의학석사 학위논문

루푸스에서의 단백뇨 악화를 예측하는 새로운 소변 생체표지자로써의 비타민D 결합단백

Urinary Vitamin D-Binding Protein as a Novel Biomarker for Lupus Nephritis Predicts the Development of Proteinuric Flare in Systemic Lupus Erythematosus

2016년 8월

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이 논문을 의학 석사 학위논문으로 제출함

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Abstract

Urinary Vitamin D-Binding Protein as a Novel Biomarker for Lupus Nephritis Predicts the Development of Proteinuric Flare in Systemic Lupus Erythematosus

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Background: Lupus nephritis (LN) is a major complication of systemic lupus erythematosus (SLE), which occurs in up to 50% of patients. Conventional biomarkers for assessing renal disease activity are imperfect in predicting clinical outcomes associated with LN. The aim of this study is to identify urinary protein biomarkers that reliably reflect disease activity or predict clinical outcomes.

Methods: A quantitative proteomic analysis of pooled urine samples was performed to identify urinary protein biomarker

candidates that can differentiate between SLE patients with and without LN. Selected biomarker candidates were further verified using urine samples from a larger cohort of SLE patients (n = 121) presenting with LN (n = 62) and without LN (n = 59) by enzyme-linked immunosorbent assay (ELISA) to investigate their predictive values for LN activity measure. Furthermore, association between urinary level of selected panel of potential biomarkers and prognosis of LN was assessed with a 4-year follow-up study of renal outcomes.

Results: Urinary vitamin D-binding protein (VDBP), transthyretin (TTR), and retinol binding protein 4 (RBP4) were significantly elevated in SLE patients with LN, especially in patients with active LN (n = 21), compared to those without LN. Among them, VDBP was well correlated with severity of proteinuria (Spearman's rho = 0.661, $P < 0.001$) and renal SLE disease activity index (renal SLEDAI) (Spearman's rho = 0.520, $P < 0.001$). In the 4-year follow-up, VDBP was found to be a significant risk factor (hazard ratio 9.627, 95% CI 1.698 to 54.571, $P = 0.011$) for the development of proteinuric flare in SLE patients without proteinuria (n = 100) after adjustments of multiple confounders.

Conclusion: Urinary VDBP was not only correlated with proteinuria and renal SLEDAI, but also predicted the development of proteinuria in SLE patients.

Keywords: Systemic lupus erythematosus, Lupus nephritis, Vitamin D-binding protein, Urinary biomarker, Proteinuric flare

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that can cause multi-organ failure. Approximately 50% of patients with SLE develop clinical renal disease (1). Given that lupus nephritis (LN) contributes to the increased morbidity and mortality of SLE patients (2, 3), early diagnosis and subsequent immunosuppressive therapy are crucial to prevent progression into renal insufficiency (4, 5). Renal biopsy is often required to confirm the diagnosis of LN and to determine the treatment strategy according to histologic features. However, it is rather invasive, occasionally leading to procedure-related complications like bleeding, pain, and arteriovenous fistula (6, 7). Although anti-double-stranded DNA (anti-dsDNA) antibodies and complement component 3 and 4 (C3 and C4) levels, as serologic biomarkers for systemic disease, are associated with the presence of LN, they have insufficient sensitivity and specificity for reflecting disease activity of LN (8). Therefore, there is a need for non-invasive biomarkers that could provide accurate information about disease activity of LN.

Urine is an ideal biological sample for biomarkers of LN since it is easy to harvest and urine proteins may be more specific for renal inflammation than serum proteins (9). Several urinary protein biomarkers, such as neutrophil gelatinase-associated lipocalin (NGAL) (10, 11), monocyte chemoattractant protein-1

(MCP-1) (12), tumor necrosis factor-like weak inducer of apoptosis (TWEAK) (13), and prostaglandin D synthase (PTGDS) (14, 15) have been reported as potential predictors of renal disease activity of SLE. However, the prognostic values of these biomarkers have not yet been fully evaluated for predicting LN flares.

Recent advance of mass spectrometry-based proteomics has facilitated highly sensitive measurement of urinary biomarker candidates for LN (16). Here, we used a global proteomic profiling experiment to identify potential biomarkers for predicting LN using pooled urine samples of SLE patients with or without LN. Moreover, a panel of candidate urine proteins was further quantified in clinical practice. We then investigated the association between the selected biomarker candidates and prognosis of LN in regard to response or flare of proteinuria.

