

Annexin A2 and FTH1 are potential biomarkers for lupus nephritis

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Received May 11, 2018; Accepted July 20, 2018

DOI: 10.3892/etm.2018.6686

Abstract. Lupus nephritis (LN) occurs in ~50% of patients with systemic lupus erythematosus and is a major cause of morbidity and mortality of the affected individuals. Therefore, identification of novel and predictive biomarkers for the early diagnosis and progression of LN is required. The present study included 10 patients with LN whose diagnoses were confirmed by renal biopsy and 5 healthy participants as control subjects. Sera were collected both from patients with LN and healthy controls. Subsequently, mesangial cells were treated with these sera for 24 h. Differential proteins between groups were detected by two-dimensional difference gel electrophoresis (2D-DIGE) and matrix-assisted laser desorption/ionization time of flight mass spectrometry analysis. 2D-DIGE maps of cellular proteins were obtained for LN and normal control groups. A total of 45 proteins were characterized, and 2 low-abundance proteins were identified. Compared with the normal human sera group, expression level of Annexin A2 was elevated in patients with LN, while the expression of the

ferritin heavy chain (FTH1) decreased in the LN group; the analysis was carried out by DeCyder version 7.0 automatically. The results of the present study suggest that Annexin A2 and FTH1 contributed to the progression of LN and could serve as potential biomarkers for this disease.

Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by abnormal immune response leading to malfunction in several organs (1-3). Lupus nephritis (LN) occurs in ~50% of patients with SLE and is a major cause of morbidity and mortality among the affected individuals. Immunosuppressants are used for the treatment of LN; however, these are effective in only 50% of the affected patients (4,5). The therapy is usually associated with severe adverse effects, including increased risk of infertility and sepsis (6). Despite the overall improvement in the care of patients with LN during the past two decades, almost 20% of patients progress to end-stage renal disease within 10 years after the onset (7). Therefore, in addition to exploring more effective, less toxic drugs, it is essential to elucidate the molecular mechanisms underlying the pathogenesis of LN.

Preliminary studies have demonstrated the important role of autoantibodies, proinflammatory cytokines and toll-like receptors in the pathogenesis of LN (8-11). Previous studies also indicated that immune and inflammatory reactions in the glomerular mesangial cells (MCs) primarily lead to LN progression (12,13). However, the contributing mechanisms remain unclear. An *in vitro* model of LN was developed in the present study. MCs were treated with sera from patients with LN confirmed by renal biopsy. This model (derived from LN patient sera samples) mimics autoantibodies and other biological mediators, including anti-double stranded DNA antibodies, interleukin (IL)-12 and IL-18 cytokines that stimulate MCs leading to an immune response and inflammatory reactions. Previous studies focused on specific pathogenic factors in LN progression (14-16). The present study used a quantitative proteomic approach to elucidate the global alterations in protein abundance in MCs simulated by sera from patients with LN.

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Abbreviations: LN, lupus nephritis; SLE, systemic lupus erythematosus; MC, mesangial cell; 2D-DIGE, two-dimensional difference gel electrophoresis; MS, mass spectrometry; MALDI-TOF/TOF, matrix-assisted laser desorption/ionization time of flight tandem; IPG, immobilized pH gradient; NH, normal human

Key words: lupus nephritis, cellular proteomics, Annexin A2, ferritin heavy chain, biomarker

Several proteomics techniques have been used previously to investigate LN (17,18). Among these, two-dimensional gel electrophoresis, followed by mass spectrometry (MS) analysis is the most widely used method to analyze the expressions of different proteins; however, it exhibits low reproducibility and is time-consuming (19). Furthermore, this assay has low sensitivity for the detection of low abundance proteins with low molecular weight (LMW) <20 kDa. These LMW proteins may include important mediators which are expected to be involved in the progression of renal disease, including chemokines, cytokines and growth factors. By contrast, two-dimensional difference gel electrophoresis (2D-DIGE) is an assay that separates proteins according to their isoelectric point and molecular weight. With an internal standard, the 2D-DIGE technologies can be used to determine and quantify the proteins accurately, and the reproducibility of this method reduces the required number of biological replicates (20).

In the current study, 2D-DIGE combined with matrix-assisted laser desorption/ionization time of flight tandem (MALDI-TOF/TOF) MS was used to detect the differentially expressed proteins in MCs stimulated by sera of patients with LN. These proteins are candidate biomarkers of LN.

Patients and methods

Patients. A total of 10 patients with LN were recruited from the Division of Nephrology, First Affiliated Hospital of Guangzhou University of Traditional Chinese Medicine (Guangzhou, China). LN was confirmed according to the 1999 World Health Organization criteria (21). The classification of LN was based on the International Society of Nephrology and the Renal Pathology Society criteria established in 2003 and revised in 2004 (22). In addition, 5 healthy age- and sex-matched volunteer participants were included as normal controls. Based on the SLE disease activity index (SLEDAI) score, 5 class I LN (LN-I) patients with an SLEDAI score of 10-14 were collected, which indicated intermediate activity. Furthermore, 5 class IV LN (LN-IV) patients with an SLEDAI score of >15 indicated high activity. Written informed consent was obtained from each donor prior to enrollment in the study. The protocol was approved by the Ethics Committee of The First Affiliated Hospital of Guangzhou University of Traditional Chinese Medicine.

Serum sample collection. A total of 5 ml whole blood was collected from each subject and centrifuged at 2,200 x g for 10 min at 4°C (Heraeus™ Fresco™ 21 Microcentrifuge; Thermo Fisher Scientific, Inc., Waltham, MA, USA) (23). Sera were collected, filtered with serum filters (EMD Millipore, Billerica, MA, USA) and preserved at -80°C.

