Screening of Autoantibodies in Serum of Renal Cancer Patients Based on Human Proteome Microarray

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Research

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Abstract

Background: The autoantibody in the patient's serum can be used as a marker for the diagnosis of cancer, and the differences of autoantibodies are closely related to the changes of their target proteins. The human proteome microarray platform can be used for the screening of autoantibodies.

Methods: In this study, 16 renal cancer (RC) patients were taken as a disease group, and the same number of healthy people was selected as a healthy control (HC) group. The protein microarray containing 16,152 proteins was used to detect the possible differences of autoantibodies IgG and IgM in the sera between the RC group and HC group. The screening criteria for autoantibodies and their target proteins are: fold change > 1.2, p-value < 0.05, positive ratio of RC > 30% and positive ratio of HC < 10%. Then the screened target proteins were used for cluster analysis of functions and pathways by PANTHER, DAVID and STRING.

Results: Through the comparative analysis of the microarray results, there were 139 types of IgG and 43 types of IgM autoantibody significantly higher in RC than in the HC, and the highly responsive autoantibodies can be candidate biomarkers, such as anti-BCAS4-IgG and anti-RCN1-IgM. There were 159 IgG and 261 IgM autoantibodies that were significantly changed between the RC and the HC. The target proteins BCAS4 and RCN1 may be RC-related antigen and proteins such as GAP43 and CCT8 may be RC-related or RC-specific antigen. The functional clustering results showed those target proteins were mainly directed against the MAPK signaling pathway, Antigen processing: ubiquitination & proteasome degradation, Cargo recognition for clathrin-mediated endocytosis, etc.

Conclustions: The high-content human proteome microarray platform can effectively screen autoantibodies in serum as candidate markers for renal cancer, and their corresponding target proteins can lay the foundation for the study of renal cancer.

Background

The risk of renal cancer is ranked in the top 10, whether male or female [1]. The 5-year survival rate of patients with early-stage renal cancer can reach 93%. 80% of renal cancer patients are asymptomatic in the early stage, and 34.8% of patients have had cancer metastases when they were diagnosed. Surgery is currently the most effective treatment, but the recurrence rate of renal cancer patients 3 years after surgery is up to 31.1%[2]. Based on this, early diagnosis and prognostic monitoring of renal cancer are very necessary. Screening RC-related tumor markers from serum is a good direction.

Changes in protein expression, structure and function are key factors leading to the progression of renal cancer. In previous studies, mass spectrometry methods were used to discover markers and signal pathways closely related to renal cancer [3–6]. The discovery of these markers provides new ideas for the diagnosis, prognosis monitoring and drug treatment of renal cancer. Overexpression, underexpression, degradation or mutation of proteins in tissues can produce tumor-specific or tumor-related antigens, which are released from tumor tissues into the blood so that the patient's immune system produces

autoantibodies. Autoantibodies can be used as tumor markers in serum for diagnosis and postoperative monitoring of tumors [7–10].

There were highly expressed proteins in renal cancer tissues, such as livin and arrestin-1, and further studies have found that the autoantibodies of the two target proteins are significantly higher than those of healthy controls [11, 12]. This is a routine protocol for studying individual autoantibody. In order to screen autoantibodies in the sera of renal cancer patients on a large scale, the SEREX (serological analysis of recombinant cDNA expression libraries) method was developed. 65 renal cancer-related antigens were found and the autoantibodies that reacted with them were explored [13]. Beyond that, there were few reports on systemic autoantibody screening for renal cancer. With the development of microarray technology, high-content human proteome microarray contains almost all human recombinant proteins, which have the advantages of high sensitivity, high specificity, low sample volume, simple operation, and short time-consuming. It is widely used in the screening of autoantibodies against autoimmune diseases and various tumors [14–17]. In the research of tumor autoantibody screening, the normal recombinant human protein immobilized on the microarray is beneficial to the screening of over-expressed TAA (Tumor-associated antigen) corresponding highly response autoantibodies, but there is no relative description on the low-expressed TAA or mutated TSA (Tumor-specific antigen) corresponding lowly response autoantibodies.