PATIENTS AND METHODS

SLE patients with and without LN

We collected urine samples from two sets of independent cohorts in the Rheumatology Clinic of Seoul National University Hospital (SNUH): 1) 8 SLE patients with (n = 4) and without (n = 4) LN for proteomics analysis, 2) 121 SLE patients who fulfilled the 1997 American College of Rheumatology (ACR) criteria (17) for validation of biomarker candidates by enzyme-linked

immunosorbent assay (ELISA). LN was classified according to the 1997 ACR criteria for renal involvement, and biopsy-proven LN was further classified according to the World Health Organization (WHO) classification (18). SLE patients were divided into three groups: active LN (renal SLE disease activity index [SLEDAI] ≥ 4 (19) with random urine protein-creatinine ratio [UPCR] ≥ 0.5 mg/mg, equivalent to 24h urine protein ≥ 500 mg), inactive LN (UPCR < 0.5 mg/mg with previous history of active LN), and SLE without LN (UPCR < 0.5 mg/mg without previous history of active LN). The demographic and clinical characteristics including the laboratory findings within one month of urine sampling date (baseline) were obtained.

The patient's medical records were reviewed to examine the further occurrences of remission or flare of proteinuria after initial urine sampling for approximately 4 years. Remission of proteinuria was defined as return to random UPCR < 0.5 mg/mg in SLE patients with active LN (UPCR ≥ 0.5 mg/mg) (20). The definition of flare was the development of significant proteinuria, defined by UPCR ≥ 1.0 mg/mg or 24h urine protein ≥ 1.0 g, in SLE patients without definite proteinuria (UPCR < 0.5 mg/mg) (21, 22). This study was approved by the Institutional Review Board of SNUH (IRB No. 1208-086-422) and informed consent was obtained from study participants.

Urinary protein extraction for proteomics assay

Equal volumes (5 mL aliquot) of urine samples from patients were pooled for proteomics analysis. Following the general urinary proteomic methods (23), proteins were extracted from the pooled urine samples using the methanol precipitation. The protein pellets were suspended with 5 mM ethylenedinitrilo tetraacetic acid disodium salt (EDTA) in 50 mM Tris/HCl (pH 7.5) including protease inhibitor, and the protein amount was measured using the Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Then, albumin was depleted from the extracted proteins (1 mg each) using an Affinity Removal Spin Cartridge (Agilent Technologies, Wilmington, DE, USA). Following the manufacturer's protocol, urine protein samples were diluted 3-fold with depletion Buffer A (Agilent Technologies) and filtered through a 0.22 μm spin filter (Agilent Technologies). The diluted urinary protein samples were loaded onto the Spin Cartridge and centrifuged at 100 g for 1.5 min. The flow-through fraction was collected and concentrated using an Amicon 3 kDa molecular weight cutoff filter (Millipore, Bedford, MA). Depleted urinary protein concentration was measured again using the Micro BCA Protein Assay Kit (Thermo Scientific).

SDS-PAGE fractionation and in-gel digestion

Equal amounts of albumin-depleted proteins (100 μg) were boiled in an LDS sample buffer (Novex, San Diego, CA, USA), separated on a 4−12% Bis−Tris NuPAGE gel (Novex), and

stained with Coomassie Brilliant Blue (Sigma-Aldrich, St Louis, MO, USA). The entire gel lane was cut into 12 pieces and subjected to in-gel tryptic digestion following the general protocol (24). Briefly, excised protein bands were destained, reduced with 20 mM dithiothreitol (DTT), and then alkylated with 55 mM iodoacetamide. After dehydration with acetonitrile (ACN), the proteins were digested with 12.5 ng/μL modified trypsin (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate overnight at 37°C. Peptides were extracted from the gel slices with 50% (v/v) ACN in 0.1% (v/v) formic acid (FA). The eluates were dried under a Centrivap concentrator (Labconco, Kansas City, MO, USA) and stored at −20°C until use.

Mass spectrometry and database search

Extracted peptide samples were suspended in solvent A (0.1% formic acid in water, Optima LC/MS grade, Fisher Scientific, Pittsburgh, PA), loaded onto an EASY-Spray C18 column (75 μm × 50 cm, 2 μm) and separated with a 2-35% gradient of solvent B (0.1% formic acid in ACN) for 65 min at a flow rate of 300 nL/min. Mass spectrometry (MS) spectra were recorded on a Q-Exactive hybrid quadrupole-Orbitrap MS (Thermo Fisher Scientific, San Jose, CA) interfaced with a nano-ultra-HPLC system (Easy-nLC1000; Thermo Scientific, Waltham, MA). The Q-Exactive was operated in a data-dependent mode with one survey MS scan in the mass range of 300 - 1400 m/z using an

