이 학 석 사 학 위 논 문

MRL/MpJ-*Fas*^{lpr}/J 마우스를 이용한 조기 루푸스 신염의 소변 바이오마커 발굴

Urine biomarkers for early detection of Lupus nephritis in MRL/MpJ-*Fas*^{lpr}/J Mice

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이 논문을 이학 석사학위 논문으로 제출함

2023 년 08 월

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Abstract

Background. Lupus nephritis (LN) is systemic lupus erythematosus (SLE) with kidney injury. The prognosis of LN remains poor if proper treatment is not provided in the early stage of LN. However, there are patients of early staged LN who are limited to renal biopsy, such as patients with normal proteinuria. Therefore, non-invasive biomarkers for early stages of LN are needed.

Objective. To discovery urine biomarker candidates and evaluate the usefulness of the candidates as biomarkers for early detection of LN.

Methods. Using SWATH LC-MS analysis, we found urine biomarker candidates that differentiate early-stage LN from SLE patients without nephritis. The candidates were measured in the urine of MRL/MpJ-*Fas*^{lpr}/J (MRL/lpr) mice at 13 and 23 weeks. Hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) and Masson Trichrome staining (MT) was performed on the kidney of 23-week-old mice for histological examination. The expression of ORM1 and SERPINC1 of the kidney was confirmed by immunofluorescence staining.

Results. Urine ORM1, SERPINC1, Transferrin and albumin were elevated in SLE patients with newly diagnosed LN compared with SLE patients without LN. Levels of urine SERPINC1 and ORM1 were also significantly higher in MRL/lpr mice than in MRL/MpJ mice at 13 and 23 weeks. In contrast, a considerable difference in urine transferrin and albumin between the two groups was only observed at 23 weeks, not at 13 weeks. Regarding the kidney pathology of MRL/lpr mice, urine ORM1 and albumin, but not urine SERPINC1, were positively correlated with the activity index and chronicity index.

Conclusions. We propose that urine SERPINC1 and ORM1 are novel biomarkers to detect early-stage LN. Additionally, we suggest that urine ORM1 could be useful for prediction of kidney chronicity and activity index in suspected LN patients that are limited to kidney biopsy.

Keywords: Lupus nephritis, Systemic lupus erythematosus, Biomarker, Proteome analysis, Serpin family C member 1 (SERPINC1), Alpha-1-acid glycoprotein (ORM1), Transferrin (TF), Interleukins

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1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease with organ involvement. SLE with kidney involvement is lupus nephritis (LN), a leading cause of mortality and morbidity [1]. A major feature of SLE is the production of autoantibodies such as anti-dsDNA antibody and anti-C1q antibody [2]. In LN, the autoantibodies form immune complexes with antigens such as apoptotic debris and deposit them in the kidneys. The deposited immune complexes (ICs) contribute to inflammation and kidney injury by activating complement, increasing leukocyte interactions, increasing autoantibodies, inducing cytokines and chemokines, and recruiting inflammatory cells [3].

Range of LN differ from silent LN that has normal proteinuria and renal function to acute LN that has severe proteinuria and renal failure. Nevertheless, Most of LN patients have mild proteinuria and/or hematuria is manifested by the appearance of proteins and red blood cell casts in the urine [4]. Thus, in many SLE classification criteria, renal involvement is defined through 24-hr urine protein excretion or urine protein-creatine ratio or presence of urine sediment such as red blood cell cast [5]. However, urinalysis is affected by collecting error and physical activity and does not necessarily reflect the severity of the histologic features of LN [6]. Kidney biopsy is the gold standard of LN diagnosis and confirms histological patterns that characterize LN subtype and determine chronicity and activity index. However, kidney biopsy cannot be performed in all suspected patients and risk of complications should be considered [7]. Especially, the renal biopsy is limited in suspected LN patients with normal proteinuria. Therefore, as a non-invasive method, discovery of novel biomarkers for early stage of LN is continuously needed.

Many SLE mouse model develop for last four decades including NZB/NZW F1, BXSB, Pristane-induced model. MRL/MpJ-*Fas*^{lpr}/J (MRL/lpr) mice are one of the spontaneous models of SLE. Background Genetics of the mice is complex, but there is lpr mutation of Fas receptor gene, which plays critical role in apoptosis of T cells and B cells. However, *Fas*^{lpr} simply accelerates development of disease does not cause in MRL/lpr mice. Nevertheless, MRL/lpr mice are used extensively for lupus nephritis experiment because they exhibit multiple manifestations of LN such as increasing autoantibodies, proteinuria, skin lesions, vasculitis, and fast disease progression [8, 9].

Traditionally, Biomarkers of LN have been selected based on the LN-associated pathophysiological pathway [10, 11]. For example, Autoantibody against dsDNA is one of the traditional LN biomarkers and has been used in clinical because of prove results that anti-dsDNA antibody is correlated with development of LN [12, 13]. Complements is critical role of LN pathogenesis that deposit immune complexes (ICs). Thus, C3 that is produced via alternative pathway and C4 that is produced via classical pathway are also traditional LN biomarker as complement activation marker. Proteinuria as a manifestation of LN patients has been used widely in clinical [14]. However, the biomarkers are unsatisfactory for detecting early stage of LN and estimating the progress of treatment [15]. LN prognosis is poor if proper treatment is not provided in the early stages of the disease [16-18]. Thus, there is a need to develop biomarkers to detect early-stage LN.

Profiling proteins by proteome analysis is an unbiased way to identify biomarkers [19]. Moreover, the techniques for high-throughput proteomic analysis have been greatly developed. Thus, proteome analysis has been applied in studies of biomarker and drug target discovery [20, 21]. Of the various quantitative proteomic analysis method, sequential windowed acquisition of all theoretical fragment ion mass spectra (SWATH-MS) is a specific acquisition method of the data-independent acquisition (DIA). SWAHT-MS can quantitatively analyze against peptides covering 1,000 proteins, and the results have a high quantitative consistency and accuracy. Therefore, it is ideal to study that use a lot of samples and require reproducible and accurate quantification for the main fraction of the expressed proteome or peptidome in each sample [22]. In addition, SWATH-MS combined with liquid chromatography is SWATH LC-MS, a novel mass spectrometry-based proteome analysis, has been used as a method that discovery biomarkers for diagnosis of disease [23].