Cell culture and treatment. Human glomerular MCs were purchased from Shanghai Enzyme Research Biotechnology Co., Ltd. (Shanghai, China; cat. no. CC-Y1261; www.elisakits.cn/Index/productInfo/cid/153/id/1311.html). The cell culture was maintained according to the procedures described previously (24). Briefly, MCs were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F12 containing 5% fetal bovine serum (both Gibco; Thermo Fisher

Scientific, Inc.). After serum starvation for 24 h, MCs were treated with 7 ml DMEM/f12 and 3 ml sera from different individuals, which comprised the 30% sera. MCs were then cultured at 37°C for 24 h. Normal control MCs were treated with 7 ml DMEM/f12 and 3 ml calf serum (Gibco; Thermo Fisher Scientific, Inc.), which also comprised 30% serum. The subsequent experimental design is illustrated in Fig. 1.

Protein purification and determination. Whole-cell lysates were prepared using 2-D Clean-up kit (GE Healthcare Life Sciences, Uppsala, Sweden) to deplete salt, lipid and polysaccharides from the samples. The concentrations of protein samples were determined using Ettan™ 2-D Quant kit (GE Healthcare Life Sciences), according to the manufacturer's protocol.

Protein labeling. Sample labeling was performed using CyDye DIGE Fluor minimal dyes (GE Healthcare Life Sciences), according to the manufacturer's protocol. Briefly, 50 µg protein from each sample was mixed with 400 pmol of either CyDye DIGE Fluor Cy3 or Cy5. The internal standard was prepared by mixing equal volumes of different protein samples and labeling with CyDye DIGE Fluor Cy2. The labeling reactions were carried out on ice in the dark for 30 min and stopped by adding 1 µl of 10 mM lysine.

Protein separation by 2D-DIGE. Two DIGE gels were prepared including gel A and gel B. In gel A, the samples of normal calf serum (NC) group were labeled with Cy3 and samples of the normal human (NH) group with Cy5. In gel B, the lupus nephritis class I (LN-I) group samples were labeled with Cy3 and those of the lupus nephritis class IV (LN-IV) group with Cy5. The labeled protein samples were placed in 24-cm immobilized pH gradient (IPG) gel strips with pH 3-10. Each sample contained 50 µg Cy2-, Cy3- or Cy5-labeled proteins. The IPG strip was hydrated at 30 V for 12 h and the subsequent program was performed as follows: 100 V for 0.5 h, 500 V for 0.5 h, 1,000 V for 1 h and 5,000 V for 1 h, and then stabilized at 8,000 V under isoelectric focusing for 8.5 h. Following one-dimension electrophoresis, the IPG was stabilized in solution A (6 mmol/l Urea, 2% SDS, 75 mmol/l Tris-HCl pH8.8, 29.3% glycerol, 1% DTT, Bromophenol blue) for 15 min and then treated with solution B (6 mmol/l Urea, 2% SDS, 75 mmol/l Tris-HCl pH 8.8, 29.3% glycerol, 2.5% iodoacetamide) for 15 min. Following this, two-dimension electrophoresis was performed with 12.5% SDS-PAGE.

Gel scanning and image analysis. Typhoon 9400 scanner (GE Healthcare Life Sciences) was used to scan the gels following 2D-DIGE. The Cy2-, Cy3- and Cy5-labelled samples were scanned at wavelengths of 488/520, 532/580 and 633/670 nm, respectively. Samples were stained 0.25% Coomassie Brilliant Blue R-250 staining at room temperature for 2-4 h. Gels were then prefixed in 50% MeOH, 10% HoAc and 40% H₂O for 30 min to overnight. Staining was considered complete when the gel was no longer visible in the dye solution. Prior to complete staining, the gels appeared lighter against the dark staining solution. Samples were de-stained at room temperature using 5% MeOH,

