L-arginine-glycine amidinotransferase, betaine-homocysteine s-methyltransferase, and neuropolypeptide h3 are diminished in renal clear cell carcinoma of humans

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ABSTRACT

الأهداف: التحقق من وجود العلاقة بين المؤشرات الحيوية مثل البروتينات وسرطان الخلايا الكلوية.

الطريقة: أُجريت هذه الدراسة في قسم المسالك البولية بمستشفى رينمين التابع لجامعة وهان، وهان، الصين وذلك خلال الفترة من يناير 2008م إلى سبتمبر 2009م. شملت الدراسة 12 عينة من الأنسجة التي تم جمعها من المرضى المصابين بسرطان الخلايا الكلوية (مجموعة الدراسة)، هذا بالإضافة إلى عينات الأنسجة الطبيعية (مجموعة الشاهد). لقد تمت الاستعانة بكل من عملية الهجرة الكهربائية ثنائية الأبعاد باستخدام هلام بولي أكريلاميد، وجهاز (MALDI-TOF-MS) من أجل التحري عن التغيرات التي تحصل في الأنسجة وعمل مقارنة بين مجموعة الدراسة ومجموعة الشاهد. كما تم استخدام اختبار ويسترين بلوت وتقنية التفاعل التسلسلي المبلمر ذو االنسخ العكسي من أجل تحليل مستويات البروتينات والمؤشرات الحيوية الأخرى المرتبطة بالمرض.

النتائج: أشارت نتائج الدراسة إلى زيادة ظهور بقعة واحدة من البروتينات، وانخفاض ظهور 13 بقعة، وغياب ظهور 22 بقعة من البروتينات في الأنسجة المصابة بسرطان الخلايا الكلوية. ولقد كانت البروتينات الغائبة في الأنسجة المصابة كالتالي: ناقلة أميدين غليسين- أرجينين الإل (AGAT)، وناقلة ميثيل الإس هوموسيستئين- بيتائين (BHMT)، وكيتوهيسكوكيناز (KHK)، وعديد الببتيد العصبي إتش3 (NPh3). أظهر التفاعل التسلسلي المبلمر ذو النسخ العكسي مدى انخفاض الحمض النووي الريبي المرسال (mRNA) في بروتينات AGAT، وBHMT و NPh3 وذلك في كافة الأنسجة بمجموعة الدراسة. بالإضافة إلى ذلك فقد أظهر اختبار ويسترين بلوت غياب كلاً من البروتينات AGAT في 11/12 عينة، وBHMT في 9/12، وNPh3 في 5/12، وذلك في أنسجة مجموعة الدراسة.

خاتمة: أظهرت الدراسة مدى ارتباط تطور سرطان الخلايا الكلوية بغياب بروتينات AGAT، وBHMT، و NPh3، وهكذا يمكن اعتبار هذه البروتينات من المؤشرات الحيوية التي قد تسهم في تشخيص المرض وعلاجه، بالإضافة إلى التنبؤ بسير المرض.

Objectives: To identify renal clear cell carcinoma-associated marker proteins.

Methods: Twelve patients with renal cell carcinoma (RCC) were collected and processed in the Department of Urology, Renmin Hospital, Wuhan University, China, between January 2008 and September 2009. Two-dimensional polyacrylamide gel electrophoresis and matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) were employed to investigate differentially expressed protein spots between RCC tissues and adjacent normal tissues, then reverse transcription polymerase chain reaction and western blot were employed to confirm the proteomic results.

Results: One protein spot was upregulated, 13 were downregulated, and 22 were absent in RCC tissues. Four of the absent proteins were L-arginine-glycine amidinotransferase (AGAT), Betaine-homocysteine S-methyltransferase (BHMT), Ketohexokinase (KHK), and Neuropolypeptide h3 (NPh3). The reverse transcriptase-polymerase chain reaction analysis demonstrated mRNA expression of AGAT, BHMT, and Nph3 was significantly decreased in 12 RCC tissues. In addition, Western blot analysis showed AGAT protein was absent in 11/12, BHMT in 9/12, and Nph3 in 5/12 RCC tissues.

Conclusion: Absence of AGAT, BHMT, and Nph3 is common events in clear cell RCC; hence, it may be involved in the development of RCC; therefore, they have the potential to be tumor markers for diagnosis, treatment, and prognosis of RCC patients.