In this study, we developed biomarkers for initial LN by validating biomarker candidates in human urine via proteomic analysis and evaluating the biomarkers in murine model of LN, MRL/lpr mice.

2. Materials and Methods

2.1 Patients and Samples collection for proteome analysis

Urine samples from 25 healthy controls (HCs) and 77 patients with SLE at Asan Medical Center, Seoul, Korea, were collected between January 2019 and August 2020. All patients with SLE met the 2012 Systemic Lupus International Collaborating Clinics classification criteria for SLE. The patients with SLE were classified into three groups: (i) SLE patients without nephritis (isolated microscopic hematuria and pyuria were not considered nephritis), (ii) initial LN patients (iLN): SLE patients with LN newly diagnosed at the time of urine sample collection, and (iii) LN patients: SLE patients previously diagnosed with LN. The Institutional Review Board of Asan Medical Center approved the study (IRB No. 2013-0405). Written informed consent was obtained from all patients. Urine was stored with Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific™) at -80℃.

2.2 Urinary sample preparation and proteome analysis

We first sought potential urine biomarkers for LN in humans by quantitative proteomics. Methods for urine sample preparation and proteome analysis were described in previous study [23]. In brief, 1 ml of urine samples were dried using CentriVap benchtop vacuum concentrators (Labconco) and reconstituted using 500ul of 5% sodium dodecyl sulfate buffer with 50mM triethylammonium bicarbonate (pH 8.5). After bicinchoninic acid assay, peptization was performed using S-trap mini (Profiti) as manufacture's instruction except trypsin/LysC (1:25 of protease: protein) setting. *In-house* urinary proteome library was generated using the pooled sample of peptide from all groups as described in previous study [23]. SWATH LC-MS analysis for individual urine sample using quadrupole time of flight mass spectrometry (TripleTOF® 5600+ System, Sciex) coupled with microflow LC (NanoLC 425,

Sciex) was performed using pre-described parameters. And resulting spectrum data was processed using DIA-NN (version 1.7.10) with *in-house* urine proteome spectral library to get relative quantitative information and identification. Resulting proteome quantities of each sample were normalized using creatine value of each sample. Receiver operating characteristics (ROC) analysis with normalized abundance of proteome was conducted using MedCalc (MedCalc Software Ltd, version 20.115) and additional ontology analysis and protein-interaction analysis using STRING (https://string-db.org/) was performed using the differentially expression proteins (DEPs).

2.3 Mice

A total of twenty-four MRL/MpJ (n=13) and MRL/lpr (n=13) mice were bred and maintained under the specific pathogen free (SPF) condition. Blood was incubated for 1 hour at room temperature (RT) and centrifuged for 15 minutes at 5,000 g and separated serum. Serum was collected every two weeks from 6 to 18 weeks and at 21 weeks Urine was collected from each mouse at 13 and 23 weeks. In 23 weeks, the mice were sacrificed, and kidney tissue was obtained. All animal experiments were performed in accordance with the guidelines for animal care of the Animal Experimentation Committee of Asan Institute for Life Sciences (IRB No: 2020-14-099).

2.4 Enzyme-Linked Immunosorbent assays (ELISA)

All ELISA were performed in accordance with the manufacturer's instruction. Serum anti-dsDNA antibodies were measured with a mouse anti-dsDNA antibody total IgG ELISA kit (Alpha Diagnostic). Briefly, all steps are performed at room temperature (RT). Diluted sample and standards were incubated in immobilized ds-DNA on a plate for 60 minutes and washed

wells 4 times. Next, diluted anti-mouse Ig HRP was added to each well and incubated for 30 minutes. After washing 5 times, the wells were incubated with TMB substrate for 10 minutes in dark. Finally, stop solution was added and measured absorbance of each well at a wavelength of 450 nm.

Levels of orosomucoid 1 (ORM1) in urine of mice are determined with mouse ORM1 ELISA kit (Abbexa). All incubation of procedures were carried out at 37℃. Diluted samples incubated in a microplate for 90 minutes and then discarded. Detection reagent A working solution added to each well and incubated for 1 hour and then washed 3 times. The plate incubated with Detection reagent B working solution for 30 minutes and washed 5 times. TMB substrate added and incubated away from light exposure for 20 minutes. Stop solution added, and the plate was read at 450 nm.

Mouse urine serpin family C member 1 (SERPINC1) was measured using mouse antithrombin III ELISA kit (abcam). The plate was incubated with diluted samples and standards for 2 hours and washed 5 times. Diluted biotinylated antithrombin III antibody was added to each well and incubated for an hour. The plate was washed with diluted wash buffer 5 times and incubated with SP conjugate for 30 minutes. After washing the plate, chromogen substrate was added to the wells and incubated in ambient light for 10 minutes. The plate with stop solution was read at 450 nm.

Transferrin (TF) levels in urine of mice were confirmed with mouse Transferrin ELISA kit (LSbio). Diluted samples, standards and blank were loaded to each well and incubated for 1 hour at 37℃. The wells incubated with detection reagent A for 1 hour at 37℃ after removing liquid and washed 3 times. Detection reagent B was added to each well. After incubating them for 30 minutes at 37℃, the plate was washed 5 times, then TMB substrate was added to washed wells followed by 10 minutes of incubation at 37℃. Finally, stop solution was added and measured absorbance of the plate at a wavelength of 450 nm.

Mouse urine albumin was measured using mouse albumin ELISA kit (abcam). Urine sample was 2000-fold diluted with diluent N. Diluted sample and standards were added to the microplate. After 2 hours of incubation, the wells were washed 5 times, then Biotinylated albumin antibody was added to each well and incubated 1 hour followed by washing the plate 5 times. The plate with SP conjugate was incubated for 30 minutes and then washed 5 times. Chromogen substrate was added to each well and incubated for 20 min in dark. Last, stop solution was added to the plate and detected absorbance at 450 nm. Creatinine of mouse urine was determined with Creatinine Colorimetric Detection kit (Enzo). Diluted samples, standards and creatinine detection reagent were added to the microplate and incubated for 30 minutes. The plate was measured absorbance of each well at 490 nm. Level of ORM1, SERPINC1, Transferrin and Albumin in the urine of mice were adjusted by creatinine concentrations.

