Metabolism-related enzyme alterations identified by proteomic analysis in human renal cell carcinoma

Abstract: The renal cell carcinoma (RCC) is one of the most common types of kidney neoplasia in Western countries; it is relatively resistant to conventional chemotherapy and radiotherapy. Metabolic disorders have a profound effect on the degree of malignancy and treatment resistance of the tumor. However, the molecular characteristics related to impaired metabolism leading to the initiation of RCC are still not very clear. In this study, two-dimensional electrophoresis (2-DE) and mass spectra (MS) technologies were utilized to identify the proteins involved in energy metabolism of RCC. A total of 73 proteins that were differentially expressed in conventional RCC, in comparison with the corresponding normal kidney tissues, were identified. Bioinformatics analysis has shown that these proteins are involved in glycolysis, urea cycle, and the metabolic pathways of pyruvate, propanoate, and arginine/proline. In addition, some were also involved in the signaling network of p53 and FAS. These results provide some clues for new therapeutic targets and treatment strategies of RCC.

Keywords: renal cell cancer, metabolism, two-dimensional electrophoresis, proteome

Introduction

The early stage diagnosis of renal cell carcinoma (RCC) in many countries is probably associated with the observed plateau in RCC mortality in the US and in many European countries. Nevertheless, ~50% of patients diagnosed across all stages of this disease die within the first 5 years after diagnosis. Conventional chemotherapy and radiotherapy does not exert a significant long-term benefit on RCC; instead, it has been found to decrease the length or quality of life. RCC is not a single disease; rather, it is a compilation of several types of cancer that occur in the kidney. The poor prognosis of RCC is largely due to the effects of different oncogenes, each having a different histology and response to therapy. Metabolic control analysis is useful in assessing the influence of metabolic pathways on the course and treatment of complex diseases. Since the metabolic environment influences the rate-controlling steps of enzymes in metabolic pathways, the management of complex disease phenotypes is largely dependent on the expression of the entire collection of genes involved than on any particular gene or enzyme. This means that the management of complex disease phenotypes relies on a collection of system-wide interconnected processes that involve glycolysis and respiration. Successful manipulations of metabolic networks can lead to restoration of order and adaptive behavior in disordered states that involve complex gene–environment interactions.

Metabolic control analysis is especially important in kidney cancer management, because disorder and abnormal energy metabolism are characteristics of RCC. However, there are few specific studies that identify the tumor-related metabolic proteins in RCC. In the present study, a comprehensive bioinformatics approach was applied to tissue proteomic data to identify those metabolic steps and networks.
that have a role in RCC onset and development. In kidney cancer, the expression of proteins involved in metabolism, cell growth, morphology, and the heat shock response is deregulated. Therefore, we hypothesize that the defects in identified pathways should serve as targets for the development of effective and long-lasting kidney cancer therapies that will be superior to those presently in use.

Materials and methods

Tissue samples
Surgical specimens of five patients from the Navy General Hospital, obtained after radical nephrectomy, were used to prepare tissue samples of conventional RCC and the surrounding noncancerous kidney tissues. The mean age of the patients was 55.8 years. The tumor stage of the patients ranged from pT1 to pT3. Macroscopic cell type of samples (benign or cancer) was examined histologically. The tumor stage was determined according to the 1997 TNM (tumor, node, metastasis) criteria. The samples were not necrotic.

Table 1 shows a summary of detailed clinicopathologic data of patients included in the study. Institutional Ethics Committee of the Navy General Hospital approved this project, and informed consent were obtained from all patients, or their relatives, prior to commencing the study. A pathologist examined all specimens. The samples were immediately frozen in liquid nitrogen and stored at –80°C until use.

2-DE and image analysis
Two-dimensional electrophoresis (2-DE) was performed as described previously. Briefly, cells were lysed in the lysis buffer (8 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, and 0.2% pH 3–10 ampholyte; Bio-Rad Laboratories Inc., Hercules, CA, USA) containing a protease inhibitor. After sonication and centrifugation, the supernatant was retrieved, and protein concentrations were determined using the DC protein assay kit (Bio-Rad Laboratories Inc.). Two-dimensional electrophoresis (2-DE) was performed as described previously. Mass spectra were acquired using a quadrupole time-of-flight mass spectrometer (Micromass, Manchester, UK) fitted with an electrospray ionization or matrix-assisted laser desorption/ionization source (Micromass). The MS/MS data were acquired using ProteinLynx 2.2.5 software (Waters Corp, Milford, MA, USA). The pkl files were analyzed using the MASCOT search engine (http://www.matrixscience.com). The following search parameters were used: database, Swiss-Prot, taxonomy, Homo sapiens, enzyme, and trypsin. One missed cleavage was allowed. Carbamidomethylation was selected as a fixed modification, and oxidation of methionine was set as the variable. The peptide and fragment mass tolerance were set at 0.1 Da and 0.05 Da, respectively. Positively identified proteins had at least one peptide exceeding their