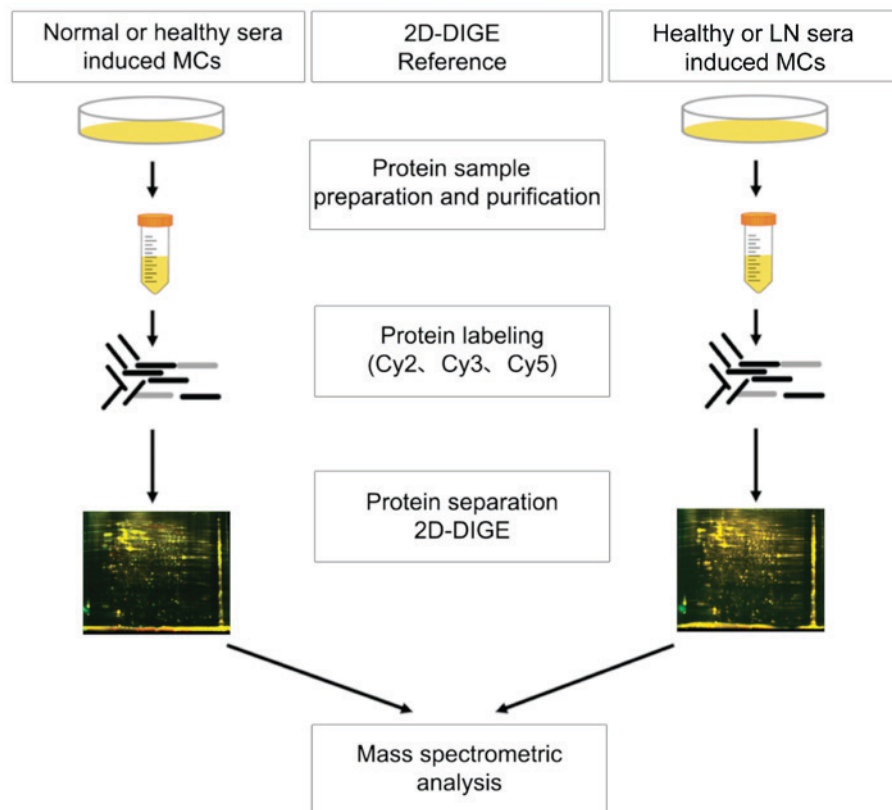


Figure 1. Schematic representation of the experimental protocol. MCs were treated with either normal calf, normal human sera or sera from patients with lupus nephritis patients for 24 h. Cells were subsequently harvested and proteins extracted. Proteins were labeled with CyDye DIGE tags following trypsin digestion. Matrix-assisted laser desorption/ionization time of flight tandem was used for peptide mass fingerprinting. LN, lupus nephritis; MC, mesangial cell; 2D-DIGE, two-dimensional difference gel electrophoresis; Cy, cyanine.

7.5% HOAC, 87.5% H₂O until the backgrounds were clear for 4-24 h. Bands began to appear in 1-2 h. This method detects as little as 0.1 µg/band. Gels were then stored in 7% HOAC. The Photomultiplier Tube (PMT) value with maximum gray level with respect to the whole gel or within the region of interest was in the range of 60,000-90,000 standards. (20) DeCyder 2D software (version 7.0; GE Healthcare Life Sciences) was used to analyze the 2D-DIGE gel images. The differential protein spots were identified based on the >1.5-fold difference in size (>1.5-fold upregulated or downregulated). After 2D-DIGE, proteins were digested with trypsin. The selected gel particles were collected using Ettan™ Spot Picker (GE Healthcare Life Sciences) and cryopreserved in 500 µl Eppendorf Tubes® at -20°C for subsequent MS.

MS analysis. The aforementioned gel particles were subjected to peptide mass fingerprinting (PMF) analysis by MALDI-TOF/TOF (ABI 4800 Proteomic Analyzer; Applied Biosystems; Thermo Fisher Scientific, Inc.). PMF was assimilated when protein content was within a range of 800-4,000 Da. Subsequently, 10 most intense peaks were selected to obtain tandem mass spectrometry (MS/MS) data. Conjunction search was conducted with MS and MS/MS. Results with a total score >64 and a match of >4 peptide fragments (best ion score >30; P<0.05) were accepted. The bioinformatics data of PMF by MS and MS/MS were searched by Mascot engine (Version 2.1, Matrix Science, Ltd., London, UK) in MSDB and Swiss-Prot database (25).

Properties of proteins. WoLF PSORT software (version of PSORT II; Piscataway, NJ, USA) was utilized to analyze the molecular function of proteins.

Search Tool for the Retrieval of Interacting Genes (STRING) protein-protein analysis. The STRING protein interaction database (version-10-5) was used to analyze associations among proteins. STRING is a protein-protein analysis database program that generates a network of interactions from a variety of sources, including different interaction databases, text mining, genetic interactions and shared pathway interactions. We used this search Tool for the Retrieval of Interacting Genes with a confidence cut-off of 0.6.

Western blot analysis. MC Protein expression was analyzed by western blotting as described previously (26). The following primary antibodies were used: Anti-Annexin A2 (cat. no. ab178677), anti-ferritin heavy chain (FTH1; cat. no. ab75972; both Abcam, Cambridge, MA, USA) and anti-α-tubulin (cat. no. T9026; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany).

Statistical analysis. Data are presented as the mean ± standard deviation. Comparisons between groups were made using one-way analysis of variance followed by Student-Newman-Kuels test. P<0.05 was considered to indicate a statistically significant difference. The statistical analyses were performed using SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA). In-gel difference analysis was performed

Table I. Clinical characteristics and demographic data of the study subjects.

Characteristic	NH group	LN-I group	LN-IV group
Individuals (n)	5	5	5
Age (years)	33.32±3.07	34.36±3.26	32.12±2.81
Sex (M/F)	1/4	1/4	1/4
Proteinuria (g/24 h)	0.05±0.02	1.59±0.45 ^a	3.39±0.85 ^b
Urine erythrocyte (10 ³ /ml)	0.2±0.1	34.8±14.1	61.4±20.2 ^b
BUN (mmol/l)	4.16±0.40	6.04±1.56 ^a	8.38±1.54 ^b
Scr (μmol/l)	43.2±11.6	108.7±56.3 ^a	123.4±76.5
ANA positive (%)	0	40	100
Anti-dsDNA positive (%)	0	40	100
Complement C3 (g/l)	1.10±0.15	0.72±0.11 ^a	0.47±0.13 ^b
Complement C4 (g/l)	0.50±0.05	0.18±0.06 ^a	0.13±0.04 ^b
SLEDAI (score)	0	10.6±2.7	19.8±2.6 ^b
Duration of LN (months)	0	9.2±1.3	7.2±2.5

^aP<0.05 compared with NH group, ^bP<0.05 compared with LN-I group. dsDNA, double stranded DNA; NH, normal human sera; LN-I, lupus nephritis class I; BUN, blood urea nitrogen; Scr, serum creatinine; ANA, anti-nuclear antibody; SLEDAI, systemic lupus erythematosus disease activity index.

