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An in-depth analysis of proteomics expression profiling in rat glomeruli utilizing LC-MS

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Glomeruli are an essential functional element of renal filtration. The majority of renal diseases caused by glomerular sclerosis or fibrosis may result in renal dysfunction. A fomulate protein profile, a comprehensive analysis of glomeruli of normal rats was conducted in this study via protein spectrum. Functional annotation and classification of these proteins were performed and it was found that 26 had the same glomerule (endothelial cells, podocytes and mesangial cells) markers with proteins.

glomeruli, protein spectrum, multidimensional protein identification technology, glomerule marker, homologene

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Glomeruli are a critical element of the kidneys, and functions' urine filtration. Glomerular dysfunction due to sclerosis or fibrosis is a common cause of the end stages of renal diseases. The molecular pathogenesis mechanism of renal diseases associated with glomerular sclerosis or fibrosis, remain uncertain. A variety of proteins may be involved in the initiation and development of glomerular sclerosis or fibrosis. Therefore, it is necessary to examine the proteome profile of glomeruli at the physiological level, which may significantly contribute to understanding the machinery of the development of glomeruli sclerosis or fibrosis.

The proteomic profile of kidneys has been investigated related to chronic disease of kidneys (CDK), because it is able to provide information about pattern switch of proteins expression in kidney as the process of CDK, and might potentially identify key proteins or drug targets which trigger or represent events. A relatively complete proteome database of biomarkers in CDK has been developed by the Human Kidney and Urine Proteome Project which login in 2005. Most investigations focused on chronic kidney disease (CDK) have proposed to analyze the proteins in the urine, which may represent the plasma protein instead of native proteins of kidneys in terms of the abnormal tubule infiltration, secretion and reabsorption. Few studies had been carried out by proteomic analysis based on the anatomy of kidneys. Zhao et al. [1] described the proteome profile of the renal cortex of mice, which was characterized by 1967 kinds of proteins, most of which were consistent with the protein identified in urine. By using one-dimension separation (SDS-PAGE), two-dimension separation (IEF-SDS) and liquid chromatography-mass spectrometry (LC-MS), Miyamoto et al. [2] found 6686 kinds of proteins in the glomeruli of normal renal tissue from a urethral carcinoma patient. However, proteomic studies with kidney disease in rats, as the primary model, have rarely been reported.

Multi-dimension protein identification technology (MDP-IT) was developed to analyze the proteome by Yates et al. [3]. The protein complex is cleaved into polypeptide fragments by certain proteases and the polypeptide fragments are analyzed and identified by cationic exchange and reverse-phase two-dimension LC/MS. The previous methods, including SDS-PAGE and IEF-SDS, separate the protein in the gel, subsequently isolate and assay the polypeptide

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fragments. MDPIT, compared with the latter two approaches, may identify the low-abundance or hydrophobic membrane proteins, and proteins with a broader isoelectric point or a low molecular weight.

Regarding the advantages of MDPIT, in this study, it was utilized to detect and identity the protein expression in the normal glomeruli from rats. The results of this study might significantly enhance the understanding of the protein profile related to normal kidney function, and facilitate screening and targeting proteins, which might alter the expression pattern in the process of CDK, enabling it to clarify the pathogenesis of CDK as well.

1 Materials and methods

1.1 Subjects

The male Sprague Dwaley (SD) rats were purchased from Vital River Lab Animal Technology Co., Ltd., aged from 8 to 10 month, weight from 180 to 200 g. These rats were bred in a Specific Pathogen Free (SPF) environment with free diet and drinking. The urinary sample of 24 h was harvested up to 3 d, and a 2-mL urine sample of each animal was collected for the quantitative measurement of protein and systolic blood pressure was monitored and recorded. The animals were intraperitoneally injected with 2% pentobarbital sodium (40 mg/kg) for anesthesia, a blood sample was obtained from the abdominal aorta, nephridial tissue was isolated and stored at –80°C.

1.2 Main reagents

BCA protein concentration detection kit (Bio-Rad, USA), iodoacetamide, DTT, ammonium bicarbonate, carbamide, protease inhibitor (Sigma, USA), sequencing grade trypsin (Promega, USA), ultra-pure water produced by the Milli-Q system (Millipore, USA), acetonitrile and formic acid (JT Baker Phillipsburg, USA), C18 filler (particle size 3.5 μm, bore diameter 120-Å, the Great Eur-Asia Sci & Tech Development Co., Ltd, China), SCX filler (particle size 5 μm, bore diameter 300 Å, Phenomenex, USA).

1.3 Monitoring of the basic conditions of rats

(i) Detection of renal functions. The Bradford method was used to detect the protein concentration in urine, which was multiplied by the urinary production in 24 h to obtain the 24-h urine production.

(ii) Detection of systolic blood pressure. An MRB2 III A computer rat blood pressure and heart rate meter (produced by the Shanghai Institute of Hypertension) was used to measure the systolic blood pressure in rat tail arteries.

(iii) PAS stain of the nephridial tissues of rats. Nephridial tissue was fixed with 10% formalin overnight, subsequently dehydrated and lucidificated, embedded in paraffin. The tissue blocks were sectioned and dewaxed. Periodic acid-Schiff stain was applied to observe the tissue pathology, with images captured by a microscope with a camera.

1.4 Purification and separation of the glomeruli

The glomeruli of normal rats were isolated as described by Krakower et al. [4]. The renal cortex was sterilely obtained and cut into thin strips which were grounded, and filtrated through an 80-mesh wire sieve. The glomeruli and smudge cells below the mesh were collected, with further purification of the glomeruli made utilizing a 150-mesh wire sieve.

