Glomerular Proteomic Profiles in the NZB/W F1 Hybrid Mouse Model of Lupus Nephritis

Lupus nephritis is one of the most serious complications of systemic lupus erythematosus (SLE) and is associated with patient mortality. This study aimed to investigate the proteomic profiles of the glomerulus in the NZB/W F1 hybrid mouse model of mild and severe lupus nephritis using two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) combined with matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS).

Material/Methods:
Female NZB/WF1 mice (n=60) at 28 weeks of age were divided into the mild proteinuria group (+1), the moderate proteinuria group (+2), and the severe proteinuria group (+3) using paper strip urine testing, and then later divided into a mild (≤1+) and severe (≥3+) proteinuria group to allow comparison of upregulation and down-regulation of proteins between the two groups. Renal glomeruli were isolated following renal perfusion with magnetic beads. Protein expression was determined by Western blot, immunohistochemistry, 2D-DIGE, and MALDI-TOF-MS.

Results:
A total of 56 differentially expressed proteins were identified from 133 protein spots, of which 18 were upregulated and 23 were downregulated between groups 1 and 2. Expression of the proteins Ras-related GTP-binding protein B (RRAGB), serine/threonine-protein kinase 1 (SMG1), angiopoietin 2 (ANGP2), methylmalonate semialdehyde (MMSA), and ATP beta chain (ATPB) were identified by Western blot and SMG1, ANGP2, and MMSA were identified by immunohistochemistry.

Conclusions:
In a mouse model of lupus nephritis, expression of SMG1, MMSA, and ATPB were down-regulated, and RRAGB and ANGP2 were upregulated.

MeSH Keywords:
Lupus Nephritis • Proteomics • Two-Dimensional Difference Gel Electrophoresis

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Background

Systemic lupus erythematosus (SLE) is an autoimmune disease that is associated with the production of autoantibodies that result in immune damage to several organs, and commonly affects the kidneys [1–3]. The etiology and pathogenesis of SLE remain unclear, but genetic, epigenetic, and environmental factors have been described [4–6]. Between 40–70% of patients with SLE have clinical lupus nephritis [7]. Lupus nephritis is an immune complex-mediated glomerulonephritis, and the severity of renal damage determines the prognosis of lupus nephritis [8]. Up to 10% of patients with lupus nephritis develop end-stage renal disease, which is a leading cause of patient morbidity and mortality due to SLE [9].

Proteins are key players in cellular processes, and human diseases can be caused by abnormal protein expression at multiple levels. Therefore, understanding disease in proteomic terms can help to explain the molecular basis of disease, with the aim of disease prevention, diagnosis, and treatment. Mass spectrometry (MS)-based proteomics has made a significant contribution to understanding these basic biological processes and is able to measure hundreds of thousands of proteins in any biological system test platform [10]. During the past decade, with the application of MS-based proteomic technologies, discoveries have been made that have provided insight into the pathogenesis of some important diseases. Also, the development of proteomics databases has provided information about peptide and protein amino acid sequence and quantitative information for research studies [11,12]. SLE and lupus nephritis have also been studied with the use of proteomics techniques with urine being most commonly used in proteomics studies [13], followed by peripheral blood mononuclear cells [14], plasma [15], and renal cortical tissue [16]. However, the changes in protein expression at the level of the glomerulus in lupus nephritis remain unknown.

A new method has been developed that uses magnetic beads to isolate renal glomeruli from mice, which provides the opportunity for the proteomic profiling of the glomerulus [17]. However, this method can be expensive to use, and so a new and simpler technique that uses fewer magnetic beads but can obtain the same amount of protein has provided new opportunities for proteomic studies of the glomerulus [18]. Therefore, this study aimed to investigate the proteomic profiles of the glomerulus in the NZB/W F1 hybrid mouse model of mild and severe lupus nephritis using two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) combined with matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS).

Material and Methods

Animals

Specific pathogen-free (SPF) 12-week-old female NZB/W F1 hybrid mice (n=60) were purchased from the Jackson Laboratory (Bar Harbour, ME, USA) and SPF 12-week-old female C57BL/6 mice (n=60) were purchased from Beijing Vitonghua Laboratory Animal Technology Co., Ltd. NZB/W F1 hybrid mice were chosen because they spontaneously developed lupus nephritis. The mice were bred in a Chinese Medical University SPF animal room, at 23°C (±3°C), with a humidity of 50% (±20%), a 12-hour light and dark cycle, and free access to food and drink, in accordance with the guidelines of the Chinese National Standard (GB 14925-2001). The study complied with the protocols approved by the Institutional Animal Care and Use Committee at China Medical University (Approval Date: Dec14, 2016) (IACUC Issue No. 2016101). At the end of the study, all mice were euthanized with sodium pentobarbital anesthesia.