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Renal cell carcinoma (RCC) is the most malignant tumor of the kidney, and it accounts for approximately 2-3% of all adult cancers in Western countries.[1] Moreover, the morbidity and mortality of renal carcinoma has steadily increased in the past 20 years. Histopathologically, RCC is classified into 4 subtypes, with 80% of cases comprising the clear cell subtype.[2] Due to its insidious onset, patients frequently have advanced disease at the time of clinical diagnosis, drugs and newly discovered markers cannot significantly increase the survival of these patients.[3] In our study, our objective is to develop new specific biomarkers of RCC by proteomic analysis. The proteomic approach is a powerful tool for identifying disease-associated protein markers to assist in diagnosis or prognosis, and for selecting potential targets for specific drug therapy. This approach has already been used in RCC to identify changes including the repressed expression of ubiquinol cytochrome reductase and mitochondrial NADH-ubiquinone oxidoreductase complex I,[4] as well as the up-regulation of several glycolytic enzymes, vimentin, and heat shock protein 27.[5] However, the identified proteins have been shown to be either non-specific or insensitive for detecting RCC, no biological markers that could be screened or used as target therapy have yet been established. Furthermore, there was poor repeatability of these data among individual laboratory. In our study, we found 4 proteins that were absent in the clear cell RCC by the analysis of 2D-PAGE. Matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) identified the 4 absent proteins as L-arginine-glycine amidinotransferase (AGAT), Betaine-homocysteine S-methyltransferase (BHMT), Ketohexokinase (KHK), and Neuropolypeptide h3 (Nph3). Except KHK, which has previously been found to be diminished in human clear cell RCC,[6,7] the other 3 proteins had not yet been identified and reported in detail in RCC. Furthermore, we made specific primers and antibodies to verify the altered expression of AGAT, BHMT, Nph3 in RCC using RT-PCR and western blot analysis, and found decreased mRNA and protein expression in RCC tissues compared with normal tissues.

Methods. Surgical specimens were obtained from 12 patients after radical nephrectomy in Renmin Hospital of Wuhan University, Wuhan, China between January 2008 and September 2009. Informed consent was obtained from all participants and the study protocol was approved by the Human Subject Research Committee of Wuhan University, Wuhan, China. According to classification of malignant tumors (TNM),[8] the 12 patients (4 women and 8 men) were classified as: stage I (n=3), stage II (n=7), and stage III (n=2). The mean age of the patients was 59.2 years (ranging 48-72). The samples from all patients were examined histologically to verify clear cell type. Human RCC and normal tissues were homogenized in lysis buffer (9M urea, 4% CHAPS, 65mM DTT, Rose' cocktail enzyme inhibitor). For one-dimensional isoelectric focusing, the immobilized pH gradient gel strips (IPG, pH 4-7, 18cm; Amersham Biosciences, Sunnyvale, CA) were rehydrated in a swelling solution containing 500 μg protein for 12 hours at 20°C. Isoelectric focusing (IEF) was performed on IPG using the IPGphor system (EttanDM IPGphor IITM, Amersham Biosciences, Sunnyvale, CA, USA) at 20°C in 4 steps, at 30V (12 hours), 500 V (one hour), 1000 V (one hour) and then 8000 V (6 hours). For 2-dimensional gel electrophoresis, the equilibrated IPG gel strip was laid on top of a prefabricated gel (8-16% gradient) and covered with a 0.5% agarose solution. The gel board was put in an electrophoretic apparatus (SE-600, Amersham Biosciences, Sunnyvale, CA, USA), and the gel electrophoresis was carried out at 16°C at 40mA (constant current) for 20 minutes, and then at 60 mA until the bromophenol blue reached the bottom of the gel. The gel was then fixed and stained using Owl's silver stain kit (Owl Separation Systems, Portsmouth, NH, USA) for protein detection.

