Proteomic analysis of clear cell renal cell carcinoma

Identification of potential tumor markers

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ABSTRACT

الأهداف: التعرف على بروتينات محددة في الورم الحديث لسرطان الخلية الكلوية (RCC) باستخدام التحليل البروتيني.

الطريقة: استأصل أنسجة عدد=9 ورم لسرطان الخلية الكلوية و أجريت العمليات الجراحية خلال الفترة من يونيو 2007م حتى سبتمبر 2008م في قسم المسالك البولية – مستشفى جامعة سوتشي – سوتشو – محافظة جيانغسو – الصين. ثم تم حفظ أنسجة السرطان وأنسجة الجارات الكلوية الطبيعية في سائل النتيروجين. في هذا التقرير، قمنا بتحليل أنسجة سرطان الخلية الكلوية والأنسجة الطبيعية للجارات باستخدام جل الرحلان الكهربائي بولي أكريلاميد (2D-PAGE). تم تحليل ظهور 16 بقع بروتينية باستخدام المصفوفة المساعدة لتأين الليزر، وقياس ثقل الجزيئات وتركيبها النووي، ومطياف الكتلة الترادفي بقيمة إحصائية تبلغ $p<0.05$.

النتائج: انخفض 6 بروتين وازداد 10 بروتين في سرطان الخلايا الكلوية الظاهرة مقارنة مع أنسجة الكلية الطبيعية. كانت البروتينات المنخفضة هي كالبندين، وأنزيم أستير C11orf54، ونازعة هيدروجين الكحول، وامسير 1 المشابه للبروتين، وأنزيم الترانسلوكاز ADP/ATP، وعنصر LYR المحتوي على البروتين الخامس، بينما تزايدت البروتينات التالية، عائلة مجموعة HIG1، وأنزيم جلوتاتيون، والثيروكسين المعتمد على أنزيم البروكسيد، والبيروكسيدين- 6، وCD2 المرتبط بالبروتين، والانكسين A5 وانيولاز غاما، وأنزيم الهيدروجين الشبكي، وفيمنتين، وأنزيم الغاما غلوتاميل 2.

خاتمة: تعطي بياناتنا علامات الورم الظاهرة لتشخيص سرطان الخلية الكلوية RCC.

Objectives: To identify new tumor specific proteins of renal cell carcinoma (RCC) using proteomic analysis.

Method: Nine renal cell carcinomas were resected and these surgical operations were carried out from June 2007 to September 2008 in the Urology Department of the Second Affiliated Hospital of Soochow University, Suzhou, Jiangsu Province, China. The cancer tissues and para-cancer normal tissues were preserved in liquid nitrogen. We analyzed the RCC tissues and para-cancer normal tissues by 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The 16 differentially expressed protein spots ($p<0.05$ by Student t-test) were identified by matrix assisted laser desorption ionization/time of flight mass spectrometry and tandem mass spectrometry.

Results: Six proteins were down regulated and 10 proteins were up regulated in clear cell RCC compared with corresponding normal kidney tissue. The down regulated proteins were calbindin, ester hydrolase C11orf54, alcohol dehydrogenase, ammecr1-like protein, adenosine diphosphate (ADP)/adenosine-5'-triphosphate (ATP) translocase 3 and leucine-tyrosine-arginine (LYR) motif-containing protein 5. The up regulated proteins were hypoxia inducible domain family member 1A, glutathione S-transferase P, thioredoxin-dependent peroxide reductase, peroxiredoxin-6, CD2-associated protein, annexin A5, gamma-enolase, retinal dehydrogenase 1, vimentin, and protein-glutamine gamma-glutamyltransferase 2.

Conclusion: The data provide potential tumor markers for diagnosis of RCC.

Saudi Med J 2010; Vol. 31 (5): 525-532

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Received 6th January 2010. Accepted 10th March 2010.

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Disclosure. This study was supported by the Natural Science Fund for Colleges and Universities in Jiangsu Province (Grant #09KJB310013), and Jiangsu Planned Projects for Postdoctororal Research Funds (Grant #0702053C).