This study intends to use high-content human proteome microarray to discover potential autoantibodies and their corresponding target proteins in the serum of patients with renal cancer, and to provide more new biomarkers for the diagnosis and even targeted therapy of renal cancer.

Methods

Collection of serum samples

Serum samples of patients and healthy controls were obtained from the Department of Urology, Shenzhen Second People's Hospital. The study has been approved by the ethics committee, and all patients and volunteers signed informed consent. The sera of 16 renal cancer patients and 16 healthy people were prepared according to standard procedures. In simple terms, blood collection tubes without anticoagulant are used to collect 5 ml of whole blood, placed at room temperature for 30 minutes, and centrifuged in a refrigerated centrifuge at 4 °C for 10 minutes at a speed of 2000 × g. The upper serum is divided into 0.5 mL each tube and stored at -80 °C.

Human proteome microarray

The human proteome microarray (HuProt array version 3.1, CDI Laboratories, Baltimore, MD, USA) was constructed as described earlier. It contained 16,152 proteins, which covered 81% of human proteins. The fusion proteins with N-terminal glutathione S-transferase (GST) tags were expressed by *Saccharomyces cerevisiae* and purified with glutathione agarose beads, and then printed on the modified substrate by the spotting robot. In addition to the recombinant protein, histone H3 and H4 were used as positive controls,

and BSA and biotinylated BSA were used as negative controls. Two spots were repeated for each protein. The human proteome microarray was stored at -80 °C.

Protein microarray for serum analysis

The microarray was taken out from - 80 °C and restored to room temperature, placed in PBST (PBS with 0.5% Tween-20) containing 3% BSA, and incubated at room temperature for 3 hours to complete the blocking of the microarray. 25 µL of serum samples were diluted into 5 mL of PBST containing 1% BSA, and then the blocked microarray was placed in the serum diluent and incubated on a lateral shaker at 40 rpm and 4 °C for 12 hours. The microarray was washed 3 times with PBST for 10 minutes each time. Goat anti-human IgG (Cy3 labeled) and donkey anti-human IgM (Cy5 labeled) were diluted at a dilution ratio of 1:1000 into 5 mL PBST. Then, the microarray was placed in the fluorescence-labeled antibodies dilution solution and incubated at room temperature in dark for 1 hour. The microarray was washed 3 times with PBST for 10 minutes each time and then washed with ddH₂O for 10 seconds. After the microarray was dried by the spin dryer, the microarray scanner was used to read the signal value of the microarray, then Genpix Pro 6.0 was used to analyze the foreground and background values of the points, SNR value = foreground value (F)/background value(B) of each point, and the SNRs of two repeated points were averaged.

Data analysis of microarray

The normalization between microarrays was performed according to the median value of all protein spots to eliminate the systematic errors caused by experimental samples and experimental operations. The 32 groups of samples were statistically analyzed to screen out autoantibodies those can distinguish the RC from HC, including significantly high response(Up)autoantibodies and significantly low response (Down) autoantibodies. If the IgG-SNR ≤ 4 and IgM-SNR ≤ 5 of all samples in the RC group and HC group on a certain protein, it was judged as a negative spot and directly excluded. Screening criteria of Up autoantibodies and their target proteins were as follows: RC positive ratio > 30% and HC positive ratio < 10% and the positive ratio was calculated as the number of RC or HC positive reactivity to its sum [18]. The p-value of t-test was < 0.05. For any protein, calculate the ratio of the mean value of the RC to the HC, namely fold change (FC), which was used to indicate the degree of the RC higher than HC, $FC \geq 1.5$. In addition, screening criteria of Down autoantibodies were as follows: HC positive ratio > 30%, RC positive ratio < 10%, $FC < 2/3$ and the p-value of t-test < 0.05.

