Serum protein profiling to identify biomarkers for small renal cell carcinoma

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Diagnostic biomarkers for early detection of renal cell carcinoma (RCC) are in great need. In the present study, we compared the serum protein profiles of patients with small RCC to those of healthy individuals to identify the differentially expressed proteins with potential to serve as biomarkers. Serum samples were collected from 10 patients with small RCC and 10 healthy individuals. The serum protein expression profiles were analyzed by two-dimensional (2-D) gel electrophoresis. Twenty-seven proteins with differences in expression levels between RCC patients and healthy volunteers were identified. Of these, 19 were expressed at different levels and eight were expressed in serum from the RCC group, but not from the control group. Six differentially expressed proteins identified by using mass spectrometry included coagulation factor XIII B, complement C3 and its precursor, misato homolog 1 (isoform CRA_b), hemopexin, and alpha-1-B-glycoprotein. Some of these serum proteins are known regulators of tumor progression in human malignancies. In conclusion, we successfully applied 2-D gel electrophoresis and identified six serum proteins differentially expressed between patients with small RCC and healthy volunteers. These proteins may provide novel biomarkers for early detection and diagnosis of human RCC.

Keywords: Renal cell carcinoma; Serum biomarkers; Two-dimensional gel electrophoresis

For most human malignancies, the survival of patients depends on the early detection of the disease. Cancer biomarkers are a class of molecules identified to help achieve this goal. Ideally, cancer biomarkers should have the following features. First, the presence/absence or level change of a certain biomarker has strong implication for a certain type of cancer and preferentially for the early stage of the cancer. Second, a cancer biomarker should be detected in the blood or other body fluids easily accessible in a non-invasive manner. Third, cancer biomarkers should present optimal sensitivity and/or specificity, allowing themselves to become screening or diagnostic tools in clinical settings. Given the heterogenous nature of most human malignancies, it becomes more and more obvious that a single biomarker may not hold promise, but instead biomarker profiles may provide a higher level of diagnostic accuracy. Proteomic approaches have become valuable tools in mapping biomarker expression patterns. So far, the workhorse for proteomics is to combine the two-dimensional polyacrylamide gel electrophoresis (2-DE) with protein identification by mass spectrometry (MS).

Renal cell carcinoma (RCC), the most common neoplasm in the kidney, accounts for approximately 3% of adult malignancies and 90% of kidney cancers. Although the cure rate for localized RCC without distant metastasis can be as high as 89%, the lack of obvious clinical symptoms and efficient detection approaches results in approximately 30% of patients presenting with metastatic disease, whose five-year survival rate is lower than 10%. Besides, correlation study has suggested that for tumors with a diameter of >3.0 cm, there was a sharp increase in the incidence of negative prognostic parameters. Therefore, catching the tumor in the early stage is highly important for improving the survival of patients with RCC. At present, the diagnosis of RCC...
in clinic mainly relies on imaging techniques such as computed tomography (CT), the advances of which significantly improve the incidental identification rate of renal mass with a diameter of ≥ 1 cm. However, CT has poor capability in differentiating small RCC and benign renal tumors. In addition, the relatively high price and irradiation associated with CT also makes it less optimal for large-scale population screening.

2-DE was first reported in 1975 by O’Farell. With a history of more than 30 years and in face of promising alternative approaches, such as multidimensional protein identification technology and protein arrays, 2-DE is still the workhorse for proteomics. 2-DE separates complex protein mixtures in a 2-DE gel based on isoelectric point (pI), molecular weight (Mr), solubility and relative abundance, allowing the parallel quantitative comparison of protein profiles between two or more samples. Technically, however, 2-DE is still challenging. Any factors that may interfere with sample preparation, isoelectric focusing and polyacrylamide gel electrophoresis could eventually affect the protein profiling.

To identify potential serum biomarkers associated with the early development of RCC, in the current study, we combined the 2-DE with surface enhanced laser desorption/ionization time-off light mass spectrometry (SELDI-TOF MS) and compared the serum protein profiles between healthy individuals and patients with early-stage RCC.

