Proteomic Analysis of Colorectal Cancer Reveals Alterations in Metabolic Pathways

MECHANISM OF TUMORIGENESIS*

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Colorectal cancer is the second leading killer cancer worldwide and presently the most common cancer among males in Singapore. The study aimed to detect changes of protein profiles associated with the process of colorectal tumorigenesis to identify specific protein markers for early colorectal cancer detection and diagnosis or as potential therapeutic targets. Seven pairs of colorectal cancer tissues and adjacent normal mucosa were examined by two-dimensional gel electrophoresis at basic pH range (pH 7–10). Intensity changes of 34 spots were detected with statistical significance. 16 of the 34 spots were identified by MALDI-TOF/TOF tandem mass spectrometry. Changes in protein expression levels revealed a significantly enhanced glycolytic pathway (Warburg effect), a decreased gluconeogenesis, a suppressed gluconoronic acid pathway, and an impaired tricarboxylic acid cycle. Observed changes in protein abundance were verified by two-dimensional DIGE. These changes reveal an underlying mechanism of colorectal tumorigenesis in which the roles of impaired tricarboxylic acid cycle and the Warburg effect may be critical. *Molecular & Cellular Proteomics 5:1119–1130, 2006.

Colorectal cancer (CRC)1 is the third most common type of cancer in both men and women and the second leading cause of cancer death in developed countries. In the United States, about 145,290 new cases of CRC are expected in 2005 with about 56,290 people expected to die of the disease, accounting for about 10% of cancer deaths.2 In Singapore, CRC has overtaken lung cancer as the most common malignancy in the male population in recent years. Statistical data have shown that CRC is the leading cancer and accounts for 15.3% of all cancers in males and 14.7% in females during the period of 1988–1992 (1). On the other hand, it has also been shown that ~90% of the patients can be cured by surgery if the cancer is detected at an early stage. Therefore, the early diagnosis is important for proper control of CRC (2).

The elucidation of signaling pathways involved in cancer progression; identification of biomarkers for early detection, prognosis, and response to treatment; determination of novel targets for drug discovery; and therapeutic intervention are important goals for cancer research by multiplex technologies (3). Early detection and diagnosis of CRC and defining treatment targets rely on the understanding of molecular mechanism of carcinoma development (4). Proteomic approaches are promising tools for the discovery of new cancer biomarkers and therapeutic drug targets (4).

Several groups have carried out proteomic studies of CRC previously (5–12). A number of proteins with altered expression levels were identified. However, most studies focused on the proteins in the pH range of 4–7 due to the difficulties to achieve a good resolution of basic proteins (pH 7–10) by isoelectric focusing (13). However, mitochondrial proteins, histones, and ribosomal proteins, which have important cellular functions such as energy metabolism, apoptosis, DNA replication, transcription, and translation, are among the proteins with basic pl (14). The study of mitochondrial protein function is particularly important in cancer research as it is involved in oxidative phosphorylation, energy production, apoptosis, and release of reactive oxygen species, all of which have been associated with tumorigenesis (14). Most of the glycolytic enzymes and mitochondrial proteins such as tricarboxylic acid cycle enzymes were proven to have alkaline pl (15). There is increasing evidence linking mitochondrial dysfunction to tumorigenesis. Fumarate hydratase and succinate dehydrogenase were shown to play a role in tumorigenesis (16–18). So far, the only proteomic study of the basic proteins of colon tissue was two-dimensional gel electrophoresis (2-DE) separation of crypt proteins without any comparison with tumor samples (15). Thus a more comprehensive study of cancer samples above pl 7.0 needs to be further explored.
This study reports the 2-DE analysis of basic proteins (pH 7–10) from paired CRC tissues and corresponding adjacent normal controls. Several metabolic pathway alterations were found to be related to colorectal tumorigenesis that were verified by 2-D DIGE. Pathway analysis revealed that impaired tricarboxylic acid cycle and the Warburg effect may be critical in colorectal tumorigenesis.

**EXPERIMENTAL PROCEDURES**

**Samples**—Colonic tissues including tumor and adjacent mucosa from seven patients with Stage 3 CRC were collected from freshly isolated surgical resections in the operating room of the Singapore General Hospital. All tissues were immediately snap frozen in liquid nitrogen after the pathological examination. Frozen tissues were mounted on Tissue-Tek® tissue freezing medium (O.C.T.), and 10-μm sections were prepared using a Leica cryostat at −25 °C. Random sections from different portions of the tumor and mucosa tissues were mixed to make up to a total weight of ~200 mg.

**Protein Extraction**—Approximately 200 mg of a tissue sample were homogenized using an Ettan sample grinding kit (Amersham Biosciences). The sample was homogenized in an equal volume of 40 mM Tris-HCl, pH 8 (200 μl) together with 4 μl of 10 μM Halt protease inhibitor mixture (EDTA-free) and 2 μl of endonuclease (Sigma). The sample mixture was vortexed and incubated for 30 min at room temperature. Three volumes of modified lysis buffer (9.5 mM urea, 4% CHAPS, 2 mM tributylphosphine) were added thereafter according to Galvani et al. (19). Furthermore the sample was vortexed and incubated for 1 h at room temperature followed by centrifugation at 20,000 × g for 20 min. The supernatant was then subjected to ultracentrifugation at 75,000 rpm at 20 °C for 1 h.