In-gel digestion
In-gel digestion of proteins was performed using mass spectrometry grade Trypsin Gold (Promega Corporation, Madison, WI, USA). Briefly, the spots were cut out of the gel (1–2 mm diameter) using a razor blade and destained twice with 100 mM NH₄HCO₃/50% acetonitrile (ACN) at 37°C for 45 minutes in each treatment. After drying, the gels were pre-incubated in 10–20 μL trypsin solution for 1 hour. Following, 15 μL digestion buffer was added (40 mM NH₄HCO₃/10% ACN) to cover each gel and incubated overnight at 37°C. Tryptic digests were extracted using Milli-Q water initially, followed by two 1 hour repeat extractions with 50% ACN/5% trifluoroacetic acid. The combined extracts were dried in a vacuum concentrator at room temperature. The samples were then subjected to mass spectrometric analysis.

MS/MS analysis and protein identification
Mass spectra were acquired using a quadrupole time-of-flight mass spectrometer (Micromass, Manchester, UK) fitted with an electrospray ionization or matrix-assisted laser desorption/ionization source (Micromass). The MS/MS analysis was performed as described previously. The MS/MS data were acquired and processed using MassLynx V 4.1 software (Micromass) and converted to PKL performed using ProteinLynx 2.2.5 software (Waters Corp, Milford, MA, USA). The pkl files were analyzed using the MASCOT search engine (http://www.matrixscience.com). The following search parameters were used: database, Swiss-Prot, taxonomy, Homo sapiens, enzyme, and trypsin. One missed cleavage was allowed. Carbamidomethylation was selected as a fixed modification, and oxidation of methionine was set as the variable. The peptide and fragment mass tolerance were set at 0.1 Da and 0.05 Da, respectively. Positively identified proteins had at least one peptide exceeding their

Table 1 Clinicopathologic features of renal cell carcinoma samples

| No | Sex | Age (years) | Clinicopathologic features | TNM stage |
|----|-----|------------|---------------------------|-----------|
| 1  | Male| 52         | Clear cell renal cell carcinoma | T1N0M0   |
| 2  | Male| 52         | Clear cell renal cell carcinoma | T1N0M0   |
| 3  | Male| 60         | Clear cell renal cell carcinoma | T3N0M1   |
| 4  | Female| 54       | Clear cell renal cell carcinoma | T2N0M0   |
| 5  | Male| 61         | Clear cell renal cell carcinoma | T2N0M0   |

Abbreviation: TNM, tumor, node, metastasis.
score threshold \(P<0.05\), and their molecular weight and isoelectric point consistent with the gel regions from which the spots were excised. The spectra of proteins identified by a single peptide, and with a score > 40 (lower were discarded) were manually inspected.

**Immunoblot**
The radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl [pH 7.4], 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1 mg/mL aprotinin, 1 mM Na\(_3\)VO\(_4\), and 1 mM NaF) was used to break open the cells. The proteins were then suspended in the Lammli sample buffer and centrifuged at 15,000 rpm for 30 minutes. The supernatant was recovered for analysis. Each protein sample of 10 \(\mu\)g was loaded per well and separated with 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins inside the gel were electroblotted onto polyvinylidene fluoride membranes (EMD Millipore) by wet blotting. After incubation in the blocking buffer (1× Tris-buffered saline, 0.1% Tween-20, and 5% w/v dry nonfat milk) for 1 hour at room temperature, the membranes were incubated by primary antibodies. Following, the membrane were incubated with secondary antibodies for 45 minutes at room temperature. Enhanced chemiluminescence was used to detect reactive bands (Amersham Biosciences Corp, Piscataway, NJ, USA).

**Bioinformatics and statistical analysis**
Gene Ontology search was used to classify and determine the functions of identified proteins. Pathway data were obtained from the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg) – a collection of online databases dealing with genomes, enzymatic pathways, and biological chemicals.\(^{13}\) Protein–protein interactions were identified with the search tool STRING database. Both direct (physical) and indirect (functional) protein associations were examined.\(^{14,15}\)

The two-tailed Student’s \(t\)-test was used to determine the significant differences between the control and the exposure groups. Statistical analysis was performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA), and \(P<0.05\) was considered statistically significant.