Renal cell carcinoma (RCC) represents the most common neoplasia of the kidney and accounts for approximately 3% of all adult malignancies in western countries.[1] Renal cell carcinoma is a histologic diverse disease with an often unpredictable course. It is one of the most malignant tumors in urology, and the therapies currently available are of limited success for the treatment of metastatic RCC. Due to its insidious onset patients frequently have advanced disease at the time of clinical presentation. Thus, early detection is crucial in the management of RCC. Today many methods are used to search for new diagnosis and prognosis molecule markers. With the development of proteomics technology, the 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry are used more widely to search for differentially expressed proteins in tumor and normal tissue. In this study, our objective was to search for new tumor specific proteins of RCC by proteomic analysis.

Methods. Tissue samples of conventional RCC and the surrounding non-cancerous kidney tissues were prepared from surgical specimens of 9 patients after radical nephrectomy. These surgical operations were carried out from June 2007 to September 2008 in the Urology Department of the Second Affiliated Hospital of Soochow University, Suzhou, Jiangsu Province, China. Ethic approval for this study was obtained from the University Ethics Committee. The specimens received pathological examination. The remaining parts of the samples for subsequent protein extraction were placed in ice-cold RPMI medium containing a complete mini protease inhibitor cocktail tablet for transport to the laboratory, washed in ice-cold phosphate buffer solution (PBS), cut into small blocks (~100 mm^3), rinsed with ice-cold isotonic sucrose (250 mM) and snap-frozen in liquid nitrogen or embedded in optimum cutting temperature™ (OCT) embedding medium prior to storage in liquid nitrogen. The frozen tissues were flushed through the hepatic vein with a perfusion buffer (PBS 1 0.02% EDTA) to remove excessive blood contamination. They were immediately washed with a cold washing buffer (25 mM sorbitol +10mM Tris-HCl pH 7.4), and then sliced into pieces within a washing buffer. The sliced pieces were then homogenized gently 3 times to release the cells from the tissue. The cell solution was then filtered through a 150 x 200 meshes per cm^2 sieve, sequentially. The resulting solution, containing mainly individual cells, was then centrifuged and washed 3 times at 1000 rpm, each for 5 minutes. The cell pellet was suspended in a lysis buffer containing 8 M urea, 40 mM Tris, 4% 3-cholamidopropyl)dimeth ylammonio)-1-propanesulfonic acid (CHAPS), 65 mM dithiothreitol (DTT), 1% pharmalyte pH 4-7, 5mM Na_3VO_4, 1 mg/ mL aprotinin, 1 mg/mL leupeptin, and 5 mM phenylmethanesulfonyl fluoride (PMSF) (the ratio of pellet to lysis buffer was approximately 1:2). The suspension was placed on ice for 2 hours with gentle vortex every 15 minutes. The suspension was aliquoted and stored at -80°C until use. The protein concentration was determined by the Coomassie brilliant blue (CBB) method.[2] The 2D-PAGE was essentially carried out as described in the 2D-PAGE handbook from Amersham Corporation using the Amersham Ettan IPGphor and Ettan DALT 6 systems (Amersham Corporation, Uppsala, Sweden). Briefly, each sample of 1mg protein was loaded on an immobilized 18 cm, pH 4-7 linear strip. Re-hydration was performed at 30 V for 12 hours. The sample focusing condition was as follows: 100 V for one hour, 500 V for one hour, 1000 V for one hour, 8000 V gradient for 30 minutes, and 8000 V for 60000 V hour. The 2-D separation was performed on 12% sodium dodecyl sulphate (SDS)-PAGE. The gels were stained with CBB R350. Electric images of the gels were obtained using the MagicScan densitometer (Amersham Biosciences, Uppsala, Sweden) at the same scanning parameters. The tiff format images were subsequently used for image analysis.[2] The 2D-PAGE digital images were analyzed using ImageMaster 2-D Elite software (Amersham Biosciences, Uppsala, Sweden). Briefly, protein spots were automatically detected first and then refined manually; subsequently the gels were normalized and matched. For each spot, the ratio between its volume and the sum of all the spots volume in the gel was calculated and used for quantitative comparison. Each inter-group comparison of the samples between control and test was carried out on 3 separate paired gels. For changes in protein spots to be considered a genuine difference in actual protein expression, we applied the following criterion: show similar qualitative changes in all 3 paired repeats of a given comparison and the expression change was at least the p-value <0.05. We then employed one-way ANOVA to analyze the protein expression change between 2 groups. We tried to identify those proteins with similar expression patterns between the 2 groups. The results were analyzed by student t-test and by using Microsoft Office Excel software (Microsoft Inc, Seattle, Washington, USA). Protein spots were excised manually from the stained gels, rinsed 3 times with Milli-Q water, and then destained in 25 mM NH_4HCO_3/50% acetonitrile until the gel pieces became transparent. Prior to the addition of trypsin, the clear gel pieces were dehydrated with 100% acetonitrile for 5 minutes and dried. The dried gel pieces were then incubated in 10 µL trypsin solution (trypsin concentration, 10 ng/µL; total 100 ng) at 37°C overnight. Afterwards, 1 µL trypsin-