1.5 Identification of glomerular purity

After the collected glomeruli were resuspended in the PBS, one droplet was placed on a glass slide. The purity was observed with a 200× light microscope and images were captured. An appropriate amount of glomeruli were taken and the cell lysate was achieved utilizing a RIPA buffer (containing 50 mmol/L of pH 7.5 Tris-HCl, 150 mmol/L of NaCl, 0.5% deoxycholic acid, 1% Nonidet P-40, 0.1% SDS, 1 mmol/L PMSF, a variety of protease inhibitors: 1 μg/mL). After schizolysis, it was placed at room temperature for 15 min, centrifuged at 12000 r/min for 20 min at 4°C. The supernatant was obtained to determine the concentration within a BCA kit. The protein samples were denatured by SDS, 80 μg protein was run in SDS-PAGE, and transferred to the PVDF membrane. The membrane was blocked by 5% skim milk in TBST at 4°C overnight, incubated with E-Cadherin polyclonal antibody with 1:100 dilution (Goat anti-rats, SantaCruz, USA) at room temperature for 4 h, rinsed 3 times by 1 × TBST, incubated with anti-goat HRP conjugated secondary antibody at room temperature for 1 h. Color was developed by an ECL chromogenic system. The Alpharmimage 2002 system was used for scan and a gray-scale analysis was performed.

1.6 Preparation of MS protein samples

Purified glomeruli were resuspended by MilliQ ultra-pure water, rinsed 3 times to remove the PBS solution. The sample (800 g) was centrifuged for 5 min to remove the excess water. Glomeruli (10 mg) was dissolved in a 1-mL buffer with 7 mol/L carbamide and 2 mol/L thiocarbamid, was sonicated, centrifuged, discarding the pellets and retaining the supernatant. Protein concentration was determined by BCA assay. To break down the disulfide bond, 100 μg of glomerular protein was treated by 10 mmol/L DTT at 56°C for 1 h, cooled down to room temperature, 50 mmol/L IAM was rapidly added to close hydrosulphonyls and the mixture was left undisturbed at room temperature in a dark room for 45 min. DTT (40 mmol/L) was added to the sample and the sample was kept at room temperature for 15 min to quench the excess IAM. Subsequently, the sample was diluted with

25 mmol/L ammonium bicarbonate to 5 times the original volume. The sample was trypsinized at a 1:1000 ratio at 37°C for 12 h followed by further trypsinization with the same amount of trypsin 37°C for 6 h. FA (0.1%) was introduced to terminate the reaction. The sample was centrifuged by 13000×*g* for 10 min to remove the insoluble and the supernatant was collected and stored at –80°C for a mass spectrometry test.

1.7 Nanoliter two-dimensional liquid chromatography-mass spectroscopy analysis

The multidimensional protein identification system is composed of an LTQ-ion trap (ThermoFisher Scientific, Waltham, USA) mass spectrometer with an electrospray ionization (nano-ESI) source, which was modified as described by Yates et al. [5–7].

This apparatus consisted of three parts, i.e., a shunt device, a two-phase fractioning capillary chromatographic column, and a C18 anti-phase analytical capillary chromatographic column. The shunt device achieved a flow rate by 400–800 nL/s; the two-phase separation capillary chromatographic column contained 3 cm C18 column packing (5 μm, 120-Å, SunChrom, Germany) and 3 cm ion-exchange packing; and the C18 anti-phase analytical capillary chromatographic column was comprised of a tip head made by a capillary machine by pulling a quartz capillary with an internal diameter of 100 μm, and C18 packing was filled into the capillary column with an effective column length of about 10 cm by means of high-pressure nitrogen gas.

The analysis process of the polypeptide samples were as follows: the polypeptide mixture was filled into the self-made quartz capillary column by means of high-pressure nitrogen gas. The mobile phases of two-phase chromatographic fraction: mobile phase A: 5% ACN/0.1% FA; mobile phase B: 80% ACN/0.1% FA; mobile phase C for the 800 mmol/L ammonium acetate/5% ACN/0.1% FA. The two-phase capillary column was used to desalinize for 45 min in the mobile phase A, followed by the elution of the two-phase columns in 6 steps in a step-type gradient according to the concentration of ammonium acetate (0–800 mmol/L) in mobile phase C. The specific steps were as follows: step 1 involved 0–100% mobile phase B for 100 min. Steps 2 to 6 involved the following in order: 100% mobile phase A for 3 min, X% mobile phase C for 5 min, 0–10% mobile phase B in gradient for 5 min, 10%–45% mobile phase B in gradient for 77 min, 45%–100% mobile phase B for 10 min, and 100% mobile phase B of the balanced column for 10 min. X% mobile phase C represents the concentration of ammonium acetate of 10%, 25%, 40%, 70% and 100%. All of the two-phase column eluate was directly injected into the C18 anti-phase analytical capillary chromatographic column, and the liquid effluent was directly used for mass chromatographic analysis after electrospray ionization. The electrospray voltage was 2.0 kV, and the temperature of the ion transfer tube was 200°C. The mass-spectrometric data was collected in the Data Dependent Acquisition (DDA) model, i.e. after a primary all-ion mass spectrometry scan (400 –2000 *m/z*), 5 parent ions with the strongest signals in the primary mass spectrometry were selected for MS/MS analysis. The parameters for acquisition of MS/MS data were as follows: collision energy for ion fragmentation, 35%; ionic strength threshold, 2000 counts; activation energy (*Q*-value) 0.25; activation time, 30 ms; dynamic exclusion time, 30 s. The gradients and the MS scan functions of the HPLC solution were controlled by an XCalibur software system (Thermo Fisher).

1.8 Data analysis

The MS data was retrieved by SEQUESTv.28 of Bioworks 3.31 from the on-line database of NCBI Rat Refseq, Version 2009.10.05. The false positive rate was calculated using the reverse database [8], and the retrieval parameter was trypsin. The number of restriction sites of enzyme was set to be 2. The mass tolerance of the parent ion was ±2 Da (monoisotopic), the mass tolerance of the fragment ion was ±1 Da (monoisotopic); parameters for modification of amino acid residue: the variable modification comprised iodoacetylation of cysteine (57.02 Da); oxidation of methionine (15.99 Da). The peptide possibility of the data retrieved was calculated by Bioworks, and after filtration of XCorr, sp, Rsp, DeltaCn and peptide possibility, the false positive rate FDR for identification of peptide fragments =1%. Protein combination and calculation of protein coverage were performed by a protein coverage summarizer using the identified peptide.