**Results**

**2-DE profiling of differentially expressed proteins**
Protein expression in RCC tissues and the corresponding normal kidney tissues was examined by 2-DE. Figure 2 shows a pair of representative 2-DE maps. Proteins extracted from RCC tissues and the corresponding normal kidney tissues was resolved by 2-DE and visualized by CBB R-250 staining. Those protein spots with a \(P\)-value < 0.05 according to the Student’s \(t\)-test and reproducible changes in intensity > 1.5-fold were identified. The analysis resulted in a total of 90 protein spots that were differentially expressed between RCC tissues and the corresponding normal kidney tissues; of those, 73 proteins were successfully identified by MS/MS (Table 2). Twenty-six proteins were downregulated and 47 proteins were upregulated in RCC tissues (Figures 1 and 2).

**Protein identification and functional classification**
Seventy-three proteins were identified (Figure 1) and are listed Table 2. The MS/MS data, which included the mass and intensity values, and the charge of the precursor ions, were compared against the SWISS-PROT protein database using a licensed copy of the MASCOT 2.0 program. Figure 3 shows a representative MS/MS map of spot #9. Among them, HIBCH was downregulated in RCC tissues in comparison with the adjacent nonmalignant tissues \((P<0.05)\). Furthermore, MS/MS analysis revealed 12 matching peptides, with 44% sequence coverage and a MOWSE score of 714 (Figure 3).

**Immunoblotting validation for differentially expressed proteins**
Two altered proteins, EIF5A and PKM2, were further validated by Western blotting. As shown in Figure 4, EIF5A and PKM2 were upregulated in RCC tissue in comparison with adjacent nonmalignant tissue, which was consistent with the 2-DE results \((P<0.05)\).

**Network, pathway, and process analyses of significantly changed proteins**
Table 2 lists differentially expressed proteins in RCC and the corresponding normal tissues, as confirmed by mass spectrometry. Their molecular function and biological processes are included in Table 2. Interactions exist among these proteins, and most of them are a part of a biological network, as illustrated by STRING (Figure 5). Out of 73 identified proteins, 63 were interconnected and ten proteins did not show any type of connection at the selected confidence level (STRING score = 0.4). The following significant functions are associated with this network of proteins: metabolism, transcription, proteolysis, electron transport, and molecular chaperoning. ENO1, ENO2, AKR1A1, PGAM1, and PGA are important proteins in gluconeogenesis, while...
Table 2 Identification results of proteins differentially expressed between RCC and the corresponding normal tissues

