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Original Article

Study on protein differential expression and pathological mechanism of IgA nephropathy treated by leeches

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Abstract: IgA nephropathy is characterized by deposition of the IgA antibody in the mesangium and adjacent capillary walls of the glomerulus, causing glomerulonephritis and inflammation, and leading to blood stasis in the kidney. Although there has been a large body of research focusing on IgA nephropathy, the pathological mechanisms of IgA nephropathy and protein differential expression for diagnosis are still unclear. This study used the iTRAQ technique combined with LC-MALDI-TOF/TOF to determine the differentially expression pattern of proteins in the serum and the urine of an IgA nephropathy rat model with and without drug treatment, and analyzed these proteins with pathway mapping via GO and KEGG databases. We found 4 upregulated molecules, including polymeric immunoglobulin receptor (PIGR), endothelial cell-selective adhesion molecule (ESAM), gamma-glutamyl hydrolase (GGH) and yip1 domain family member 3 (YIPF3); along with 3 downregulated molecules including attractin (ATRN), lactate dehydrogenase a (LDHA), and protein S100-A4 (S100a4) in the urine: all of which can be used as protein molecules to evaluate the efficacy of leeches in the treatment of IgA nephropathy. The leeches can improve the degradation of proteins and other biomolecules and can be used for diagnosis of the state of the leeches after treatment of IgA nephropathy. Prednisone and leeches share the same DE protein in the treatment of IgA nephropathy, which belongs to the category of platelet activation and can affect IgA nephropathy caused by dysfunction of platelet activation pathway in serum. Systematic biological studies based on proteomics could assist in the standardization of diagnosis and treatment of IgA nephropathy in traditional Chinese medicine.

Keywords: IgA nephropathy, blood stasis, iTRAQ, proteomics analysis

Introduction

Immunoglobulin A (IgA) nephropathy is a primary glomerular disease with high incidence worldwide [1]. It is characterized by the deposition of the IgA antibody in the mesangium and adjacent capillary walls of the the glomerulus [2, 3]. This abnormal deposition causes glomerulonephritis and inflammation, which leads to a high concentration of plasma protein in the micro-circulation, slow blood flow and microthrombus in the kidney [4]. The progression of IgA nephropathy usually results in glomerular hemodynamic abnormalities, narrowing of renal arterioles, renal arteriosclerosis and chronic kidney failure [5, 6]. Enormous research has focused on this problem, but the pathological mechanism of IgA nephropathy remains unclear.

Clinically, prednisone is used for the treatment of IgA nephropathy [7-9]. Prednisone is a synthetic corticosteroid drug and has particular effectiveness as an immunosuppressive agent, but has some side effects include high blood glucose levels, depression, Cushing's syndrome, steroid dementia syndrome and weight gain [7, 10, 11]. These side effects severely limit its clinical application. In the theory and application of traditional Chinese medicine (TCM), the pathological mechanism of IgA nephropathy is related to the syndrome of blood stasis in the kidney, and leeches are commonly used to treat blood stasis and activate blood flow [12]. Modern pharmacological studies have shown that hirudin in the leech is a natural inhibitor of thrombin, which is critical for the coagulation system and the inflammation process. The TCM concept and treatment could

help to uncover the biological mechanism of IgA nephropathy and identify the bio-markers.

In the mechanism study and molecular bio-markers discovery, proteomics-based approaches have been widely used and show many advantages. Furthermore, proteomics-based system biological studies could assist in standardization of diagnosis and treatment in TCM. Several poly-peptides in the urine were identified to specifically discriminate IgA nephropathy from other membranous nephropathies [13-15]. The different expression levels of 6 proteins in the urine were discovered to define the pathological grades. In serum, 7 proteins were identified as bio-markers of blood stasis related to IgA nephropathy. However, the identification and analysis were limited to the urine or the serum, which could not systematically demonstrate the regulation pathways behind the disease. Compared with other proteomics-based techniques, an isobaric tag for relative and absolute quantitation (iTRAQ) has been widely used to identify bio-markers of diseases [16-18], because it allows the protein samples to be pooled post labeling without increasing the complexity of measurement, and makes it possible to identify and quantify proteins simultaneously [19].

In this study, the iTRAQ technique combined with LC-MALDI-TOF/TOF was used to analyze the differential expression patterns of proteins in the urine and the serum of the rat IgA nephropathy model with or without drug treatment. We identified the differentiated protein in the urine for evaluating drug efficacy and revealed the pharmacological effects of prednisone and leeches in treatment of IgA nephropathy. Further, we systematically summarized the correlations between the protein expression patterns in the urine and in the serum.