1.9 Classification of the functions of glomerular protein

Classification of the functions of glomerular protein was achieved through combination of the online tools PANTHER (Protein Analysis Through Evolutionary Relationships) [9] and http://www.uniprot.org. The IDs and names of genes were obtained from the RGD (Rat Genome Database).

2 Results and discussion

2.1 The basic conditions of rats

Twenty-four hour' UPQ: 9.12 ± 0.32, normal; tail artery systolic pressure: 14.2576 ± 0.3857 kPa (normal range: 10.906 – 15.96 kPa) [10].

2.2 PAS staining of rat kidneys

No hyperplasia occurred in the glomerular mesangium (mesangial cells, mesangial matrix), the capillary loops opened well, no infiltration of inflammation cells was found in

mesenchymes, no cast occurred in the renal tubules, and no rupture appeared in the basal side (Figure 1).

2.3 Glomerular purity test

The glomerular purity directly affects the identification results of glomerular protein. Reducing pollution of glomeruli by renal tubules and the interstitial tissue of kidneys may contribute to identifying the specific glomerular proteins. We purified glomeruli by following the protocol described by Krakower et al. [4]. The purity of glomeruli reached over 98% by visualization under the microscope. The glomeruli were morphologically normal, as shown in Figure 2.

E-cadherin is normally expressed in epithelial cells and renal tubules other than glomeruli. To rule out the contamination by epithelial cells of renal tubules during purification, Western blot was performed to quantitatively measure the level of E-cadherin in the samples. β-actin was chosen to be the internal control. Figure 3 shows E-cadherin expression with a high abundance in tubules, whereas a low abundance in glomeruli, which indicates that the purity of the glomeruli is sufficient to be subjected to protein analysis.

2.4 Results of 2D-LC-MS/MS identification of glomerular proteins

Approximately, 20 μg of purified glomerular protein was gone through 6-step 2D-LC-MS/MS detection, with the total ion current shown in Figure 4. The result of the 6 steps of separation was uniform.

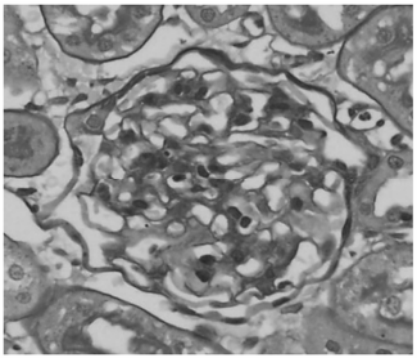

Figure 1 PAS staining of rat kidney (×400).

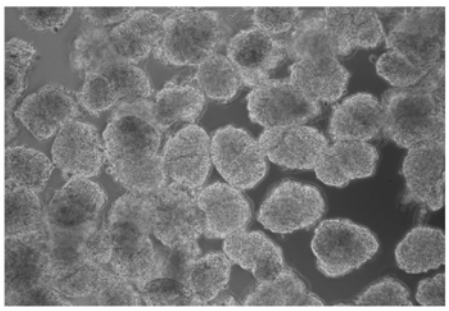

Figure 2 Detection of the purity of isolated glomeruli with a light microscope (×200).

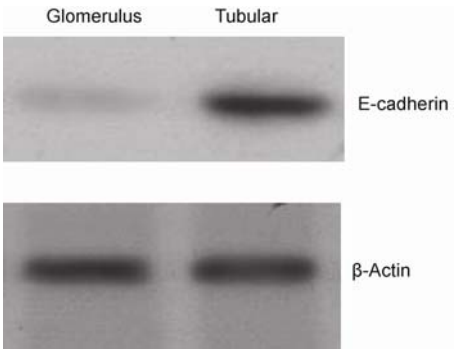

Figure 3 Detection of the expression of E-cadherins with Western blotting.

The MS result was merged and filtered by retrieving the reversed database with the filter parameters: peptide possibility <0.5, sp> 500, Rsp ⩽ 5, DeltaCN = 0 or > 0.08. A total of 19160 kinds of protein were identified, in which 99 were from the reversed database, 19062 from the real database. The false positive rate, i.e., the ratio of retrieved results from the reversed database/retrieved results of the real database, was 0.517%. The non-repeat peptide fragments (11249 pieces) were identified among 19062 effective mass chromatograms. Peptide.xls in Supporting Information 1 lists all of the peptide fragments and the various parameters. Through the protein coverage summarizer software, the 19062 pieces of peptide fragments were merged into proteins and the protein coverage was calculated. A total of 1795 kinds of proteins were identified, including 2740 kinds of proteins with two or more peptide fragments. These proteins represented 3109 non-duplicated genes. See Supporting Information 2: "Protein annotation.xls" for the basic information and results of classification about proteins. The amount of information in this research was more abundant than that in previous investigations. As early as in 1997, Magni et al. [11] used 2DE separation techniques of proteins in the human renal cortex, founding 89 kinds of proteins and 74 pieces of polypeptide subtype. It was subsequently reported 85 proteins were identified in a 170-point protein block in bovine kidney tissues via 2DE [12]. Glomerular cells are divided into 3 types, i.e., mesangial cells, podocytes, and endothelial cells. Each has a unique marker, and the study of biomarkers is theoretically and practically significant, which facilates understanding of the molecular functions of kidney cells and the pathogenesis of renal diseases. Utilizing MDPIT, we identified a variety of protein markers of glomeruli through simultaneously using a small number of samples. The main spherulous cell markers identified in the experiments are listed in Table 1. Thirteen kinds of endothelial cell markers were identified with 3 types, including nucleolin, thrombomodulin, von Willebrand factor, ICAM-1. Podocyte markers were characterized by 11 proteins, including podocin, ezrin, synaptopodin. It appeared