| Spot no | Protein description                                      | Gene name*   | Function   | Accession no* | Theoretical MW/pl | Score* | Coverage* | Fold change* |
|---------|----------------------------------------------------------|--------------|------------|---------------|-------------------|--------|-----------|--------------|
| 1       | Phosphoenolpyruvate carboxykinase [GTP]                 | PCK2         | Metabolism | Q16822        | 71,438/7.57       | 233    | 22%       | ↓            |
| 2       | 78 kDa glucose-regulated protein                        | GRP78        | Molecular chaperone | P11021   | 72,402/5.07       | 58     | 6%        | ↓            |
| 3       | Delta-1-pyruvoly-5-carboxylate dehydrogenase            | ALDH4A1      | Metabolism | P30038        | 62,137/8.25       | 224    | 21%       | ↓            |
| 4       | Alpha-aminoadipic semialdehyde dehydrogenase            | ALDH7A1      | Metabolism | P49419        | 59,020/8.21       | 255    | 22%       | ↓            |
| 5       | Glycine aminotransferase                                | GATM         | Metabolism | P50440        | 48,938/8.26       | 167    | 42%       | ↓            |
| 6       | Medium-chain specific acyl-CoA dehydrogenase            | ACADM        | Metabolism | P11310        | 47,015/8.61       | 302    | 26%       | ↓            |
| 7       | Argininosuccinate synthase                              | ASS          | Metabolism | P00966        | 46,786/8.08       | 230    | 33%       | ↓            |
| 8       | Fructose-1,6-bisphosphatase I                           | FBP1         | Metabolism | P09467        | 37,218/6.54       | 1,094  | 56%       | ↓            |
| 9       | 3-Hydroxyisobutyryl-CoA hydrolase                       | HIBCH        | Metabolism | Q6NYY1        | 43,797/8.38       | 714    | 44%       | ↓            |
| 10      | Acetyl-CoA acetyltransferase, mitochondrial              | ACAT1        | Metabolism | P24752        | 45,456/8.98       | 585    | 39%       | ↓            |
| 11      | Ester hydrolase C1orf54                                 | C1orf54      | Metabolism | Q9H0WV9       | 35,608/6.23       | 240    | 31%       | ↓            |
| 12      | Glycerol-3-phosphate dehydrogenase [NAD+]               | GPD1         | Metabolism | P21695        | 38,171/5.81       | 863    | 60%       | ↓            |
| 13      | Complement component 1 Q subcomponent-binding protein   | C1QBP        | Immune regulation | P07021   | 31,749/4.74       | 2,109  | 46%       | ↓            |
| 14      | Agmatinase, mitochondrial                              | AGMAT        | Metabolism | Q9B5E5        | 38,206/7.55       | 707    | 41%       | ↓            |
| 15      | Calbindin                                                | CALB1        | Metabolism | P05937        | 30,291/4.7        | 531    | 46%       | ↓            |
| 16      | Glutathione peroxidase 3                                | GPX3         | Metabolism | P22352        | 25,763/8.26       | 491    | 29%       | ↓            |
| 17      | Cytochrome b–c1 complex subunit Rieske                 | UQCRFS1      | Electron transport | P47985   | 29,934/8.55       | 405    | 37%       | ↓            |
| 18      | ES1 protein homolog                                      | C21orf33     | Metabolism | P30042        | 28,495/8.5        | 201    | 38%       | ↓            |
| 19      | Transgelin                                              | TAGLN        | Structural component | P01995  | 26,653/8.87       | 158    | 57%       | ↓            |
| 20      | Nucleoside diphosphate kinase B                         | NME2         | Metabolism | P22392        | 17,401/8.52       | 205    | 40%       | ↓            |
| 21      | Nucleoside diphosphate kinase A                         | NME1         | Metabolism | P15531        | 17,309/5.83       | 246    | 53%       | ↓            |
| 22      | Peptidyl-prolyl cis–trans isomerase B                    | PP1B         | Metabolism | P22384        | 23,785/9.42       | 511    | 41%       | ↓            |
| 23      | Transthyretin                                            | TTR          | Hormone-binding protein | P02766  | 15,991/5.52       | 122    | 40%       | ↓            |
| 24      | Cytochrome c oxidase subunit 5A, mitochondrial          | COX5A        | Electron transport | P20674  | 16,923/6.3        | 435    | 47%       | ↓            |
| 25      | Fatty acid-binding protein, liver                       | FABP1        | Lipid transport | P07148  | 14,256/6.6        | 368    | 53%       | ↓            |
| 26      | 10 kDa heat shock protein                               | HSPE1        | Metabolism | P61604        | 10,925/8.89       | 533    | 52%       | ↓            |
| 27      | Serum albumin                                           | ALB          | Metabolism | P02768        | 71,317/5.92       | 195    | 16%       | ↑            |
| 28      | Retinal dehydrogenase 1                                | ALDH1        | Metabolism | P00352        | 55,454/6.3        | 1,196  | 59%       | ↑            |
| 29      | Alpha-enolase                                           | ENO1         | Metabolism | Q6GMP2        | 47,481/7.01       | 2,799  | 65%       | ↑            |
| 30      | Glycine aminotransferase, mitochondrial                 | GATM         | Metabolism | P50440        | 48,938/8.26       | 288    | 49%       | ↑            |
| 31      | Pyruvate kinase isozymes M1/M2                          | KPYM         | Metabolism | Q9BVW85       | 58,480/7.96       | 259    | 29%       | ↑            |
| 32      | Septin-2                                                | SEPT2        | Structural component | Q15019  | 41,689/6.15       | 519    | 40%       | ↑            |
| 33      | Fumarlylactoacetase                                     | FAH          | Metabolism | P16930        | 46,743/6.46       | 425    | 30%       | ↑            |
| 34      | Gamma-enolase                                           | ENO2         | Metabolism | P09104        | 47,581/4.91       | 771    | 7%        | ↑            |
| 35      | Phosphotriesterase-related protein                      | PTER         | Metabolism | Q96B5V5       | 39,506/6.07       | 201    | 59%       | ↑            |
| 36      | Alpha-soluble NSF attachment protein                    | NAPA         | Electron transport | P54920  | 33,667/5.23       | 228    | 66%       | ↑            |
| 37      | Annexin A4                                              | ANXA4        | Calcium ion binding | P09525  | 36,092/5.84       | 985    | 54%       | ↑            |
| 38      | Phosphoserine aminotransferase                         | PSAT1        | Metabolism | Q9Y617        | 40,796/7.56       | 249    | 44%       | ↑            |
| 39      | Aldose reductase                                        | ALDR1        | Metabolism | P15121        | 36,230/6.51       | 293    | 68%       | ↑            |
| 40      | Annexin A2                                              | ANXA2        | Calcium ion binding | P07355  | 38,808/7.57       | 359    | 46%       | ↑            |