Materials and methods

Study subjects

All of the experimental procedures involving animals were approved by the Institutional Animal Care and Usage Committee (IACUC) of the Guangxi University of Chinese Medicine Institutional Review Board. The rats were purchased from Guangxi Institute for Food and Drug Control. Animals were maintained in a 12 h light/dark photoperiod with food and water *ad libitum*.

Grouping and drug treatment

Six-week-old Wistar male rats weighing 180-200 g were used in this experiment. The experimental IgAN model was established using bovine serum albumin (BSA) + lipopoly-saccharide (LPS) + carbon tetrachloride (CCl4). Immunofluorescence showed obvious IgA deposition in the renal tissue, the abnormal albuminuria and hematuria, and the significant increase in shear rate and plasma viscosity in blood rheology, indicate the success of modeling. After successful modeling, the rats were randomly divided into the model group, the prednisone-treated group, the high-dose leech-treated group and the low-dose leech-treated group (n = 20 for each). The model group did not undergo any intervention until the end of the 14th week. Prednisone-treated group: 2 ml of prednisone suspension was given every morning after successful modeling until the 14th week by intragastric administration. Prednisone was administered at a daily dose of 5.5 mg/kg (Prednisone tablets (5 mg/tablet) were provided by Zhejiang Xianju Pharmaceutical Co., Ltd.). The leech-treated groups received 2 ml of prednisone suspension every morning, 2 ml of leech suspension in the afternoon by gavage, until the 14th week. The high-dose leech-treated group was administered at 1.2 g/kg per day, and the low-dose leech-treated group was administered at a dose of 0.3 g/kg per day (Leech TCM extraction granules were produced by Jiangyin Tian Jiang Pharmaceutical Co. Ltd. Every 10 g granules were equivalent to 10 g of crude drug). Blood samples and urine samples were collected at the end of the 14th week (**Figure 1**).

Sample collection

The urine samples of rats were collected 24 hours before the end of experiment, and cooled to 4°C. Cells and debris were centrifuged for 30 min at 5,000 g and then the samples were stored at -80°C. After adding acetone (-80°C) to the urine samples (10 mL), the protein was precipitated by centrifugation for 30 min at 14,000 g. The precipitation was re-dissolved by cell lysate (1 mL), and the protein was enriched in the supernatant after centrifugation for 10 min at 14,000 g. The concentration of protein was determined by the Bradford method for protein quantitation.

Figure 1. Experimental design flow chart.

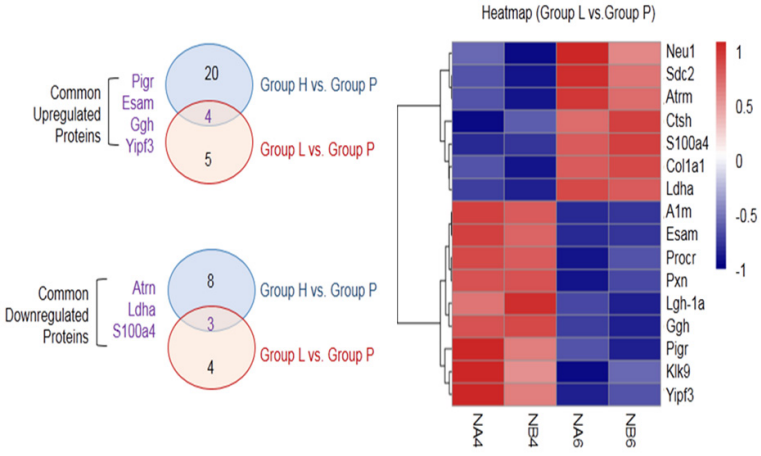

Figure 2. Differentially expression proteins in urine in comparison with the leech-treatment group and the prednisone-treatment group. A. Venn diagrams illustrate up-regulated and down-regulated proteins listed in the leech group vs prednisone group (the high-dose leech group: Group H; the low-dose leech group: Group L; the prednisone group: group P). B. Heatmap showing the clustering result of the protein expression pattern between the leech and prednisone-treated samples.