digested peptide mixture mixed with 0.6 µL matrix solution (50 mg/mL 2,4-Dihydroxybenzophenone purum (DHB) in 50% acetonitrile/0.1% trifluoroacetic acid(TFA) was loaded onto a stainless steel 100-well mass spectrometry (MS) plate and air dried. The samples were then analyzed on an Autoflex matrix assisted laser desorption ionization/time of flight mass spectrometry (MALDI TOF [Bruker Daltonics, Billerica, MA, USA]) to obtain the MS and MS/tandem mass spectrometry (MS/MS) data for these proteins. Spectra were calibrated with a trypsin-fragment peak serving as an internal standard. Subsequently, protein identification was determined by searching in the SWISS-PROT database using the MASCOT search engine (http://www.matrixscience.com).[3] The searching parameters were set up as follows: the mass tolerance was ±0.3 Da; the number of missed cleavage sites was allowed up to 1; modifications were carbamidomethylation (fixed) and oxidation of methionine (variable); the peptide ion was $[M + H]^+$; and the monoisotopic masses were used.

To explore the RCC related proteins, we compared the protein expression profiling of RCC tissues and para-cancer normal tissues by 2D-PAGE. Total protein was first separated on pH 4-7 immobilized pH gradients (IPG) strips 3 times under the same conditions. The number of protein spots in 3 gels analyzed by software was 1238 ± 19 in normal kidney tissues and 1324 ± 26 in RCC samples, and the average matching rate between these 2 groups of gels was 84.1%. For the assessment of differentially expressed proteins only protein spots altered in all tumor samples were considered. The integrated protein intensity was determined for each identified spot in 3 gels each from the RCC tissues and corresponding normal kidney tissues.[2] According to software analysis, those spots possessing over 2-fold changes in percentage volume (vol%) after normalization were regarded as showing a notable difference. Those spots showing consistently different expression in all 3 parallel experiments were excised and subjected to subsequent MALDI-TOF/MS analysis. As for each protein spot, MS/MS analysis was carried out after MALDI-TOF/MS to verify the identification by PMF. All of the identities of the candidate proteins were determined by searching databases with both PMF data and MS/MS fragmentations.

Table 1 - Clinical and pathological features of the patients with clear cell renal cell carcinoma.

No.	Gender	Age (year)	Cell type	TNM Stage
1	Female	56	Clear cell	T1N0M0
2	Male	73	Clear cell	T1N0M0
3	Male	59	Clear cell	T2N1M0
4	Male	62	Clear cell	T1N0M0
5	Male	58	Clear cell	T3N0M0
6	Female	51	Clear cell	T2N0M0
7	Male	56	Clear cell	T1N0M0
8	Male	49	Clear cell	T1N0M0
9	Female	86	Clear cell	T1N1M0

TNM - tumor node metastasis

Results. The patients ranged from 49-86 years old, and the mean age was 61.1 year old. The tumor stage of the patients ranged from pT1 to pT3a. **Figure 1** shows the histological examination of these samples to verify the macroscopic cell type (benign or malignant). **Table 1** shows the clinical and pathological features of these patients with clear cell RCC. **Figure 2** shows the result of 2D-PAGE and 16 proteins spots differentially expressed in the normal kidney samples and RCC samples were determined through software analysis and refining manually.