(Continued)
### Table 2 (Continued)

| Spot no | Protein description                  | Gene name* | Function                        | Accession no b | Theoretical MW/pl | Score* | Coverage* | Fold change* |
|---------|-------------------------------------|------------|---------------------------------|----------------|-------------------|--------|-----------|-------------|
| 41      | Proteasome activator complex subunit 2 | PSME2      | Proteolysis                     | Q9UL46         | 27,555/5.54       | 584    | 66%       | ↑           |
| 42      | Proteasome subunit alpha type-3      | PSMA3      | Proteolysis                     | P25788         | 28,643/5.19       | 224    | 14%       | ↑           |
| 43      | S-formylglutathione hydrolase         | ESD        | Metabolism                      | P10768         | 31,956/6.54       | 276    | 47%       | ↑           |
| 44      | Voltage-dependent anion-selective channel protein 2 | VDAC2     | Electron transport              | P45880         | 32,060/7.49       | 235    | 40%       | ↑           |
| 45      | Nicotinamide N-methyltransferase     | NNMT       | Metabolism                      | P40261         | 30,011/5.56       | 648    | 50%       | ↑           |
| 46      | Glutathione S-transferase P          | GSTP1      | Metabolism                      | P09211         | 23,569/5.43       | 1,197  | 85%       | ↑           |
| 47      | Proteasome subunit beta type-4       | PSMB4      | Proteolysis                     | P28070         | 29,242/5.72       | 436    | 38%       | ↑           |
| 48      | Phosphoglycerate mutase I            | PGAM1      | Metabolism                      | P18669         | 28,900/6.67       | 197    | 45%       | ↑           |
| 49      | Triosephosphate isomerase            | TPI        | Metabolism                      | P60174         | 26,943/6.45       | 1,109  | 55%       | ↑           |
| 50      | Superoxide dismutase [Mn]            | SOD2       | Electron transport              | P04179         | 24,722/8.35       | 442    | 59%       | ↑           |
| 51      | Proteasome subunit beta type-8       | PSMB8      | Proteolysis                     | P28062         | 30,677/7.67       | 273    | 27%       | ↑           |
| 52      | Actin, cytoplasmic 1                 | ACTB       | Structural component            | P60709         | 42,052/5.29       | 194    | 7%        | ↑           |
| 53      | Alpha-I-antitrypsin                  | AAT        | Metabolism                      | P01009         | 46,878/5.37       | 44     | 3%        | ↑           |
| 54      | Sorcin                               | SR1        | Calcium ion binding             | P30626         | 21,947/5.32       | 237    | 47%       | ↑           |
| 55      | Ferritin heavy chain                 | FTH1       | Metabolism                      | P02794         | 21,383/5.5        | 110    | 22%       | ↑           |
| 56      | Haptoglobin                          | HP         | Metabolism                      | P00738         | 45,861/6.13       | 139    | 14%       | ↑           |
| 57      | Alpha-crystallin B chain             | CRYAB      | Metabolism                      | P02511         | 20,146/6.76       | 772    | 72%       | ↑           |
| 58      | Hippocalcin-like protein 1           | HPCAL1     | Metabolism                      | P37235         | 22,413/5.21       | 108    | 29%       | ↑           |
| 59      | Ferritin light chain                 | FTL        | Metabolism                      | P02792         | 20,064/5.51       | 391    | 41%       | ↑           |
| 60      | Eukaryotic translation initiation factor 5A-1 | EIF5A     | Translation regulation          | P63241         | 17,053/5.08       | 303    | 42%       | ↑           |
| 61      | Matrilysin                           | MMP7       | Metabolism                      | P09237         | 29,829/7.74       | 181    | 35%       | ↑           |
| 62      | Cofilin-I                            | COF1       | Signal transduction             | P23528         | 18,723/8.22       | 364    | 31%       | ↑           |
| 63      | Peptidyl-prolyl cis–trans isomerase A | PPIA       | Protein folding                 | P62937         | 18,233/7.68       | 548    | 64%       | ↑           |
| 64      | Annexin A3                           | ANXA3      | Calcium ion binding             | P12429         | 36,527/5.63       | 87     | 4%        | ↑           |
| 65      | Fatty acid-binding protein, epidermal | FABP5     | Structural component            | Q01469         | 15,497/6.6        | 291    | 43%       | ↑           |
| 66      | Histidine triad nucleotide-binding protein 1 | HINT1     | Metabolism                      | P49773         | 13,905/6.43       | 477    | 65%       | ↑           |
| 67      | Glutathione S-transferase theta-I    | GSTT1      | Metabolism                      | P30711         | 27,489/7.01       | 125    | 13%       | ↑           |
| 68      | Small nuclear ribonucleoprotein F    | SNRF      | Metabolism                      | P62306         | 97,76/4.7         | 161    | 40%       | ↑           |
| 69      | SH3 domain-binding glutamic acid-rich-like protein 3 | SH3BGR1 | Metabolism                      | P9H29        | 10,488/4.82       | 194    | 66%       | ↑           |
| 70      | Protein S100-A4                       | S100A4     | Calcium ion binding             | P26447         | 11,949/5.85       | 367    | 49%       | ↑           |
| 71      | Protein S100-A11                      | S100A11    | Calcium ion binding             | P31949         | 11,847/5.6       | 1,792  | 73%       | ↑           |
| 72      | Beta-2-microglobulin                 | B2M        | Immune regulation               | P61769         | 13,820/6.06       | 402    | 37%       | ↑           |
| 73      | Ubiquitin-40S ribosomal protein S27a | RPS27A     | Metabolism                      | P62979         | 18,296/9.68       | 78     | 24%       | ↑           |