The protein patterns in the gel were recorded as digitized images using a high-resolution scanner (GS-710 Calibrated Imaging Densitometer, Bio-Rad, Hercules, CA, USA). Three gels were run for each sample. The scanned gel images were analyzed using a standard protocol for Imagemaste software (Amersham Biosciences, Sunnyvale, CA, USA). The mean intensity of each spot was calculated by the difference in silver staining intensity between the RCC tissues and the normal tissues. Protein spots with a ratio of $\geq$1.5 were taken to be differently expressed. The MALDI-TOF/TOF MS (Bruker-Daltonics AutoFlex, Bruker, Germany) was employed to identify the proteins. Protein identification was based on the public sequence databases (NCBI).[6]

Specimens from 12 patients were tested using reverse transcriptase PCR (RT-PCR) for the detection of AGAT, BHMT, Nph3, and mRNA level.[9,10] To prepare polyclonal antibodies against AGAT, BHMT, Nph3 proteins, the cDNA sequence of AGAT, BHMT, Nph3 was inserted into a pet28a plasmid to construct the prokaryotic expression plasmids: pet28a-AGAT, pet28a-BHMT, or pet28a-Nph3. The expression plasmids were transformed into *Escherichia coli* BL21. The His-tagged fusion proteins were expressed with the induction of isopropyl beta-D-1-thiogalacto-pyranoside (IPTG) and purified with a Ni (2+)-NTA affinity chromatography column.[11] Purified proteins were used to immune the rabbit. Antisera were obtained

from rabbits immunized with each respective antigen. The titer of polyclonal antibody was detected by enzyme-linked immunosorbent assay (ELISA), and the specificity of each polyclonal antibodies were identified by western blot. Then antibodies with high titer and specificity were used to detect AGAT, BHMT, Nph3 protein levels in RCC, and normal tissues. Western blot was performed as described.[11]

Statistical analysis was performed using the Statistical Package of Social Sciences version 10.0 (SPSS In,c Chicago, IL, USA). Data are expressed as mean (standard deviation). Statistical differences between 2 groups were determined by t-test. A p-value of <0.05 was considered significantly different.

Results. The average number of protein spots detected was 1324±24 from RCC tissues and 1386±18 from the corresponding normal kidney tissues. Four specific regions containing differentially expressed proteins in RCC and normal tissues were further analyzed by comparing their expression patterns. We identified 36 protein spots differentially expressed in normal kidney and RCC tissues (**Figure 1**). One protein was overexpressed, 13 were repressed, and the remaining 22 spots were revealed to be largely decreased or even absent in clear cell RCC tissues. Of the 22 largely decreased spots, 4 absent spots were identified by MALDI-TOF MS. A typical mass spectrum of one of the differently expressed proteins (ID 623) (**Figure 2**). An advanced database search showed that these decreased proteins in RCC were: AGAT in ID 611, BHMT in ID 623, KHK in ID 1031, and Nph3 in ID 1561. The expression level of each identified protein in both RCC and normal kidney tissues was determined from the density

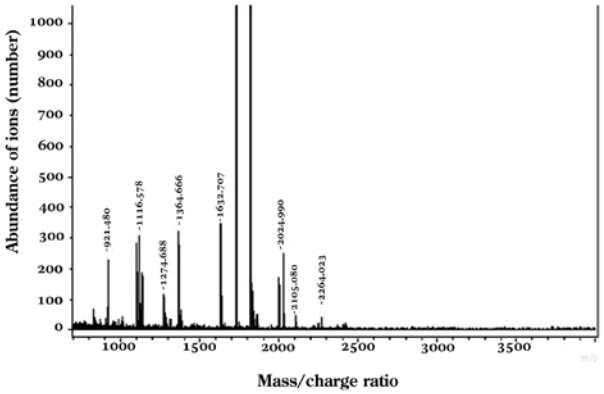

Figure 2 - Typical spectrum of the Betaine-homocysteine S-methyltransferase (BHMT) protein (ID 623) obtained from peptide mass fingerprinting of a gel spot. The abscissa represents the mass/charge ratio of ions detected in the matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) experiments. The ordinate represents the relative intensity of the averaged ion current in arbitrary units.