isolation width of 2.0 m/z, followed by 12 tandem mass spectrometry (MS/MS) scans, and the duration time of dynamic exclusion was 15 sec. Collected MS/MS raw files were converted to mzXML files using the Trans-Proteomic Pipeline (version 4.4) and analyzed using the Sequest (version 27) algorithm in the SORCERER (Sage-N Research, Milpitas, CA) platform. The search was performed using the IPI human database (version 3.83, 186578 entries). Full tryptic specificity and up to two missed cleavage sites were allowed. Mass tolerances for precursor ions and fragment ions were set to 10 ppm and 1 Da, respectively. Fixed modification for carbamidomethyl-cysteine and variable modifications for methionine oxidation were used. All proteins with a ProteinProphet probability of ≥ 99% with minimum two peptides and a PeptideProphet probability of ≥ 95% (FDR ≤ 0.15%) were identified (25). Relative protein quantitation was accomplished using spectral counting. The MS/MS data were normalized to compare the abundances of proteins between samples using Scaffold (version 4.5.3; Proteome Software, Portland, OR). The normalized spectral counts from duplicate liquid chromatography (LC) coupled with MS/MS (LC-MS/MS) runs were compared using the R program with power law global error model (PLGEM) software (http://www.bioconductor.org) in order to identify differentially expressed proteins (DEPs) (26).

Enzyme-linked immunosorbent assay (ELISA)

Potential biomarker candidates, selected from proteomic approach, were again quantified by ELISA. The urinary concentrations of vitamin D-binding protein (VDBP) and transthyretin (TTR) were measured by sandwich ELISA methods, using mouse monoclonal anti-VDBP antibody (Abcam, Cambridge, MA) and mouse monoclonal anti-TTR antibody (Abcam, Cambridge, MA), respectively. An indirect ELISA was performed to measure the urinary prostaglandin D2 synthase (PTGDS) concentration, using antibody solution of pre-mixed rat monoclonal anti-PTGDS antibody (Cayman chemical, Ann Arbor, MI). Urinary retinol-binding protein 4 (RBP4) concentrations were measured with a human RBP4 ELISA kits (R&D systems, Minneapolis, MN) in accordance with the manufacturer's instruction. A researcher who performed ELISA was blinded to the clinical data. Concentrations of urinary biomarkers were derived from an average of the duplicated assay and standardized to creatinine (Cr) concentrations in the same urine.

Statistical analysis

Mann-Whitney U test (median [interquartile range]) or student's t-test (mean ± standard deviations) were used to compare continuous variables, and chi-square or Fisher's exact test were used when comparing categorical variables, as appropriate. The relationships between urinary biomarkers and laboratory findings in LN were assessed with Spearman's rank correlation test.

Receiver operating characteristic (ROC) curves were used to calculate the area under the curve (AUC) for urinary biomarkers and to determine the cut-off values for prognostic prediction. Logistic regression and Cox proportional hazard regression with stepwise forward selection were used to predict the occurrence of remission and flare according to multiple covariates, respectively. Kaplan-Meier curves and log-rank tests were implemented to estimate the proteinuria-free survival rate with respect to low versus high level of urinary biomarkers. A P value < 0.05 was considered statistically significant. SPSS software version 22.0 (SPSS, Inc., Chicago, IL, USA) was utilized for data analysis.

RESULTS

Urinary proteome profiling for identification of biomarker candidates

To identify potential urinary protein biomarkers for differentiating between SLE patients with and without LN, we performed quantitative urinary proteome profiling of pooled urine samples in 4 patients with and without LN, respectively. After duplicated LC-MS/MS analysis, we merged the protein database search results and identified a total of 1373 proteins (Protein Probability $\geq$ 99.0%, minimum two peptides, Peptide Probability $\geq$ 95.0%, Peptide FDR $\leq$ 0.15%). Label-free quantitative proteome analysis based on spectral counting of identified proteins using PLGEM

software resulted in 153 differentially expressed proteins (DEPs) (P-value $\leq$ 0.001). Among them, 56 proteins were up-regulated (Table 1) and 97 proteins were down-regulated in patients with LN compared to patients without LN.

Considering the extensive level of divergence between SLE with LN and without LN, VDBP (signal-to-noise [STN] = 34.5, $P <$ 0.0001), TTR (STN = 38.6, $P <$ 0.0001), and RBP4 (STN = 104.6, $P <$ 0.0001) were selected for further evaluation using ELISA assay. Although urinary level of PTGDS (STN = - 9.6, $P =$ 0.001) is down-regulated in the SLE patients with LN compare to those without LN in our quantitative proteome analysis, which contradicts to its known expression in the urine of LN patients (27, 28), we included PTGDS as a reference in our ELISA-based verification experiment.

