using elution buffers with increasing ionic strength; *R* raw tissues extracts, *M* marker, *E1* elution with 200 mM sodium phosphate buffer, *E2* elution with 700 mM sodium phosphate buffer, *E3* elution with 1 M sodium phosphate buffer, *N* normal colon tissues, *C* cancerous colon tissue
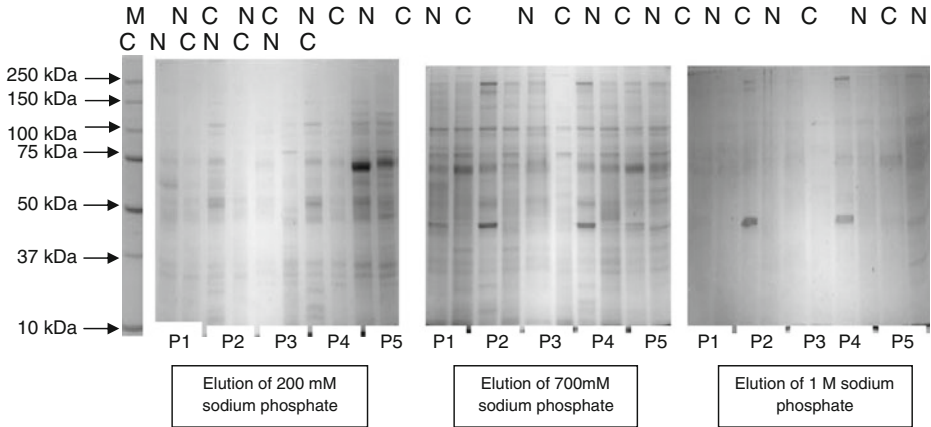
Fig. 3 Comparison of protein elution profiles for five different colorectal cancer patients (*P1* to *P5*); *M* marker, *N* normal colon tissues, *C* cancerous colon tissues

due to tissue heterogeneity as well as an inherent variability associated to the patient peculiarities. Only the proteins which were consistently expressed in all the five patients were considered in this study.

All of the protein bands on the gels were excised in pair of normal and cancer and subjected to mass spectrometric analysis. The intensities of the peptides ions of the proteins from normal and cancerous tissues were compared in order to term the protein as up- or downregulated protein. Figure 4 shows an example of the comparison of area under peak of peptides for alpha-actinin-4 extracted from normal and cancerous tissues, the peak area for the peptide isolated from cancerous tissues was with higher abundance compared to that of normal tissues, and this is consistent with other peptides of alpha-actinin-4 (data not shown), therefore alpha-actinin-4 was termed upregulated proteins. Using this approach, we have identified a list of upregulated and downregulated proteins as listed in Table 1. Among the proteins listed in Table 1, four of the proteins were presented with extreme basic pH (pH>8), these proteins were brain mitochondrial carrier protein 1, SOSS complex subunit B2, protein jade-1 and transgelin, Except for transgelin, the other three proteins, and T-cell surface glycoprotein CD1a have never been reported in 2D gel analysis.

Brain mitochondrial carrier protein 1 (BMCP1) was detected as a downregulated in our present study; it is a highly basic protein with pH of 9.66. BMCP1 is a novel mitochondrial uncoupling protein (UCP 5) that had been investigated only at transcript level [31]. It was reported to be involved in the proton transfer at mitochondrial membrane [32]. Recently, there was evidence showed the correlation of BMCP1 and human tumorigenesis where mitochondrial was said to be the key cancer-associated organelle [32].