**Reduction and Alkylation**—5 mM tributylphosphine was added to the sample, and the sample was vortexed and incubated at room temperature for 1 h with constant shaking followed by the addition of 15 mM iodoacetamide to alkylate the free thiol groups. The sample mixture was vortexed and kept in the dark at room temperature for 1.5 h with constant shaking. Alkylation was stopped by adding an equal volume of sample buffer (7 M urea, 2 M thiourea, 4% CHAPS) and incubated for another 5 min. Interfering components were removed by the 2-D Clean-Up Kit™ (GE Healthcare). Protein concentration was determined using the RC DC protein assay (Bio-Rad) using bovine γ-globulin as the standard.

**Two-dimensional Gel Electrophoresis**—IEF was performed using a Protean IEF Cell (Bio-Rad) with a 17-cm ReadyStrip, pH 7–10 (Bio-Rad). 100 μg of protein sample diluted in 300 μl of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG buffer pH 6–11) were loaded with each IPG strip and in-gel rehydrated overnight. The sample without reduction and alkylation, 12 μl/ml DeStreak reagent (GE Healthcare) was added before focusing.

The IPG strip was rehydrated and focused at 20 °C, starting with passive rehydration for 4 h followed by 12 h active rehydration at 50 V. The focusing was started at 250 V for 15 min (rapid voltage ramping), 500 V for 1 h, and 1,000 V for 1 h followed by linearly ramping to 10,000 V over 3 h and then kept at 10,000 V until 60,000 V-h were reached.

After IEF, the IPG strips were immediately stored in a −80 °C freezer or equilibrated with 0.375 M Tris-HCl, pH 8.8, 6 M urea, 20% glycerol, 2% SDS. For those samples without reduction and alkylation, the IPG strip was first treated with 130 mM DTT for 10 min followed by 135 mM iodoacetamide for 10 min with constant shaking. The equilibrated strip was transferred to the top of a 12% SDS-polyacrylamide gel and fixed with 1% low melting agarose in SDS-Tris-glycine running buffer with a trace of bromphenol blue. Electrophoresis was carried out using a Protean II Xli system (Bio-Rad) with constant current (30 mA/gel) at 15 °C for ~4.5 h until the dye front had reached the bottom of the gel.

**Gel Staining and Image Analysis**—All gels were fixed in a fixation solution (40% methanol, 10% acetic acid) for at least 1 h and then stained according to the modified silver staining method of Blum et al. (20). Briefly gels were sensitized in 0.8 mM sodium thiosulfate for 1 min, rinsed with water for 1 min, and then incubated for 20 min in 0.2% silver nitrate with 0.02% (v/v) formaldehyde. The gels were then soaked for 3–5 min in developing buffer (3% Na2CO3 with 0.05% (v/v) formaldehyde and 0.01 mM sodium thiosulfate), and the development was stopped in 1.4% Na2-EDTA. The gels were scanned in a Bio-Rad GS-710 densitometer using PDQUEST 7.3 software (Bio-Rad). Image analysis was carried out with PDQUEST 7.31 2D software package (Bio-Rad) including the quantitative analysis. At least four replicates of each sample pair were compared, and the gel images were normalized based on the total spot volume of each gel.

**2-D DIGE and Analysis**—50 μg of each of normal mucosa and tumor protein extracts were minimally labeled with Cy3 and Cy5 fluorescent dyes according to the manufacturer’s instructions (GE Healthcare). An internal standard pool generated by combining equal amounts of extracts from all seven pairs of mucosa and tumor tissues (in total 14 samples) was labeled with Cy2 fluorescent dye. The labeling reaction was performed on ice for 30 min in the dark and quenched with 10 mM lysine for 10 min on ice in the dark. Equal amounts (50 μg) of quenched Cy3-labeled normal mucosa and Cy5-labeled tumor samples from each patient together with the aliquoted 50 μg of Cy2-labeled internal standard pool (described above) were focused using IPG strips (ReadyStrip, Bio-Rad, pH 7–10, 17 cm) in the Protean IEF Cell (Bio-Rad) as mentioned above 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. The second 12% SDS-PAGE was then carried out for all seven gels simultaneously as described above.

The Cy2- (mixed internal standard), Cy3- (normal), and Cy5- (tumor) labeled proteins in each gel were visualized using a Typhoon 9410TM (GE Healthcare) fluorescence scanner at 480/530 nm for Cy2, 520/590 nm for Cy3, and 620/680 nm for Cy5 dyes. Gels were then silver-stained as described above. Image analysis was carried out with DeCyder 5.01 software (GE Healthcare). The DeCyder differential in-gel analysis module was used for pairwise comparisons of each pair of normal and tumor samples with its corresponding internal mixed standard present on each gel and for the calculation of normalized spot average abundance changes (12).