Baseline characteristics of SLE patients in a validation study

The demographic and clinical characteristics of patients (n = 121) included in ELISA assay are summarized in Table 2. The mean age of SLE patients was 41.9 ± 12.6 years and 14 (11.6%) were male. LN had been diagnosed according to the 1997 ACR criteria in 62 (51.2%) patients, including 46 (74.2%) patients with biopsy-proven LN. Patients with LN had a higher proportion of male, lower estimated glomerular filtration rate (eGFR) assessed using the Modification of Diet in Renal Disease formula (29) and

higher SLEDAI than did those without LN. Comparing with inactive LN, active LN (n = 21) was associated with younger age, short disease duration of SLE or LN, higher anti-dsDNA titer, and lower C3 level.

Levels of urinary biomarkers according to LN status

The ELISA analysis showed higher urine concentrations for each of the biomarker candidates in the patients with active LN (n = 21) when compared with those with inactive LN (n = 41); VDBP median 211.36 (interquartile range 15.19-609.71) ng/mgCr vs. 2.68 (0.61-13.76) ng/mgCr, TTR 69.48 (3.39-308.57) ng/mgCr vs. 0.54 (0.08-11.42) ng/mgCr, RBP4 1086.80 ng/mgCr (164.46-5859.26) vs. 90.72 (62.70-339.47) ng/mgCr, and PTGDS 2306.84 (961.45-19336.52) ng/mgCr vs. 701.22 (340.02-1250.76) ng/mgCr (Figure 1). Interestingly, urine concentrations of biomarker candidates except VDBP in patients with inactive LN (n = 41) were also significantly higher compared with those in patients without LN (n = 59); TTR 0.54 (0.08-11.42) ng/mgCr vs. 0.13 (0.01-4.53) ng/mgCr, RBP4 90.72 ng/mgCr (62.70-339.47) vs. 55.27 (12.68-129.92) ng/mgCr, and PTGDS 701.22 (340.02-1250.76) ng/mgCr vs. 335.60 (132.32- 709.07) ng/mgCr (Figure 1). When we analyzed patients with biopsy- proven LN (n = 46) only, the comparison results of urinary biomarker levels according to LN status were similar to the above-mentioned findings (Figure 2).

Association between urinary biomarkers and laboratory findings in LN

The concentrations of each of the biomarker candidates were analyzed for correlation with disease activity of SLE patients with LN (n = 62) (Figure 3). They increased significantly in direct proportion to the severity of proteinuria, as estimated by UPCR in a random urine sample, and renal SLEDAI (range 0-16). In particular, VDBP was best correlated with UPCR (Spearman's rho = 0.661, $P < 0.001$) and renal SLEDAI (Spearman's rho = 0.520, $P < 0.001$). RBP4 (Spearman's rho = -0.500, $P < 0.001$) showed an inverse correlation with eGFR, but other biomarker candidates (VDBP, TTR, and PTGDS) did not. Urine concentrations of any biomarkers were not associated with serum anti-dsDNA titer and complement levels.

Prediction of remission of proteinuria in active LN

Having demonstrated the performance of the biomarker candidates in differentiating LN status and reflecting disease activity, we sought to assess the predictability of remission in active LN patients (n = 21) treated with immunosuppressive therapy. Seven patients were treated with cyclophosphamide (CYC) pulse therapy, one patient was treated with mycophenolate mofetil (MMF), and 4 patients were treated with tacrolimus. All patients received corticosteroids (CS) and 13 patients received

angiotensin converting enzyme (ACE) inhibitor or angiotensin receptor blocker (ARB). One patient was lost to follow-up.

Urine concentrations of RBP4 were lower in patients who achieved remission within 12 months (n = 9) than in those who did not (n = 11) (223.78 [98.17-514.01] ng/mgCr vs. 3068.81 [1086.80-18210.22] ng/mgCr, P = 0.020). Other biomarker candidates (VDBP, TTR, and PTGDS) were not significantly different between the two groups (Figure 4). An ROC curve showed that urine concentration of RBP4 had a good predictive value with an AUC of 0.808. A cut-off value for RBP4 of 800 ng/mgCr was chosen, based on optimization of sensitivity and specificity (sensitivity 0.82 and specificity 0.89). In univariate logistic regression, elevated urine concentration of RBP4 was associated with remission failure of proteinuria (odd ratio 0.028, 95% confidence interval [CI] 0.002 to 0.367, P = 0.007). However, it was not significant after adjusting for covariates, such as age, sex, disease duration of LN, comorbidities, anti-dsDNA titer, serum complements, and induction treatment regimens (Table 3).