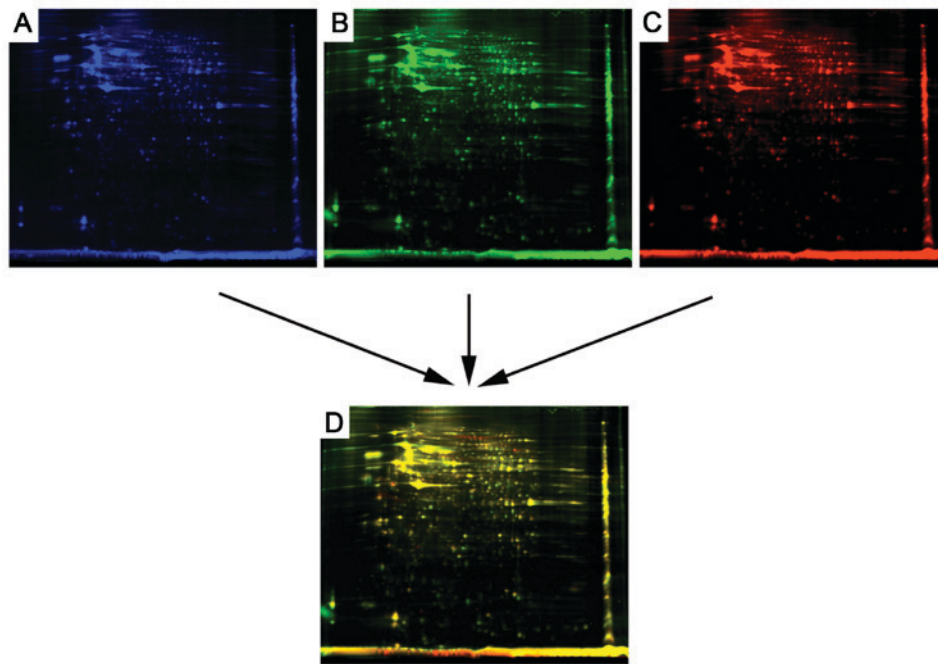


Figure 2. 2D-DIGE analysis of cellular proteins from the NC and NH groups. (A) Cy2 (blue) image of proteins from an internal standard (equal amounts of NC and NH samples). (B) Cy3 (green) image of proteins from the NC group. (C) Cy5 (red) image of proteins from the NH group. (D) False-colored DIGE gel image of cellular proteins from the NC and NH groups. The overlay image shows yellow spots containing proteins that exhibited similar expression levels in the two samples, red spots containing proteins with high expression and green spots with downregulated proteins. NC, normal calf sera; NH, normal human sera; Cy, cyanine; 2D-DIGE, two-dimensional difference gel electrophoresis.

using DeCyder 2D software (version 7.0) automatically; results were compared between gels.

Results

Clinical characteristics. Patients and normal healthy donors were well matched for sex and age (Table I). Active SLE patients (LN-IV) presented high levels of proteinuria, while

inactive SLE patients (LN-I) showed intermediate levels. SLE is more prevalent among females (27) and, therefore, there were more females than males included in the present study.

Proteomics results. The paired analyses of DIGE dye-labeled gels were shown in Figs. 2 and 3. The proteomic analysis revealed 56 differential protein spots between all groups (Fig. 4). Compared with the NH group, there were

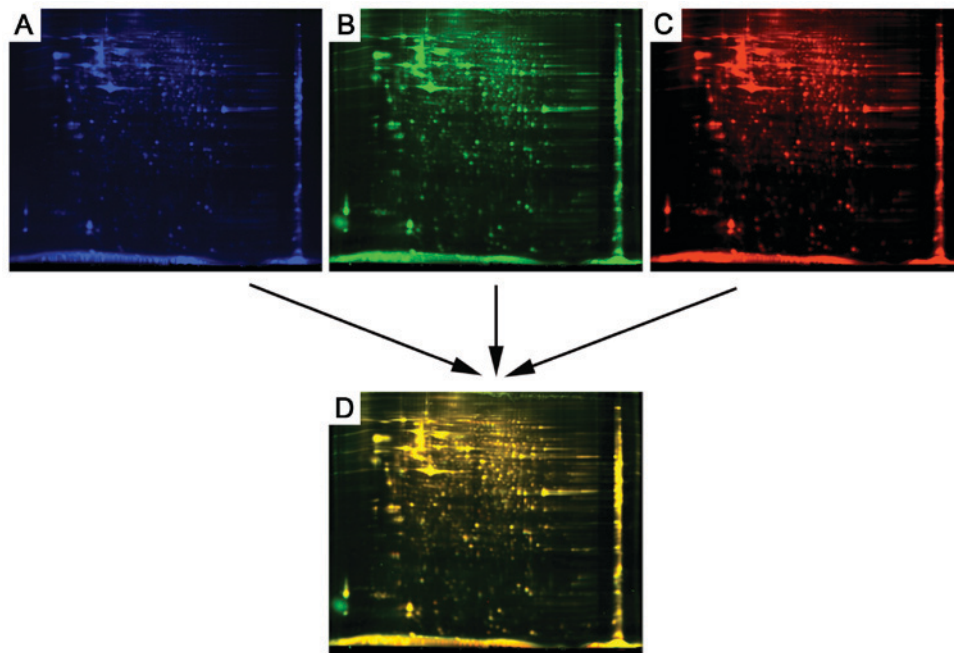


Figure 3. 2D-DIGE analysis of cellular proteins from the LN-I and LN-IV groups. (A) Cy2 (blue) image of proteins from an internal standard (equal amounts of LN-I and LN-IV samples). (B) Cy3 (green) image of proteins from the LN-I group. (C) Cy5 (red) image of proteins from the LN-IV group. (D) False-colored DIGE gel image of cellular proteins from LN-I and LN-IV groups. The overlay image shows yellow spots containing proteins that have similar expression levels in the two samples, red spots containing proteins with higher expression and green spots with downregulated proteins. LN-I, lupus nephritis class I; LN-IV, lupus nephritis class IV; Cy, cyanine; 2D-DIGE, two-dimensional difference gel electrophoresis; PI, isoelectric point.