**Trypsin Digestion and MALDI-TOF/TOF MS and MS/MS Analysis**—Spots of interest were excised and digested with sequencing grade modified porcine trypsin (Promega) as described previously (21). Briefly gel pieces were washed in 100 mM ammonium bicarbonate buffer and then destained in potassium ferricyanate. The gel pieces were then dehydrated in acetonitrile and dried in a SpeedVac before being rehydrated on ice for 40 min in trypsinization buffer (12.5 ng/μl trypsin, 20 mM NH4HCO3, pH 8.0). Proteins were digested overnight at 37 °C. Peptides were extracted with 20 mM NH4HCO3, 50% acetonitrile, and 0.1% trifluoroacetic acid (v/v) sequentially. All extracts were saved, pooled, then lyophilized, and redissolved in 0.1% trifluoroacetic acid. After desalting with a Millipore Zip plate (Millipore Corp.), samples were finally dissolved in 5 mg/ml α-cyano-4-hydroxycinnamic acid matrix in 50% acetonitrile, 0.1% trifluoroacetic acid. MS and MS/MS spectra were obtained using the ABI 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems) operating in a result-dependent acquisition mode. Peptide mass maps were acquired in reflectron mode (1-keV accelerating.
voltage) with 1,000 laser shots per spectrum. 6 external standards (mass standard kit for the 4700 Proteomics Analyzer calibration mixture, Part Number 4333604, Applied Biosystems) were used to calibrate each spectrum to a mass accuracy within 50 ppm. The MS/MS data were acquired with stop conditions so that 3,000–6,000 laser shots were accumulated for each spectrum. The MS together with MS/MS spectra were searched against the International Protein Index (IPI) human database Version 3.10 (57,478 sequences; 25,254,519 residues) using the software GPS Explorer™ Version 3.0 and MASCOT 2.0 (Matrix Science). Searches were performed without restriction of protein Mr or pI and with mandatory carbamidomethylation of cysteines and variable oxidation of methionine residues. One trypsin miscleavage was allowed. Peptide mass tolerance and fragment mass tolerance were set to 150 ppm and 0.4 Da, respectively. High confidence identifications have statistically significant search scores (greater than 95% confidence interval, equivalent to MASCOT expect value <0.05), are consistent with the protein experimental pI and Mr, and account for the majority of ions present in the mass spectra.

Data Analysis—Matched gel images by PDQUEST were imported into the in-house developed bioinformatics program Systematic Proteomics Laboratory Analysis & Storage Hub (SPLASH) (on-line demonstration available at oncoproteomics.nus.edu.sg/login) and were analyzed statistically. Gel spots with intensity changes greater than 2.0-fold with a confidence interval above 95% (two-sample t test; p < 0.05) were considered as statistically significant changes.

RESULTS

The Basic 2-D Map of Colorectal Tumor Tissue—We were able to optimize the 2-D conditions for the high pI range protein profiling of the CRC tissues with (a) alkylation of the
# Proteomic Analysis of Colorectal Cancer

**Table I**  
List of proteins from human CRC tissue resolved on pH 7–10 2-DE gels and identified by MALDI-TOF/TOF MS  
SNARE, soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors; ALB, albumin; cds, coding sequence.