Among the excised spots, 16 differentially expressed proteins were successfully identified in pH 4-7 gels, among which 10 proteins were up regulated (Y1-Y10, **Table 2**) and 6 proteins were down regulated (C1-C6, **Table 2**) in clear cell RCC tissues compared with normal kidney tissues. The identified differentially expressed protein spots were labeled in 2D-PAGE gels, as shown in **Figures 2 & 3**. The protein spot numbers, Swiss-Prot ID, molecular mass weights (MW), the value of isoelectrical point (pI), MASCOT search scores and sequence coverage percentages are summarized in **Table 2**. The predicted values of isoelectric point pI of some proteins summarized in **Table 2** were not completely compatible to those acquired from the gels, probably due to posttranslational modifications that induce change in the protein charge.

Discussion. Renal cell carcinoma is one of the most radiation- and chemotherapy-resistant tumors, and surgical resections are only effective in organ-defined disease.[4] As the current therapies are inefficient and the 5-year survival rates are very low, it is urgent to find novel diagnostic, prognostic, and therapeutic markers for this disease. In the recent years, the proteomics technology has been a powerful tool for identifying RCC related proteins. The newly discovered RCC-related markers may potentially lead to a better subclassification of RCC, a diagnostic marker or a potential therapeutic target of this disease. In this paper, 16 proteins were shown to have a different expression level (≥2 fold) in RCC, among which 6 proteins were down regulated in RCC. Among the 6 proteins, 4 proteins (ester hydrolase C11orf54, alcohol dehydrogenase, ammecr1-like