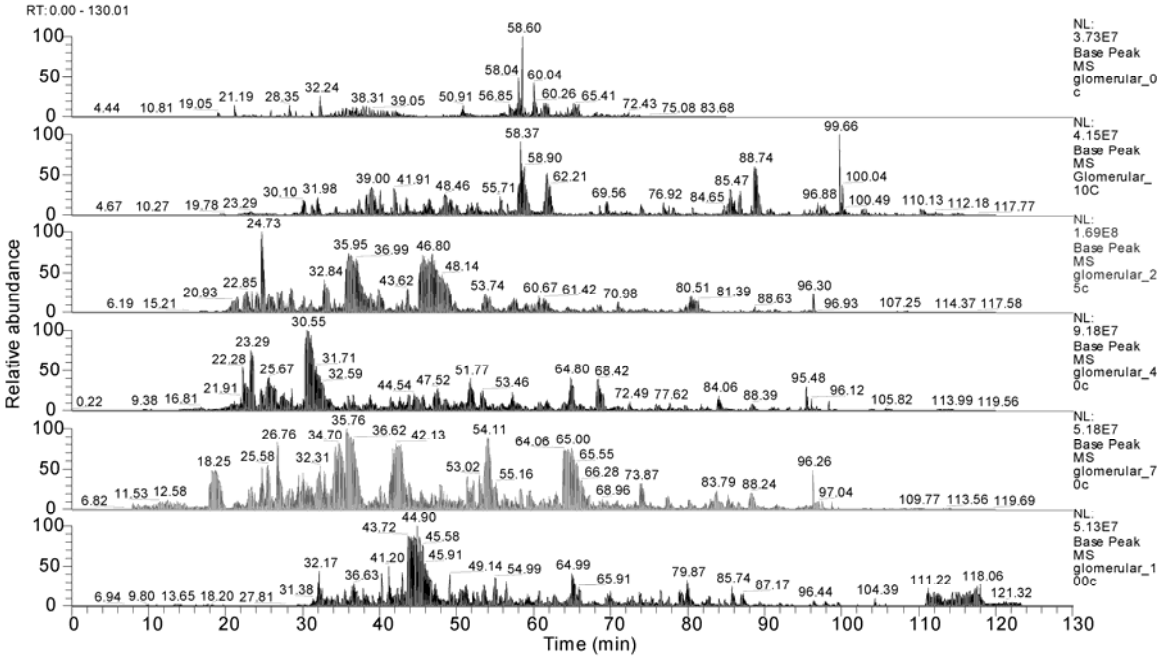

Figure 4 Chart of the total ion current in the 6-step SCX-RP.

that there were no typical marker proteins for mesangial cells as they are a type of myofibroblast, but we also detected relatively classical marker proteins, such as Desmin, alpha-actin, Thy1. Hemodialysis patients with chronic kidney diseases often experience vascular endothelial cell dysfunction, which is a key factor to induce atherosclerotic cardiovascular disease. Local von Willebrand factors (vWF) in glomeruli are a primary marker of vascular endothelial cell injury. Therefore, detection of vW factors has clinical significance in the prevention of cardiovascular diseases and chronic kidney diseases [13–15].

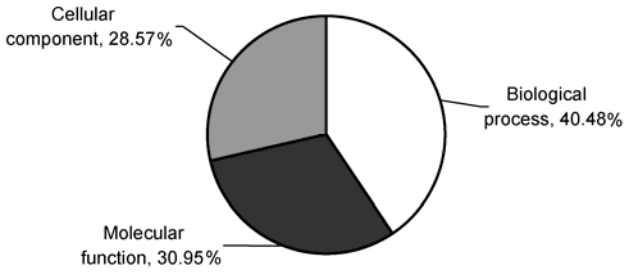

Figure 5 General chart of GO classification.

2.5 GO classification of proteins

The proteins obtained were submitted to the UniProt Knowledgebase for GO (Gene Ontology) classification. The Uniprot identified the GI numbers of 3086 proteins. The GO classification includes: (1) molecular function classification; (2) cellular component classification; and (3) biological processes classification (Figure 5).

Figure 6 illustrates GO molecular function classification. The results suggested that the majority of proteins have multiple functions: binding activity, catalytic activity and transport activity. The binding function referred to the capability of a certain protein in selectively binding with one or more specific sites of its partner protein. About 50% of the proteins possessed this property and were able to associate with proteins, metal ions, nucleotides, ATP, RNA, actins and lipids. About 32% of proteins have catalytic activity. Transport proteins, which are capable of transporting specific substrates or related substrate groups across cell membranes, account for 5% of all the proteins in this category. As shown in Figure 6, 100 proteins (3.7%) were structural proteins. The proteins (3%) were respectively associated with enzyme regulation and signal transduction. The 2 types of proteins are vital factors in cell proliferation, differentiation and apoptosis. In addition, it is also compromised proteins with ionophore activity, transcription modulation, anti-oxidation, channel regulatory proteins, translation regulatory proteins. The diversity of molecular function suggested that the LC-MS approach had the capacity to assay extensive, multi-dimensional and unbiased protein expression in tissues.

Figure 7 shows the analytic results of the cellular components in the GO classification. Most of the proteins function as cell components, organelles, and membrane components, which respectively represent 37%, 25%, 13% and 12%. In addition, we also found the extracellular proteins and synapse proteins. This result shows that LC-MS identifies the structures of not only the organelles and membrane proteins of the dominant portion but also the extracellular proteins and intramembrane proteins.

Figure 8 shows the analytical results of the cell processes

Table 1 The markers of the 3 types of glomerular cells (mesangial cells, podocytes, endothelial cells)