Prediction of future proteinuric flare in SLE without proteinuria

We also assessed the predictability of flare in patients with inactive LN (n = 41) and without LN (n = 59). These patients (n = 100) were followed up for a mean duration of 43.1 ± 6.9 months. Twenty-four patients received additional

immunosuppressive treatments with CS; CYC in 3, MMF in 3, azathioprine (AZA) in 6, cyclosporine A (CsA) or tacrolimus in 14 patients. ACE inhibitor or ARB were given to 15 patients and hydroxychloroquine (HCQ) was given to 75 patients. In patients who developed proteinuric flare (n = 15), mean duration until flare was 23.7 ± 12.7 months.

SLE patients with flare had a higher urine concentrations of VDBP (4.32 [2.02-28.34)] ng/mgCr vs. 1.14 [0.23-9.94] ng/mgCr, P = 0.024), RBP4 (141.09 [68.25-333.21] ng/mgCr vs. 68.37 [20.19-172.39] ng/mgCr, P = 0.036), and PTGDS (795.46 [343.99-2486.83] ng/mgCr vs. 414.27 [181.71-803.61] ng/mgCr, P = 0.008) than those without flare. According to ROC analysis, the optimal cut-off values for predicting proteinuric flare were 1.90 ng/mgCr for VDBP (AUC 0.683, sensitivity 0.80, and specificity 0.60), 51.85 ng/mgCr for RBP4 (AUC 0.671, sensitivity 0.93, and specificity 0.42), and 294.20 ng/mgCr for PTGDS (AUC 0.715, sensitivity 0.93, and specificity 0.40), favoring higher sensitivity as screening assay (Figure 5). Using these cut-off values in the Kaplan-Meier analysis, patients with higher urine concentrations of biomarker candidates showed a considerably increased rate of flare compared with those with lower concentrations (Figure 6). In multivariate Cox regression, higher urine VDBP (hazard ratio 9.627, 95% CI 1.698 to 54.571, P = 0.011) was significantly associated with future development of significant proteinuria (UPCR ≥ 1.0 mg/mg) after adjustment for age, sex, disease

duration of SLE, previous history of active LN, comorbidities, anti-dsDNA titer, serum complements, and immunosuppressive treatment. Younger age was also at enhanced risk of developing proteinuria in SLE (Table 4).

DISCUSSION

In the present study, we described the identification of urinary biomarker candidates for LN using proteomics and verified their clinical utility in SLE patients by ELISA-based quantification. When analyzed with quantitative measurement in a larger subset of 121 SLE patients, increased levels of these biomarker candidates could indicate the presence of active LN. Considering the robust correlation with UPCR or renal SLEDAI, these proteins were eligible for organ-specific biomarkers of LN. Increased urine VDBP was a risk factor predicting proteinuric flare in SLE patients.

Of the four biomarkers in our panel, VDBP, TTR, and RBP4 proteins were found to be highly elevated in the pooled urinary proteome analysis of SLE patients with LN compared to those without LN. Among them, VDBP and TTR have not been reported about the clinical significance in LN. Although PTGDS is known as a urinary biomarker elevated in the urine of patients with active LN (27), we unexpectedly detected decreased level of PTGDS excretion in our pooled urine samples of SLE patients

with LN. However, ELISA-based PTGDS abundance measurement was the reverse of the result obtained by the proteome analysis, which is consistent with other studies (14, 15). One possible explanation for the contradictory quantification readouts between the two methods could be that the antibody binding epitope or PTGDS protein isoform recognized by PTGDS antibody in the ELISA assay is not necessarily identical with the proteolytic peptide read-out in a mass spectrometry-based proteome analysis (30).