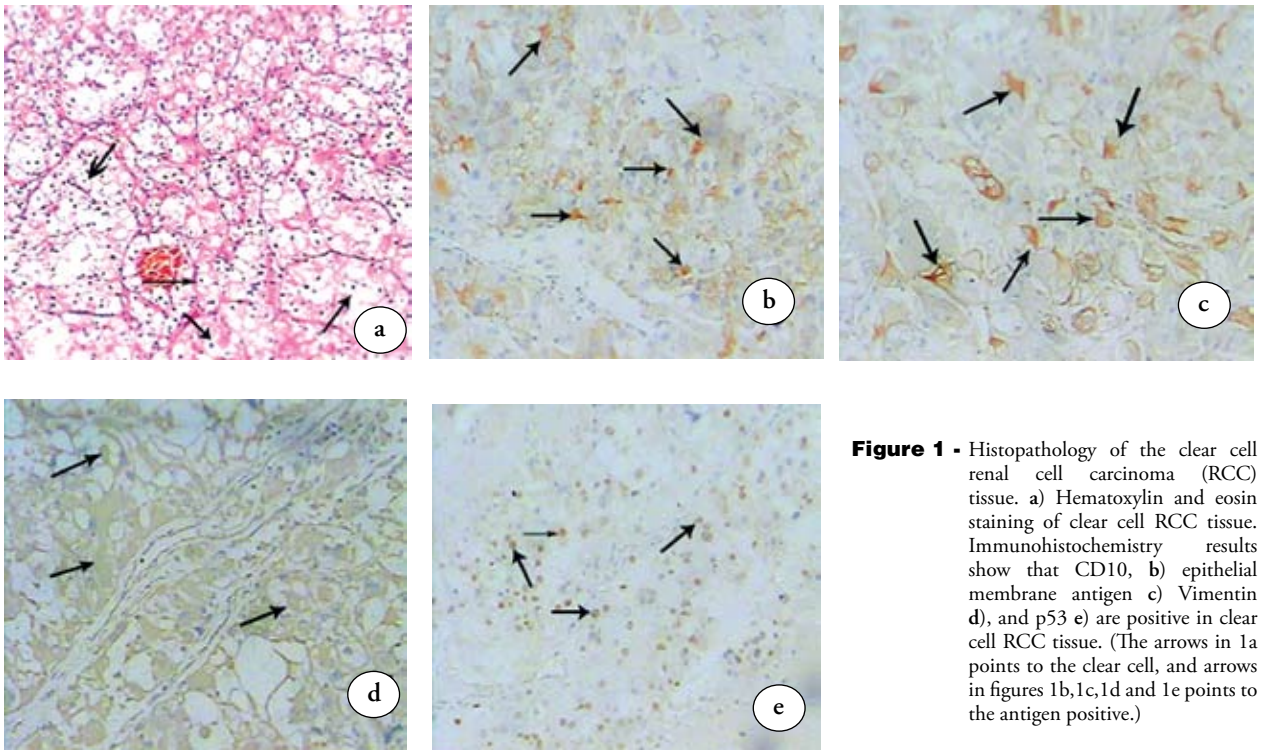

Figure 1 - Histopathology of the clear cell renal cell carcinoma (RCC) tissue. **a**) Hematoxylin and eosin staining of clear cell RCC tissue. Immunohistochemistry results show that CD10, **b**) epithelial membrane antigen **c**) Vimentin **d**), and p53 **e**) are positive in clear cell RCC tissue. (The arrows in 1a points to the clear cell, and arrows in figures 1b,1c,1d and 1e points to the antigen positive.)

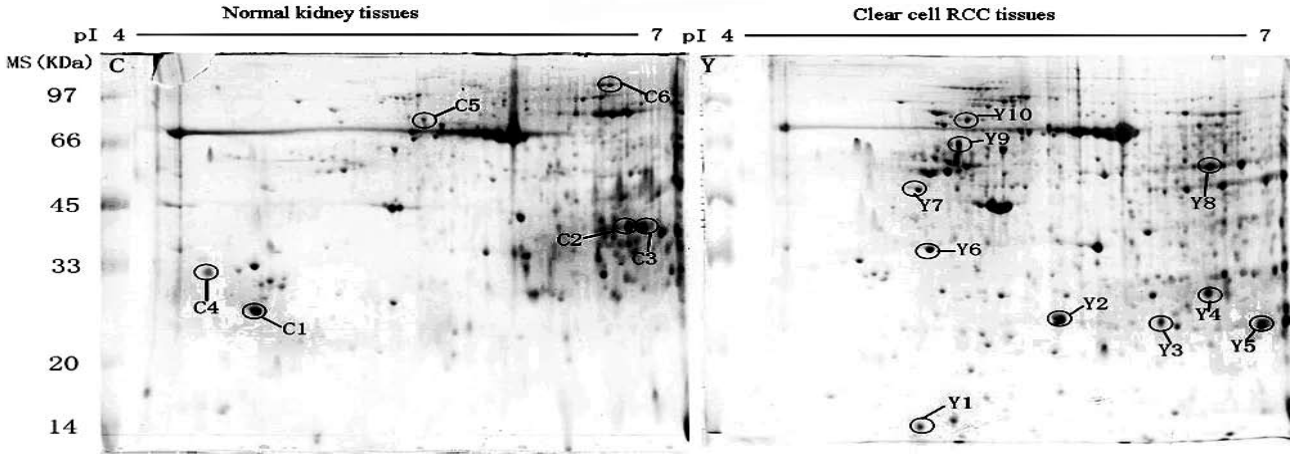

Figure 2 - Two-dimensional polyacrylamide gel electrophoresis images of protein profiles of the clear cell renal cell carcinoma tissues and the corresponding normal kidney tissues. Isoelectric focusing at pH 4-7 was carried out at the first dimension using immobilized pH gradients strips, and a 7.5-17.5% gradient sodium dodecyl sulphate gel electrophoresis was followed at the second dimension. Protein spots identified by mass spectrometry were marked with numbers.

preferentially expressed in cancer stem cells and could more specifically characterize the tumorigenic liver cancer stem cells (CSC) population. However, Abdulrahman et al[26] indicated that retinal dehydrogenase had no effect on cell growth and motility in the RCC cell line.