Materials and Methods

Patients and samples

Twenty patients with small renal cell carcinoma (the tumor diameter of ≤ 3 cm, SRCC group) (15 males and 5 females, average age (56.2 ± 8.4) yrs), and twenty age- and gender-matched healthy individuals (control group) (15 males and 5 females, average age (55.8 ± 8.1) yrs) registering at the Blood Bank, Shanghai Municipal Center for Disease Control & Prevention (Shanghai, China) were recruited into this study. The diagnoses for SRCC were confirmed by pathological examinations. By Robson staging criteria, these SRCC were all stage I tumors. Peripheral blood was collected from healthy individuals and all patients before the surgical resection in the morning after overnight fast. Samples were kept at room temperature for 30 min and centrifuged at 4 000 rpm for 5 min at 4°C to isolate serum. The serum was aliquoted and stored at –80°C until further use. This study was approved by the Ethical Committee of Hua Shan Hospital, Fudan University, and informed consents were obtained from all blood donors.

Removal of high abundance serum proteins

For both the SRCC and control groups, 100-μL serum from each individual was pooled together and centrifuged at 4°C (× 12 000 g) to remove the serum lipids. To minimize the interference from high-abundance proteins, such as serum albumin and immunoglobulins, the serum samples were first passed through 0.22-μm filters (Millipore, USA), followed by the Multiple affinity removal column (Agilent Technologies, USA) and the dialysis tube with a cut-off of 5 000 Da (Millipore, USA) for further desalting and concentration.

Protein determination, two-dimensional gel electrophoresis

The total protein concentration in the depleted serum fractions was determined using the Bio-Rad protein assay reagent (Bio-Rad, USA), following the manufacturer’s instructions. Two-dimensional gel electrophoresis (Pharmacia IPGphor systems) was used to separate proteins. Total protein (300 μg) was run in the first dimension isoelectric focusing (IEF) with non-linear gradient IPG-strips (13-cm) pH 3-10 and a programmed voltage gradient. After the IEF run, strips were equilibrated with gentle shaking in equilibration buffer I (1% DTT, 50 mM, Tris-HCl pH 6.8, 6 M urea, 30% glycerol, 2% SDS and bromophenol blue), followed by equilibration buffer II which was identical to buffer I, except that the 1% DTT was replaced with 2.5% IAA. Thereafter, the IEF strips were run in 12.5% acrylamide gels in the second dimension at 30 mA for 3 h till the bromophenol blue front reached the bottom of the gel.

Silver staining, imaging and analysis

After SDS-PAGE, the gels were sequentially incubated with a fixation buffer (40% ethanol and 10% glacial acetic acid) for 30 min, sensitizing solution (30% ethanol, 0.2% sodium thiosulfate and 6.8% sodium acetate) for 30 min, staining buffer (0.25% silver nitrate and 0.4% methanol) for 20 to 30 min, and developing solution (2.5% sodium carbonate and 0.2% methanol) for 2 to 5 min. When the staining was optimal, the color development was stopped with 5% glacial acetic acid. The stained 2-D gel was scanned with Amersham Pharmacia Scanner.
In-gel trypsin digestion

Spots for differentially expressed proteins were excised and completely destained with 100 mM of ammonium bicarbonate in 30% (v:v) acetonitrile. The gel pieces were then frozen dried, added to 2.5–10 ng/µL trypsin and allowed to rehydrate at 4°C for 30 to 60 min. Excess trypsin was removed and replaced with 20–30 µL of 25 mM ammonium bicarbonate (pH 7.8 to 8.0). The gel pieces were then digested at 37°C overnight. The digested peptides were extracted by sonication in 60% acetonitrile (v:v) and 0.1% trifluoroacetic acid (TFA, v:v) and prepared for mass spectrometry (MS) analysis.