Accession no.	Gene symbol	Protein description	Percent coverage (%)	Function	Location
NP_113863.2	*Actn4*	Alpha actinin 4	73.765	Interacts with integrins and strengthens the podocyte-GBM interaction thereby stabilizing glomerular architecture and preventing disease	Podocyte [16]
NP_602308.1	*Agt*	Angiotensinogen	3.983	Essential component of the renin-angiotensin system (RAS), a potent regulator of blood pressure, body fluid and electrolyte homeostasis	Mesangial cell [17]
NP_001012055.1	*Cdh16*	Cadherin 16	10.241	A factor in the organization of lateral endothelial junctions and in the control of permeability properties of vascular endothelium	Endothelial cell [18]
XP_343484.1	*Dag1*	PREDICTED: similar to Dystroglycan precursor (Dystrophin-associated glycoprotein 1)	9.854	Plays a dual role in the maintenance of the unique architecture of podocytes by its binding to the glomerular basement membrane, and in the maintenance of the integrity of the filtration slit	Podocyte [19]
NP_071976.1	*Des*	Desmin	31.13	A damaged podocyte marker	Podocyte/mesangial cell [20,21]
NP_598308.1	*Gbp2*	Guanylate binding protein 2	2.876	A novel cellular activation marker that characterizes the IC-activated phenotype of endothelial cells	Endothelial cell [22]
NP_037099.1	*Icam1*	Intercellular adhesion molecule 1 precursor	2.385	Facilitates the development of cell polarity and modulates endothelial cell migration	Endothelial cell [23]
NP_001007726.1	*Icam2*	Intercellular adhesion molecule 2	6.498	Expressed at the endothelial junctions, also mediates angiogenesis	Endothelial cell [24]
NP_997489.1	*Kirrel1*	Nephrin 1 precursor	13.308	Podocyte membrane protein of the Ig superfamily	Podocyte [25]
NP_001029181.1	*Mcam*	Melanoma cell adhesion molecule isoform 2	8.746	Reflects endothelial remodeling more effectively than soluble CD31	Endothelial cell [26]
NP_076473.2	*Mcam*	Melanoma cell adhesion molecule isoform 1	8.179		Endothelial cell [26]
NP_036881.2	*Ncl*	Nucleolin	8.403	A specific marker of angiogenic endothelial cells within the vasculature	Endothelial cell [27]
NP_072150.1	*Nphs1*	Nephrosis 1 homolog, nephrin	18.61	The major podocyte protein, is a podocyte specific marker and is a critical factor in the filtration barrier of the glomerular podocyte	Podocyte [28,29]
NP_570841.2	*Nphs2*	Podocin	20.888	A podocyte-specific protein	Podocyte [31]
NP_620203.1	*Podxl*	Podocalyxin-like precursor	24.33	Marker specific for differentiating podocytes	Podocyte [31–33]
NP_067727.2	*Synpo*	Synaptopodin	33.382	A podocyte specific marker	Podocyte [31]
NP_113959.1	*Thbd*	Thrombomodulin	7.106	A specific marker of endothelial cell damage, is a transmembranous glycoprotein with anti-coagulant properties	Endothelial cell [34]
NP_036805.1	*Thy1*	Thy-1 cell surface antigen precursor	9.938	A signal transduction molecule in T lymphocytes and transfected B lymphocytes	Mesangial cell [35]
NP_062230.1	*Vil2*	Ezrin	40.273	The increased permeability of the filtration barrier in steroid-resistant and proteinuric glomerulopathies may be a consequence of subcellular changes in podocyte-associated proteins following decreased expression of ezrin	Podocyte [31]
NP_112402.1	*Vim*	Vimentin	52.361	The podocyte intermediate cytoskeletal protein	Podocyte [36]
XP_001066203.1	*Vwf*	PREDICTED: similar to von Willebrand factor	1.387	A glycoprotein produced uniquely by endothelial cells and megakaryocytes, is routinely used to identify vessels in tissue sections	Endothelial cell
XP_342760.3	*Vwf*	PREDICTED: similar to von Willebrand factor	1.387		Endothelial cell [37–39]
NP_001099736.1	*ZO-1*	Tight junction protein 1	15.176	A junctional protein involved in the assembly and proper function of a number of tight junctions and is also expressed at the junction of podocytes with the slit diaphragm	Podocyte [40]
NP_001100180.1	*EMILIN1*	Elastin microfibril interfacer 1	3.111	To be the only endothelial lineage-specific cell surface glycoprotein identified to date	Endothelial cell [41]

(*to be continued*)

(*Continued*)

Accession no.	Gene symbol	Protein description	Percent coverage (%)	Function	Location
NP_001100877.1	*CDH5*	Cadherin 5	1.289	Endothelial-specific cadherin that is a factor in the organization of lateral endothelial junctions and in the control of permeability properties of vascular endothelium	Endothelial cell [18]
NP_001101892.1	*PLXDC2*	Plexin domain containing 2	1.887	A novel protein whose cell surface expression is essential during endothelial cell capillary morphogenesis	Endothelial cell [42]

in the GO classification. The modulation of the glomerular filtration function was varied by mesangial cells, endothelial cells. This process involves complex biological processes, including energy metabolism, intercellular communication and ion transport. Therefore, a large number of proteins involved in biological processes are concentrated in cell processes, metabolism and biological regulation, respectively accounting for about 24%, 19% and 10%.

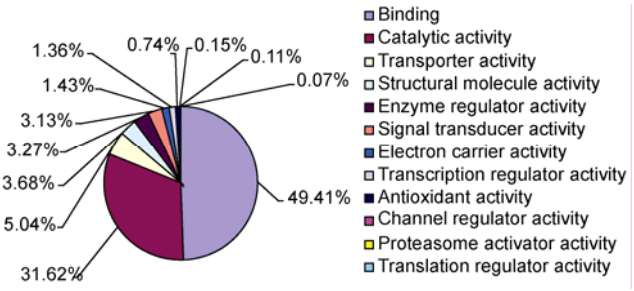

Figure 6 Results of molecular function analysis in GO classification.

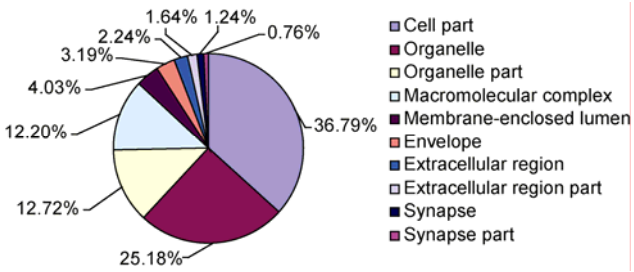

Figure 7 Results of cell component analysis after GO classification.