Vimentin is a widely known diagnostic marker for tumors. Previous studies reported that vimentin was expressed differently in oncocytoma, chromophobe RCC and conventional RCC, and this molecule might be a useful diagnostic marker to discriminate the oncocytoma from RCC mimics.[27,28]

Protein-glutamine gamma-glutamyltransferase 2 plays a role in diverse biological functions, including extracellular matrix formation, integrin-mediated signaling, and signal transduction involving 7-transmembrane receptors. Multiple studies have shown

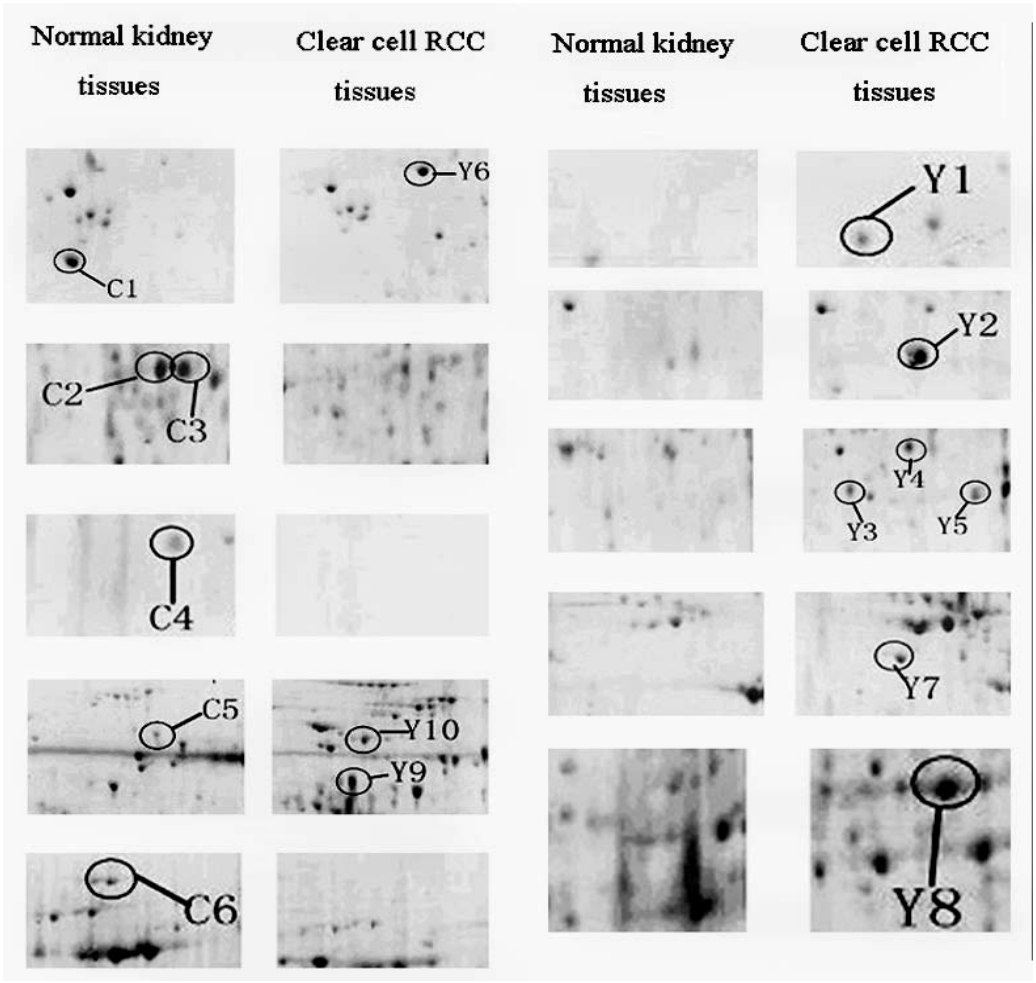

Figure 3 - Enlarged pictures of the significant differences between clear cell RCC tissues and corresponding normal kidney tissues. RCC - renal cell carcinoma.