Bioinformatics analysis

PANTHER (Protein Analysis through Evolutionary Relationships) was used to classify target proteins corresponding to significantly up-regulated and down-regulated autoantibodies. Gene symbols were used as input for the classification system [19]. The target proteins were then analyzed to determine if any types of proteins are over-represented. GO (Gene ontology) analysis [20] was performed using the web-accessible program DAVID Bioinformatics Resources 6.8 [21]. The default Homo sapiens proteome was selected as the background list. The significance of the enrichments was statistically evaluated with a modified Fisher's exact test (EASE score), and a *P*-value for each term was calculated by applying a

Benjamini–Hochberg false discovery rate correction [22]. The differential target protein interaction networks were produced by STRING [23].

Results

We used 32 human proteomic microarrays to profile serum samples from 16 healthy persons (HC) and 16 renal cancer patients (RC), and anti-human antibodies with different fluorescence are used to mark the IgG or IgM which can bind to the recombinant proteins of the microarray, where goat anti-human IgG (labeled by Cy3) antibody in green, the donkey anti-human IgM (labeled by Cy5) antibody is in red, and the workflow is shown in Fig. 1a. Comparing RC and HC, for IgG or IgM, there are 3 possible results on the microarray, 1) Unchange, autoantibodies of RC and HC have the same response to the majority of target proteins, 2) Up, autoantibodies from RC are more responsive than HC to some target proteins, 3) Down, autoantibodies from RC serum are less responsive than HC to the remaining proteins of the microarray. According to these three situations, we found the corresponding target proteins in the experimental results (Fig. 1b).

We performed a statistical analysis of the signal of each protein on the microarray and set criteria for screening autoantibody and their target proteins in the "Methods" section. The volcano plot shows all Up, Down and Unchange autoantibodies of IgG and IgM in RC (Fig. 2a, 2b). Among them, 420 autoantibodies have changed significantly, and the positive rate, FC and p-value of all target proteins are shown in Table S1. Compare RC to HC, Up autoantibodies included 139 types of IgG and 43 types of IgM and Down autoantibodies included 20 types of IgG and 218 types of IgM. Among IgG autoantibodies, Up autoantibodies are significantly more than Down autoantibodies, while in IgM autoantibodies, the statistical results are just the opposite. Some up autoantibodies can be applied as candidate biomarkers, for example, anti-BCAS4 IgG and anti-RCN1 IgM have high positive rates, 43.75% (7/16) and 31.25% (5/16), suggesting that they may be RC-related autoantibody target proteins (Fig. 2d, 2f). Besides, down autoantibodies are hardly be used as candidate biomarkers, but their targeted proteins are closely related to renal cancer. For example, anti-GAP43-IgG and anti-CCT8-IgM in RC are significantly lower than HC (Fig. 2c, 2e). Among the 420 differential target proteins that were screened out, 10 were present in both IgG and IgM. After removing duplicate counts, the remaining 410 differential target proteins were analyzed by bioinformatics.

To understand the biological relevance of the target proteins, we applied the online protein classification tool, PANTHER, to the 410 screened target proteins to identify enrichment for specific biological process, cellular component and molecular function. We found that the target proteins could be classified into 12 biological process groups (Fig. 3a), the largest of which was the cellular processes group (GO: 0009987), followed by metabolic processes (GO: 0008152). In terms of cellular component, the candidates could be classified into 11 groups. Finally, the candidates could be classified into 8 protein class groups, the top 3 of which were binding (GO: 0005488), catalytic activity (GO: 0003824), and molecular function regulator (GO: 0098772).

To gain insight into possible functional roles of the identified targeted proteins, the enrichment of ontology terms and components of molecular pathways of the candidates were analyzed using DAVID in comparison with their occurrence. The candidate proteins were examined for enrichment, the GO terms of $P < 0.05$ were shown in Fig. 3b. There is a significant number of the candidates localizing in Mitogen-activated protein kinase ($P = 0.001180$). Besides zona pellucida receptor complex ($P = 0.013122$), other significantly enriched terms were IGF-1 Signaling Pathway ($P = 0.003730$) and DNA damage response ($P = 0.035909$).