| Spot no. | Protein name | IPI_Human accession no. | Molecular mass Da | pl | No. of matched peaks | Protein score | Ion score | Sequence coverage | Highest ranking non-homologous protein score |
|----------|--------------|-------------------------|-------------------|----|----------------------|---------------|-----------|------------------|---------------------------------------------|
| 1        | β-Globin gene from a thalassemia patient, complete cds | IPI00382950 | 19,204             | 6.28 | 11 | 367 | 274 | 65 | 50 |
| 2        | Phosphatidylethanolamine-binding protein | IPI00219446 | 21,027             | 7.42 | 9 | 169 | 128 | 40 | 40 |
| 3        | ALB protein | IPI00216773 | 46,442             | 5.77 | 7 | 116 | 102 | 13 | 52 |
| 4        | Peptidyl-prolyl cis-trans isomerase A | IPI00419585 | 18,098             | 7.82 | 8 | 85 | 41 | 42 | 57 |
| 5        | β-Globin gene from a thalassemia patient, complete cds | IPI00382950 | 19,204             | 6.28 | 8 | 374 | 294 | 46 | 40 |
| 6        | β-Globin gene from a thalassemia patient, complete cds | IPI00382950 | 19,204             | 6.28 | 8 | 313 | 250 | 46 | 51 |
| 7        | Peptidyl-prolyl cis-trans isomerase A | IPI00419585 | 18,098             | 7.82 | 12 | 232 | 166 | 45 | 100 |
| 8        | Cofilin, non-muscle isoform | IPI00012011 | 25,543             | 8.51 | 13 | 218 | 110 | 57 | 44 |
| 9        | 28-kDa Golgi SNARE protein | IPI00029447 | 29,062             | 9.36 | 8 | 68 | 68 | 16 | 41 |
| 10       | Hemoglobin δ chain | IPI00473011 | 16,028             | 7.97 | 4 | 88 | 46 | 28 | 44 |
| 11       | Profilin-1 | IPI00216691 | 15,085             | 8.48 | 7 | 132 | 98 | 28 | 42 |
| 12       | Peroxiredoxin 1 | IPI00000874 | 22,324             | 8.27 | 13 | 451 | 358 | 55 | 39 |
| 13       | 28-kDa Golgi SNARE protein | IPI00029447 | 29,062             | 9.36 | 8 | 68 | 68 | 16 | 41 |
| 14       | Hemoglobin δ chain | IPI00473011 | 16,028             | 7.97 | 4 | 88 | 46 | 28 | 44 |
| 15       | Profilin-1 | IPI00216691 | 15,085             | 8.48 | 7 | 132 | 98 | 28 | 42 |
| 16       | Mn-Superoxide dismutase, mitochondrial precursor | IPI00022314 | 24,878             | 8.35 | 4 | 188 | 156 | 22 | 49 |
| 17       | Mn-Superoxide dismutase, mitochondrial precursor | IPI00022314 | 24,878             | 8.35 | 6 | 93 | 66 | 25 | 43 |
| 18       | Triosephosphate isomerase | IPI00643696 | 26,937             | 6.45 | 13 | 218 | 110 | 57 | 44 |
| 19       | Splice isoform 1 of heat shock cognate 71-kDa protein | IPI00038365 | 71,082             | 5.37 | 22 | 466 | 405 | 24 | 58 |
| 20       | Heat shock 70-kDa protein 1A | IPI00514377 | 70,280             | 5.48 | 7 | 430 | 365 | 15 | 51 |
| 21       | Glyceraldehyde-3-phosphate dehydrogenase | IPI00219018 | 36,070             | 8.58 | 3 | 140 | 131 | 12 | 36 |
| 22       | Annexin A2 | IPI00455315 | 38,677             | 7.56 | 8 | 344 | 260 | 23 | 40 |
| 23       | Glyceraldehyde-3-phosphate dehydrogenase | IPI00219018 | 36,070             | 8.58 | 3 | 129 | 114 | 12 | 38 |
| 24       | Glyceraldehyde-3-phosphate dehydrogenase | IPI00219018 | 36,070             | 8.58 | 3 | 62 | 43 | 8 | 42 |
| 25       | Phosphodiesterase 5A isoform 3 | IPI00066543 | 94,996             | 5.74 | 9 | 56 | 17 | 42 |
| 26       | α enolase | IPI00465248 | 47,350             | 6.99 | 10 | 311 | 253 | 36 | 45 |
| 27       | α enolase | IPI00465248 | 47,350             | 6.99 | 10 | 314 | 233 | 32 | 52 |
| 28       | β-Globin gene from a thalassemia patient, complete cds | IPI00382950 | 19,204             | 6.28 | 4 | 223 | 200 | 29 | 39 |
| 29       | Glyceraldehyde-3-phosphate dehydrogenase | IPI00552282 | 37,086             | 8.08 | 1 | 58 | 58 | 4 | 35 |
| 30       | Heterogeneous nuclear ribonucleoprotein H1 (HNRPH1) | IPI00026230 | 49,517             | 5.89 | 6 | 126 | 107 | 16 | 32 |
| 31       | Glyceraldehyde-3-phosphate dehydrogenase | IPI00219018 | 36,070             | 8.58 | 4 | 75 | 63 | 12 | 45 |
| 32       | 53-kDa protein (pyruvate kinase 3) | IPI00383237 | 58,314             | 7.58 | 14 | 373 | 304 | 36 | 49 |
| 33       | Glyceraldehyde-3-phosphate dehydrogenase | IPI00219018 | 36,070             | 8.58 | 5 | 91 | 61 | 17 | 43 |
| 34       | Glyceraldehyde-3-phosphate dehydrogenase | IPI00219018 | 36,070             | 8.58 | 5 | 377 | 355 | 23 | 48 |
| 35       | Fructose-bisphosphate aldolase C | IPI00418262 | 39,699             | 6.46 | 4 | 125 | 111 | 15 | 43 |
| 36       | Transketolase variant | IPI00644970 | 68,547             | 7.9 | 8 | 153 | 96 | 20 | 39 |
| 37       | Voltage-dependent anion-selective channel protein 1 | IPI00216308 | 30,737             | 8.63 | 9 | 290 | 225 | 40 | 44 |
| 38       | Phosphoglycerate kinase 1 | IPI00169383 | 44,854             | 8.3 | 10 | 256 | 209 | 35 | 41 |
| 39       | Phosphoglycerate kinase 1 | IPI00169383 | 44,854             | 8.3 | 10 | 381 | 311 | 37 | 42 |
| Spot no. | Protein name | IPI_Human accession no. | Molecular mass Da | pl | No. of matched peaks | Protein score | Ion score | Sequence coverage % | Highest ranking non-homologous protein score |