17 upregulated and 9 downregulated differential protein spots in the LN-I group, and 25 proteins were upregulated and 19 downregulated in the LN-IV group. Furthermore, 14 differential protein spots were detected between the LN-I and IV groups. Therefore, a total of 45 proteins were characterized by mass spectrometry (Table II).

Properties of proteins. To elucidate the physiological roles of these proteins in LN, a subcellular localization software WoLF PSORT was used to analyze the molecular functions of these differentially expressed proteins (data not shown). The majority of differential proteins identified in the present study shuttle between the cytoplasm and nucleus, and may serve roles in the regulation of cellular immunity and inflammation during the process of LN.

Search Tool for the Retrieval of Interacting Genes (STRING) protein-protein analysis. STRING protein interaction database (version 10-5) was used to analyze the associations among proteins. STRING is a protein-protein analysis database program that generates a network of interactions from a variety of sources, including different interaction databases, text mining, genetic interactions, and shared pathway interactions. This analysis aided systematic understanding of cellular events in LN process. The networks formed by interacting proteins provided insights into the potential mechanisms of immunity and inflammation that may affect the etiology of LN. The STRING analysis revealed functional connections among 29 significantly regulated proteins in the HC and LN groups, LN-I and LN-IV groups (Fig. 5).

Validation of selected proteins. Since the primary aim of the present study was to identify proteins that may contribute to

the LN process, protein expression levels of Annexin 2 and FTH1 were determined by western blotting. Western blotting confirmed the results obtained from DIGE. Consistent with the aforementioned proteome analysis, protein expression of Annexin 2 and FTH1 significantly increased and decreased, respectively in MCs treated with sera from patients with LN compared with the NH group (Fig. 6).

Discussion

Alterations in the expression of specific proteins in the normal physiological state or during renal disease progression may be used to characterize the pathogenic states occurring during each phase of LN and provide information for diagnostic and prognostic purposes. The present study demonstrated the feasibility of 2D-DIGE combined with MALDI-TOF/TOF-MS to screen serial alterations in the cellular proteome of LN. A total of 56 differential protein spots were detected using 2D-DIGE, of which, 4 proteins could not be recognized in NC, NH LN-I and LN-IV groups, so a total of 51 were identified by MALDI-TOF/TOF-MS and 1 was not. Six protein spots were identified to be the same proteins. As a result, 45 differential proteins among normal human and LN groups were characterized. Some of these proteins were highly abundant in plasma and had been previously used in clinical diagnosis (28). Therefore, the present study focused on examining the proteins with low abundance and LMW in the cellular proteome in LN progression. In addition, the present study indicated that Annexin A2 and FTH1 were differentially expressed during different phases of LN.

Annexin A2 is a 36 kDa protein composed of an N-terminal domain and has a conserved C-terminal domain

Table II. Identification of differential proteins by MALDI-TOF-TOF MS^a.

Spot ^b	Protein name	Accession no.	Protein MW	Protein PI	Score CI%	Average ratio		
						LN-I/NH	LN-IV/NH	LN-IV/LN-I
799	Nucleoporin 210kDa	Q8TEM1	20589.5	6.33	76.144	1.27	1.62	1.28
829, 1299	Keratin, type II cytoskeletal 1	P04264	66170.1	8.15	100	-1.16	1.74	2.02
835	Elongation factor G, mitochondrial	Q9GRP9	84102.7	6.58	100	-1.19	1.64	1.95
990	SH2B adapter protein 2	O14492	68380.1	5.85	35.455	1.34	1.65	1.23
1060	78 kDa glucose-regulated protein	P11021	72402.5	5.07	100	1.57	2.39	2.13
1070	Serotransferrin	P05186	79280.5	6.81	100	1.22	-1.6	1.31
1255	Serum albumin	P02768	71317.2	5.92	100	-1.21	-1.86	-1.57
1650	Serine protease HTRA2, mitochondrial	O43464	48868	10.07	81.983	-1.58	-1.78	-1.12
1771	RuvB-like 2	Q9Y230	51295.6	5.49	100	1.28	1.52	1.19
1851	Calumenin	O43852	37197.2	4.47	96.522	-1.45	-2.02	-1.39
1969	Vimentin	P08670	53676.1	5.06	100	-1.47	-1.74	-1.19
1912	Proliferation-associated protein 2G4	Q9UQ80	44101.3	6.13	100	-1.17	1.32	1.54
2068	SPARC	P09486	35465	4.73	99.987	1.94	1.26	-1.54
2157	Poly(rC)-binding protein 1	Q15365	37987.1	6.66	100	1.53	1.26	-1.21
2196, 2282	Isocitrate dehydrogenase subunit alpha	P50213	40022.2	6.47	100	-1.37	-1.63	-1.19
2235	Syntaxin-18	Q9P2W9	38820.7	5.36	36.124	1.39	1.69	1.22
2295	Annexin A2	P07355	38807.9	7.57	99.939	1.52	1.85	1.51
2303	Elongation factor 1-delta	P29692	31216.8	4.9	56.538	-2.44	-2.44	-1
2320	F-actin-capping protein subunit alpha-1	P52907	33073.4	5.45	100	1.36	1.65	1.21
2359	60S acidic ribosomal protein P0	P05388	34422.9	5.71	99.571	1.54	1.71	1.11
2371	F-box/LRR-repeat protein 15	Q9H469	33547.7	7.13	90.54	-1.43	-1.56	-1.09
2394	Serine/threonine-protein phosphatase 2A	Q9Y5P8	65760.5	5.01	73.701	1.35	1.5	1.11
2495	Annexin A5	P08758	35971.4	4.94	100	-1.18	1.38	1.62
2591	Thiosulfate sulfurtransferase	Q16762	33635.9	6.77	13.19	-1.03	1.8	1.85
2598	CTP synthase 2	Q9NRF8	66320.3	6.45	51.215	-1	1.79	1.79
2602	Tropomyosin beta chain	P07951	32944.6	4.66	48.916	1.37	1.51	1.1
2649, 3581	Histone H2A type 1-B/E	P04908	14127	11.05	92.252	-1.42	1.18	1.67
2664, 3012	Proteasome subunit alpha type-1	P25786	29822	6.15	100	-1.82	-1.02	1.79
2826	NADH dehydrogenase ubiquinone 2	P19404	27659.1	8.22	100	1.66	1.43	-1.17
2949	Stromal cell-derived factor 2-like protein 1	Q9HCN8	23811.7	6.52	100	1.59	2.28	1.43
3044	Cerebellar degeneration-related protein	Q86X02	53377.2	5.7	80.918	-1.78	-1.13	1.57
3045	CoA hydroxylase-interacting protein	Q92561	38118.6	6.53	28.106	1.047	1.88	1.28
3059, 3094	Ferritin heavy chain	P02794	21383.4	5.3	99.816	-2.02	-2.44	-2.02
3108, 3170	Myosin regulatory light chain 12B	O14950	19823.5	4.71	99.952	1.77	1.58	-1.12