2.5 Histologic analysis

Mice were anesthetized and performed perfusion through cardiac with PBS. To analysis kidney histologically, kidneys were fixed in 10 % neutral buffered formalin solution, followed by embedded in paraffin and sectioned into slice of 4-μm thickness. The sections were stained hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) and Masson Trichrome staining (MT). Standard protocols were used for H&E, PAS, MT. Pathologic lesions of the kidney were evaluated blind by an animal pathologist, based on National Institutes of Health (NIH) activity and chronicity indices [24].

2.6 Immunofluorescence staining

Using the Opal method (Perkin Elmer), two primary antibodies were applied sequentially to a single slide. After deparaffinization in xylene and rehydration in ethanol, antigen was retrieved by microwave treatment in citrate buffer (pH 6.0). Primary rabbit antibodies for ORM1 (1:200, LSbio) were incubated for 1 hour in a humidified chamber at room temperature (RT), followed by detection using Polymer HRP Ms + Rb. Visualization of ORM1 was accomplished using fluorescein opal 520 (1:100), after which the slide was placed in citrate buffer (pH 6.0) and heated by microwave. The slides were then incubated with primary rabbit antibody for SERPINC1 (1:100, LSbio) for 1 hour in a humidified chamber at RT, followed by detection using Polymer HRP Ms + Rb. SERPINC1 was visualized using opal 690 (1:100). Finally, the slides were again placed in citrate buffer (pH 6.0) and heated by microwave. Nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI) (1:500) and the sections were mounted to coverslips with mounting medium (Enzo). The stained slides were scanned with a SLIDEVIEW VS200 and OlyVIA (Olympus) and measured with HALO software (Indica Labs).

2.7 Statistical analysis

All analysis was performed using GraphPad Prism 8.4.3 (GraphPad software). Mann-Whitney U tests were performed for two-group comparisons. All result is represented as mean ± standard error of the mean (SEM). P values less than 0.05 were considered statistically significant.

3. Results

3.1 ORM1, SERPINC1 and Transferrin were more expressed in initial LN than SLE.

Initially, biomarker candidates in urine samples from 25 healthy controls (HCs) and 77 SLE (SLE without nephritis; 37, iLN; 17, LN; 23) patients were validated. Of the SLE patients, 62 patients (78.5%) were women. The median ages of each group were 51.0 years (IQR 37.5-56.3), 43.0 (IQR, 23.0-49.5), 39.0 (IQR, 22.0-50.0) for SLE without nephritis, iLN and LN patients, respectively, and the corresponding levels of proteinuria were 92.2 mg/g, 696.2 mg/g and 970.9 mg/g. Three of 17 (17.6%) patients in the iLN group had proteinuria less than 500 mg/g.

From quantitative proteome analysis for 4 groups of urine sample using SWTAH LC-MS, we selected proteins which increase in iLN group compared to HC group and SLE group. Considering that iLN is initial stage of lupus nephritis, proteins which increase in the urine sample at early stage of lupus nephritis patients can be a diagnosis biomarker. To select iLN urine biomarker candidates, hierarchical clustering analysis using fold change values of the quantified urinary proteins was performed for 5 kinds of combination (iLN/HC, LN/HC, SLE/HC, LN/SLE and iLN/SLE), respectively. From the resulting 4 clusters, cluster 4 showed protein expression patterns which increased in iLN group compared to HC and SLE group (**Figure 1A**). Protein interaction network analysis showed that of 23 proteins which increased in iLN group than SLE group, 19 proteins including SERPINC1, ORM1 and Transferrin (TF) showed a protein interaction network related to acute phase response, acute inflammatory response, and platelet degranulation (**Figure 1B**).

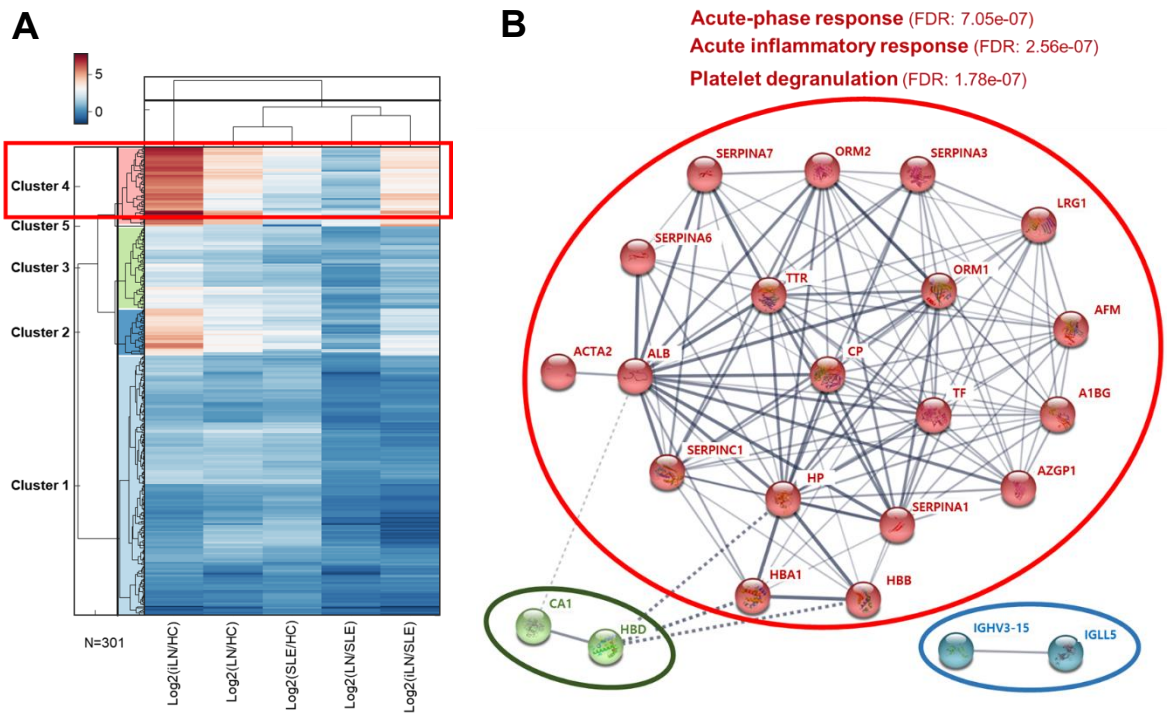

Figure 1. Quantitative urinary proteome analysis.