The serum samples of rats were collected after fasting for 12 hours at the end of experiment and stored at -80°C. Then the serum samples (100 μL) were mixed with Buffer A (300 μL), and filtered via sterile membrane filter (0.22 μm). The 14 highest abundance proteins in the serum were removed via Human 14 Multiple Affinity Removal System Columns (Agilent). The remaining samples were concentrated and desalted via the ultrafiltration centrifuge tube.

iTRAQ labeling

The proteins in the urine and serum samples were subjected to iTRAQ analysis. Each sample was digested and labeled according to the protocol. The freeze-dried samples (100 μg) were mixed with the dissolving buffer (20 μL), the denaturing reagent (2% SDS,) and the reducing reagent (2 μL). The mixture was incubated at 60°C for 1 hour. After the cysteine blocking reagent (1 μL) was added, the mixture was incubated at room temperature for 10 minutes. After the addition of trypsin (5 μg), the mixture was continuously incubated at 37°C for 16 hours. The iTRAQ reagent (AB sciex) was mixed with the ethanol (70 μL) at room temperature. The samples of the four groups were labeled using reagents (114, 115, 116 and 117). After confirmation of iTRAQ labeling, the samples were mixed with the equal amounts and dried by freezing.

LC-MS/MS analysis

The labeled samples were diluted by 10 times using SCX

Table 1. GO analysis based on the functional annotation of the differentially expressed genes (group H vs. group P)

Term	*P* value	Fold Enrichment
GO: Biological Process		
Wound healing	0.020792	13.04688
Response to nutrient	0.024303	11.99932
Regulation of cysteine-type endopeptidase activity involved in apoptotic process	0.026203	73.0625
Hemophilic cell adhesion via plasma mebrane adhesion molecules	0.028743	10.95938
proteolysis	0.049925	4.643803
GO: CC		
Extracellular exosome	2.55E-13	5.090359
Extracellular space	9.82E-10	6.823248
Blood micro-particle	0.001093	19.02414
Extracellular region	0.007127	4.716068
Lysosome	0.008453	9.200199
Cell surface	0.020392	4.585066
Go: MF		
Protein binding	0.005840781	3.04208368
Cell adhesion molecule binding	0.007219962	22.6942299
Serine-type carboxypeptidase activity	0.024941538	76.7281106
Calcium ion binding	0.035014297	3.858453838

Note: GO, Gene ontology; BP, Biological process; CC, Cellular component; MF: Molecular function. Red are shared by both Group H vs Group P and Group L vs Group P.

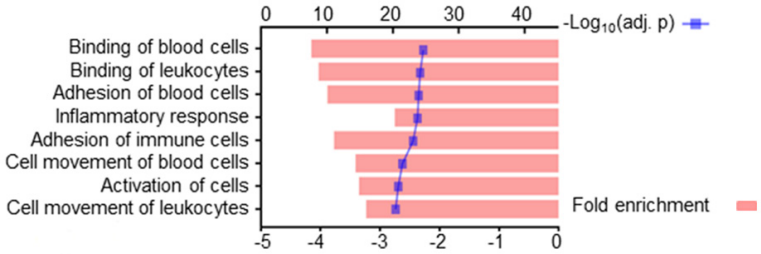

Figure 3. Schematic diagram of GO analysis based on the functional annotation of the differentially expressed genes (group H vs. group P).

buffer A and were injected into the SCX preinstalled column. After washing using buffer A and KCl buffers at different concentrations (35, 50, 75, 100, 125, 150, 175, 200, 250 and 300 mM), polypeptides at different salt concentrations were collected and eluted in a gradient by the Magic C18AQ column (Michrom). Then, the labeled peptides were submitted to the MALDI-TOF/TOF system (AB sciex, 5800) for the proteomics identification and the relative quantitative analysis.

Proteomics analysis

The MS/MS data was searched in the SWISSPROT database using Protein Pilot 2.0 for peptide identification and quantification [14, 20]. The identified proteins were filtered by the confidence with a cutoff of 0.95. Relative quantification was based on area under peak of the samples labeled with reagents (114, 115, 116 and 117). The ratios of 114:117, 115:117 and 116:117 were reported and the result was filtered by *p*-value with a cutoff of 0.05. The identified proteins were classified using the enrichment analysis algorithm, and mapped with the Gene Ontology database (Biological Process, Cellular component and Molecular Function) [21]. The involved pathways were analyzed using Kyoto Encyclopedia of Genes and Genomes (KEGG) [22].