|---------|--------------|-------------------------|------------------|----|---------------------|---------------|-----------|---------------------|---------------------------------------------|
| 40      | Phosphoglycerate kinase 1 | IPI00169383 | 44,854 | 8.3 | 5 | 105 | 83 | 15 | 13 | 39 |
| 41      | Fructose-bisphosphate aldolase C | IPI00418262 | 39,699 | 6.46 | 3 | 110 | 93 | 12 | 41 |
| 42      | Fructose-bisphosphate aldolase A | IPI00465439 | 39,720 | 8.39 | 6 | 60 | 16 | 17 | 43 |
| 43      | Fructose-bisphosphate aldolase A | IPI00465439 | 39,720 | 8.39 | 11 | 100 | 65 | 19 | 55 |
| 44      | α2 globin variant | IPI00410714 | 15,328 | 8.72 | 1 | 67 | 56 | 10 | 36 |
| 45      | Galectin-3 | IPI00465431 | 26,098 | 8.61 | 7 | 205 | 152 | 17 | 42 |
| 46      | Keratin type II cytoskeletal 1 | IPI00556624 | 66,198 | 8.16 | 2 | 70 | 66 | 1 | 57 |
| 47      | ALB protein | IPI00216773 | 46,442 | 5.77 | 7 | 57 | 29 | 16 | 36 |
| 48      | Transgelin (smooth muscle protein) | IPI00216138 | 22,522 | 8.88 | 22 | 298 | 160 | 73 | 45 |
| 49      | Complement C3 precursor | IPI00164623 | 188,586 | 6.02 | 12 | 63 | 44 | 8 | 49 |
| 50      | Complement C3 precursor | IPI00164623 | 188,586 | 6.02 | 2 | 58 | 53 | 1 | 44 |
| 51      | Catalase | IPI00465436 | 59,816 | 6.95 | 12 | 211 | 138 | 32 | 66 |
| 52      | Glyceraldehyde-3-phosphate dehydrogenase | IPI00219018 | 36,070 | 8.58 | 4 | 80 | 64 | 11 | 68 |
| 53      | Hypothetical protein | IPI00448985 | 25,790 | 6.14 | 3 | 94 | 77 | 21 | 70 |
| 54      | Glyceraldehyde-3-phosphate dehydrogenase | IPI00219018 | 36,070 | 8.58 | 3 | 59 | 49 | 8 | 52 |
| 55      | Keratin 8 variant | IPI00418411 | 53,705 | 5.52 | 6 | 114 | 101 | 13 | 46 |
| 56      | Heterogeneous nuclear ribonucleoprotein A2/B1 isoform A2 (HNRPA2B1) | IPI00477522 | 37,332 | 9.08 | 8 | 170 | 88 | 24 | 41 |
| 57      | Flavin reductase | IPI00219910 | 22,751 | 7.12 | 2 | 89 | 78 | 17 | 37 |
| 58      | 22-kDa protein | IPI00218414 | 22,316 | 8.38 | 22 | 142 | 112 | 36 | 40 |
| 59      | Creatine kinase, ubiquitous mitochondrial precursor | IPI00024638 | 47,406 | 8.6 | 6 | 212 | 170 | 18 | 41 |
| 60      | 71-kDa protein | IPI00479291 | 71,833 | 8.41 | 9 | 58 | 18 | 42 |
| 61      | 34-kDa protein | IPI00412577 | 34,598 | 8.84 | 3 | 55 | 34 | 9 | 43 |
| 62      | GTP-AMP phosphotransferase, mitochondrial | IPI00465256 | 25,419 | 9.16 | 6 | 126 | 74 | 34 | 43 |
| 63      | Single-stranded DNA-binding protein mitochondrial precursor | IPI00029744 | 17,249 | 9.59 | 3 | 155 | 111 | 26 | 44 |
| 64      | β actin | IPI00550724 | 42,078 | 5.29 | 3 | 82 | 74 | 13 | 38 |
| 65      | Malate dehydrogenase, mitochondrial precursor | IPI00291006 | 35,965 | 8.92 | 3 | 89 | 73 | 15 | 36 |
| 66      | Carbonyl reductase (NADPH) 1 | IPI00295386 | 30,510 | 8.55 | 3 | 73 | 48 | 14 | 48 |
| 67      | Keratin 1 | IPI00556624 | 66,198 | 8.16 | 4 | 63 | 51 | 9 | 40 |
| 68      | Neutropilopeptide h3 (prostatic binding protein) | IPI00219446 | 21,072 | 7.12 | 3 | 74 | 58 | 21 | 40 |
| 69      | Heat shock 70-kDa protein 8 isoform 1 variant | IPI00643188 | 71,083 | 5.28 | 7 | 57 | 44 | 9 | 35 |
| 70      | Glyceraldehyde-3-phosphate dehydrogenase | IPI00219018 | 36,070 | 8.6 | 7 | 188 | 161 | 16 | 77 |
| 71      | Transgelin | IPI00216138 | 22,522 | 8.9 | 12 | 189 | 121 | 29 | 43 |
| 72      | Glyceraldehyde-3-phosphate dehydrogenase, liver | IPI00219018 | 36,070 | 8.6 | 3 | 160 | 125 | 12 | 39 |
| 73      | 57-kDa protein (keratin 10) | IPI00479902 | 56,699 | 5.1 | 4 | 66 | 27 | 10 | 42 |
| 74      | Glyceraldehyde-3-phosphate dehydrogenase | IPI00219018 | 36,202 | 8.6 | 3 | 90 | 64 | 12 | 47 |
| 75      | Malate dehydrogenase, mitochondrial | IPI00291006 | 35,965 | 8.9 | 8 | 253 | 224 | 15 | 39 |
| 76      | Malate dehydrogenase, mitochondrial | IPI00291006 | 35,965 | 8.9 | 2 | 107 | 94 | 7 | 41 |
| 77      | Hypothetical protein FLJ39660 | IPI00176356 | 34,561 | 8.1 | 7 | 62 | 20 | 51 |
| 78      | Carbonic anhydrase II | IPI00218414 | 29,285 | 6.9 | 2 | 115 | 96 | 11 | 46 |
| 79      | UDP-glucose 6-dehydrogenase | IPI00306420 | 55,674 | 6.7 | 18 | 476 | 354 | 25 | 57 |
| 80      | Phosphoenolpyruvate carboxykinase, mitochondrial precursor | IPI00294380 | 71,447 | 7.6 | 4 | 73 | 31 | 7 | 43 |
| 81      | UTP-glucose-1-phosphate uridylyltransferase 2 | IPI00395676 | 55,813 | 7.7 | 10 | 79 | 34 | 15 | 49 |
reduced thiol groups prior to IEF (22) and (b) the use of in-gel sample rehydration. We chose the pH 7–10 strips for profiling of the basic proteins from CRC samples. We established the reference 2-D map of colorectal tumor tissue. In the pH 7–10 range, a total of 991 spots were clearly detected by silver staining (Fig. 1). Major spots were excised and identified via mass spectrometry (Fig. 1 and Table I). Among the identified proteins, most of them were glycolytic enzymes and mitochondrial proteins, involved in the tricarboxylic acid cycle and ATP energy metabolism, and metabolite carrier channels, etc. This is concordant with the 2-DE analysis of colon crypt by Li et al. (15).