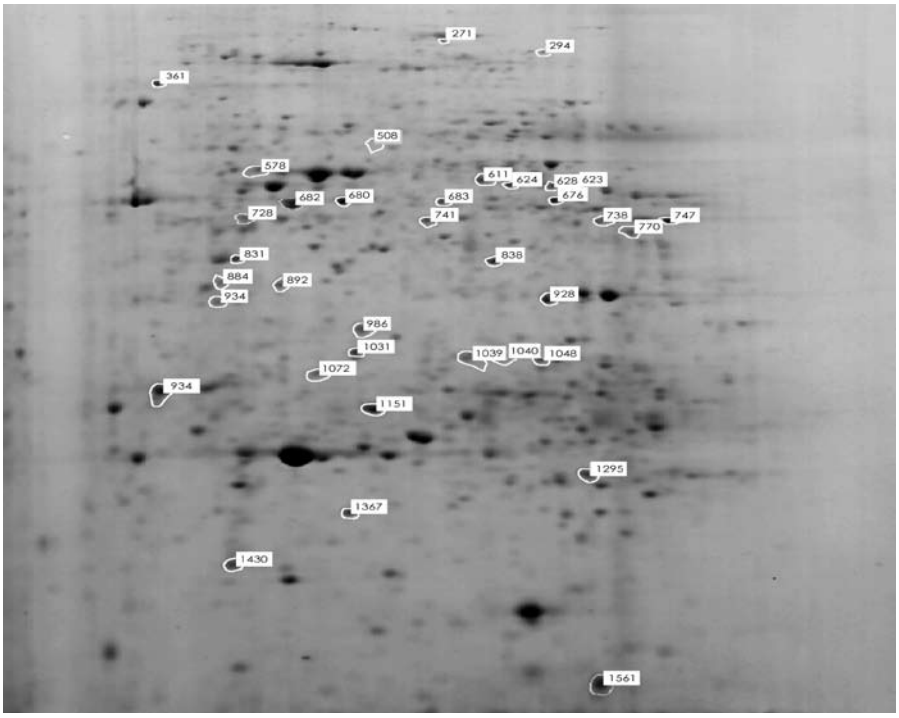

Figure 1 - The match analysis of 2-dimensional gel electrophoresis images indicates that there are 36 differently expressed protein spots between renal cell carcinoma (RCC) and normal kidney tissues (p<0.05). The IDs of the differently expressed protein spots are provided in the figure.