Table II. Continued.

Spot ^b	Protein name	Accession no.	Protein MW	Protein PI	Score CI%	Average ratio		
						LN-I/NH	LN-IV/NH	LN-IV/LN-I
3131	Trafficking protein particle complex subunit	Q9Y5R8	16934.6	9.23	51.141	-1.64	-1.42	1.16
3149	Thioredoxin domain-containing protein 12	O95881	19364.6	5.24	99.178	2.02	2.21	1.09
3150	Translocon-associated protein subunit delta	P51571	19157.7	5.76	99.996	1.19	1.67	1.4
3167	Protein canopy homolog 2	Q9Y2B0	20981.3	4.81	99.932	1.84	1.6	-1.15
3288	Eukaryotic translation initiation factor 5A-1	P63241	17291.9	5.76	95.725	-2.26	-2.5	-1.1
3294	Stathmin	P16949	17291.9	5.76	95.725	-2.26	-2.5	-1.1
3411	Mucin-16	Q8WXX17	2359682.5	5.65	70.554	1.85	1.03	-1.8
3417	Myosin light polypeptide 6	P60660	17090.2	4.56	100	1.62	1.64	1.01
3422	Aftiphilin	Q6ULP2	102935.2	4.4	33.692	1.67	1.76	1.06
3545	Protocadherin Fat 4	Q6V017	546287.2	4.77	83.754	3.19	1.41	-2.26
3640	Amphoterin-induced protein 2	Q86SJ2	58865.9	8.73	72.266	-1.4	-1.61	-1.15

^a Spots for which the volume ratio was >1.5 based on DeCyder software analysis; ^b Spots referring to Fig. 4. CI, confidence interval; MALDI-TOF/TOF MS, matrix-assisted laser desorption/ionization time of flight tandem mass spectrometry; NH, normal human sera; MW, molecular weight; LN-I, lupus nephritis class I.

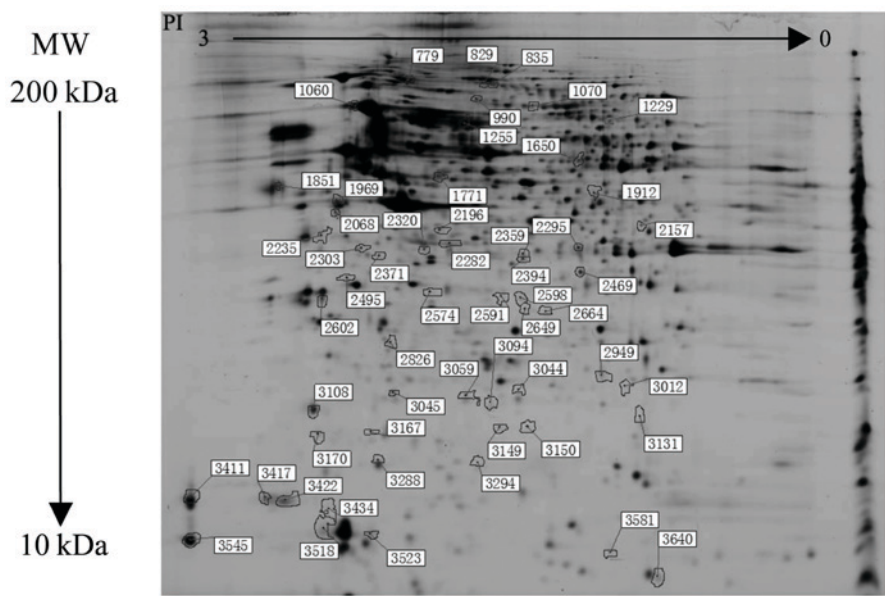


Figure 4. Two-dimensional electrophoresis (2-DE) protein profile of differential protein spots in four groups (Coomassie Brilliant Blue R-250 staining). The circles show the 56 differential protein spots. Spot numbers correspond to those in Table II. MW, molecular weight; PI, isoelectric point.