SOSS complex subunit B2 is a multiproteins complex that promotes DNA repair and G2/M checkpoint. It acts as a sensor for co-binding of single-stranded DNAs. Binding of SOSS complex with DNA lesions influences diverse endpoint in cellular DNA damage responses, which include cell-cycle checkpoint activation, recombinational repair, and maintenance of genomic stability [33]. SOSS complex subunit B2 was detected as an upregulated protein in our present study. Its upregulation in cancerous tissues indicated the operating of regulatory and maintenance proteins to resist changes of cell into cancerous. Protein Jade-1, or also known as PHD finger protein 17, is a protein that promotes apoptosis or a renal tumor suppressor protein. It was detected as an upregulated protein in the present study.
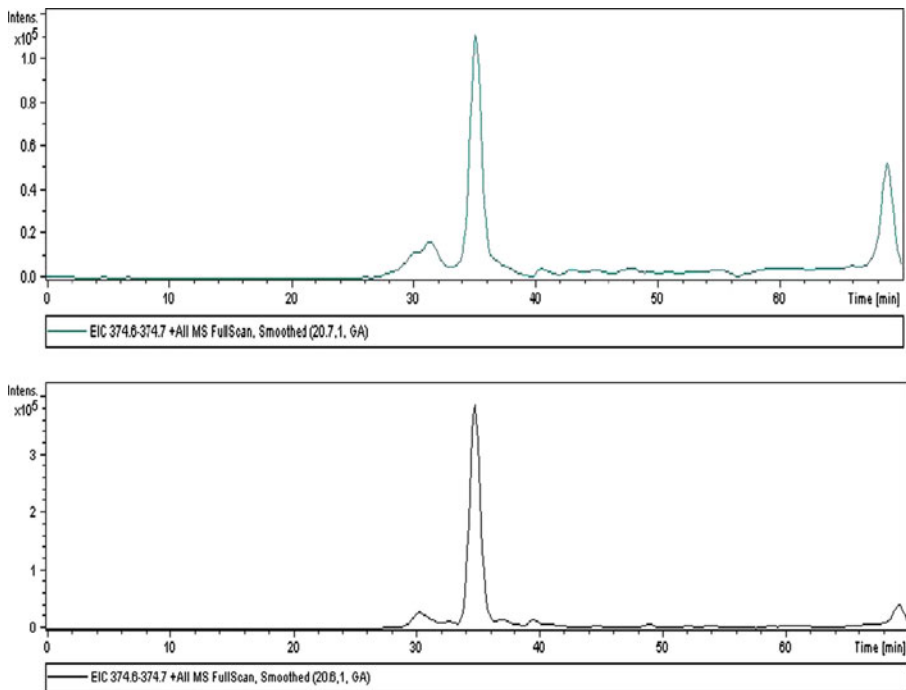
Fig. 4 Extracted ion chromatogram of peptides from alpha-actinin-4. *Upper panel* peptide isolated from normal tissues with 8131725 peak area. *Lower panel* the same peptide isolated from cancerous tissue with 26089163 peak area

Transgelin (TAGL) also named smooth muscle protein 22 (SM22). It is a tumor-suppressive protein that functions via methylation of gene promoters to inhibit transcription [34]. TAGL is an abundant protein of submucosal layer of healthy colon. Nevertheless, its expression was significantly reduced following the onset of carcinogenesis in colorectal cancer and also other types of cancer, i.e. lung, breast and renal carcinoma [35, 36]. The significant change of TAGL level in colorectal cancer was implicated as an indication of early or intermediate stage of colorectal cancer carcinogenesis [35, 37]. The use of TAGL as marker for colorectal cancer was said to be better than the existing carcinoembroynic antigen (CEA) for diagnosis of the early stage colorectal tumorigenesis [35]. The expression of transgelin had been linked with higher survival rate of the patients [34] in different tumor types, stage, and experimental model [38]. However, combination of a panel of biomarkers with transgelin is needed for better prognosis of the disease [34].

In this study, we have also detected T-cell surface glycoprotein CD1a, an upregulated protein which had not previously reported by 2D gel analysis. T-cell surface glycoprotein CD1a is an antigen-presenting protein that presenting bound antigen to T-cell receptors on natural killer T cell [39, 40], this specific function of T-cell surface glycoprotein may associate its upregulation in cancerous tissues as a channel of destroying transformed cells in the tissue.

Among the seven cytoskeletal proteins identified in the present study, filamin-A, alpha-actinin-4, and actin aortic smooth muscle were upregulated proteins, whereas, vimentin, vinculin, transgelin, and desmin were downregulated proteins.