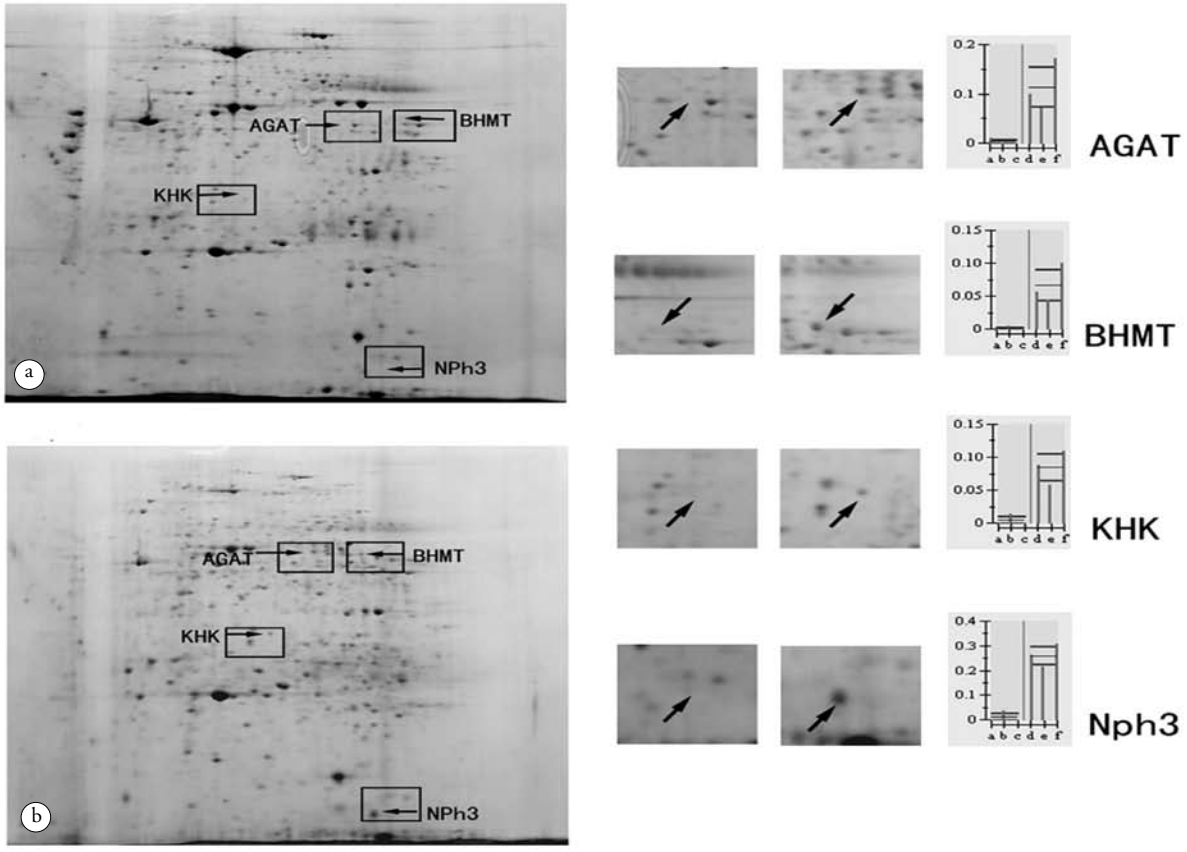

Figure 3 - Four differently-expressed protein spots between **a**) renal cell carcinoma (RCC) and **b**) normal kidney tissues were identified by matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS), L-arginine-glycine amidinotransferase (AGAT), Betaine-homocysteine S-methyltransferase (BHMT), Ketohexokinase (KHK), and Neuropolypeptide h3(Nph3), that were dominantly expressed in normal kidney tissues, but absent in RCC tissues. Histograms indicate the relative amount of the same protein obtained from 62-dimensional gel electrophoreses of tissues from a patient. Markers a, b, c (in the right column) diagram represent the 3 two-dimensional gel electrophoreses of RCC tissues markers d, e, f (in the right column diagram) represent the 3 two-dimensional gel electrophoreses of normal kidney tissues.

values (**Figure 3**). The 4 absent proteins in RCC include proteins involved in metabolism (AGAT, KHK), DNA methylation (BHMT), and signal regulation (Nph3).

To investigate mRNA changes in 3 of the absent proteins, we used RT-PCR to examine the expression of AGAT, BHMT, Nph3 in 12 patients. L-arginine-glycine amidinotransferase, BHMT, and Nph3 mRNA were significantly decreased in all analyzed tumor tissues compared with normal tissues. In some tumor tissues, almost none mRNA expression was detectable (**Figure 4**).

To validate our proteomic data, we developed antibodies against AGAT, BHMT, Nph3, and used these antibodies to detect protein expression in 12 normal and RCC tissues by western blot (**Figure 5**). Results clearly showed a complete lack of AGAT protein expression in 11 of the 12 tumors, in contrast to its abundant expression in the corresponding normal tissues. In one RCC sample (patient #5), we found a very weak expression of AGAT, which corresponded approximately to 7.8% of the level that was present in normal tissue. S-methyltransferase expression was absent in 9 of 12 RCC tissues, in which the BHMT expression of the normal tissues expression level in 3 samples was decreased to 6.5% in patient #4, 12.4% in patient #5, and 30.8% in patient #9. Neuropolypeptide h3 expression was absent in 5 of 12 RCC tissues; in which the Nph3 expression of the normal tissues expression level in 6 samples decreased to 39% in patient #3, 8.7% in patient #4, 29.5% in patient #5, 35% in patients #8, 8.2% in patient #9, and 11.2% in patient #12. In one patient (patient #5), we observed a moderate (50%), but significant loss of Nph3 expression.

Discussion. Renal cell carcinoma is relatively resistant to chemotherapy and radiotherapy.[12] Recent advances in drug development are providing novel agents for the treatment of RCC, but the 5-year survival rates are still very low.[13] In addition, there is an urgent need to identify diagnostic and prognostic markers for