Hierarchical clustering analysis of the expression of proteins in the iLN group compared with the HC, SLE, and LN (A). The protein expression pattern of cluster 4 was higher in the iLN group than HC and SLE. Protein interaction map generated using DEPs that increased more than 2-fold with $p < 0.05$ in the iLN group compared with the SLE group, and their gene ontology (B). The red circle indicates a network of 19 proteins assigned to 3 biological processes (acute-phase response, acute inflammatory response, and platelet degranulation)

Abbreviations; HC, healthy controls; SLE, systemic lupus erythematosus; iLN, initial lupus nephritis; DEPs; differentially expressed proteins; FDR, false discovery rate.

In comparative analysis between iLN group and SLE group indicated that SERPINC1, ORM1 and TF showed iLN-increased expression pattern compared to SLE group. Statistically, *p* value SERPINC1 and ORM1 between iLN and SLE group was 0.006 and 0.003, respectively. *p* value TF between iLN and SLE group was less than 0.001. Area under curve (AUC) of receiver operating characteristics (ROC) for SERPINC1, ORM1 and TF between iLN group and SLE group was 0.8912, 0.886 and 0.941, respectively (**Figure 2A, B, C**).

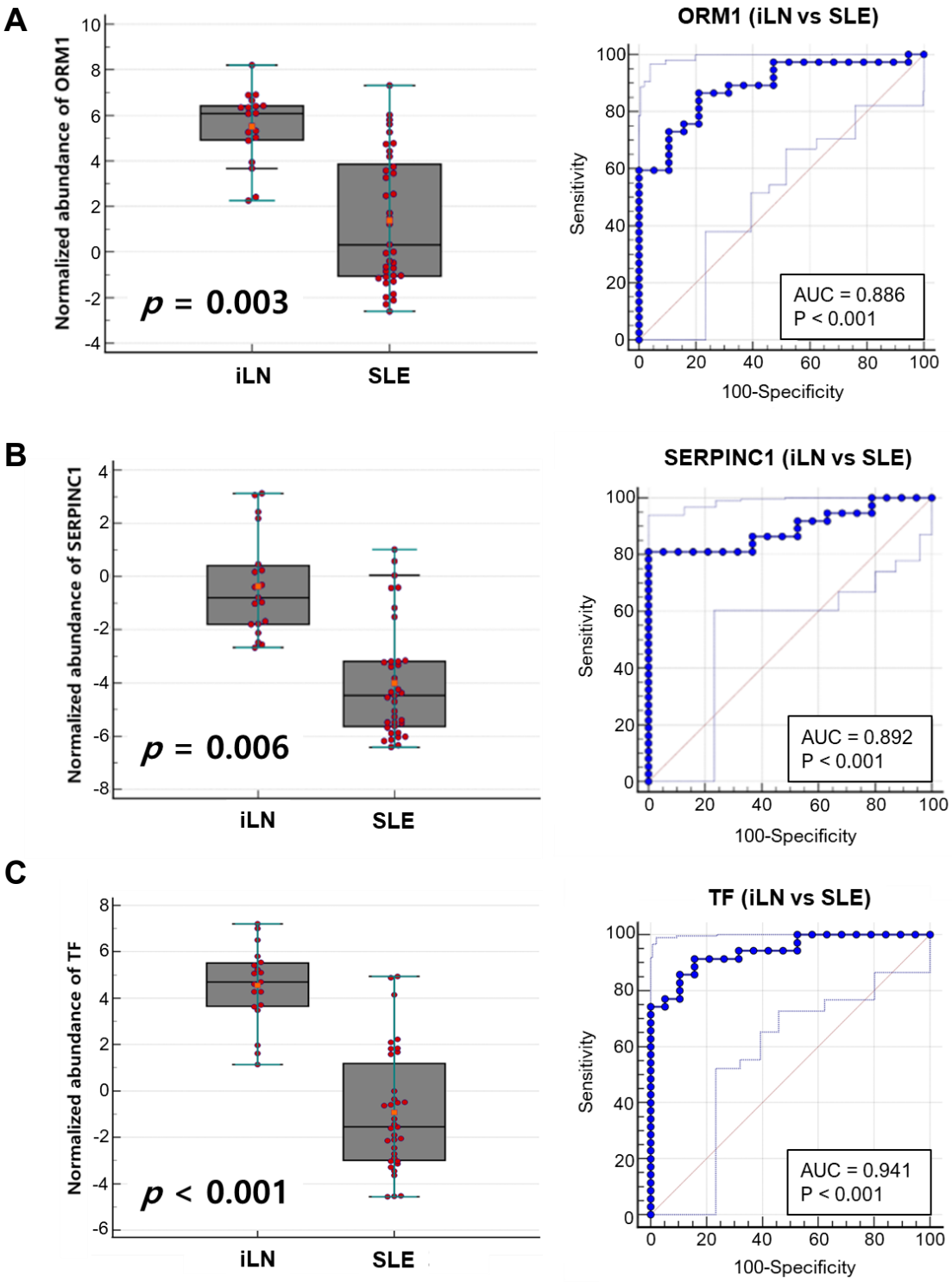
A
Normalized abundance of ORM1
p = 0.003
iLN
SLE
ORM1 (iLN vs SLE)
Sensitivity
100-Specificity
AUC = 0.886
P < 0.001
B
Normalized abundance of SERPINC1
p = 0.006
iLN
SLE
SERPINC1 (iLN vs SLE)
Sensitivity
100-Specificity
AUC = 0.892
P < 0.001
C
Normalized abundance of TF
p < 0.001
iLN
SLE
TF (iLN vs SLE)
Sensitivity
100-Specificity
AUC = 0.941
P < 0.001

Figure 2. Comparative urinary proteome analysis between the iLN and SLE group.