Statistical analyses

Statistical analysis was performed using SPSS 17.0. The measurement data was expressed as mean ± standard deviation. One-way ANOVA was used for statistical analysis among multiple groups, and the difference of the data between the two groups was compared by

Table 2. KEGG pathway analysis of the leech and prednisone-treated samples (group H vs. group P)

rno04514 Cell adhesion molecules (CAMs) (4)
rno05418 Fluid shear stress and atherosclerosis (3)
rno01100 Metabolic pathways (3)
rno04921 Oxytocin signaling pathway (2)
rno04922 Glucagon signaling pathway (2)
rno00620 Pyruvate metabolism (2)
rno04151 PI3-Akt signaling pathway (2)
rno04015 Rap1 signaling pathway (2)
rno04722 Neurotrophin signaling pathway (2)
rno05205 Proteoglycans in cancer (2)
rno04371 Apein signaling pathway (2)

Note: KEGG, Kyoto Encyclopedia of Genes and Genomes. Red text indicates pathways shared by both group H vs group P and group L vs group P.

using the post-hoc Dunnett test. A *P* value less than 0.05 is considered statistically significant.

Results

Identification of differentiated protein for leech treatment

To identify differentiated protein for IgA nephropathy treatment by leech, urine samples of "high-dose leech + prednisone" group, the "low-dose leech + prednisone" and the prednisone group were collected and analyzed via iTRAQ and MS analysis. In total, 35 proteins were differentially expressed between the "high-dose leech + prednisone" group and the prednisone group, of which 24 proteins were significantly up-regulated and 11 proteins were significantly down-regulated ($P < 0.05$, **Figure 2A**). Compared with the prednisone group, the "low-dose leech + prednisone" group exhibited 16 proteins differentially expressed, of which 9 proteins were up-regulated and 7 proteins were down-regulated significantly (**Figure 2A** and **2B**). Encouragingly, we combined these two comparisons and found that they shared 4 upregulated genes, including polymeric immunoglobulin receptor (PIGR), endothelial cell-selective adhesion molecule (ESAM), gamma-glutamyl hydrolase (GGH) and yip1 domain family member 3 (YIPF3); as well as 3 downregulated proteins, including attractin (ATRN), lactate dehydrogenase a (LDHA) and protein S100-A4 (S100a4). These can be used as protein molecules to evaluate the efficacy of leech in the treatment of IgA nephropathy.

We described molecular function of the protein sets with GO analysis [23, 24], in which all of the identified proteins were mapped to terms in the GO database (**Table 1** and **Figure 3**). The results showed these proteins belonged to several categories that grouped into major clusters including biological process, cellular components and molecular function. The common pathways shared by the two comparisons were proteolysis, extracellular exosome, extracellular space, lysosome, and protein binding pathways. These observations indicated that leech treatment improved the degradation of protein and other biomolecules, which was crucial for the diagnosis of the IgA nephropathy state after drug treatment.

We analyzed the biological pathways of these DE proteins using KEGG database [25, 26]. A summary of overall KEGG pathways associated with DE proteins was provided (**Table 2** and **Figure 4**). KEGG pathway analysis of all DE proteins found 2 enriched pathways including cell adhesion molecules and proteoglycans in cancer, which were consistent with the pharmacological functions of the leech. These DE proteins were also clustered in network analysis (**Figure 4**) supporting the GO and KEGG analysis.

Pharmacological function of the combination of prednisone and leech

To study the pathological mechanism of prednisone treatment, the samples from the serum of the model group, the "high-dose leech + prednisone" group, and the "low-dose leech + prednisone" were collected and analyzed via iTRAQ and MS analysis. In total, 64 proteins were expressed differentially between the "high-dose leech + prednisone" group and the model group ($P<0.05$, **Figure 5A**). While, compared with the model group, the "low-dose leech + prednisone" group exhibited 6 proteins differentially expressed (**Figure 5A** and **5B**). We further combined the 2 comparisons and found that they shared 3 DE proteins, including human platelet glycoprotein V (GP5), haptoglobin (Hp) and brain-specific angiogenesis inhibitor 1-associated protein 2 (BAIAP2). These identified proteins may be used to explain the pharmacological function of drug treatment.