**Notes:** *the proteins gene name and ID from ExPaSy database; theoretical molecular weight (kDa) and pl from the ExPaSy database; probability-based MOWSE scores; number of unique peptides identified by MS/MS sequencing and sequence coverage; expression level in RCC compared with the corresponding normal tissues. ↑, increase; ↓, decrease.

**Abbreviations:** MW, molecular weight; pl, isoelectric point; MS, mass spectra; RCC, renal cell carcinoma.

PSME2, PSMA3, PSMB4, and PSMB8 are involved in proteasome-related proteolysis. The proteins were divided into several classes as a result of bioinformatic analysis based on the Kyoto Encyclopedia of Genes and Genomes pathway, which included: gluconeogenesis, the urea cycle and amino acid metabolism, proteasome, fatty acid metabolism, glutathione (GSH) metabolism, and so forth (Table 3).

**Discussion**
Identifying the changes in protein expression in cancer cells is a useful predictor of potential changes in the functional
pathways, which are, in turn, directly related to the basic mechanism of cancer onset and progression. Analysis at the proteome level enables the identification of proteins that are differentially expressed in RCC and adjacent normal tissues. These RCC-specific protein biomarkers might facilitate more efficient subclassification and early diagnosis of RCC.\(^{16,17}\) In this study, we analyzed the expression of proteins in eleven pairs of RCC tissues and matching normal kidney tissues from RCC patients utilizing two-dimensional electrophoresis and matrix-assisted laser

\[\text{Figure 1} \text{ Representative 2-DE gel images of RCC tissue compared to adjacent nonmalignant tissue.}\
\text{Notes:} \text{ The gels were stained with Coomassie brilliant blue R250. Differentially expressed protein spots were labeled with numbers.}\
\text{Abbreviations:} \text{ RCC, renal cell carcinoma; 2-DE, two-dimensional electrophoresis.}\]

\[\text{Figure 2} \text{ The enlargement of six selected regions as examples of protein spots that are dysregulated in this study.}\
\text{Notes:} \text{ Protein spot discrepancies were labeled with arrows and marked with numbers (to the left of the images).}\]
We found that 47 proteins were overexpressed and 26 proteins underexpressed in RCC. An altered expression of some of these proteins has previously been observed in RCC. The analysis of biochemical pathways conducted in this study has led to identification of protein networks, which play important roles in oncogenesis or progression of clear cell RCC (ccRCC).

![Figure 3](image1)

**B**

HIBCH HUMAN  Mass: 43797  Score: 714  Matches: 47  Sequences: 12  emPAI: 1.90  3-hydroxyisobutyryl-CoA hydrolase, mitochondrial  OS = Homo sapiens  GN = HIBCH  PE =1  SV =2

1  MGQREMWRLM SRFNAFKRTN TILHHLRMSK HTDAEEVLL EKKGCTGVIT
51  LNRPKFLNAL TLNMIROIQYP QLKKWEQDPE TFILIIGGAG GKAFCAGGGI
101  RVISEAEKAK QKIAPFFERE EYMLNNAVGCS CQKPYVALIH GITMGGGVL
151  SVHQFQFRVAT EKCLFAMPET AIGLFPVDGG GYFLPLRLQGG LGYFLALTGF
201  RLRGVRDVYRA GIATHFVDSE KLAMLEDLL ALKSPSKEJNI ASVLNYHTE
251  SKIDRDKSFII LEEHDMDKINS CFSAINTVEEI IENLQQDGSSE FALEQLKVIN
301  KMSMTSIFKT LRQLMEGSSK TLQEVLMTEY RLSQACMRGH DFHEGVRAVL
351  IDQDSPKWK PADLKEVTDE DLNHFKSLG SSDLKF

Figure 3  Identification of protein spot #9.