Mass spectrometry

All mass spectra were acquired on an AutoFlex MALDI-TOF/TOF mass spectrometer with LIFT technology (Bruker Daltonics, Bremen, Germany). Trypsin-digested peptides were prepared on an AnchorChip sample plate (Bruker Daltonics, Bremen, Germany) according to the manufacturer’s protocol. Both MS and MS/MS data were acquired with a N2 laser at 25-Hz sampling rate. PMF data and MS-MS data were combined using FlexAnalysis and the combined data set was submitted to MASCOT for protein identification. The National Center for Biotechnology non-redundant (NCBI/nr) database, with Homo sapiens (human) as taxonomy was selected. Other parameters for searching included enzyme trypsin, one missed cleavage, fixed modifications of carbamidomethyl (C) and variable modifications of oxidation (Met). Peptide tolerance of 100 ppm, fragment mass tolerance of ± 0.5 Da and peptide charge of 1+ were selected. Only significant hits, as defined by the MASCOT probability analysis (p<0.05) were accepted.

Results

Nineteen proteins showed differential expression levels between the control and SRCC groups

To search for serum biomarkers associated with the early stage of RCC, we collected serum samples from both healthy individuals (control group) and patients with a renal cell carcinoma of no more than 3.0 cm in diameter (SRCC group) and compared the serum protein profiles by 2-DE. As shown in Fig. 1A and B, the overall protein expression patterns between these two groups were similar. After data process with ImageMaster software, 19 differentially expressed proteins were identified (Fig. 1C). By statistical analysis, these proteins showed significantly different expressions between the two groups (P < 0.05) and the ratio of expression levels was >1.5. Among the 19 proteins, eleven were up-regulated and eight down-regulated in the SRCC group as compared to the control group (Fig. 2).

Eight “all or non” spots were identified between the control and SRCC groups

Besides the 19 proteins that showed differentially expressions between the two groups, by manual

Fig. 1—2-DE revealed 19 differentially expressed proteins between the control and the SRCC groups [Serum samples were pooled from all ten individuals within each group, pre-treated to remove high-abundance proteins and run on 2D gels. (A) Representative 2D gel picture from the control group; (B) Representative 2D gel picture from the SRCC group; and (C) Representative 2D gel picture from the control group with 19 differentially expressed proteins as identified by ImageMaster software circled and labeled on the gel. Each spot was assigned an ID number by the software.
screening of the gel, we identified another eight spots showing “all or none” expression patterns, which were clustered in three areas (D-1 to D-3 in Fig. 3A). For the purpose of explanation, we named these spots based on the area they were identified and the relative position to other “all or none” spots within that area from left to right. As shown in Fig. 3B, all eight protein dots were not visible on the 2D gels from the control group, but became detectable on those from the SRCC group.

Several proteins were identified as differentially expressed between the control and SRCC groups

To characterize the proteins that showed differential or “all or none” expressions between the control and SRCC groups, we set out to examine the identities of the 27 differentially expressed proteins by MALDI-TOF-MS (Fig. 4). Following the Mascot analysis, six proteins returned a significant and qualified score (>66), which were factor XIII B, complement C3 and its precursor, misato homolog 1 CRA_b, hemopexin and alpha-1-B-glycoprotein, respectively (Table 1).

Discussion

To identify biomarkers from serum samples, the first potential problem associated with protein profiling is individual difference, which could dramatically confound further analysis. To resolve this, we pooled together equal volume of serum sample from each individual within the same group and repeated the next-step experiments three-times, which significantly reduces the workload for analyzing each single sample within a group, and also improves the reliability and reproducibility of acquired data.
A second challenge associated with identifying serum biomarkers is that the majority of “biologically important” proteins within a serum sample are usually expressed at relatively low levels. In contrast to these low-abundance biomarkers, there are quite a few high-abundance proteins within the serum, such as albumin, immunoglobulins (Igs), transferring, fibrinogen, macroglobulins and etc., among which, albumin and Igs could account for approximately 60 to 97% of total serum proteins. The presence of the high-abundance proteins not only limits the loading of low-abundance proteins on to 2-D gels, but also dramatically masks the identification of low-abundance proteins with similar molecular weights and therefore running close to these high-abundance proteins on the gel. On the other hand, some high-abundance proteins are known to function as carriers for low-abundance proteins in the serum, thus the complete removal of the former may unavoidably reduce the detection of some low-abundance proteins. In the current study, we applied the Agilent multiple affinity removal column that targets approximately 94% of total serum proteins, mainly the high-abundance proteins. Preliminary experiments suggest that after going through the column, the majority of high-abundance proteins are removed, while the overall protein migration pattern on the gels is not affected.