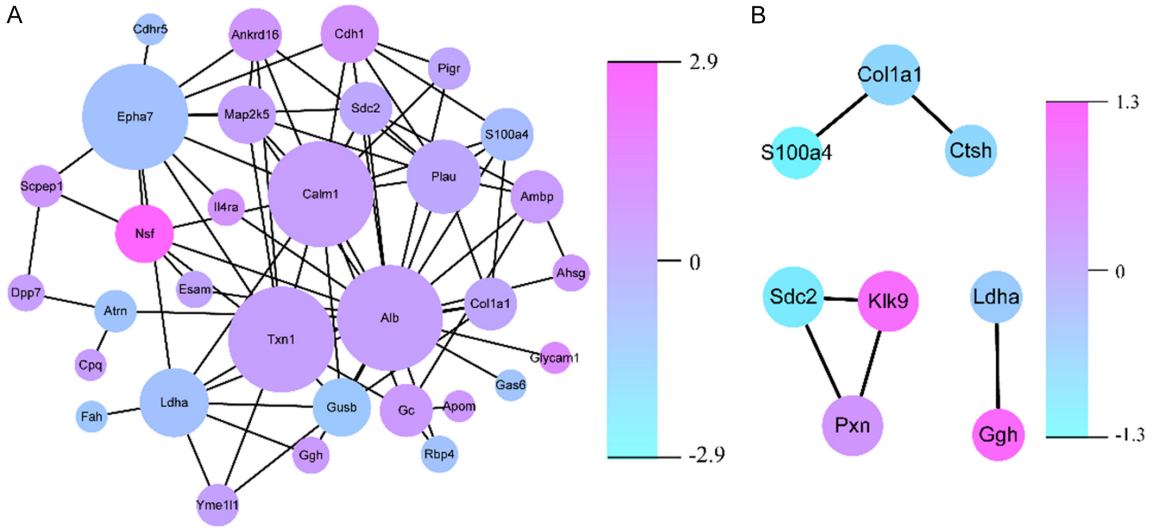

Figure 4. Network analysis showing the regulatory pathway and key components. A. Group H and Group P. B. Group L and Group P.

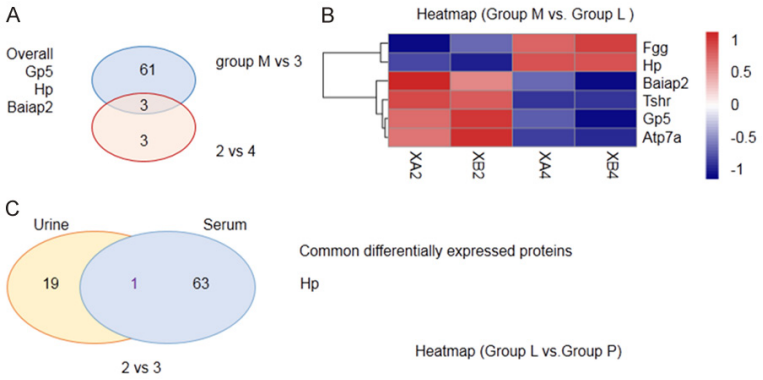

Figure 5. Differentially expression proteins in serum in comparison with the model group (group M) and the leech-treatment groups (group H and L). A. Venn diagrams illustrate differentially expressed proteins listed in group M vs. group H and L. B. Heatmap showing the clustering result of the protein expression pattern between the group H and group M. C. Venn diagrams display the differentially expressed protein list in group M vs. group H.

The results showed that some of these proteins belonged to platelet activation category indicating that pathological mechanism of drug treatment was related to platelet activation. Disorders of the platelet activation process potentially resulted in hemostasis and obstructive clotting (thrombosis).

Interestingly, we compared the DE proteins in the serum and in the urine after drug treatment and found that Hp protein was shared in both of the two protein groups (**Figure 5C**). These observations in the urine support the conclusion that dysfunction of the platelet activation pathway may lead to IgA nephropathy.

To elucidate the specific contribution of leeches in drug treatment, the samples from the serum of the "high-dose leech + prednisone" group, the "low-dose leech + prednisone" and the prednisone group were collected and analyzed via iTRAQ and MS analysis. In total, expression of 85 proteins were different between the "high-dose leech + prednisone" group and the prednisone group ($P<0.05$, **Figure 6A**). While, compared with the model group, the "low-dose leech + prednisone" group exhibited 9 proteins differentially expressed (**Figure 6A** and **6B**). The combination of the 2 comparisons suggested that they shared 4 common DE genes which were heat shock protein 90 alpha family class B member 1 (HSP90AB1), 5-hydroxytryptamine receptor 4 (HTR4), PR domain containing 5 (PRDM5) and myosin light polypeptide 6 (MYL6) (**Figure 6C**). These identified proteins may serve the pharmacological function behind leech treatment. In addition, the DE proteins in the serum and in the urine after drug treatment showed that 5 proteins were common in the two protein groups, including albumin (ALB), LDHA, alpha-2-HS-glycoprotein (AHSG), Cucurbitadienol synthase (CPQ) and ATRN. These observations pro-