Box plots and ROC curves for ORM1 (A), SERPINC1 (B), TF (C). The 3 proteins showed protein expression pattern which higher in iLN than SLE. In ROC curve, AUC of ORM1, SERPINC1 and TF was 0.886, 0.892 and 0.941, respectively.

Abbreviations; ROC, receiver operating characteristics; iLN, initial lupus nephritis; SLE, systemic lupus erythematosus; ORM1, orosomucoid 1; SERPINC1, serpin family C member 1; TF, Transferrin.

3.2 ORM1 and SERPINC1 were increased in early-stage urine of MRL/lpr mice, but not Transferrin.

A major characteristic of LN is an increase in the level of anti-dsDNA autoantibody. Therefore, we measured anti-dsDNA autoantibody levels of mice to estimate the clinical activity for the mice. The autoantibody levels in mice were elevated in the MRL/lpr than MRL/MpJ mice from 8 weeks and increased over time in the MRL/lpr mice (**Figure 3**).

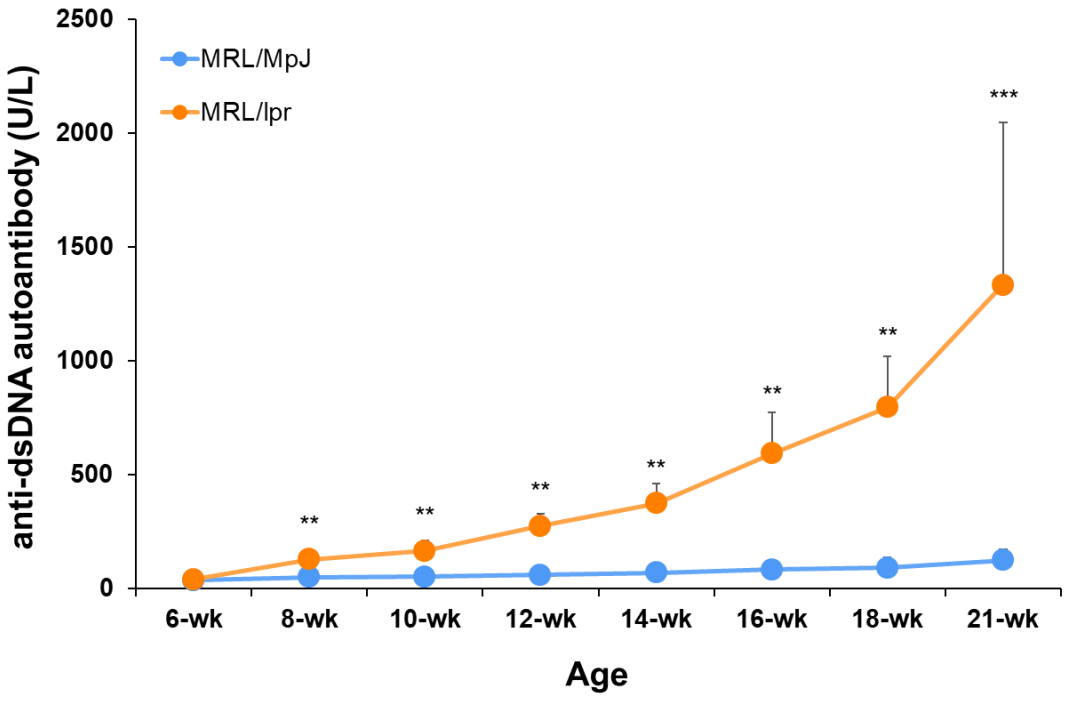

Figure 3. The levels of anti-dsDNA autoantibody in mice.

The autoantibody levels were determined every 2 weeks to 18 weeks and at 21 weeks. Individual dots represent the average of serum anti-dsDNA autoantibody levels in each group. From 8 weeks of age, autoantibody levels were higher in MRL/lpr than MRL/MpJ mice. The data are represented as mean with SEM in each group (** $p < 0.01$, *** $p < 0.001$).

To examine the usefulness of biomarker candidates that were validated via proteomics for early detection of lupus nephritis, their levels in the mice urine were analyzed with ELSIA at 13 and 23 weeks. The levels of ORM1 and SEPRINC1 in the mice urine were significantly higher in the MRL/lpr than the MRL/MpJ mice at 13 and 23 weeks (**Figure 4A, B**). However, the urine Transferrin levels were increased in the MRL/lpr mice at 23 weeks (**Figure 4C**). In addition, urine albumin was detected a significant difference between the two groups at 23 weeks (**Figure 4D**).