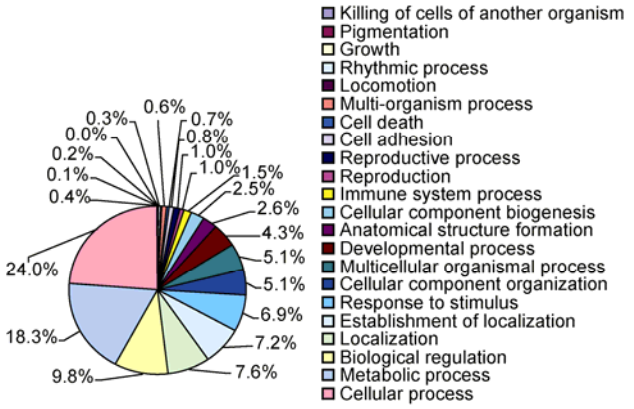

Figure 8 Results of biological process analysis in GO classification.

2.6 Enzyme classification

A total of 2009 proteins were involved in the 6 major categories of the biological enzyme system, with hydrolytic enzymes and oxidoreductases accounting for the largest portion, wherein, hydrolases (182) and oxidoreductases (142) together accounted for about 59%. Glomeruli are a primary an important functional unit in the filtration function of kidneys, and the filtration process consumes large amounts of energy. The energy metabolism is closely related to hydrolysis or oxidation-reduction of proteins. Therefore, as compared with renal tubules, glomeruli need more hydrolases and oxidoreductases when involved in this process [43] (Figure 9).

2.7 Pathway analysis

A total of 32 metabolic pathways were involved, including the 2 pathways of lipid metabolism (24) and carbohydrate degradation (20) containing the largest number of proteins (Table 2). The 2 pathways are mainly responsible for glycolysis and fatty acid metabolism to provide energy for cells. Glomeruli mainly functions in withstanding the pressure in blood rheology [44], filtration and metabolism of a variety of materials, and incretion [45]. Therefore, the multiple metabolic pathways are very active. A number of metabolism-related genes, such as enolases and PFKI, in the glycolysis pathway, are retrieved in the data table. When mesangial cells were incubated with high glucose, they had a relatively high level of energy metabolism, which detected the upregulation of Enolase [46]. Active metabolic pathways

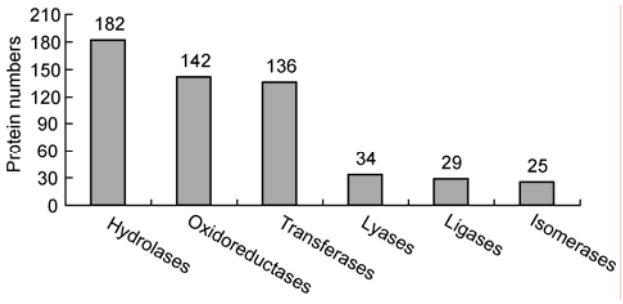

Figure 9 Enzyme classification and analysis of 3086 proteins.

Table 2 List of pathway analysis

No.	Pathway name	Gene number
1	Lipid metabolism	24
2	Carbohydrate degradation	20
3	Amino-acid biosynthesis	9
4	Amino-acid degradation	9
5	Purine metabolism	8
6	Carbohydrate metabolism	7
7	Carbohydrate biosynthesis; gluconeogenesis	6
8	Cofactor biosynthesis	6
9	Protein modification 1	5
10	Amine and polyamine biosynthesis	4
11	Energy metabolism	4
12	Fermentation; pyruvate fermentation to lactate; (S)-lactate from pyruvate	4
13	Porphyrin metabolism	3
14	Metabolic intermediate biosynthesis; 5-phospho-alpha-D-ribose 1-diphosphate biosynthesis; 5-phospho-alpha-D-ribose 1-diphosphate from D-ribose 5-phosphate (route I): step 1/1	2
15	Metabolic intermediate metabolism	2
16	Nitrogen metabolism; urea cycle	2
17	Polyol metabolism	2
18	Secondary metabolite metabolism	2
19	Sulfur metabolism	2
20	Alcohol metabolism; ethanol degradation; acetate from ethanol	1
21	Amine and polyamine degradation; sarcosine degradation; formaldehyde and glycine from sarcosine	1
22	Amino-sugar metabolism; N-acetylneuraminate degradation; D-fructose 6-phosphate from N-acetylneuraminate	1
23	Cofactor metabolism; retinol metabolism	1
24	Glycerolipid metabolism; triacylglycerol degradation	1
25	Glycolipid biosynthesis; glycosylphosphatidylinositol-anchor biosynthesis	1
26	Ketone metabolism; succinyl-CoA degradation; acetoacetyl-CoA from succinyl-CoA	1
27	Membrane lipid metabolism; glycerophospholipid metabolism	1
28	One-carbon metabolism; tetrahydrofolate interconversion	1
29	Phospholipid metabolism; CDP-diacylglycerol biosynthesis; CDP-diacylglycerol from sn-glycerol 3-phosphate	1
30	Pyrimidine metabolism; CTP biosynthesis via de novo pathway; CTP from UDP: step 2/2	1
31	Steroid biosynthesis; estrogen biosynthesis	1

provide energy for cells and also produce a large number of free radicals. In the anti-Thy1 rat model of glomerulonephritis, the large quantities of ROS generated in the glomeruli were key pathogenic factors [57,58].