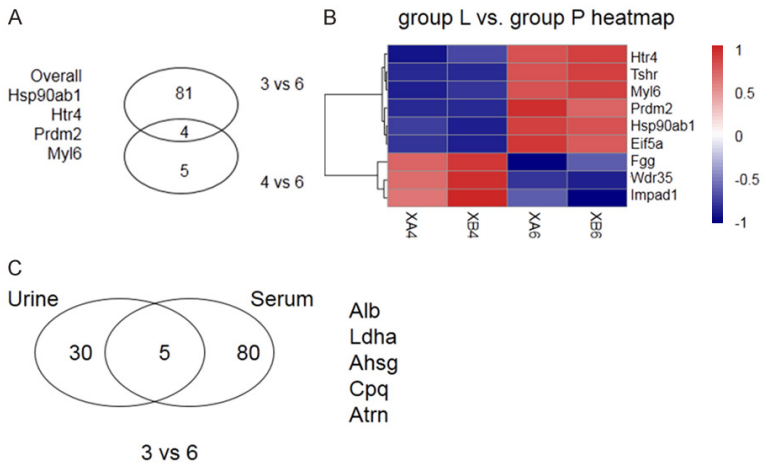

Figure 6. Differentially expressed proteins in serum in comparison with the leech-treatment groups (group H and L) with the prednisone-treatment group (group P). A. Venn diagrams illustrate differentially expressed proteins listed in group H and L vs. group P. B. Heatmap showing the clustering result of the protein expression pattern between the group L and group P. C. Venn diagrams display the differentially expressed protein list in group P vs. group H.

vided hints for studying on the pharmacological function of leech treatment.

Discussion

IgA nephropathy is characterized by abnormal deposition of the IgA antibody in the mesangium, which results in slow blood flow. Prednisone is an immunosuppressant drug that decreases inflammation and increases blood flow. In TCM, the leech could be combined with prednisone to treat IgA nephropathy. In order to study the mechanism of the disease and discover the differentiated proteins for diagnosis, proteomics-based approaches have been widely used. Several poly-peptides in serum and urine were identified as differentiated proteins of IgA nephropathy related to blood stasis. However, the identification and analysis were limited to the urine or the serum of the disease model.

We first compared the protein expression patterns of the disease model group and the treatment group. According to the GO annotations and KEGG pathway analysis, the complement and coagulation pathway and platelet activation pathway were potentially involved in IgA nephropathy. In addition, the complement and coagulation pathway were also enriched consistently. The complement system can be recruited by the immune system, including phagocytosis, inflammation and membrane attack. The inflammation and slow blood flow indicate the IgA nephropathy and disorders of the platelet activation process potentially results in hemostasis and thrombosis.

A variety of poly-peptides in urine have been identified as differentiated proteins for IgA nephropathy. Seven proteins in serum were identified as differentiated proteins of IgA nephropathy related to blood stasis. But drug treatment was not involved in these studies. The differentiated proteins for diagnosis and pathological mechanisms of drug treatment were not elucidated. However, in this study, we found that the combination of prednisone with leech, a TCM medicine, had many advantages in the treatment of IgA nephropathy. Drug treatment improved the complement pathway and platelet activation pathway.

Furthermore, in TCM, the syndrome represents a comprehensive response of the body to the pathogenic factors at a certain stage of the disease. Proteomics provides a technical platform for regulating TCM syndrome differentiation, pathogenesis study and model establishment. In syndrome proteomics, the studies on protein differential expression and post-translational modification have largely revealed the pathological mechanism of the syndrome.

In previous studies, the identification and analysis of differentiated proteins were confined the urine or the serum and it was impossible to connect these 2 groups of differentiated proteins together and systematically demonstrate the regulation pathways behind the disease. In our study, we found that the identified differentiated proteins in the urine were related to the DE proteins in the serum after drug treatment. This observation supports the pathological mechanisms behind drug treatment.

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Disclosure of conflict of interest

None.

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