Table 1 List of differentially expressed proteins in colorectal tissues

Upregulated proteins

No	SwissProt no.[a]	Protein name	Score[b]	Sequence coverage[c]	MW (Da)[d]	pI[e]	GRAVY[f]	Molecular class
1	P21333	Filamin-A/Actin-binding protein 280	394	14	280,607	5.70	−0.318	Cytoskeletal protein
2	P18206	Alpha-actinin-4	226	19	123,668	5.51	−0.408	Cytoskeletal protein
3	P17066	Heat shock 70 kDa protein 6	93	9	71,028	5.81	−0.460	Heat shock protein
4	P11142	Heat shock cognate 71 kDa protein	159	20	70,766	5.37	−0.460	Heat shock protein
5	Q5R7D3	Heat shock 70 kDa protein 1A/1B	123	16	69,921	5.48	−0.387	Heat shock protein
6	P02768	Serum albumin	219	37	66,472	5.67	−0.395	Transport/Cargo protein
7	P09104	Gamma-enolase	81	7	47,137	4.91	−0.193	Enzyme: Enolase
8	P06733	Alpha-enolase	94	17	47,037	6.99	−0.226	Enzyme: Enolase
9	P13929	Beta-enolase	69	7	46,800	7.73	−0.198	Enzyme: Enolase
10	P62738	Actin, aortic smooth muscle	70	11	41,774	5.24	−0.246	Cytoskeletal protein
11	P63103	14-3-3 protein zeta/delta	64	15	27,745	4.73	−0.621	Adaptor molecular protein
12	P60174	Triosephosphate isomerase	108	16	26,538	6.51	−0.126	Enzyme: Isomerase
13	P68871	Hemoglobin subunit beta	37	8	15,867	6.81	0.001	Transport/Cargo protein
14	Q15084	Protein disulfide isomerase A6	53	11	12,567	5.87	−0.397	Chaperone protein
15	P06126	*T-cell surface glycoprotein CD1a	39	2	35,413	6.44	−0.196	Protein-binding protein
16	Q96AH0	*SOSS complex subunit B2	38	5	22,423	9.63	−0.602	Protein-binding protein
17	Q61E81	*Protein Jade-1	38	2	95,533	8.20	−0.710	Protein-binding protein
Downregulated proteins								
1	P18206	Vinculin	53	9	123,668	5.51	−0.408	Cytoskeletal protein
2	P08670	Vimentin	55	12	53,520	5.06	−0.829	Cytoskeletal protein
3	P17661	Desmin	37	14	53,404	5.21	−0.728	Cytoskeletal protein
4	P01009	Alpha-1-antitrypsin	49	15	44,324	5.37	−0.302	Endopeptidase Inhibitor protein
5	P00738	Haptoglobin	70	9	43,349	6.13	−0.527	Transport/Cargo protein
6	Q01995	Transgelin	45	17	22,479	8.88	−0.597	Cytoskeletal protein
7	Q95258	*Brain mitochondrial carrier protein 1	49	6	36,201	9.66	0.305	Binding protein

The asterisk (*) symbol indicates the proteins that had not been previously detected by 2D electrophoresis

[a] Protein accession number as SwissProt at http://www.expasy.org/uniprot

[b] MASCOT score from MASCOT protein database search at http://www.matrixscience.com, where the score >35 is statistically significant ($p<0.05$)

[c] Sequence coverage (%) calculated from Mascot search result at http://www.matrixscience.com

[d] Molecular weight (Da) calculated from ProtParam of ExPASy Proteomics Server at http://www.expasy.org

[e] Theoretical isoelectrical point (pI) calculated from ProtParam of ExPASy Proteomics Server at http://www.expasy.org

[f] Grand average of hydropathicity (GRAVY) calculated from ProtParam of ExPASy Proteomics Server at http://www.expasy.org

Filamin-A (FLN-A) is a multifunctional protein which involves in structural formation of cell, cell proliferation, adhesion, motility, and blood vessels formation [41]. The binding of FLN-A to carcinoembryonic antigen-related cell adhesion molecule (CEACAM1) has led to motility of malignant cell [42].