Animal groups

The onset of renal disease was monitored by weekly testing of fresh urine specimens with protein test strips. Mice were grouped according to the degree of proteinuria as +1 (10 mg/dl), +2 (100 mg/dl), +3 (300 mg/dl), and +4 (>1,000 mg/dl). Mice with proteinuria of 300 mg/dl or more in repeated tests were regarded as having severe proteinuria [19]. At the age of 28 weeks, the mice were divided into two groups based upon their degree of renal disease: light lupus nephritis (proteinuria ± – 4) and severe lupus nephritis (proteinuria ± 3 – ± 4).

The use of magnetic beads to separate the glomeruli

Isolation of the mouse renal glomeruli through the thoracic aorta was performed according to the method previously described, but with some modifications [17]. Briefly, ligation of the distal abdominal aorta and inferior vena cava was performed, followed by ligation of the superior mesenteric artery and the peritoneal artery. Thoracotomy was performed to insert an intravenous indwelling needle. A 0.3 cm opening was made close to the ligation of the inferior vena cava, and phosphate-buffered saline (PBS) at 4°C was used to perfuse the kidney.

Magnetic beads (Dynal Company, Union City, CA, USA) were used to perfuse the kidney, at a rate of 7.2 ml/min/gm of kidney tissue and using 4×10⁶ beads/ml. The renal tissue was moved and placed on ice and the renal cortex was sectioned into 1 mm³ slices. The remaining renal tissue was stored at −70°C. Collagenase A was used to digest the protein at 37°C, with the lysate passed twice through a mesh with a 100 µm pore size. The washed glomeruli were isolated using a magnet, with the entire procedure was performed on ice. The glomerular
proteins were isolated using a 2-D Clean-Up Kit (GE Healthcare Life Sciences, Logan, UT, USA). The protein content was determined using electrophoresis with an Ettan TM 2-D Quant Kit (GE Healthcare Life Sciences, Logan, UT, USA), according to the manufacturer’s instructions.

**Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) of glomerular proteins**

Ettaan IPGphor III (GE Healthcare Life Sciences, Logan, UT, USA) was used to perform the isoelectric focusing (IEF) program, referred to as the first electrophoresis. For each specimen that had been sampled from mice in the proteinuria ≤1+ group and proteinuria in the ≥3+ group, 50 μg of purified glomerular protein was labeled with 1 μl (400 pmol) of CyDye (GE Healthcare Life Sciences, Logan, UT, USA), and an internal standard (the equivalent mixture of the above two glomeruli protein samples) (50 μg), which was labeled with the same content of Cy2 on the same 2D gel. The fluorescent dye was incubated in the dark for 30 minutes. Then the gel was placed in the Typhoon TRIO scanner (Billerica, MA, USA) for image acquisition. SYPRO® Ruby protein gel staining (Thermofisher Scientific, Waltham, MA, USA) was used to run the second dimension of the 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein electrophoresis system (GE Healthcare Life Sciences, Logan, UT, USA), according to the manufacturer’s instructions. A score of >50 and fold change >1.5 times indicated a significant match and accurate identification of the proteins.

**Western blot**

The extraction method of glomeruli protein was the same as described above. The BCA method was used to determine the concentration of glomerular protein. SDS-PAGE protein electrophoresis was performed (the loading quantity of the sample was 35 μg) with a voltage of 15V for 2 h on the polyvinylidene fluoride membrane (PVDF), incubated in 5% dried skimmed milk powder (prepared with TBST) and mixed for 1 h. After 1 h, the membranes were washed three times with TBST for 10 min each, followed by incubation in the primary antibody, diluted 1: 200, overnight at 4°C. The membranes were then incubated with horse-radish peroxidase (HRP)-conjugated IgG secondary antibodies to Ras-related GTP-binding protein B (RRAGB), serine/threonine-protein kinase 1 (SMG1), angiopoietin 2 (ANGP2), methylmalonate semialdehyde (MMASA), and ATP beta chain (ATPB), and Annexin A5 (ANXAS) at a dilution of 1: 1000. The PVDF membrane was washed with TBST for 10 min and then incubated for 2 h in the secondary antibody. The images were captured using Beyo ECL Plus reagent (Beyotime, Shanghai, China). Western blot was performed in triplicate, and representative photomicrograph images were obtained. Chemiluminescent gel imaging system MicroChemi (DNR Co., Israel) was used to quantify the relative expression levels of the candidate proteins.