**Notes:** (A) Peptide mass fingerprinting (PMF) of protein HIBCH. (B) HIBCH was identified by searching the MS/MS database using the MASCOT program. The matching peptides are shown in bold red.

**Abbreviations:** HIBCH, 3-Hydroxyisobutyryl-CoA Hydrolase; MS, mass spectra.

![Figure 4](image2)

**Figure 4**  Western blot detection of EIF5A and PKM2 expression in RCC tissue (T) and adjacent non-malignant tissue (N).

**Notes:** (A) EIF5A and PKM2 were upregulated in RCC tissue. (B) Western blot data were quantified densitometrically and β-actin was used as the loading control. Data are expressed as mean ± SD from three independent experiments. *P <0.05, compared with adjacent non-malignant tissue.

**Abbreviation:** RCC, renal cell carcinoma.
The finding that glycolysis enzyme levels are most significantly altered in ccRCC is in accordance with the results of other independent studies conducted in different types of cancers.\textsuperscript{19–21} Increased aerobic glycolysis in cancer, a phenomenon known as the Warburg effect, is characterized by increased metabolism of glucose to lactate in the presence of sufficient oxygen. There is a strong connection between this effect and malignant transformation, as evidenced from studies conducted on various tumor cells.\textsuperscript{22–24} p53 and c-myc are considered to be the key tumor genes and the master regulators of metabolism.\textsuperscript{25,26} The pyruvate kinase (PK) gene, which encodes a protein that converts phosphoenolpyruvate to pyruvate with release of an adenosine triphosphate, is the target gene of Myc and HIF-1.\textsuperscript{27} The dimeric form of M2-PK is another protein specific for tumor cells (known as tumor M2-PK), the dimerization seems to be caused by the interaction of M2-PK with certain oncoproteins. It is believed that this adaptive mechanism allows tumor cells to survive in environments where the levels of oxygen and nutrients are not constant.\textsuperscript{28} The interconversion of glycerate-3-phosphate and glycerate-2-phosphate is catalyzed by the glycolytic enzyme phosphoglycerate mutase, while enolase catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate. The expression of enolase is regulated both developmentally and specifically within the tissues. Proteome analysis reported in a recent study has shown that

\textbf{Figure 5} Signaling networks/functional analysis of dysregulated proteins in RCC.\textsuperscript{14} In this map, the network nodes represent proteins. The edges represent predicted functional associations. An edge may be drawn with several different lines. These lines represent the existence of several types of evidence used in predicting the associations.\textbf{Abbreviation:} RCC, renal cell carcinoma.
Table 3 Enriched processes and pathways identified with the kegg database using proteins

| Pathway                                    | Count | Gene                              | P-value          |
|--------------------------------------------|-------|-----------------------------------|------------------|
| Glycolysis/gluconeogenesis                  | 9     | ENO1, ENO2, AKR1A1, PGAM1, PGAM4, TP1, PCK2, ALDH7A1, and FBP1 | 6.86E–16         |
| Urea cycle and amino metabolism             | 4     | GATM, ALDH7A1, ASS1, and AGMAT    | 9.96E–08         |
| Propanoate metabolism                       | 4     | ALDH7A1, ACADM, HIBCH, and ACAT1  | 2.24E–07         |
| Pyruvate metabolism                         | 4     | AKR1B1, PK2, ALDH7A1, and ACAT1   | 4.85E–07         |
| Valine, leucine, and isoleucine degradation  | 4     | ALDH7A1, ACADM, HIBCH, and ACAT1  | 7.77E–07         |
| Proteasome                                  | 4     | PSME2, PSMA3, PSMB4, and PSMB8    | 9.25E–07         |
| PPAR signaling pathway                      | 4     | FABP5, PCK2, ACADM, and FABP1     | 4.25E–06         |
| Beta-alanine metabolism                     | 3     | ALDH7A1, ACADM, and HIBCH         | 5.92E–06         |
| Arginine and proline metabolism             | 3     | GATM, ALDH4A1, and ASS1           | 2.35E–05         |
| Fructose and mannose metabolism             | 3     | AKR1B1, TP1, and FBP1             | 2.35E–05         |
| Fatty acid metabolism                       | 3     | ALDH7A1, ACADM, and ACAT1         | 4.63E–05         |
| Glycerol lipid metabolism                   | 3     | AKR1B1, AKR1A1, and ALDH7A1       | 5.28E–05         |
| Glutathione metabolism                      | 3     | GSTP1, GSTT1, and GPX3            | 6.36E–05         |
| Antigen processing and presentation         | 3     | PSME2, B2M, and HSP5              | 3.53E–04         |
| Bile acid biosynthesis                      | 2     | ALDH7A1 and SOAT1                 | 9.10E–04         |
| Butanoate metabolism                        | 2     | ALDH7A1 and ACAT1                 | 0.001562         |
| Glycine, serine, and threonine metabolism   | 2     | GATM and PSAT1                    | 0.001996         |
| Trypsophan metabolism                       | 2     | ALDH7A1 and ACAT1                 | 0.001996         |
| Metabolism of xenobiotics by cytochrome P450| 2     | GSTP1 and GSTT1                   | 0.005199         |
| Drug metabolism – cytochrome P450           | 2     | GSTP1 and GSTT1                   | 0.005491         |
| Pyrimidine metabolism                       | 2     | NME2 and NME1                     | 0.009003         |
| Insulin signaling pathway                   | 2     | PCK2 and FBP1                     | 0.019038         |
| Oxidative phosphorylation                   | 2     | UQCRFS1 and COX5A                 | 0.019297         |
| Purine metabolism                           | 2     | NME2 and NME1                     | 0.022799         |