2-DE Identification of Proteins That Exhibited Alterations Consistent among All Seven Cancer Patients—Seven pairs of tumor and normal mucosa were resolved by pH 7–10 2-DE and analyzed by PDQUEST. Gel images of four pairs of samples could be matched by the software. The gel images of the remaining three pairs required manual intervention to match to the other four pairs.

In total, 38 gels from four patients, 2443, 2446, 2466, and 2498, containing at least four replicates for each sample, were compared with each other. 420 spots matched across all the gels while spot count for each gel varied from 639 to 991. After statistical analysis of the normalized quantities of matched spots, we identified 17 spots that were significantly up-regulated and 17 spots that were significantly down-regulated with intensity changes greater than 2-fold with confidence intervals at least 95% (two-sample t-test, \( p < 0.05 \)).

Corresponding spots in 30 gels from the remaining three patients, 2361, 2378, and 2433, were also compared manually. These spots were also found to exhibit alterations in the same manner with similar statistical significance.

Of the total 34 spots, we identified 16 (Fig. 2, a and b), accounting for 11 proteins (Table II). Most of these proteins belonged to various biochemical pathways such as the glucuronate metabolism, glycolysis, gluconeogenesis, and the tricarboxylic acid cycle, whereas others were related to cytoskeletal remodeling such as TGLN protein (transgelin-2) and the nitrogen metabolic enzyme carbonic anhydrase II (CA2) (Table II).
To simplify the image analysis process and to avoid gel-to-gel variation, we further carried out the 2-D DIGE to verify protein alterations found by the traditional 2-DE. Major spot changes found by DIGE were consistent with those found by silver-stained 2-DE analysis. Previously found alterations in spots 83, 101, 78 and 79, and 70 and 72 were clearly visualized in color (Fig. 3).

DISCUSSION

In this study, we focused on the analysis of proteins with basic pI values, most of which were missed by most researchers in previous proteomic studies of CRC (7, 10, 12). Although there are equal amounts of proteins with basic (>7) or acidic (<7) pI values in most eukaryotic organisms (13), most important cytosolic glycolytic and mitochondrial tricarboxylic acid cycle enzymes are basic proteins as demonstrated by the proteomic analysis of human colon crypt (15) and further confirmed by our study.

Glycolysis has been shown to be elevated in almost all cancers, the so-called “Warburg effect” (23). And many cancers show dysfunction of mitochondria (14, 23, 24). The
increased aerobic glycolysis for ATP generation in cancer cells is frequently associated with mitochondrial respiration defects and hypoxia (25, 26). A recent report showed that inhibition of glycolysis in colon cancer cells could overcome drug resistance (against common anticancer agents) associated with mitochondrial respiratory defect and hypoxia (27). The mitochondrial enzyme succinate dehydrogenase, which links tricarboxylic acid cycle dysfunction to oncogenesis via hypoxia-inducible factor (HIF)-1α was also demonstrated recently (18).

Proteomics is a powerful approach to identify signaling proteins and to decipher the complex signaling circuitry or pathways involved in tumor growth. Together with genomics, proteomics is able to characterize different types of tumors and thus to define new therapeutic targets for future drug treatment (28). Our 2-DE data showed up-regulation of glycolytic enzymes such as aldolase A, enolase 1, GAPDH, etc., thus providing a proteomic evidence that the Warburg effect had occurred in CRC. Meanwhile the key regulatory enzyme phosphoenolpyruvate carboxykinase in gluconeogenesis was down-regulated. The up-regulation of enolase 1 and GAPDH were confirmed by a separate study using ICAT LC-MS analysis.3 The ICAT results also revealed some other up-regulated glycolytic enzymes: phosphoglycerate kinase 1, pyruvate kinase 3 isoform 1, triosephosphate isomerase 1, and dihydrolipoyl dehydrogenase. Down-regulation of UDP-glucose 6-dehydrogenase (UGDH) and UDP-glucose pyrophosphorylase 2 indicated a suppressed glucuronic acid anabolism. The down-regulation of UGDH was also confirmed by the ICAT studies. The 2-DE data also revealed an impaired tricarboxylic acid cycle in CRC as evidenced by down-regulation of enzymes at the early entrance steps such as aconitase and aconitate hydratase and up-regulation of malate dehydrogenase at the exit step. These findings suggested extensive alterations in metabolic pathways that have not been well defined before (Table II). It has the potential for the design of marker panels to assist in early diagnostics and therapeutic strategies in CRC (11, 29–31).