**Immunohistochemistry**

Paraffin sections of mouse kidney were deparaffinized with xylene and rehydrated in a graded series of alcohols. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 10 min. The sections were incubated with primary antibodies to ANGPT2, SMG1, and ANXA5 overnight at 4°C. HRP-conjugated and biotinylated secondary antibodies were added for 20 min at room temperature, incubated at 37°C for 30 min, and washed three times for 5 min each in PBS. Then, 100 μl of the brown chromogen, 3,3’-diaminobenzidine (DAB) (Solarbio Science & Technology Co. Ltd., Beijing, China) was added and incubated on the tissue section until a brown reaction developed. Tissue sections were counterstained, washed, dehydrated, and mounted for viewing by light microscopy. Histochemical stains used to study the histology of
the mouse model and control kidney tissue included hematoxylin and eosin (H&E), Periodic acid Schiff (PAS), and Masson's trichrome (Figure 1).

**Results**

**Histology of the kidney tissue of the NZB/W F1 hybrid mouse model of lupus nephritis**

The main histological changes of lupus nephritis of the NZB/W F1 hybrid mice were glomeruli mesangial cell proliferation and mesangial matrix hyperplasia. Compared with the mild proteinuria group, the severe lupus nephritis F1 mice showed glomerular mesangial cell proliferation, and glomerular sclerosis, cell nuclear fragmentation, and nuclear shrinkage (pyknosis), and neutrophil infiltration.
with neutrophil infiltrates. The glomerular basement membrane was thickened, and some tubules were necrotic (Figure 1).

Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) imaging

The gels were analyzed using a Typhoon Trio scanner (GE Healthcare Life Sciences, Logan, UT, USA) for two-dimensional differential gel electrophoresis (2D-DIGE) imaging. The dyed blue map using Cy2 was an internal standard group, the dyed green map using Cy3 is the severe proteinuria group, the dyed red map using Cy5 is the mild proteinuria group. The final image was created by the superposition of three fluorescent channels (Figure 1). If the color was yellow, the protein expression levels were close to each other; if it was red, the protein (Cy3) was upregulated; and if the color was green, the protein was down-regulated (Figure 2).

A total of 1630 protein points were matched, and a total of 56 differentially expressed proteins were obtained from 133 protein spots identified by mass spectrometry. After removing the damaged colloidal particles using an Ettan spot picker and comparing the two groups, there were 13 upregulated proteins and 15 down-regulated protein expression. Details of the proteins identified are shown in Tables 1 and 2.

Western blot analysis of representative candidate proteins

According to the 2-D and MS results, the following were selected, as the candidate proteins, Ras-related GTP-binding protein B (RRAGB), serine/threonine-protein kinase 1 (SMG1), angiopoietin 2 (ANGP2), methylmalonate semialdehyde (MMSA), and ATP beta chain (ATPB). Annexin A5 (ANXA5) was a positive control. Western blot was used to confirm these proteins (Figure 3).

Immunohistochemistry

Using positive staining for ANXA5, in mice in group 2 the protein expression was greater than for group 1. There were differences in expression levels of MMSA and ANGP2 between the two groups (Figure 4).
### Table 1. Comparison of upregulated protein expression in the renal glomeruli of the NZB/W F1 hybrid mouse model of lupus nephritis between the mild proteinuria group (group 1) (≤1+) and the severe proteinuria group (group 2) (≥3+).