Previously, the clinical utility of elevated urine VDBP level was mainly investigated in diabetic nephropathy and non-lupus glomerulonephropathies (31-33). In this study, urinary VDBP was closely correlated with disease severity in LN. When we divided LN into active LN and inactive LN, the correlation between urine VDBP and UPCR was significant in active LN patients with proteinuria (Spearman's rho = 0.623, P = 0.004), but not in patients with inactive LN (Spearman's rho = 0.273, P = 0.152). In addition, urine VDBP was predictive of future development of proteinuria (UPCR $\geq$ 1.0 mg/mg) in SLE patients without proteinuria. VDBP, a multi-functional protein synthesized in the liver, plays a role in binding and transporting vitamin D metabolites to target tissues through the circulation (34). Normally, VDBP is nearly undetectable in urine. Glomerular filtration and subsequent proximal tubular reabsorption of VDBP are known to be critical processes to convert 25-hydroxyvitamin

D (25(OH)D) into its active metabolite, 1,25-dihydroxyvitamin D (1,25(OH)2D) via megalin/cubilin-mediated endocytosis (35). In patients with chronic kidney disease (CKD), increase of urine VDBP was associated with tubulointerstitial inflammation, independently of albuminuria (33). Thus, we hypothesized that urinary loss of VDBP in SLE patients without proteinuria reflects not only glomerular capillary leak but also subclinical tubular damage, reducing reabsorption of VDBP and 1,25(OH)2D synthesis. It might stimulate renal macrophages and promote compensatory production of VDBP that can augment the complement-mediated chemotaxis of neutrophils (36, 37). Although not evaluated in our study, it was reported that serum 25(OH)D levels were inversely associated with urine VDBP concentrations in patients with pediatric SLE (38). In another study, SLE patients with active LN had significantly lower serum level of 25(OH)D with inverse correlation with UPCR (39). In vitro, 1,25(OH)2D had a regulatory effect on B lymphocyte activation and autoantibody production, closely involved in the pathogenesis of LN (40). These findings suggest that urine VDBP could be useful for the forecaster of renal flare in SLE patients. However, there were inconsistent reports on whether urinary loss of VDBP actually affects vitamin D status (31, 41). Therefore, further evaluation of serum VDBP, 25(OH)D and 1,25(OH)2D in patients with SLE may be required to demonstrate the prognostic value of VDBP.

Urine concentrations of proteins except VDBP (TTR, RBP4, and PTGDS) still remained higher in LN patients whose proteinuria improved with treatment when compared to SLE patients without LN (Figure 1). These proteins are assumed to be elevated due to remaining kidney injury after remission. In the previous study (42), urine TTR, a carrier protein of thyroxine and RBP (43), could predict the response to ACE inhibitor therapy in IgA nephropathy. In LN, TTR was associated with severity of concurrent proteinuria, but not with prognosis of future proteinuria in LN. Urinary RBP was suggested as an independent prognositc marker which could identify patients who will progress into renal failure in non-lupus glomerulonephropathies (44). Also, it was a determining factor of steroid responsiveness in patients with the idiopathic nephrotic syndrome (45). In LN, urine RBP was reported as a sensitive marker of proximal tubular dysfunction (46). In the present study, urine RBP4 level, inversely correlated with eGFR, was associated with risk of remission failure in patients with active LN in univariate regression analysis. However, it was not a significant factor after adjusting baseline characteristics and concurrent immunosuppressive treatments. There was simply not enough power to be conclusive, based on a relatively small group of 20 patients with active LN.

In the current study, we included a relatively large cross-sectional cohort (n = 121) of SLE patients in validation

with quantitative measurement and followed up to 4 years. Urine biomarkers were superior to conventional serologic markers, such as anti-dsDNA and complements, for evaluation of disease activity of LN. Their diagnostic values were significant in both LN according to the ACR criteria and biopsy-proven LN. To our knowledge this study is the first to investigate urine VDBP as long-term prognostic biomarker for renal involvement of SLE.

There are some limitations of our study. Baseline characteristics were not identical within the patient groups, even though we adjusted them in regression analysis. Assessment about histologic findings of LN was precluded as urine sampling was not performed concurrently with renal biopsy. Brunner et al. reported that urine VDBP was significantly higher in proliferative LN compared to membranous LN and might discriminate LN subtypes (47). It was not possible to trace the change of urine biomarker as a response to treatment, because the longitudinal measurement was not conducted.

In conclusion, urinary biomarkers including VDBP well reflected LN status and renal disease activity in SLE patients. Moreover, urine VDBP concentrations may allow clinicians to anticipate the occurrence of future renal flare in patients with clinically quiescent renal involvement. To confirm these findings, further prospective controlled study is warranted.