A recent report using 2-D DIGE and MS with the pH range of 4–7 found that in colon cancer some proteins such as succinate dehydrogenase subunit A, succinyl-CoA 3-ketoacid coenzyme A transferase, aldehyde dehydrogenase, and carbonic anhydrase I were down-regulated, whereas several other proteins such as triosephosphate isomerase and keratins 8 and 18 were up-regulated (10). The use of wider pH range (pH 4–9) agarose 2-DE and MS techniques for CRC tissues led to the detection of elevated expressions of various basic proteins (9) and confirmation of proteins known to exhibit altered expression levels in CRC, i.e. carbonic anhydrase 1, peptidyl-prolyl isomerase A, manganese superoxide dismutase, keratin 18, enolase 1 and pyruvate kinase 3 (7, 32–35). In addition, these investigators also found elevated expressions of the following proteins: pyruvate dehydrogenase beta, pyruvate kinase-3, triosephosphate isomerase 1, aldolase A, phosphoglycerate kinase 1, and malate dehydrogenase 2.

### Table II

| Spot no. | Protein name                                      | Average ratio (T/M) | Predicted molecular mass (kDa) | Predicted pl | Biological function                              | Ref. |
|---------|---------------------------------------------------|---------------------|--------------------------------|--------------|--------------------------------------------------|------|
| 27      | Enolase 1                                          | 2.436               | 47.1                           | 7.01         | Glycolysis                                       | 9, 36|
| 43      | Fructose-bisphosphate aldolase A (ALDA)           | 5.154               | 39.9                           | 8.49         | Glycolysis                                       | 9, 36|
| 70      | GAPDH                                             | 31.668              | 36.0                           | 8.57         | Glycolysis                                       | 36b  |
| 72      | GAPDH                                             | 60.408              | 36.0                           | 8.57         | Glycolysis                                       | 36b  |
| 102     | GAPDH                                             | 2.347               | 36.0                           | 8.57         | Glycolysis                                       | 36b  |
| 103     | GAPDH                                             | 2.487               | 36.0                           | 8.57         | Glycolysis                                       | 36b  |
| 75      | Malate dehydrogenase, mitochondrial precursor (MDH) | 2.996               | 35.5                           | 8.92         | Tricarboxylic acid cycle                         | 9    |
| 76      | Malate dehydrogenase, mitochondrial precursor (MDH) | 4.654               | 35.5                           | 8.92         | Tricarboxylic acid cycle                         | 9    |
| 71      | TAGLN protein transgeline                         | 4.406               | 22.2                           | 8.41         | Cytoskeleton/structural                          | 9, 10|
| 80      | Phosphoenolpyruvate carboxykinase, mitochondrial precursor (PEPCK) | 0.247               | 71.5                           | 7.56         | Gluconeogenesis                                  | 36b  |
| 78      | Carbonic anhydrase II (CA2)                       | 0.17                | 29.3                           | 6.87         | Nitrogen metabolism                              | d    |
| 79      | UGDH                                              | 0.208               | 55.7                           | 6.73         | Glucuronate                                      | d    |
| 81      | UDP-glucose pyrophosphorylase 2 (UGP2)            | 0.286               | 55.8                           | 7.69         | Glucuronate                                      | d    |
| 82      | UDP-glucose pyrophosphorylase 2 (UGP2)            | 0.314               | 55.8                           | 7.69         | Glucuronate                                      | d    |
| 83      | Aconitate hydratase, mitochondrial precursor      | 0.163               | 86.1                           | 7.36         | Tricarboxylic acid cycle                         | b    |
| 101     | Aconitase 2                                       | 0.164               | 86.3                           | 7.62         | Tricarboxylic acid cycle                         | b    |

* Tumor versus normal mucosa.
* Alteration in CRC reported for the first time.

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**Table II**

| Spot no. | Protein name                                      | Average ratio (T/M) | Predicted molecular mass (kDa) | Predicted pl | Biological function                              | Ref. |
|---------|---------------------------------------------------|---------------------|--------------------------------|--------------|--------------------------------------------------|------|
| 27      | Enolase 1                                          | 2.436               | 47.1                           | 7.01         | Glycolysis                                       | 9, 36|
| 43      | Fructose-bisphosphate aldolase A (ALDA)           | 5.154               | 39.9                           | 8.49         | Glycolysis                                       | 9, 36|
| 70      | GAPDH                                             | 31.668              | 36.0                           | 8.57         | Glycolysis                                       | 36b  |
| 72      | GAPDH                                             | 60.408              | 36.0                           | 8.57         | Glycolysis                                       | 36b  |
| 102     | GAPDH                                             | 2.347               | 36.0                           | 8.57         | Glycolysis                                       | 36b  |
| 103     | GAPDH                                             | 2.487               | 36.0                           | 8.57         | Glycolysis                                       | 36b  |
| 75      | Malate dehydrogenase, mitochondrial precursor (MDH) | 2.996               | 35.5                           | 8.92         | Tricarboxylic acid cycle                         | 9    |
| 76      | Malate dehydrogenase, mitochondrial precursor (MDH) | 4.654               | 35.5                           | 8.92         | Tricarboxylic acid cycle                         | 9    |
| 71      | TAGLN protein transgeline                         | 4.406               | 22.2                           | 8.41         | Cytoskeleton/structural                          | 9, 10|
| 80      | Phosphoenolpyruvate carboxykinase, mitochondrial precursor (PEPCK) | 0.247               | 71.5                           | 7.56         | Gluconeogenesis                                  | 36b  |
| 78      | Carbonic anhydrase II (CA2)                       | 0.17                | 29.3                           | 6.87         | Nitrogen metabolism                              | d    |
| 79      | UGDH                                              | 0.208               | 55.7                           | 6.73         | Glucuronate                                      | d    |
| 81      | UDP-glucose pyrophosphorylase 2 (UGP2)            | 0.286               | 55.8                           | 7.69         | Glucuronate                                      | d    |
| 82      | UDP-glucose pyrophosphorylase 2 (UGP2)            | 0.314               | 55.8                           | 7.69         | Glucuronate                                      | d    |
| 83      | Aconitate hydratase, mitochondrial precursor      | 0.163               | 86.1                           | 7.36         | Tricarboxylic acid cycle                         | b    |
| 101     | Aconitase 2                                       | 0.164               | 86.3                           | 7.62         | Tricarboxylic acid cycle                         | b    |