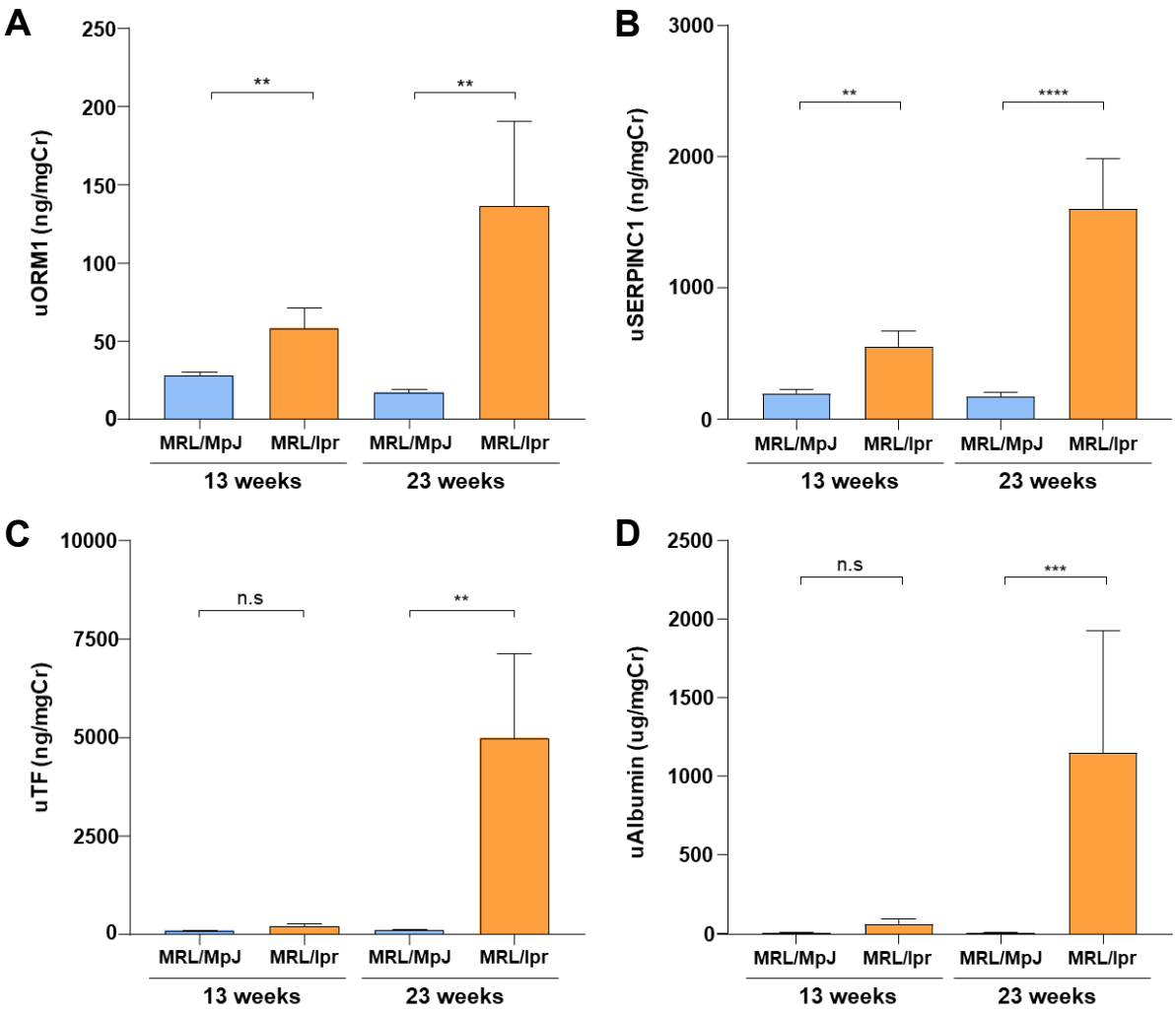

Figure 4. The levels of biomarker candidates and albumin in urine of mice.

Urine ORM1 (A), SERPINC1 (B), Transferrin (C) and albumin (D) were measured at 13 and 23 weeks of MRL/MpJ and MRL/lpr mice. Urinary levels of all candidates and albumin were increased in MRL/lpr mice at 23 weeks. At 13 weeks, only urinary ORM1 and SERPINC1 levels were increased in MRL/lpr mice. The data are represented as mean with SEM in each group (** $p < 0.01$, *** $p < 0.001$, ****$p < 0.0001$).

Abbreviations; uORM1, urine orosomucoid 1; uSERPINC1, urine serpin family C member 1; uTF, urine transferrin; uAlbumin, urine albumin.

3.3 Histologic analysis of MRL/lpr mice

We performed histology analysis of MRL/lpr mice. Interstitial inflammation and endocapillary hypercellularity, which are indicators of activity index, were observed in all kidney tissue of MRL/lpr mice. Interstitial fibrosis and global glomerulosclerosis, indicators of chronic index, were also observed in kidney tissue (**Figure 5A**). Activity index and chronicity index in MRL/lpr mice were median 8.0 (6.5-9.0), 2.0 (1.5-2.5), respectively. When we examined the expression of ORM1 and SERPINC1 in kidney tissue, SERPINC1 and ORM1 were primarily seen in the tubular membranes of immunofluorescence-stained kidney tissue (**Figure 5B**). The expression of ORM1 and SERPINC1 in kidney tissue was significantly higher in MRL/lpr than MRL/MpJ mice (**Figure 5C**).