For isoelectric focusing (IEF), the gel strips could be of different lengths (usually 7-24 cm) and pH ranges (linear or non-linear, wide or narrow range), depending on the specific goal for each experiment. Gels with linear pH range facilitate the observation of overall protein distribution, while those with non-linear pH range offer a better resolution of proteins with pI values falling into the pH range of gel strips.
Table 1—Differentially expressed proteins as identiﬁed by MALDI-TOF MS

<table>
<thead>
<tr>
<th>Spot ID</th>
<th>Protein ID</th>
<th>Mass (Da)</th>
<th>Mascot Score</th>
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<tr>
<td>422</td>
<td>Factor XIII B subunit precursor</td>
<td>77,592</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>Coagulation factor XIII, B polypeptide</td>
<td>77,723</td>
<td>97</td>
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<tr>
<td></td>
<td>Coagulation factor XIIIb</td>
<td>77,624</td>
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<tr>
<td></td>
<td>Coagulation factor XIII B subunit precursor</td>
<td>77,742</td>
<td>82</td>
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<tr>
<td>1280</td>
<td>Chain C, Human complement component C3c</td>
<td>40,204</td>
<td>150</td>
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<tr>
<td></td>
<td>Complement C3 precursor</td>
<td>45,642</td>
<td>145</td>
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<tr>
<td></td>
<td>Chain B, Structure of complement C3b</td>
<td>104,912</td>
<td>98</td>
</tr>
<tr>
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<td>Chain B, Human complement component C3b</td>
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<td>98</td>
</tr>
<tr>
<td></td>
<td>Chain B, Human complement component C3</td>
<td>114,238</td>
<td>90</td>
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<tr>
<td>1565</td>
<td>Similar to complement C3 precursor</td>
<td>45,642</td>
<td>74</td>
</tr>
<tr>
<td>D1-1</td>
<td>Misato homolog 1 (Drosophila), isoform CRA_b</td>
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<td>D1-2</td>
<td>Hemopexin precursor</td>
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<td>78</td>
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<td></td>
<td>Hemopexin</td>
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<td>Hemopexin protein</td>
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<td>D3-1</td>
<td>Alpha-1-B-glycoprotein</td>
<td>52,479</td>
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<td></td>
<td>Alpha-1B-glycoprotein precursor (Alpha-1-B glycoprotein)</td>
<td>54,809</td>
<td>73</td>
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</tbody>
</table>

Wide-range pH is good for total protein separation and analysis, while narrow-range performs better in separating target proteins with known pI. In the present study, for the purpose of systematically identifying potential serum biomarkers for SRCC, we have chosen 13 cm gel strips with non-linear pH range 3-10.

By applying 2-DE on pooled serum samples from 10 healthy individuals and 10 SRCC patients, we have identified a total of 27 serum proteins that are differentially expressed between the two groups. Among them, eleven are up-regulated, eight are down-regulated and eight become detectable in SRCC group, as compared to the control group. By further MALDI-TOF MS analysis, six of these 27 proteins are identified to be factor XIII B, complement C3 and its precursor, misato homolog 1 CRA_b, hemopexin and alpha-1-B-glycoprotein, respectively.

Factor XIII, also known as fibrin stabilizing factor (FSF) is a member of the transglutaminase family. It is the last zymogen to be activated during the blood coagulation cascade. Structurally, it is a heterotetramer composed of 2A subunits and 2B subunits. The A subunits have catalytic function, catalyzing the formation of gamma-glutamyl-epsilon-lysine cross-link between fibrin molecules, thus stabilizing the fibrin clot. One of the differentially expressed proteins identified in this study is the B subunit, which does not have enzymatic activity and may serve as a plasma carrier molecule. Both A and B subunits are essential and integral structural components of factor XIII.