| Spot ID | MW (Da) | Protein name | SWISS-PROT accession | Ratio | Protein score |
|---------|---------|--------------|-----------------------|-------|---------------|
| 1499    | 43620   | Ras-related GTP-binding protein B | RRAGB | 3.07 | 59            |
| 1476    | 40669   | cATP-dependent protein kinase catalytic subunit PRKX | PRKX | 2.47 | 53            |
| 1369    | 8930    | Protein FAM229B | F29B | 2.07 | 70            |
| 1327    | 96750   | A-kinase anchor protein 3 | AKAP3 | 1.93 | 50            |
| 1472    | 38937   | Annexin A2 | ANXA2 | 1.92 | 124           |
| 541     | 175669  | NACHT domain and WD repeat-containing protein 1 | NWD1 | 1.88 | 50            |
| 406     | 56996   | Angiopoietin-2 | ANG2 | 1.78 | 59            |
| 538     | 67839   | Moesin | MOES | 1.66 | 57            |
| 806     | 135640  | Wings apart-like protein homolog | WAPL | 1.6 | 50            |
| 795     | 129164  | Coiled-coil domain-containing protein 150 | CC150 | 1.6 | 50            |
| 1529    | 35787   | Annexin A5 | ANXA5 | 1.54 | 56            |
| 2072    | 19824   | Myosin regulatory light chain 12B | ML12B | 1.5 | 53            |
| 1588    | 36178   | Annexin A4 | ANXA4 | 1.51 | 101           |

### Table 2. Comparison of down-regulated protein expression in the renal glomeruli of the NZB/W F1 hybrid mouse model of lupus nephritis between the mild proteinuria group (group 1) (≤1+) and the severe proteinuria group (group 2) (≥3+).

| Spot ID | MW (Da) | Protein name | SWISS-PROT accession | Ratio | Protein score |
|---------|---------|--------------|-----------------------|-------|---------------|
| 1971    | 413559  | Serine/threonine-protein kinase SMG1 | SMG1 | 2.2 | 64            |
| 1377    | 89167   | Myotubularin-related protein 10 | MTMRA | 1.96 | 60            |
| 1335    | 36792   | Alcohol dehydrogenase [NADP(+)] | AK1A1 | 1.74 | 51            |
| 807     | 58335   | Methylmalonate-semialdehyde dehydrogenase | MMSA | 1.72 | 80            |
| 800     | 15119   | Profilin-1 | PROF1 | 1.66 | 60            |
| 2494    | 12035   | Pterin-4-alpha-carbinolamine dehydratase | PHS | 1.64 | 60            |
| 2628    | 149978  | Lysine-specific demethylase 3A | KDM3A | 1.64 | 52            |
| 141     | 130344  | Pyruvate carboxylase, mitochondrial | PYC | 1.61 | 66            |
| 844     | 59337   | Alpha-aminoacidic semialdehyde dehydrogenase | AL7A1 | 1.57 | 51            |
| 1331    | 39938   | Fructose-bisphate aldolase B | ALDOB | 1.56 | 64            |
| 439     | 22461   | Coiled-coil domain-containing protein 85B | CC85B | 1.54 | 54            |
| 443     | 12625   | NADH dehydrogenase [ubiquinon] a-subunit 7 | NDUA7 | 1.53 | 50            |
| 766     | 54751   | Dihydrolipoyl dehydrogenase | DLDH | 1.52 | 74            |
| 819     | 56265   | ATP synthase subunit beta, mitochondrial | ATP8 | 1.51 | 99            |
| 2625    | 61088   | 60 KDa heat shock protein, mitochondrial | CH60 | 1.51 | 84            |
Discussion

Proteomics complements genomics. Following the completion of the human genome project, scientists realized that many problems related to proteins cannot be completely explained solely by genomics. Protein ultimately implements the function of not only coding genes but also non-coding genes, including microRNA (miRNA) and long noncoding RNA (lncRNA).

The aims of this study were to investigate the proteomic profiles of the glomerulus in an NZB/W F1 hybrid mouse model of lupus nephritis using two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) combined with matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF-MS).

In this study, differences in fluorescence using 2D-DIGE combined with MALDI-TOF-MS proteomic demonstrated the protein profile of the glomerulus in a spontaneous mouse model of lupus nephritis, the NZB/W F1 mouse. A total of 56 differentially expressed proteins were screened during the development of lupus nephritis. As representative the expression of RRAG and ANGPT2 being up-regulated and the expression of SMG1, MMSA and ATPB being down-regulated were identified by western blot detection. SMG1, MMSA, and ANGPT2 were identified by Immunohistochemical method. These results suggest that these proteins may be involved in the progression of lupus nephritis.