Note: Enriched processes and pathways identified with the kegg database using proteins which were significantly altered in RCC as compared to normal tissue, with P<0.05.

Abbreviation: RCC, renal cell carcinoma.

both phosphoglycerate mutase and enolase seem to be differentially overexpressed in human lung squamous carcinoma. Our data suggest that anaerobic glycolysis-related enzyme PK, enolase, is upregulated, whereas the other carbohydrate metabolism-related enzymes, phosphoenolpyruvate carboxy kinase (PCK2) and acetyl-CoA acetyltransferase (ACAT1), are downregulated in RCC, which is consistent with the results from other laboratories.29,30 Recently, the role of agents targeting glycolytic activity and glycolysis-linked metabolic processes is being studied for reversal of Warburg effect.31,32 Proteasomes and ubiquitin (Ub) are key participants of the energy-dependent, nonsylosomal proteolytic pathway. Previous studies have indicated that cell proliferation and apoptosis are regulated by the Ub–proteasome system. The research community is focusing its efforts on identifying the potential role of certain proteasome inhibitors to act as novel anticancer agents.33 In this work, Ubiquitin-40S ribosomal protein S27a and four members of the proteasome family, PSME2, PSMA3, PSMB4, and PSMB8 were highly expressed in RCC, which is consistent with one previous study.34 Other studies have suggested that proteasomes and Ub also have important roles in various nonproteolytic functions. Proteasomes are thought to regulate the translational activities of cytoplasmic mRNAs.35 Ub has been found to have many apparently distinct roles, such as DNA repair, cell cycle progression, modification of polypeptide receptors, and biogenesis of ribosomes.36,37

GSH has multiple roles in the body; it is involved in cell differentiation, proliferation, and apoptosis, as well as antioxidant defense and nutrient metabolism.38 It has been shown that enzymes involved in GSH metabolism, particularly glutathione S-transferase and glutathione peroxidase, play a role in multistage carcinogenesis.39 Our results point to significant variations in the GSH-dependent enzyme activity in RCC and support the finding that GSH metabolism is important in RCC onset and progression. Because they have high energy demands, cancer cells are forced to tap into alternative sources of energy, such as fatty acid oxidation and other nonglycolytic pathways. Our findings suggest that the products of fatty acid metabolism have a key role in RCC metabolism. Fatty acid-binding proteins (FABPs) are involved in lipid metabolism, regulation of gene expression, cell signaling, cell growth, and differentiation.40 Moreover, FABPs also have an important role in carcinogenesis.41 Studies identifying FABP as tumor markers of RCC emphasize the significant role of fatty acid metabolism in the biology of RCC.42,43 In comparison with normal tissues, we found that liver-type FABP was expressed at lower rates in 53% of all tumors, which is consistent with the findings from other studies.44
The results of this study indicate that other pathways closely associated with gluconeogenesis, such as the urea cycle, pyruvate, pentanoate, and butanoate metabolism, as well as arginine and proline metabolism, are downregulated in ccRCC. In contrast, an increase in one of the key glycolytic enzymes, pyruvate, was observed.

Our study outlines the metabolic phenotype of RCC tissue in detail. Using proteomic analysis to determine which pathways and processes are likely involved in kidney cancer, we found that the glycolysis pathway is significantly altered in ccRCC. Alterations to these pathways will allow clinicians to identify those molecules that affect metabolic regulation, such as activators or inhibitors of HIF-1, mTOR, and AMP kinase, as well as assess the effectiveness of therapy at the molecular level.

Disclosure
The authors report no conflicts of interest in this work.

References
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