Evidence suggests that up-regulation of factor XIII contributes to the onset, development and metastasis of human malignancies, including oral cancer, ovarian cancer, leukemia and etc. It is reported that in lymph nodes with high expression of factor XIII, melanoma cells could evade immune surveillance to establish metastasis. Factor XIII is also found to promote tumor angiogenesis and tissue repair through the following mechanisms: i) it promotes complex formation between integrin αVβ3 and vascular endothelial growth factor receptor 2 (VEGFR-2); ii) it stimulates the tyrosine phosphorylation and subsequent activation of VEGFR-2; iii) it up-regulates the expressions of oncogene c-Jun and early growth response protein 1 (Egr-1); and iv) it down-regulates the expression of thrombospondin-1 indirectly. Another study indicates that factor XIII is a determinant factor for the metastatic potential of malignant tumors. By promoting the stabilization of fibrin/platelet thrombi associated with newly formed micrometastases, it protects tumor cells from natural killer (NK) cell-mediated clearance. Based on these recent observations, the up-regulation of factor XIII B identified in the current study may contribute to angiogenesis and micrometastasis evasion from immune attack during the development of RCC.

Two other differentially expressed proteins between the SRCC group and the control group (ID 1280 and 1565) are identified as complement C3 and its precursor. Complement C3 plays a central role in the activation of complement system and its activation is required for both classical and alternative complement activation pathways. Recent studies
suggest that C3 level is up-regulated in the serum of patients with certain tumors, such as pulmonary squamous cell carcinoma and pancreatic cancer. As a major regulator for inflammation, the up-regulation of C3 in those patients is also associated with higher levels of other inflammatory mediators. Another study on the serum levels of C3 and other immune factors in patients with breast cancer, ovarian cancer and alimentary tumors reveals no significant alterations as compared to healthy individuals, suggesting the immune activation mechanisms may vary with different types of tumors. So far, no group has reported on the clinical significance of circulating level of complement C3 in RCC patients, and this study has demonstrated its down-regulation as compared to healthy persons, the underlying mechanisms and biological significance of which awaits further studies.

In as early as 1978, hemopexin was reported to be up-regulated in the serum of nude mice receiving human tumor xenoplants. Hemopexin is a plasma glycoprotein that binds heme with high affinity and transports it from the plasma to the liver. The studies suggest that hemopexin may cooperate with multiple matrix metalloproteinases (MMPs) to facilitate the migration and invasion of tumor cells. Based on these observations, MMP9 inhibitor blocking the binding of hemopexin has been developed and found to inhibit the cell migration in vitro and tumor xenograft growth in vivo. Antagonizing hemopexin has been proposed as a promising anti-cancer strategy. The identification of hemopexin up-regulation in serum from SRCC patients suggests RCC may utilize hemopexin as an early mechanism to promote the invasiveness of the tumor.

Several glycoproteins including CEA, CA-50, CA-19-9, CA-125 and CA-15-3 have been implicated as biomarkers for RCC. However, studies are lacking on the relevance of alpha-1-B-glycoprotein (belonging to the immunoglobulin super-family) to RCC or other human malignancies, though its high expression is reported in saliva from patients with head-and-neck squamous cell carcinoma.

Conclusion
In this study, we combined the 2-DE and SELDI-TOF-MS technique, mapped the serum protein profiles between SRCC patients and healthy individuals, and identified six differentially expressed proteins that may function as potential biomarkers for the early development of RCC. Further characterization of remaining differentially expressed proteins would provide a more complete expression profile associated with SRCC. To improve the sensitivity and/or specificity of these biomarkers, it is desirable to include more serum samples and recruit patients with benign renal tumors to further evaluate the accuracy of these biomarkers.

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