Table 2 - Differentially expressed proteins between clear cell RCC tissues and corresponding normal kidney tissues identified by mass spectrometry.

Spot No.	Protein accession No.*	Protein identity*	Theoretical pI/Mw (Dalton)	Sequence coverage (%)§ /mascot score¶
C1	P05937	Calbindin	4.7/30291	20/84
C2	Q9H0W9	Ester hydrolase C11orf54	6.23/35608	30/135
C3	P14550	Alcohol dehydrogenase [NADP+]	6.32/36892	27/105
C4	Q6DCA0	AMMECR1-like protein	9.18/35048	13/34
C5	P12236	ADP/ATP translocase 3	9.76/33073	21/66
C6	Q6IPR1	LYR motif-containing protein 5	9.88/10604	27/28
Y1	Q9Y241	HIG1 domain family member 1A	9.79/10136	22/25
Y2	P09211	Glutathione S-transferase P	5.43/23569	30/173
Y3	P30048	Thioredoxin-dependent peroxide reductase, mitochondrial	7.67/28017	14/82
Y4	P30041	Peroxiredoxin-6	6.00/25133	28/146
Y5	Q9Y5K6	CD2-associated protein	6.07/71635	9/41
Y6	P08758	Annexin A5	4.94/35971	44/183
Y7	P09104	Gamma-enolase	4.91/47581	14/54
Y8	P00352	Retinal dehydrogenase 1	6.30/55454	16/106
Y9	P08670	Vimentin	5.06/53676	30/151
Y10	P21980	Protein-glutamine gamma-glutamyltransferase 2	5.11/78420	9/56

*Protein identity and accession number are listed according to the SWISS-PROT database, §Sequence coverage - percentage of identified sequence compared to the complete sequence of the known protein, ¶MASCOT score for SWISS-PROT database search based on PMF, Mw - molecular weight, pI - isoelectric point, RCC - renal cell carcinoma.

protein, and leucine-tyrosine-arginine motif-containing protein 5) to date have no reports on their effects in tumor progression. Interestingly, alcohol dehydrogenase was also previously reported as down regulated in RCC on screening for differentially expressed proteins in RCC by proteomic analysis,[5] but the direct correlations between alcohol dehydrogenase and the development of cancer are still unknown.

Calbindin, which was down regulated in RCC in this study, is a widely known protein that participates in many signal pathways in various cells. It is reported that calbindin could buffer cytosolic calcium and stimulate a membrane Ca(2+)-ATPase and a 3',5'-cyclic nucleotide phosphodiesterase. Calbindin has a broad tissue distribution, yet cell-type-specific expression is predominantly in distal tubular cells of the kidney and several other types of cells. Previous research reported that it was negative in RCC,[6,7] and might develop as a novel diagnostic marker. The results obtained in this study are consistent with those previous reports, and we should further investigate the relationship between calbindin and RCC. Landriscina et al[8] found that reverse transcriptase inhibitors could induce cell differentiation and enhance the immunogenic phenotype in human RCC.[8] When the cells received treatment of reverse transcriptase inhibitors, calbindin was up regulated, which meant that calbindin may participate in the signal pathway that leads to cell differentiation and immunogenic phenotype inhibition.

The ADP/ATP translocase 3 (ANT3) catalyzes the exchange of ADP and ATP across the mitochondrial inner membrane and it may participate in the formation of the permeability transition pore complex (PTPC) responsible for the release of mitochondrial products that triggers apoptosis.[9] Previous studies found that many drugs induce tumor cells apoptosis through the mitochondrial PTPC in which ANT3 is involved.[10,11] Moreover, the down regulation of ANT3 in hepatoma cells may lead the resistance of cells to camptothecin, which indicated that ANT3 might play an important role in tumors resistant to chemotherapy.[12] The ANT3 has the potential to be developed into an important diagnostic marker and therapeutic target.