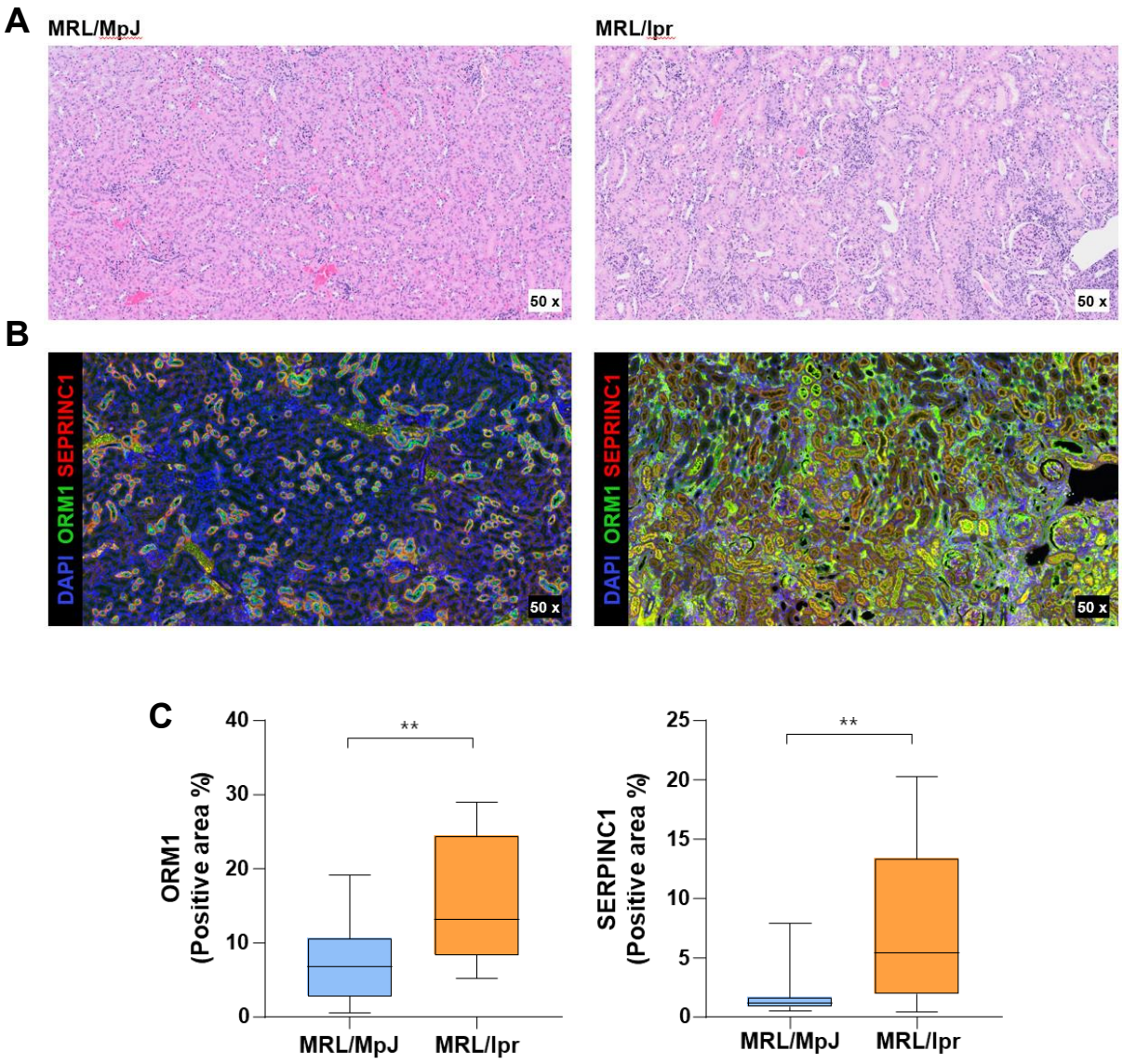

Figure 5. Histologic analysis of kidney at 23 weeks age of mice.

Hematoxylin and eosin (H&E) (A) and Immunofluorescence (B) staining of kidney tissue. The positive area of ORM1 and SERPINC1 in immunofluorescence-stained kidney tissue of mice (C). The data are represented as means with SEM in each group. (** $p < 0.01$)

Abbreviations; ORM1, Orosomucoid 1; SERPINC1, serpin family C member 1.

As shown in (**Table 1**), urine ORM1 was found to be correlated with the activity index and chronicity index in the kidney tissue of MRL/lpr mice. The correlation with the histopathology index was stronger than for urine albumin.

Table 1. Correlation between potential urine biomarkers and activity/chronicity indexes.

	Activity index			Chronicity index		
	Rho (95% CI)	R^2	P value	Rho (95% CI)	R^2	P value
Urine albumin	0.807 (0.410, 0.948)	**0.651**	**0.003**	0.869 (0.561, 0.965)	**0.755**	**< 0.001**
Urine SERPINC1	0.082 (0.545, 0.650)	0.007	0.811	0.022 (0.587, 0.619)	0.000	0.949
Urine ORM1	0.879 (0.590, 0.968)	**0.772**	**< 0.001**	0.947 (0.804, 0.987)	**0.897**	**< 0.001**

Abbreviations; ORM1, Orosomucoid 1; SERPINC1, serpin family C member 1; CI, Confidence interval.

4. Discussion

LN biomarkers have been recently discovered through different method such as genetic analysis, renal single-cell transcriptomics, and urine proteomic analysis [25, 26]. Especially, urinary biomarkers are discovered with proteomic analysis and have advantages that easily obtained non-invasive sample. These biomarkers are heavily discovered with proteomic analysis [27]. SWATH LC-MS analysis is one of the quantitative proteomic analysis methods and has several advantages, such as reproducibility, consistency, broad coverage, and sensitivity [28, 29]. Using SWATH LC-MS analysis, we found that urine level of ORM1, SERPINC1 and TF increased more in iLN than in SLE, supporting the validity of this platform and in agreement with previous studies identifying urine ORM1 and ceruloplasmin as biomarkers for active LN [30, 31]. Of the four ELISA-validated urine proteins, expression of ORM1 and SERPINC1 was significantly higher in iLN than SLE, in line with our previous study [32].

Of the 23 proteins which increased more in the iLN group than the SLE group, 19 proteins, including ORM1, SEPRINC1 and TF, were showed by protein interaction network analysis to be related to the acute phase response, acute inflammatory response, or platelet degranulation. The conclusions from signatures of the early stage of LN and that acute response pathway proteins such as ORM1 and SERPINC1 are potential biomarkers for monitoring the transition from SLE to iLN without invasive kidney biopsy.

ORM1 is the unique member of alpha-1-acid glycoprotein (AGP) as acute phase protein and activates monocytes, induces T cell proliferation, and promotes the secretion of pro-inflammatory cytokines such as tumor necrosis factor α (TNF- α), interleukin-1 (IL-1) and IL-6 [33-35]. But its biological role is poorly understood. Protein metabolic analysis of SLE patients identified 9 proteins including ORM1 and SERPINA1 that were elevated, causing modulation of the TP53 and AMPK signaling pathways [36]. Although its role in LN remains

unclear, previous proteomics studies highlighted urine ORM1 as a biomarker for active LN [30, 31]. Our present findings suggest that urine ORM1 is also a promising biomarker for early LN.

SERPINC1 that also known as Antithrombin III (ATIII) has anti-inflammatory effects by increasing the production of prostacyclin and inhibiting thrombin-induced inflammatory cascades [37]. Previous studies have identified a relationship between SERPINC1 and several kidney diseases, including nephrotic syndrome and acute kidney injury [38, 39]. However, its association with LN has not been previously recognized. We provide the first evidence that it could be used as a biomarker to reflect the underlying histology in patients with LN.

TF is an iron-bind protein and crucial role of systemic iron homeostasis as iron transporter [40, 41]. TF bound iron is filtered by glomerulus and almost reabsorbed completely by tubular epithelial cell [42]. In LN, TF bound iron are overly filtered by injured glomerulus and reabsorbed by tubular epithelial cell. As a result, excessive TF bound iron is mediated in tubular injury and renal failure [43]. In addition, urinary TF already is known as a biomarker of glomerular injury [44]. However, in our study, TF was not detected earlier in MRL/lpr mice. Kidney damage and proteinuria of MRL/lpr appears after 16 weeks [8]. It suggests that TF appears after kidney damage occurs. Therefore, urinary ORM1 and SERPINC1 is better than TF as biomarker for early-stage LN.