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Table 1. Summary of urinary proteins (n = 56) up-regulated in SLE patients with LN from proteomics screening

No	Accession number	Protein names	Molecular weight (kDa)	Spectral count				STN	*P*-value
				SLE with LN		SLE without LN			
				1st run	2nd run	1st run	2nd run		
1	IPI00553177	Alpha-1-antitrypsin	47	3294	3246	1093	1101	156.6	< 0.0001
2	IPI00022463	Serotransferrin	77	3689	3648	1789	1754	152.3	< 0.0001
3	IPI00022420	Retinol binding protein 4	23	1055	1079	227	217	104.6	< 0.0001
4	IPI00478003	Alpha-2-macroglobulin	163	932	929	295	308	75.8	< 0.0001
5	IPI00017601	Ceruloplasmin	122	1385	1363	668	655	61.1	< 0.0001
6	IPI00022429	Alpha-1-acid glycoprotein	24	821	868	321	314	59.0	< 0.0001
8	IPI00022229	Apolipoprotein B-100	516	292	283	30	38	55.0	< 0.0001
10	IPI00004656	Beta-2-microglobulin	14	267	300	85	85	49.1	< 0.0001
11	IPI00166729	Zinc-alpha-2-glycoprotein	34	833	852	491	483	42.3	< 0.0001
14	IPI00032179	Antithrombin III	53	477	496	186	176	40.2	< 0.0001
16	IPI00855916	Transthyretin	20	347	361	97	93	38.2	< 0.0001
17	IPI00555812	Vitamin D-binding protein	53	415	430	150	150	34.5	< 0.0001
21	IPI00022488	Hemopexin	52	559	544	268	253	28.8	< 0.0001
34	IPI01014563	Ferritin light chain	20	75	72	9	8	18.9	0.0003
35	IPI00215983	Carbonic anhydrase 1	29	227	219	60	54	18.8	0.0003
36	IPI00009197	Lithostathin-1-beta	19	11	15	0	0	18.1	0.0003
37	IPI00019580	Plasminogen	91	439	425	316	325	18.0	0.0003
38	IPI00019568	Prothrombin	70	192	179	79	74	17.5	0.0003
39	IPI00304273	Apolipoprotein A-IV	45	183	183	67	70	17.2	0.0003
40	IPI00217493	Myoglobin	17	71	68	8	10	15.8	0.0003
43	IPI00654755	Hemoglobin subunit beta	16	170	156	102	104	13.5	0.0007
47	IPI00006705	Uteroglobin	10	42	44	14	15	12.6	0.0007
53	IPI00032220	Angiotensinogen	53	148	152	72	78	10.5	0.0010
55	IPI00642632	Ig lambda-7 chain C	11	10	6	3	0	10.0	0.0010

This proteomic analysis was performed in urine samples from 8 subjects (4 SLE with LN and 4 SLE without LN). Signal-to-noise (STN) and *P*-value were generated by power law global error model (PLGEM) software. Retinol-binding protein 4 (RBP4), vitamin D-binding protein (VDBP), and transthyretin (TTR) were selected for further quantitative measurement by ELISA. Prostaglandin D synthase (PTGDS) was selected as a reference biomarkers because it was previously reported as a representative biomarker for LN (15, 29).

Table 2. Baseline characteristics of SLE patients (n = 121) according to LN status in the validation analysis

	SLE with LN (n = 62)	SLE without LN (n = 59)	*P*	SLE with LN (n = 62)		*P*
				Active LN (n = 21)	Inactive LN (n = 41)	
Age (years)	39.7 ± 12.1	44.1 ± 12.8	0.055	35.2 ± 11.2	42.0 ± 12.1	0.036
Sex (male)	12 (19.4%)	2 (3.4%)	0.009	7 (33.3%)	5 (12.2%)	0.086
SLE disease duration (years)	10.6 (4.5-18.4)	10.7 (2.9-16.9)	0.524	6.7 (1.4-14.8)	15.4 (6.6-19.4)	0.010
LN disease duration (years)	7.2 (2.1-17.1)	-	-	1.5 (0.1-14.0)	10.2 (4.6-17.5)	0.003
Biopsy-proven LN	46 (74.2%)	-	-	17 (81.0%)	29 (70.7%)	0.542
Hypertension	21 (33.9%)	16 (27.1%)	0.438	9 (42.9%)	12 (29.3%)	0.396
Diabetes mellitus	4 (6.5%)	5 (8.5%)	0.739	1 (4.8%)	3 (7.3%)	0.000
eGFR ($mL/min/1.73m^2$)	77.7 ± 37.0	92.8 ± 21.7	0.018	79.7 ± 47.6	76.5 ± 30.0	0.795
UPCR	0.7 (0.1-2.6)	-	-	2.4 (0.9-3.6)	0.1 (0.1-0.2)	<0.001
Anti-dsDNA (IU/mL)	8.9 (4.9-26.2)	8.9 (4.2-18.1)	0.624	17.9 (7.5-49.8)	8.0 (4.7-15.4)	0.041
C3 (mg/dL)	80.0 ± 25.0	81.9 ± 20.1	0.661	70.9 ± 21.9	84.6 ± 25.5	0.043
C4 (mg/dL)	17.4 ± 9.2	16.6 ± 7.9	0.617	17.1 ± 10.9	17.6 ± 8.3	0.829
ESR (mm/hr)	18.0 (10.5-36.0)	15.0 (8.0-26.0)	0.186	22.0 (8.8-34.3)	15.0 (10.5-37.0)	0.872
Renal SLEDAI	4.0 (0.0-4.0)	-	-	10.0 (4.0-12.0)	0.0 (0.0-4.0)	<0.001
SLEDAI	4.00 (2.0-8.0)	2.00 (0.0-4.0)	0.006	12.00 (5.0-14.5)	2.00 (0.0-4.0)	<0.001
Previous treatment						
-Cyclophosphamide	26 (41.9%)	5 (8.5%)	<0.001	7 (33.3%)	19 (46.3%)	0.418
-MMF	8 (12.9%)	0 (0.0%)	0.006	5 (23.8%)	3 (7.3%)	0.107
-CsA/Tc	7 (11.3%)	2 (3.4%)	0.164	4 (19.0%)	3 (7.3%)	0.214