In this study we found that 10 proteins were upregulated in RCC, one of which was HIG1 domain family member 1A, whose alias is hypoxia-inducible gene 1 protein, and is induced by tumor microenvironment. As the tumor microenvironment contributes to the malignant progression of tumor cells and the chemotherapy and radiation resistance, researchers hypothesize that the hypoxia-induced protein may influence clinical outcome.[13] It is reported that the expression of hypoxia-inducible factor 1œ (HIF-1œ) was greater in RCC than in benign tissue,[14] and HIF-1œ participates in RCC progression.[15] Glutathione S-transferase P (GST-Pi) can transfer the glutathione to endogenous and exogenous hydrophobic electrophiles and it has anti-apoptosis biological activity. The increased level and activity of GSTP1 will lead to tumor chemotherapy resistance. Researchers found that the increased expression of GST-Pi gene was associated with a significantly higher relapse rate, and a poorer clinical outcome.[16] Yu et al,[17] also found that the contents of mRNA coding for GST-Pi in multidrug resistance RCC cell line were higher than in the native cell line. The GST-Pi has the potential to be a target to overcome tumor multidrug resistance. Thioredoxin-dependent peroxide reductase, also named Peroxiredoxin-3, is localized in the mitochondrion. It regulates redox of the cell and protects radical-sensitive enzymes form oxidative damage by a radical-generating system. It regulates the activation of NF-kappa-B in the cytosol.[18] The high peroxiredoxin expression in tumor cells can protect cells from oxidative damage.[19] The abundantly expressed peroxiredoxin 3 in RCC may prevent the tissue undergoing oxidative damage, which may lead to activation of pathways leading to aggressive tumors. Peroxiredoxin-3 might become a therapeutic and diagnostic target.

Peroxiredoxin-6 localizes in the cytoplasm, lysosome, and cytoplasmic vesicle, which are different from peroxiredoxin-3.[20] Similarly, overexpression of peroxiredoxin 6 was found to lead a more invasive phenotype and metastatic potential in human breast cancer via regulating the levels of uPAR, Ets-1, MMP-9, RhoC, and TIMP-2 expression.[21] Annexin A5, also named Annexin V, belongs to the annexin family. This protein is an anticoagulant protein that acts as an indirect inhibitor of the thromboplastin-specific complex, which is involved in the blood coagulation cascade. Tumor cells acquire an invasive and metastatic phenotype, which is the main cause of death for cancer patients. Hayashi et al[22] performed a proteomic differential display analysis for the expression of intracellular proteins in the regressive cancer cell line, and the progressive cancer cell line and found that annexin A5 was one of the overexpressed proteins in the progressive cancer cell line. Gamma-enolase has neurotrophic and neuroprotective properties on a broad spectrum of central nervous system neurons. It binds to cultured neocortical neurons and promotes cell survival in a calcium-dependent manner. Enolase is widely known as a diagnostic marker for tumors.[23,24] Retinal dehydrogenase 1 can convert or oxidize retinaldehyde to retinoic acid. Ma et al,[25] found that retinal dehydrogenase was one of the proteins that were

that transglutaminase-2 is upregulated in cancers such as glioblastomas, malignant melanomas, and pancreatic ductal adenocarcinomas.[29] Some studies suggest that the increased expression of transglutaminase-2 in tumor tissue will promote cell survival, motility, and invasive functions of cancer cells, and so lead to drug resistance and metastasis of the tumor.[30] However, there are also reports of the down regulation of transglutaminase-2 expression in certain types of cancer.[31] Perhaps the relevance of transglutaminase-2 to cancer biology may depend upon the cancer cell type, the type of cancer, the location of the cancer, and the stage of the cancer.

In conclusion, the protein expression profiles in clear cell RCC tissue and corresponding normal kidney tissue were analyzed by 2D-PAGE and mass spectrometry, and 16 proteins were found differentially expressed. Among the 16 proteins, 6 proteins were decreased and 10 proteins were up regulated. Among these proteins, calbindin, ADP/ATP translocase 3, HIG1, glutathione S-transferase P, and thioredoxin-dependent peroxide reductase are worthy of further investigation, and they may be developed into diagnostic markers for RCC. This study clarifies the tumor specific proteins in RCC, but which among these proteins is really of diagnostic or prognostic value is unknown. In future study, we will investigate these proteins to determine their clinical value.

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