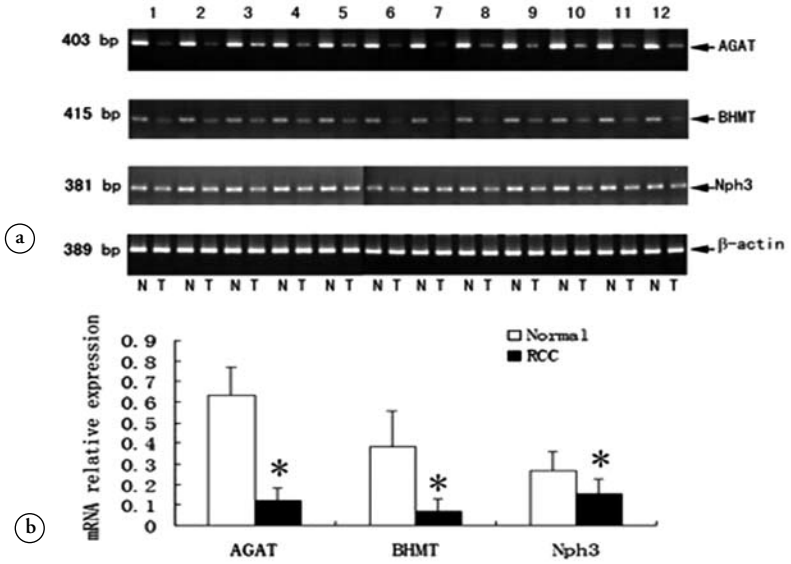

Figure 4 - Expression of human L-arginine-glycine amidinotransferase (AGAT), Betaine-homocysteine S-methyltransferase (BHMT), Neuropolypeptide h3 (Nph3) mRNA in normal and Renal cell carcinoma (RCC) tissues. Normal and RCC tissues were obtained from 12 patients immediately after radical nephrectomy. **a)** Reverse transcriptase-polymerase chain reaction analysis from corresponding normal and RCC tissues. Polymerase chain reaction fragments were subjected to gel electrophoresis. Normalization was carried out using the human ß-actin gene. **b)** Relative abundance of mRNA in normal and RCC tissues. *Significantly different compared to normal tissue. T - RCC tissues, N - normal tissues.

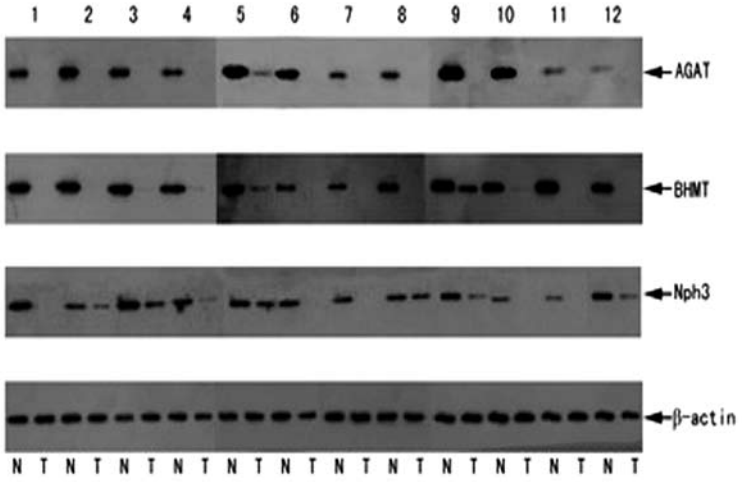

Figure 5 - Western blot analysis of human L-arginine-glycine amidinotransferase (AGAT), Betaine-homocysteine S-methyltransferase (BHMT), Neuropolypeptide h3 (Nph3) protein levels in RCC tissues (T) and normal (N) tissues. Protein (10µg) was separated on 12% SDS polyacrylamide gels. ß-actin was used as a control.

RCC.[14] The overall protein expression patterns in clear cell RCC and normal kidney tissues are similar, but the number of protein spots from the RCC tissues is obviously decreased, which is in accordance with results from other laboratories. Okamura et al,[14] identified 92 proteins that were significantly different (34 upregulated and 58 downregulated) between RCC tissues and normal tissues. Perroud et al,[15] identified 31 proteins that were differentially expressed (13 upregulated and 18 downregulated). Unwin et al,[16] found 41 protein spots that were repressed in RCC tissues. Balabanov et al[2] found 12 protein spots disappeared in RCC tissues. Sun et al,[13] found that 6 proteins downregulated and 10 proteins upregulated in clear cell RCC tissues. Yang et al,[17] identified that cyclophilin A was a potential prognostic factor for clear cell RCC by proteomic analysis. In this study, 36 proteins were shown to have different expression in RCC tissues. One protein spot was upregulated, 13 protein spots were downregulated, and the remaining 22 spots were absent in clear cell RCC tissues. We identified 4 of the absent proteins in RCC tissues by using MALDI-TOF-TOF mass

spectrometry: AGAT, BHMT, KHK, and Nph3. There are no prior reports of the repression of KHK, proteins in RCC tissues.[6,7] The RT-PCR and western blot analysis demonstrated a clearly decreased or absent amount of mRNA and protein level in RCC tissues. This was in contrast to their abundant expression in the corresponding normal tissues.