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* Tumor versus normal mucosa.
* Alteration in CRC reported for the first time.

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**Proteomic Analysis of Colorectal Cancer**

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peptidyl-prolyl isomerase A, enolase I (α), prolyl-4-hydroxylase β subunit, voltage-dependent anion channel 2, γ-lactate dehydrogenase M chain, and transgelin-2 (9) in primary CRC tissues. Our results not only confirmed alterations of expression levels of the alkaline proteins recently reported (9, 10) but also found new proteins (aconitase, phosphoenolpyruvate carboxykinase, UGDH, and UDP-glucose pyrophosphorylase 2) that exhibited altered expression level that have not been reported in CRC previously (Table II). Alterations of these proteins indicated abnormal metabolic pathways, such as impairment of the tricarboxylic acid cycle, reduction of the gluconeogenesis, and suppression of glucuronic acid synthesis, which may play critical roles in the onset of CRC.

Unwin et al. (36) were the first to demonstrate by proteomics that the elevated glycolytic pathway (Warburg effect) occurred in renal cancer tissue; this was further supported by increased glycolytic gene expression in 24 different cancers by functional genomics (24). Our results and other published protein expression pattern changes in CRC, as summarized in Fig. 4, suggest that alterations of the following major metabolic pathways are involved in the CRC tumorigenesis: elevated glycolysis, down-regulated gluconeogenesis, decreased glucuronate metabolism, and impaired tricarboxylic acid cycle (Krebs cycle). These metabolic pathways have been shown to be regulated by intracellular signaling (HIF-1α-mediated hypoxia pathway (37, 38)) and extracellular signaling (glycosaminoglycan (GAG) pathways (39), Wnt pathway (40), and vascular endothelial growth factor (41, 42)). Hereby we propose a mechanism of tumorigenesis of CRC (Fig. 4). Alteration of the expression of tricarboxylic acid cycle enzymes, i.e. down-regulation of aconitase and up-regulation of malate dehydrogenase, results in deficiency of the intermediate metabolite α-oxoglutarate (α-ketoglutarate), a critical co-substrate for Fe²⁺/α-oxoglutarate-dependent hydroxylases (prolyl hydroxylase and asparaginyl hydroxylase (factor inhibiting HIF-1)) that catalyze hydroxylation of HIF-1α subunit (43–45). In CRC, the Warburg effect and the impaired tricarboxylic acid cycle result in the accumulation of a glycolytic metabolite, pyruvate, and the tricarboxylic acid cycle intermediate oxaloacetate, both leading to inactivation of prolyl hydroxylases, independent of either 2-oxoglutarate or oxygen (46). Deficiencies in enzyme activities of prolyl hydroxylases and factor inhibiting HIF-1 result in reduced hydroxylation of Pro-402 and Pro-564 at the N-terminal transcriptional activation domain and Asn-803 at the C-terminal transcriptional activation domain of HIF-1α (47–49). Reduced hydroxylation of Pro-402 and Pro-564 diminishes the binding of the von Hippel-Lindau tumor suppressor to the oxygen-dependent degradation domain of HIF-1α (50, 51), leading to reduced ubiquitination and subsequent proteasome degradation of HIF-1α. Reduced hydroxylation of Asn-803 at the C terminus of HIF-1α facilitates its binding to CBP/p300. As a result, the stabilized phosphorylated HIF-1α translocates to the nucleus and dimerizes with its partner HIF-1β (aryl hydrocarbon re-
ceptor nuclear translocator) followed by interaction with CBP/p300 to form a CBP/p300-HIF-1 complex, promoting the expression of various HIF-1-targeted genes (52) including most glycolytic enzymes, such as aldolase, GAPDH, enolase, etc. HIF-1 also down-regulates hypoxia-responsive element-containing genes such as UGDH, a key enzyme for the conversion of UDP-glucose to UDP-glucuronate, an essential and/or critical precursor for synthesis of extracellular matrix including glycosaminoglycans, hyaluronans, proteoglycans, etc., leading to further disruption of the GAG signaling pathway (39). GAGs play an important role in the regulation of Wnt signaling, which has been shown to be involved in the onset of CRC (40).

The transcription factor HIF-1α is the key regulator of the glycolytic response and the Warburg effect in carcinogenesis (25, 53–56). Overexpression of HIF-1α, resulting from intratumoral hypoxia and genetic alterations, has been demonstrated in common human cancers including colon cancer and their metastases (37) and is correlated with tumor angiogenesis and patient mortality (38). It will be interesting to see whether the mechanism proposed above is applicable to other cancers.

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