Alpha-actinin-4 (ACTN4) is one of the four existing isoforms of alpha-actinin proteins [43]. ACTN4 involves in cell survival, regulation, proliferation, and coordination of cell morphology, focal adhesion, contractile force, as well as motility [44]. ACTN4 can be detrimental to a cell or tissue by enhancing the cell motility and metastatic potential of carcinoma cells in esophageal, breast, lung, colorectal, ovarian, and pancreatic [45]. ACTN4 was a target for oral squamous cell carcinoma gene therapy [45], where elevated expression of ACTN4 had been documented in colorectal cancer [46].

Desmin (DESN) plays a vital role in maintaining the conformation integrity of skeletal muscle [47]. In medical field, DESN was said to be useful biomarker to indicate tumors' origin [48] and also potential oncofetal serum tumor marker for colorectal cancer [49].

Enolase family (alpha-, beta-, and gamma-enolase) and triosephosphate isomerase (TPIS) are glycolytic enzymes that were associated with various types of malignancy transformation and progression [50]. In our study, enolase family and TPIS were upregulated proteins and this is in concordant with the previous report [51]. There has been substantial interest in attempting to target and validate the potency of cellular metabolic enzymes for cancer therapy [52].

In the process of carcinogenesis, hemoglobin (HBB) acts by releasing reactive oxygen species to promote cell division [53]. In contrary, haptoglobin (HPT) is protecting the cells by removing the free hemoglobin in plasma and neutralizes the toxicity of vascular under oxidative stress [54]. In this study, HBB was found upregulated while HPT was found downregulated, similar phenomena that was previously reported [55].

Serum albumin (ALBU), the most abundant transport protein in the human blood plasma, was found upregulated in cancerous tissues. ALBU had been suggested as independent prognostic marker for colorectal cancer [56].

HSP 70 isoforms, a known carcinogenic-promoting agent [57], was detected as upregulated protein in our present study. HSP 70 enhances the ability of tumor cells to alter biological functions of the cells [58].

Disulfide isomerase A6 (PDIA6) was identified as upregulated in the present study. PDIA6 is a molecular chaperone that had been shown to correlate with protein structural disorders and carcinogenesis [58].

14-3-3 Protein zeta/delta (1433Z) was detected upregulated in this study. 14-3-3 Protein zeta/delta (1433Z) belongs to 14-3-3 protein family that is involved in cell-cycle control, signal transduction, apoptosis, cytoskeleton organization, cell adhesion and motility [59, 60].

Alpha-1-antitrypsin (A1AT) was detected as downregulated in cancerous tissue. This finding is in agreement with Zhang et al. [61]. A1AT is the main serine protease inhibitor in human serum that inactivates numerous of proteolytic enzymes by forming stable complexes with the enzymes [62]. The relationship of A1AT level with various types of cancer had been previously reported [63–65]. There is growing evidence to indicate the involvement of proteolytic enzymes in cancer invasion and metastasis [66].

A detail understanding of mechanisms and cellular pathways during the cell malignant transformations provide better clues to discover the treatment for carcinoma. The literature reviews of the identified proteins in this study revealed their involvement in cancers, which include cellular communication network or interaction of protein complex within the tumor microenvironment that lead to cancer metastasis and development. We believe a panel of biomarkers may function more effectively in cancer therapy than a single biomarker.

Conclusion

In this study, we have demonstrated the use of HA column pre-fractionation, SDS-PAGE and tandem mass spectrometry for analysis of complex protein mixture in tissue extract. A few proteins, which were identified previously only at the transcription levels, had been isolated and analyzed in our present study. Although this approach cannot replace the existing 2D gel separation method, it can be used supplementary to 2D gel method for identification of proteins which cannot be separated using 2D gel electrophoresis. This work is preliminary in nature, where we have only used tissues from nine patients to demonstrate the possible use of the approach.

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