Rag GTPases (include Rag A, B, C, D) belong to the family of guanine nucleotide-binding proteins, and Rag A and B, and Rag C and D can form four dimers [20,21]. Rag GTPases played an important role in the activation of the mechanistic target of rapamycin complex 1 (mTORC1) [21]. The formation of the dimer of Rag GTPases is crucial to the activation of mTORC1, and the role of Rag A/B seems to be more important than Rag C/D in the activation of mTORC1 [20,21]. Studies have shown that in the lysosome, Rag GTPases mediate signaling of amino acids to mTORC1 [22,23]. Recently mTORC1 has been shown to affect Th17 and Treg cell differentiation, and Th17/Treg imbalance can cause renal damage in SLE [24]. A study showed that RagA/B GTPases act as important components of the lysosome and can affect the function of cardiac myocytes [25].

In the present study, the proteomic profile of NZB/W F1 mice when the group with urine protein ≤1+ and the group with urine protein ≥3+ were compared, showed that the expression of RagA increased 3.07 times with increased proteinuria. This finding may suggest that at the onset of lupus nephritis, the mTORC1 pathway may have a role. Also, mTORC1 can regulate the phosphorylation of proteins, synthesize ribosomes and lipids, and prevents autophagy. It is possible that mTORC1 has a role in SLE and lupus nephritis through Rag GTPases.
SMG-1 is a newly-described member of the phosphoinositide kinase-like kinase (PIKK) family [26]. SMG-1 can regulate the proliferation of cells and maintain the genome. The stability or maturity of PIKK family members depends in part on a molecular chaperone called tel2-tti1-tti2 (TTT) [27], which, together with HSP90, participates in the proper folding of newly synthesized PIKKs [28]. As a regulator of the DNA damage response, PIKKs promote stability and maintain the activity of mTORC1 and mTORC2 complexes, which may regulate telomere length, SMG1, and mRNA expression in response to stress [29].

SMG-1 participates in epithelial-mesenchymal transition (EMT), triggered by activation of the Wnt pathway in gastric cancer, and EMT can be associated with chronic kidney damage [30].

ATPB is a subunit of ATP that is associated with oxidative stress, which is increased in lupus nephritis [31]. Studies have shown that the expression of ATPB differs between patients with SLE with and without lupus nephritis [32]. Angiostatin, an antagonist of angiogenesis, may down-regulate endothelial cell proliferation and migration by binding to the α/β-subunits of ATP synthase on the cell surface [33]. Therefore, some factors that affect ATP synthase may also affect kidney vascular endothelial cells and may promote the development of SLE and lupus nephritis.

Angiopoietin 2 (ANGP2) can mediate endothelial cell activity and is associated with the endothelial receptor tyrosine kinase, Tie-2 [34]. Increased levels of circulating ANGP2 are associated with renal involvement in SLE, and renal expression of ANGP2 is upregulated in patients with lupus nephritis [35]. These results support the findings of the present study. Increased serum levels of ANGP2 can reflect the extent of endothelial cell activation and are correlated with renal involvement in patients with SLE [36]. The present study was the first to investigate
the expression of ANG2 in the kidney in lupus nephritis using proteomics analysis.

MMSA expression has not been previously reported in SLE but has been described in other diseases, including methylmalonic aciduria [37]. Recently, MMSA was shown to promote Th1 cell-mediated immune responses and to induce dendritic cell activation, indicating that it may have a role in the immune response, including SLE and lupus nephritis [38].

Currently, MS-based proteomics analysis has identified 241 protein markers of SLE, including Annexin A5 (ANXA5) [16, 39], which is a marker for stage IV lupus nephritis. In the present study, both 2D and MALDI-TOF-MS proteomics analysis methods were used, and ANXA5 was also identified. However, it is likely that more proteins remain to be identified in lupus nephritis.

Conclusions

In this study, the proteomic profiles of the glomerulus in an NZB/W F1 hybrid mouse model of lupus nephritis were analyzed using two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) combined with matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF-MS). A total of 56 differentially expressed proteins were identified during the development of lupus nephritis in the mouse model. The expression of Ras-related GTP-binding protein B (RRAGB) and angiopoietin 2 (ANG2) were upregulated and the expression of serine/threonine-protein kinase 1 (SMG1), methylmalonate semialdehyde (MMSA), and ATP beta chain (ATPB) were down-regulated. The findings suggest that the expression of these proteins may be involved in the progression of lupus nephritis. Further studies are required to identify the range of proteins expressed and their role in lupus nephritis.

Conflict of interest

None.

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