To create significance out of the targeted proteins, biological protein-protein interaction networks of these proteins were constructed. The candidate proteins were imported into STRING to build the network (Fig. 4a). Three pathways of ErbB, Antigen processing: ubiquitination & proteasome degradation and Cargo recognition for clathrin-mediated endocytosis were also identified (Fig. 4b-d). 4 proteins were overlapped with the MAPK pathway of papillary renal-cell carcinoma (Fig. 4e)

Discussion

In this study, human protein microarrays were used to screen autoantibodies in the serum of renal cancer patients to discover potential renal cancer markers, if multiple candidate markers are combined to form a panel, the diagnostic effect for tumors is better [14, 15]. In this study, the positive rate of anti-BCAS4 IgG was 43.75%, and the positive rate of anti-RCN1 IgM was 31.25%. If they are combined, the positive rate will increase to 62.50%. Therefore, a panel with an appropriate amount of Up autoantibodies is very favorable for the diagnosis of renal cancer.

Among the Up autoantibody, the positive ratio of anti-KCNAB2 IgG, anti-KCNAB1 IgM and anti-KCNAB2 IgM was 56.25%, 56.25% and 50% separately. The target proteins KCNAB1 and KCNAB2 are identified in potassium channel complexes that can regulate DNA damage via the creation of reactive oxygen species (ROS). In the Cancer Genome Atlas (TCGA) Kidney Clear Cell Carcinoma Illumina HiSeq data, we found that the KCNAB1 level is significantly related to the survival rate of renal cancer patients. The positive ratio of anti-RCN1 IgM was 31.25%, and RCN1 may regulate calcium-dependent activities in the endoplasmic reticulum lumen or post-ER compartment. 2D electrophoresis, mass spectrometry and western blot were used to prove that it is highly expressed in renal cancer tissues [24].

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is being regarded as a reference protein for expression quantification in tumors. In our study, the RC-positive rate of anti-GAPDH IgG was 37.50%. Interestingly, recent research has found that GAPDH was commonly up-regulated in a variety of types of cancer, including renal cancer [25].

Both IgG and IgM are autoantibody components in serum. Previous studies on autoantibodies have mainly focused on IgG, and there are few reports on IgM. Also, previous studies mainly focused on Up autoantibodies and their corresponding target proteins, and there are no relevant reports on down autoantibodies and their corresponding target proteins. We speculate that the target protein corresponding to the Up autoantibody is overexpressed in tumor tissue and is called the tumor-associated

antigen, while the target protein corresponding to the Down autoantibody may be mutated in the tumor tissue and is called the tumor-specific antigen. Anti-CCT8 IgM and anti-CCT3 IgM are down autoantibodies, and their target proteins CCT8 and CCT3 are the components of the chaperonin-containing T-complex (TRiC), a molecular chaperone complex that assists the folding of proteins upon ATP hydrolysis. The TRiC complex mediates the folding of WRAP53/TCAB1, thereby regulating telomere maintenance [26]. The alternative name of CCT8 is renal carcinoma antigen NY-REN-15, and sixty-five distinct antigens (NY-REN-1 to NY-REN-65) reactive with autologous IgG were identified by SEREX analysis of 4 renal cancer patients and were characterized in terms of cDNA sequence, mRNA expression pattern, and reactivity with allogeneic sera [13]. This report and our study both showed that CCT8 is a renal cancer antigen, and it was presumed to renal cancer-specific antigen in our study.

Analyzing candidate target proteins through bioinformatics, determined the biological functions of target proteins, signal pathways and interactions between proteins, and provided new ideas for the systematic study of renal cancer-related proteins.