2.8 Comparison of the kidney proteomes in rats, humans and mice

As the proteins of different species cannot be directly compared, HomoloGene provide a convenient way to unify the genes of different species into unique IDs for comparison. We input the gene IDs of the 3796 proteins in the glomerular proteomes in rats in the NCBI HomoloGene release 64 database and obtained 2521 unique homologene IDs. Homologene IDs (1888) of 1966 proteins in mouse renal cortical proteomes, which were consistent with Zhao et al. [1]. Miyamoto et al. [2] obtained 6686 proteins from human glomeruli. A total of 2518 homologene IDs were obtained. The number of homologene IDs was not the same as that of the proteins primarily because, except for a small number of proteins or genes which had no corresponding homologene IDs, there was a certain degree of redundancy in the protein database and it is likely that a percentage of proteins matched a single homologene ID. The IPI database was used with the human glomerular proteomes. There was greater redundancy in this database and therefore the number of homologene IDs obtained was much smaller than that of the proteins. Figure 10 shows the genetic differences in the kidney proteome in the 3 species. The result of our experiment was essentially consistent with the number of genes of human glomerular proteomes, but that of mouse genes was slightly smaller. Through comparison between the 3 species, there were a total of 639 kinds of common genes, of which the number of kinds of genes shared by rats and humans was greater than those shared by rats and mice or by humans and mice. This may be because not all of the data of the proteins from the 3 sources was matched with the Unigene IDs in the GenBank database, which resulted in the loss of a small amount of data. In addition, the data of rats and humans was based on the glomerular protein samples, but the data of mice was based on protein samples of the renal cortex, which contained not only glomeruli but also tubules and mesenchymes. Therefore, the homologene contents in the proteomic data of mice were more complex. The proteins of relatively high expression abundance in the renal tubules and the mesenchymes would mask the data of some of the glomerular proteins. Therefore, in terms of the purity of the glomeruli in the study, it might more accurately reflect the spectrum of glomerular proteomes. The results also suggest that the proteomes of renal tubules or of the renal interstitium may be separately studied in the future, which might improve the accuracy of detection and analysis and provide a platform for the research of markers of tubular

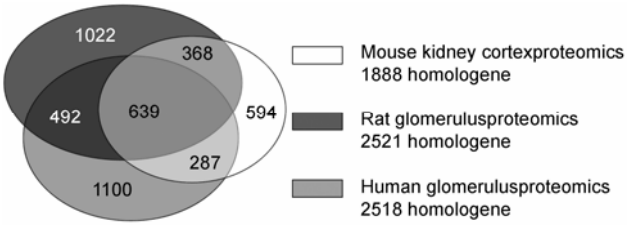

Figure 10 Comparison of the kidney proteome from the 3 species (human, rat, mouse).

or interstitial damages.

3 Conclusion

Healthy rats which were easily available were subjected to this study, and 3796 proteins were identified in the glomerular protein samples by the LC/MS System. The data was systematically analyzed and classified with the assistance of bioinformatics software and was compared with the data previously reported. The results show that the information about protein obtained in this study is more comprehensive, and the marker proteins of certain types of cells were easily obtained. Therefore, this study has laid a foundation for in-depth understanding of the structures and functions of glomeruli and has provided a stable and comprehensive platform for protein analysis to probe into the pathogenesis of CKD.

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1 Zhao Y, Denner L, Haidacher S J, et al. Comprehensive analysis of the mouse renal cortex using two-dimensional HPLC–tandem mass spectrometry. Proteome Sci, 2008, 6: 15
2 Miyamoto M, Yoshida Y, Taguchi I, et al. In-depth proteomic profiling of the normal human kidney glomerulus using two-dimensional protein prefractionation in combination with liquid chromatography-tandem mass spectrometry. J Proteome Res, 2007, 6: 3680–3690
3 Delahunty C M, Yates J R 3rd. MudPIT: Multidimensional protein identification technology. BioTechniques, 2007, 43: 563, 565, 567
4 Krakower C A, Greenspon S A. Localization of nephrotoxic antigen within isolated renal glomerulus. Azche Pathol, 1951, 51: 629–639
5 Washburn M P, Wolters D, Yates J R 3rd. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat Biotechnol, 2001, 19: 242–247
6 Romijn E P, Yates J R 3rd. Analysis of organelles by on-line two-dimensional liquid chromatography-tandem mass spectrometry. Methods Mol Biol, 2008, 432: 1–16
7 Delahunty C, Yates J R 3rd. Protein identification using 2D-LC-MS/MS. Methods, 2005, 35: 248–255
8 Elias J E, Gygi S P. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. Nat Methods, 2007, 4: 207–214
9 Thomas P D, Campbell M J, Kejariwal A, et al. PANTHER: A library of protein families and subfamilies indexed by function. Genome Res, 2003, 13: 2129–2141
10 Chen Z, Wu R. Experimental Zoology. Changsha: Hunan Science and Technology Press, 2001. 265
11 Magni G, Hijazi Z M, Pandian N G, et al. Two- and three-dimensional transesophageal echocardiography in patient selection and assessment of atrial septal defect closure by the new DAS-Angel Wings device: initial clinical experience. Circulation, 1997, 96: 1722–1728
12 D'Ambrosio C, Arena S, Talamo F, et al. Comparative proteomic analysis of mammalian animal tissues and body fluids: Bovine proteome database. J Chromatogr B Analyt Technol Biomed Life Sci, 2005, 815: 157–168
13 Foley R N, Parfrey P S, Samak M J. Clinical epidemiology of cardiovascular disease in chronic renal disease. Am J Kidney Dis, 1998, 32 (5 Suppl 3): 112–119
14 Malyszko J, Malyszko J S, Kozminski P, et al. Elevated resistin is related to inflammation and residual renal function in haemodialysed patients. Nephrology, 2007, 12: 246–253
15 Stam F, van Guldener C, Becker A, et al. Endothelial dysfunction contributes to renal function-associated cardiovascular mortality in a population with mild renal insufficiency: The Hoorn study. J Am Soc Nephrol, 2006, 17: 537–545
16 Dandapani S V, Sugimoto H, Matthews B D, et al. Alpha-actinin-4 is required for normal podocyte adhesion. J Biol Chem, 2007, 282: 467–477
17 Tanaka T, Ohkubo H, Nakanishi S. Common structural organization of the angiotensinogen and the alpha 1-antitrypsin genes. J Biol Chem, 1984, 259: 8063–8065
18 Lampugnani M G, Resnati M, Raiteri M, et al. A novel endothelial-specific membrane protein is a marker of cell-cell contacts. J Cell Biol, 1992, 118: 1511–1522
19 Vogtländer N P, Tamboer W P, Bakker M A, et al. Reactive oxygen species deglycosilate glomerular alpha-dystroglycan. Kidney Int, 2006, 69: 1526–1534
20 Nagase M, Shibata S, Yoshida S, et al. Podocyte injury underlies the glomerulopathy of Dahl salt-hypertensive rats and is reversed by aldosterone blocker. Hypertension, 2006, 47: 1084–1093
21 Smeets B, Te Loeke N A, Dijkman H B, et al. The parietal epithelial cell: A key player in the pathogenesis of focal segmental glomerulosclerosis in Thy-1.1 transgenic mice. J Am Soc Nephrol, 2004, 15: 928–939
22 Lubeseder-Martellato C, Guenzi E, Jörg A, et al. Guanylate- binding protein-1 expression is selectively induced by inflammatory cytokines and is an activation marker of endothelial cells during inflammatory diseases. Am J Pathol, 2002, 161: 1749–1759
23 Finzel A H, Reininger A J, Bode P A, et al. ICAM-1 supports adhesion of human small-cell lung carcinoma to endothelial cells. Clin Exp Metastasis, 2004, 21: 185–189
24 Huang M T, Mason J C, Birdsey G M. Endothelial intercellular adhesion molecule (ICAM)-2 regulates angiogenesis. Blood, 2005, 106: 1636–1643
25 Gerke P, Huber T B, Sellin L, et al. Homodimerization and heterodimerization of the glomerular podocyte proteins nephrin and NEPH1. J Am Soc Nephrol, 2003, 14: 918–926
26 Bardin N, Reumaux D, Geboes K, et al. Increased expression of CD146, a new marker of the endothelial junction in active inflammatory bowel disease. Inflamm Bowel Dis, 2006, 12: 16–21
27 Christian S, Pilch J, Akerman M E, et al. Nucleolin expressed at the cell surface is a marker of endothelial cells in angiogenic blood vessels. J Cell Biol, 2003, 163: 871–878
28 Wagner N, Wagner K D, Xing Y, et al. The major podocyte protein nephrin is transcriptionally activated by the Wilms'tumor suppressor WT1. J Am Soc Nephrol, 2004, 15: 3044–3051
29 Szeto C C, Lai K B, Chow K M, et al. Messenger RNA expression of glomerular podocyte markers in the urinary sediment of acquired proteinuric diseases. Clin Chim Acta, 2005, 361: 182–190
30 Vaughan M R, Pippin J W, Griffin S V, et al. ATRA induces podocyte differentiation and alters nephrin and podocin expression *in vitro* and *in vivo*. Kidney Int, 2005, 68: 133–144
31 Saleem M A, O'Hare M J, Reiser J, et al. A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression. J Am Soc Nephrol, 2002, 13: 630–638
32 Nagata M, Horita S, Shu Y, et al. Phenotypic characteristics and cyclin-dependent kinase inhibitors repression in hyperplastic epithelial pathology in idiopathic focal segmental glomerulosclerosis. Lab Invest, 2000, 80: 869–880
33 Nagata M, Nakayama K, Terada Y, et al. Cell cycle regulation and differentiation in the human podocyte lineage. Am J Pathol, 1998, 153: 1511–1520
34 Urban M, Wojtkielewicz K, Głowińska B, et al. Soluble thrombomodulin–a molecular marker of endothelial cell injury in children and adolescents with obesity (in Polish). Endokrynol Diabetol Chor Przemiany Materii Wieku Rozw, 2005, 11: 73–77
35 Richard A, Kroczek, Kurt C, et al. Thy-1 functions as a signal transduction molecule in T lymphocytes and transfected B lymphocytes. Nature, 1986, 322: 181–184
36 Nagata M, Watanabe T. Podocytes in metanephric organ culture ex-