In proteome analysis, the 23 proteins which were higher in iLN group than HC and SLE group include hemoglobin subunit beat (HBB) and afamin (AFM). However, ORM1, SERPINC1 and TF were selected as urine biomarker candidates for early staged LN. Because some of the proteins such as HBB, HBD and APOH did not have commercially available kits in mice. Also, there were proteins such as AFM which was not detected in urine and renal histological analysis of MRL/lpr mice (data not shown).

Importantly, only 17.6% of patients in the iLN group had non-significant proteinuria (UPCR<500 mg/g). This, and the fact that the guidelines for LN suggest renal biopsy in patients with proteinuria over UPCR 500 mg/g [46, 47], indicates that we were able to identify most patients with early LN. In this regard, in the murine model of LN, urine SERPINC1 and ORM1 were already different from the control group at 13 weeks, at which point urine albumin was not significantly different from the control group. Therefore, the urine biomarkers identified here could be useful for early detection of LN, before, even, the development of proteinuria.

Urine ORM1 not only identified early LN, but also was strongly associated with histologic scores for kidney pathology. There have been attempts to discover non-invasive biomarkers to reflect the renal pathology of lupus nephritis. In our study, ORM1 level was strongly correlated with the renal histopathology index, as was urine albumin, and it was primarily expressed in tubular membranes. The role of ORM1 in inflammatory cascades and the fact that glomerular inflammation is a distinct pathologic hallmark of LN [48, 49], support ORM1 as a useful biomarker for early detection of LN and for the presumption of kidney damage.

As the results, the present study showed that ORM1 and SERPINC1 were higher in newly diagnosed LN patients than SLE without lupus nephritis but also ORM1 was correlated with histological scores of mouse kidney. Nevertheless, this study needs to be improved. We evaluated the usefulness of these biomarkers in lupus nephritis animal model. Thus, further work needs to examine a longitudinal patient cohort to confirm their clinical usefulness. In addition, the biological role of ORM1 and SERPINC1 in pathogenesis of LN is unclear and needs to investigate in further study.

5. Conclusion

In this study, we carried out development of the novel biomarkers to detect early-stage of LN and evaluating the usefulness of the biomarker candidates in mouse model of LN, MRL/lpr mice. ORM1, SERPINC1 and TF were higher detected in iLN than SLE patients' urine. However, ORM1 and SERPINC1 only were detected earlier in the LN murine model than urine albumin. Urine ORM1 level was correlated with degree of activity and chronicity index. Therefore, we propose that urine SERPINC1 and ORM1 are novel biomarkers for early detection of LN and suggest that ORM1 could be useful for prediction of renal chronicity and activity index in patients of early staged LN.

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국문요약

전신홍반루푸스는 젊은 여성에게 빈번하게 발생하는 자가 면역 질환 중 하나로 주로 루푸스 발생 5년 이내에 신장 손상이 발생하는데 이를 루푸스 신염이라 한다. 루푸스 신염의 진단방법에는 소변검사와 신생검이 있으나, 소변 검사는 신체적 활동과 소변을 모으는 시기에 따라 결과가 달라질 수 있기 때문에 정확한 진단은 신생검을 통해 이루어지고 있다. 그러나 신생검은 높은 검사비용, 출혈 등의 합병증의 위험이 있고, 일부 환자의 경우, 신염의 주요 증상 중 하나인 단백뇨가 충분히 나타나지 않는 경우가 있어 신생검을 진행하기에 어려운 경우가 있다. 루푸스 신염은 초기의 치료가 중요한 만큼 유의한 단백뇨가 나타나지 않는 초기 환자에서 진단에 필요한 신생검을 시행할 수 있는 근거를 마련하기 위해 비침습적 샘플인 소변 유래 바이오마커 발굴이 필요한 실정이다.

따라서, 본 연구에서는 비침습적 샘플인 루푸스 환자의 소변샘플에서 신염 예측 가능한 바이오 마커 후보군을 발굴하고 루푸스 신염의 마우스 모델인 MRL/MpJ-Fas^{lpr}/J (MRL/lpr) 마우스 소변에서 또한 그들의 유용성을 확인하였다.

본 연구에서는 정상인과 환자 소변의 SWATH LC-MS 분석을 통해 루푸스 신염 환자의 신염발생 예측 가능한 소변 바이오 마커의 후보 단백질들을 도출하였고 이 후보 바이오마커들을 13주와 23주령 MRL/lpr 마우스 소변에서 또한 재확인하였다. 조직학적 분석을 위해 23주령 마우스 신장을 헤마톡실린&에오신 (H&E), Masson's Trichome (MT), 과옥소산시프 (PAS) 염색법으로 조직 염색하여 신장질병의 활성도와 만성 정도를 평가하였다. 또한, 23주령 마우스 신장에서 면역 형광 염색으로 ORM1과 SERPINC1을 염색하여 단백질 발현 정도를 측정하였다.

연구결과, 환자의 소변 단백질 분석에서 전신홍반루푸스 환자의 소변에 비해 진단 초기 루푸스 신염 환자의 소변에서 ORM1, SERPINC1, Transferrin가 유의적으로 증가되어 있음을 확인하였고, 이들을 소변 바이오마커 후보군으로 선정하였다. 이후 루푸스 마우스 소변에서는 ORM1과 SERPINC1이 Transferrin, 알부민과 달리 13주 MRL/lpr 마우스 소변에서 유의적으로 증가된 것을 확인하였다. 마우스 신장 조직 분석결과 또한, 소변 ORM1과 알부민은 activity index, chronicity index와 양의 상관관계가 있음을 확인하였다.

따라서, 루푸스 신염의 조기 발견을 위한 소변 바이오마커로서 ORM1과 SERPINC1을 제안하며, 신장 조직 검사가 어려운 신염 의심 환자에서 소변 ORM1 측정이 진단 및 조직 만성도와 활성도 예측에 도움을 줄 것으로 사료된다.