Anti-GAB1 IgM, anti-PIK3CA IgM and anti-Crk IgM are Down autoantibodies, and anti-HRAS IgG is an Up autoantibody. Bioinformatics analysis found that their target proteins are enriched in the MAPK signaling pathway of papillary renal cell carcinoma. GAB1 is prognostic, high expression is favorable for alive of renal cancer patients (The Human Protein Atlas), which means the expression of GAB1 is low in renal cancer. PIK3CA was identified with genetic alteration, and amplification or mutations were 5% in clear cell renal cell carcinoma [27]. HRAS is a member of the RAS family, and normal or mutated forms of HRAS are overexpressed in multiple tumors [28–31]. QPCT (Glutaminyl peptide cyclotransferase) is bound to HRAS and increases the stability of HRAS by reducing its ubiquitination degradation, thus activating the ERK signaling pathway and leading to sunitinib resistance in renal cell cancer [32]. The above results indicate that proteins abnormalities in the MAPK signaling pathway may exist in all types of kidney cancer.

Conclusion

In summary, a systematic method was performed for discovering serum diagnostic biomarkers in RC. We screened out 182 candidate autoantibodies as a biomarker library of RC. We screened out 410 corresponding target proteins of autoantibodies with a significant difference, and there are up-regulated and down-regulated proteins. Some of them are related to RC, some of them are reported regulating multiple cancers, and others of them are not reported. All of them can lay the foundation for the study of renal cancer.

Abbreviations

RC

Renal cancer

HC

Healthy control

TAA

Tumor-associated antigen

TSA

Tumor-specific antigen

Up

Highly responsive

Down

Lowly responsive

GST

Glutathione S-transferase

BSA

Bovine serum albumin

PBS

Phosphate buffer saline

TBS

Tris Buffered Saline

PANTHER

Protein Analysis through Evolutionary Relationships

GO

Gene ontology

TCGA

The Cancer Genome Atlas

ROS

Reactive oxygen species

TRiC

Chaperonin-containing T-complex

GAPDH

Glyceraldehyde-3-phosphate dehydrogenase

SEREX

Serological analysis of recombinant cDNA expression libraries

QPCT

GlutaminyI peptide cyclotransferase

Declarations

Ethics approval and consent to participate

Serum samples of patients and healthy controls were obtained from the Department of Urology, Shenzhen Second People's Hospital. The study has been approved by the ethics committee (NO. 108), and all patients and volunteers signed informed consent.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

Conceptualization, Yangyang Sun and Weiren Huang; Funding acquisition, Weiren Huang; Methodology, Yangyang Sun, Wujiao Li and Zhichao Li, Chengxi Liu; Samples collection, Aolin Li; Writing – original draft, Wujiao Li, Zhichao Li, and Chengxi Liu; Writing – review & editing, Yangyang Sun, Lei Yu and Weiren Huang. All authors have read and agreed to the published version of the manuscript.

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Figures

Figure 1

Screening of serum autoantibodies related to renal cancer based on human proteome microarray. **a**, Schematic of human proteome microarray for the screening of autoantibodies in serum. **b**, Examples of target protein-autoantibody signal on the microarray. For most target proteins, the serum autoantibodies of HC and RC have a consistent response (Unchange), while for some target proteins, RC serum autoantibodies have a higher degree of response (Up) than HC, and some are just opposite (Down).

Figure 2

Candidate autoantibodies associated with renal cancer. a, IgG - volcano plot for the comparison between RC and HC. b, IgM - volcano plot for the comparison between RC and HC. Cutoffs of fold change ≥ 1.2 , p-value < 0.05 , RC-positive ratio $> 30\%$, HC-positive ratio $< 10\%$. Unchanged autoantibodies were shown in gray color. The red color is indicative of Up autoantibodies and green is indicative of Down autoantibodies. c-f, Examples of autoantibodies were significantly different between RC and HC.

Figure 3

Functional distribution of the target proteins. a, Biological process, cellular components and molecular function, categorizations were based on information provided by the online resource PANTHER classification system. b, Function enrichment, categorizations were based on information provided by the online tool DAVID classification system.

Figure 4

The protein-protein interaction network of the target proteins. a, The 410 identified interacting proteins were imported into STRING to build the network. b-d, The representative network related to the ErbB, Antigen processing: ubiquitination & proteasome degradation and Cargo recognition for clathrin-mediated endocytosis which was generated by the online tool STRING. e, MAPK pathway was provided by the online tool DAVID classification system.

Supplementary Files

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