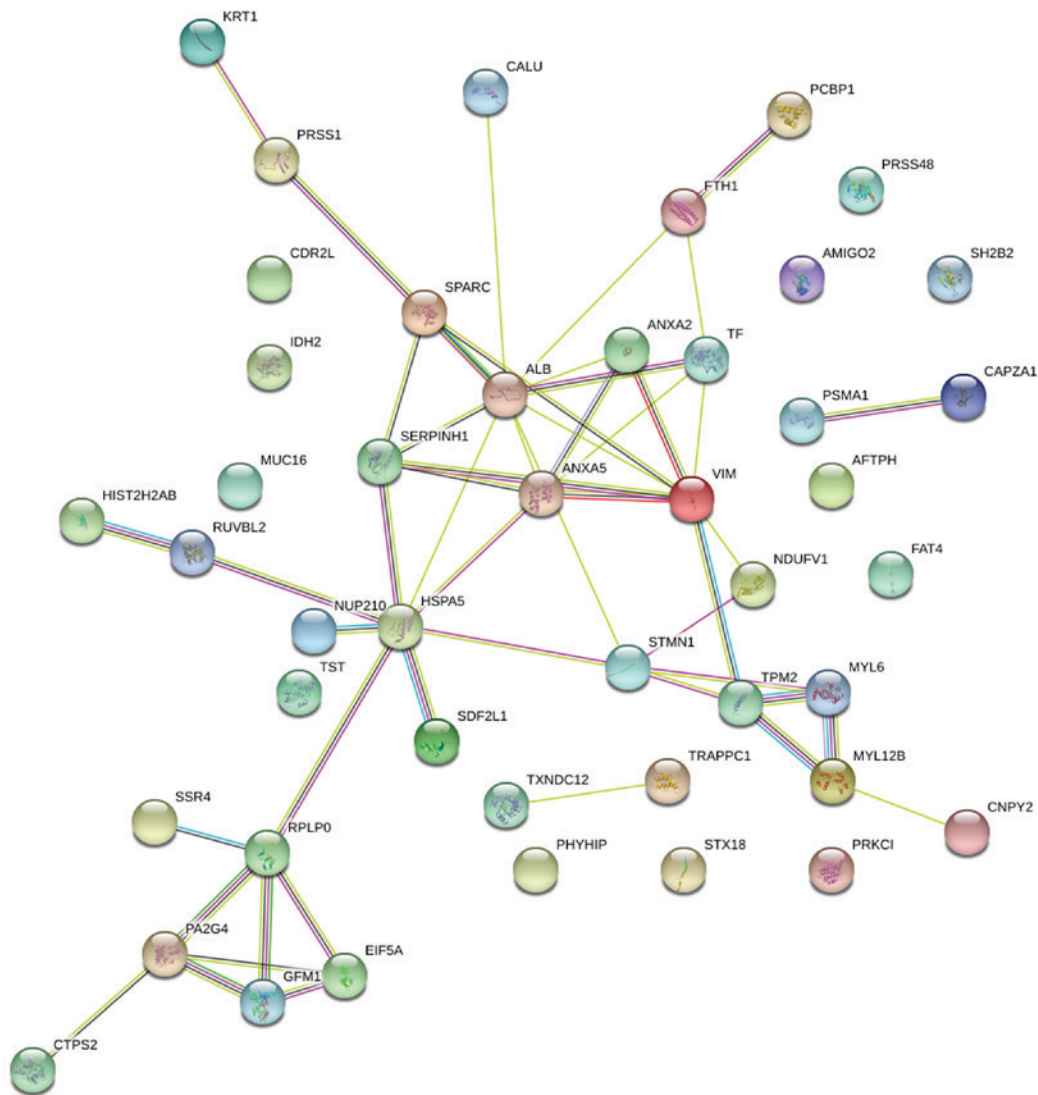


Figure 5. Predicted protein-protein interaction network between the 45 differential proteins. Interactions between proteins were mapped by Search Tool for the Retrieval of Interacting Genes with a confidence cut-off of 0.6. The thickness of edges represents the confidence level (0.6-0.9).