press characteristic *in vivo* phenotypes. Histochem Cell Biol, 1997, 108: 17–25
37 Zanetta L, Marcus S G, Vasile J, et al. Expression of Von Willebrand factor, an endothelial cell marker, is up-regulated by angiogenesis factors: A potential method for objective assessment of tumor angiogenesis. Int J Cancer, 2000, 85: 281–288
38 Blann A D. Is raised von Willebrand factor a marker of endothelial cell damage? Med Hypotheses, 1993, 41: 419–424
39 Blann A D. Von Willebrand factor antigen as an acute phase reactant and marker of endothelial cell injury in connective tissue diseases: A comparison with CRP, rheumatoid factor, and erythrocyte sedimentation rate. Z Rheumatol, 1991, 50: 320–322
40 Macconi D, Abbate M, Morigi M, et al. Permselective dysfunction of podocyte-podocyte contact upon angiotensin II unravels the molecular target for renoprotective intervention. Am J Pathol, 2006, 168: 1073–1085
41 Sanz-Moncasi M P, Garin-Chesa P, Stockert E, et al. Identification of a high molecular weight endothelial cell surface glycoprotein, endoGlyx-1, in normal and tumor blood vessels. Lab Invest, 1994, 71: 366–373
42 Wang X Q, Sheibani N, Watson J C. Modulation of tumor endothelial cell marker 7 expression during endothelial cell capillary morphogenesis. Microvasc Res, 2005, 70: 189–197
43 Burch H B, Bross T E, Brooks C A, et al. The distribution of six enzymes of oxidative metabolism along the rat nephron. J Histochem Cytochem, 1984, 32: 731–736
44 Malyszko J, Pawlak K, Borawski J, et al. Effect of ketanserin on platelet function and bleeding time in uremic patients treated with erythropoietin. Thromb Res, 1995, 78: 541–546
45 Rastaldi M P, Armelloni S, Berra S, et al. Glomerular podocytes contain neuron-like functional synaptic vesicles. FASEB J, 2006, 20: 976–978
46 Ramachandra Rao S P, Wassell R, Shaw M A, et al. Profiling of human mesangial cell subproteomes reveals a role for calmodulin in glucose uptake. Am J Physiol Renal Physiol, 2007, 292: F1182–F1189
47 Nakamura K, Oka M, Shirai M, et al. Source of reactive oxygen species in anti-Thy1 nephritis. Ren Fail, 1998, 20: 399–405
48 Zent R, Ailenberg M, Downey G P, et al. ROS stimulate reorganization of mesangial cell-collagen gels by tyrosine kinase signaling. Am J Physiol, 1999, 276: F278–F287

Supporting Information

1 Peptide.xls
2 Protein annotation.xls

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