L-arginine-glycineamidinotransferase is located in the cytosol or in the intermembrane space of mitochondria, and it is mainly expressed in kidney, pancreas, and liver. Cytosolic AGAT contains 386aa, while mitochondrial AGAT contains 391aa. In addition to our discovery that AGAT disappears in RCC tissues, AGAT is also found to decrease in Wilms' tumors[18] and in chronic renal failure.[19] L-arginine-glycine amidinotransferase catalyzes the committed step in creatine biosynthesis. Since creatine biosynthesis represents one of the main functions of renal cells, the decreased expression of AGAT may indicate that the cells in RCC tissues lose some functionality required for normal renal cells. The repression of AGAT may be a widespread phenomenon, which would justify taking repression of AGAT as a characteristic of RCC. The BHMT gene maps to 5q13.1-5q15, and it is mainly expressed in the cytosol of hepatic or renal cells. Betaine-homocysteine S-methyltransferase is a zinc metalloenzyme that catalyzes the transfer of a methyl group from betaine to homocysteine (Hcy) to produce dimethylglycine and methionine (Met), respectively. It has been reported that BHMT is repressed in several disease conditions, such as hepatocirrhosis,[20] esophageal adenocarcinoma,[21] chronic renal failure,[22] hepatic cell carcinoma.[23] Elevated concentration of Hcy in blood has been correlated to the incidence of arteriosclerotic vascular disease and thrombosis.[24] Putrescence often occurs in RCC due to the overgrowth of the carcinoma tissues. Thus, the pathological changes of blood vessels may be another important facet to the disease. The repressed expression of BHMT may be an important factor in the putrescence of RCC tissues by causing pathological changes in renal blood vessels. Another product of the BHMT catalysis reaction is Met, the sole precursor of S-adenosylmethionine, which is the primary methyl donor in the body. S-adenosylmethionine is involved in, among other things, the methylation of cytosine bases in DNA. Recent evidence suggests that enzymatic DNA methylation is an important component of gene control and may serve as a silencing mechanism for gene function.[25] Deoxyribonucleic acid hypomethylation or demethylation may activate some oncogene to arouse oncogenesis.[26,27] Thus, the downregulation of BHMT expression may be one of the initiating factors in the occurrence of RCC. Neuropolypeptide h3 is mainly expressed in the human central nervous system (CNS) and muscle system, but it is also expressed in other tissues at different levels. Neuropolypeptide h3 is a member of the phosphatidylethanolamine-binding protein (PEBP) family, which is located in the cytosol. The molecular weights of PEBPs are reported to be 21-23 kD. They are highly conserved throughout nature and have no significant sequence homology with other proteins of known structure or function. Phosphatidylethanolamine-binding protein are a kind of signal-regulating proteins, and they can inhibit cell proliferation or induce cell differentiation through regulating a range of intracellular signaling cascades.[28] The other crucial function of PEBP is to inhibit the activity of serine proteases, which can degrade components of the extracellular matrix to allow outgrowth of neuronal processes or to allow cell migration. Phosphatidylethanolamine-binding protein also act as mitogenic or survival factors.[29] It has been reported that PEBP is repressed in hepatoma cells.[30] Raf kinase inhibitor protein (RKIP) is another member of PEBP. Repressed expression of RKIP can promote the metastasis of prostate cancer[31] and also suppress cell apoptosis aroused by conventional therapies such as radiation and chemotherapeutic drugs. It may therefore produce resistance to radiotherapy or chemotherapy.[32] Phosphatidylethanolamine-binding protein may play a crucial role in tumor advancement by suspending intracellular signaling cascades or by impairing or inhibiting the activity of serine proteases. Renal cell carcinoma is one of the most resistant tumors to conventional therapies, as it grows quickly and easily metastasizes. The resistant to chemotherapy and radiotherapy could be due to the repressed expression of Nph3, a member of the PEBP family.

In conclusion, we examined the proteomes changes of clear cell RCC and the corresponding normal kidney tissues and identified four absent proteins by mass spectrometry: AGAT, BHMT, KHK and Nph3. Further RT-PCR and Western Blot analysis showed that the mRNA and protein level of AGAT, BHMT, and Nph3 was diminished or absent in RCC tissues. However, the biological role of these 3 proteins in the pathogenesis of RCC was not clear. And no biological markers for diagnosis, treatment and prognosis of RCC patients have yet been established by proteomics analysis.[3] But this technology allowed a breakthrough in the discovery of differentially expressed proteins specific to RCC. The diminished AGAT, BHMT, and Nph3 may play important roles in the occurrence of RCC.

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