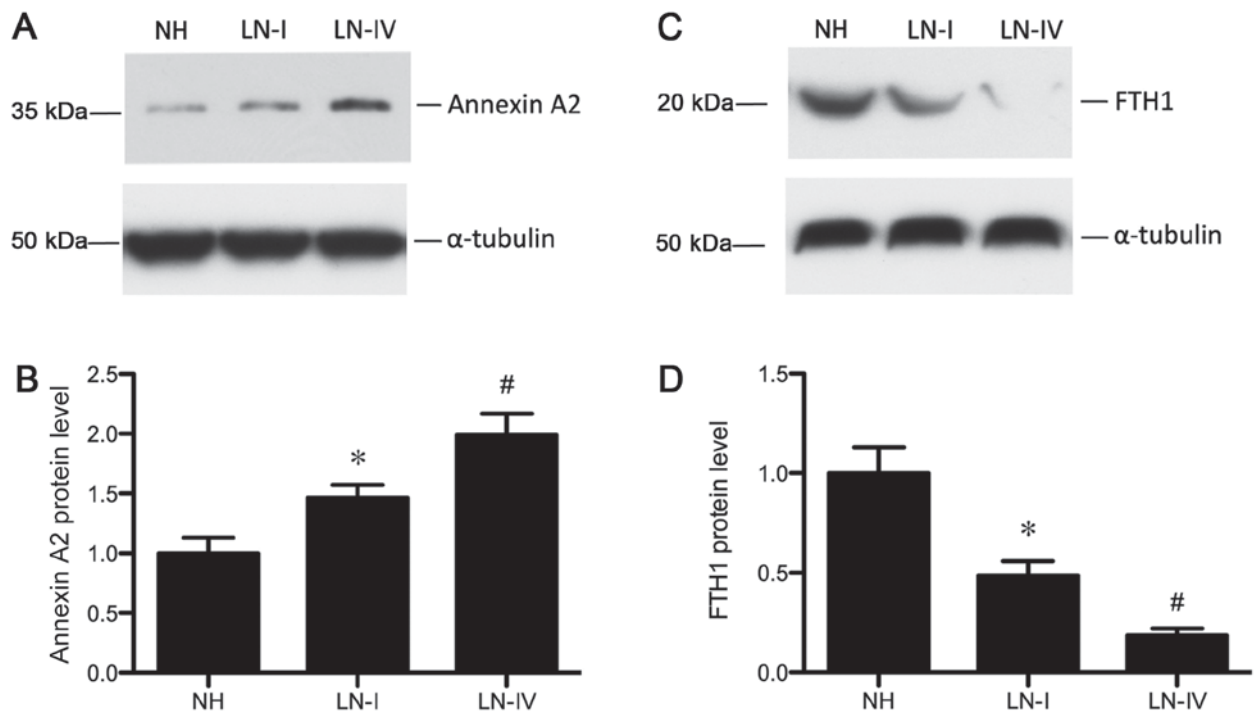


Figure 6. Validation of LN-associated proteins by western blotting. (A) A representative western blot of Annexin A2 expression. (B) Quantitative analysis indicated that treatment with sera from patients with LN increased the expression of Annexin A2 in mesangial cells compared with the NH group. (C) A representative western blot of FTH1 expression. (D) Quantitative analysis indicated that treatment with sera from patients with LN decreased the expression of FTH1 in mesangial cells compared with the NH group. * $P < 0.05$ vs. the NH group and # $P < 0.05$ vs. the LN-I group ($n = 3$). NH, normal human sera; FTH1, ferritin heavy chain; LN, lupus nephritis.

with Ca^{2+} binding sites. Annexin A2 belongs to a family of calcium-dependent phospholipid-binding proteins that serve roles in a variety of membrane-associated events including exocytosis, endocytosis, oxidative stress, apoptosis, cellular growth, cell proliferation and signal transduction (29-32). Annexin A2 has been implicated in the pathogenesis of acute kidney injury, including ischemia-reperfusion injury and folic acid-induced acute renal failure (33). Previous studies have shown that Annexin A2 serves a role in the development of renal inflammation and injury in patients with LN (34,35). Furthermore, the cellular proteome analysis of MCs induced with sera from patients with LN also demonstrated that, compared with the NIH group, the expression of Annexin A2 was significantly elevated. Furthermore, the induction of Annexin A2 increased with the progression of LN. Compared with the LN-I group, Annexin A2 exhibited a 1.63-fold increase in the LN-IV group, suggesting that it may participate in the development and severity of LN. The above results were further confirmed by western blotting. Western blot analysis supported the hypothesis that Annexin A2 could serve as a biomarker of LN.

Ferritin is an iron storage protein complex with two distinct types of chains: Light chain (L-ferritin) and heavy chain (H-ferritin). FTH1 is a 21 kDa subunit of the ferritin complex (36) FTH1 exhibits ferroxidase activity, which serves an essential role in catalyzing the conversion of the ferrous ions (Fe^{2+}) to the ferric form (Fe^{3+}) (37). FTH1 has been shown to protect proximal tube epithelial cells and kidneys against the activity of free iron in reactive oxygen species generation (38). Furthermore, a previous study also indicated that FTH1

suppressed the immune activity in autoimmune diseases in humans (39); the immunosuppressive function was dependent on IL-10 induction. Consistently, in the present study, FTH1 was downregulated in MCs stimulated with sera from patients with LN compared with the NH group. In addition, the expression of FTH1 in the LN-IV group was lower compared with the LN-I group, indicating that FTH1 may be associated with the progression of LN. Contrastingly, another study found that patients with SLE exhibited a high level of serum ferritin (40), which could be attributed to tissue specificity and immune activity. Therefore, the LMW protein FTH1 may be a suitable biomarker for LN.

In conclusion, the characterization of the dynamic alterations in protein expression at the cellular level provided an in-depth insight into the molecular pathophysiology of LN. The present study identified 45 differential proteins in MCs that were treated with different LN sera. Of these proteins, Annexin A2 and FTH1 may be associated with the progression of LN. However, additional studies on these proteins are essential in order to determine the level of sera or urine in patients with LN at different phases.

Acknowledgements

Not applicable.

Funding

The present study was supported by a grant from the National Science Foundation of China (grant no. 81600531), the Fujian

Provincial Science Foundation (grant no. 2017J01371) and Fujian Provincial Health and Family Planning Program for Young and Middle-Aged Talents Project (grant no. 2017-ZQN-92).

Availability of data and materials

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

Authors' contributions

YZ and LX performed the experiments, participated in data collection and drafted the manuscript. ST performed the statistical analysis and designed the present study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from each donor before enrollment in the study. The protocol was approved by the Ethics Committee of The First Affiliated Hospital of Guangzhou University of Traditional Chinese Medicine.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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