SLE, systemic lupus erythematosus; LN, lupus nephritis; eGFR, estimated glomerular filtration rate; UPCR, urine protein creatinine ratio; C3, complement component 3; C4, complement component 4; ESR, erythrocyte sedimentation rate; SLEDAI, SLE disease activity index; MMF, mycophenolate mofetil; CsA, cyclosporine A; Tc, tacrolimus

Data are expressed as the median (25% percentile-75% percentile), mean ± standard deviation (SD), or n (%). $P < 0.05$ was considered statistically significant. Baseline was the time of urine sampling for quantitative measurement of urine biomarkers. eGFR was assessed using the Modification of Diet in Renal Disease formula, GFR (mL/min/1.73 m^2)=$175x(S_{cr})^{-1.154}$ x $(Age)^{-0.203}$ x (0.742 if female) x (1.212 if African American). In patients with LN, renal biopsy was performed when indicated on the clinician's decision. UPCR and renal SLEDAI were not routinely evaluated in SLE patients without LN.

Table 3. Urinary biomarkers and clinical characteristics predicting remission of proteinuria (UPCR < 0.5) within 12 months in SLE patients with active LN

	Univariate logistic regression analysis			
	B	Wald	OR (95% CI)	*P*
LN disease duration (> 1 year)	- 2.757	6.061	0.063 (0.007-0.570)	0.014
Renal insufficiency (eGFR < 60)	- 21.791	0.000	0.045 (0.002-0.952)	0.046
Urine RBP4 (> 800 ng/mgCr)	- 3.584	7.397	0.028 (0.002-0.367)	0.007
	Multivariate logistic regression analysis			
	B	Wald	OR (95% CI)	*P*
LN disease duration (> 1 year)	- 3.807	6.270	0.022 (0.001-0.437)	0.012

RBP4, retinol binding protein 4

In multivariate analysis, adjusted covariates were age, sex, disease duration of SLE and LN, hypertension, diabetes, renal insufficiency with eGFR < 60 mL/min/1.73 m^2, serologic biomarkers (anti-dsDNA and serum complements), type of induction treatment (cyclophosphamide pulse therapy, mycophenolate mofetil, cyclosporine or tacrolimus), and use of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers.

Table 4. Urinary biomarkers and clinical characteristics predicting future development of proteinuric flare (UPCR > 1.0) in SLE patients without proteinuria (UPCR < 0.5)

	Univariate Cox regression analysis			
	B	Wald	HR (95% CI)	*P*
Sex (male)	1.626	6.172	5.083 (1.409-18.331)	0.013
Age (< 40 years)	2.449	10.375	11.576 (2.608-51.371)	0.001
Previous history of active LN	1.846	8.175	6.337 (1.787-22.471)	0.004
Urine VDBP (> 1.90 ng/mgCr)	1.677	6.738	5.348 (1.508-18.965)	0.009
Urine RBP4 (> 51.85 ng/mgCr)	2.256	4.748	9.543 (1.255-72.593)	0.029
Urine PTGDS (> 294.20 ng/mgCr)	2.133	4.245	8.439 (1.109-64.200)	0.039
	Multivariate Cox regression analysis			
	B	Wald	HR (95% CI)	*P*
Age (< 40 years)	2.809	9.538	16.587 (2.790-98.590)	0.002
Urine VDBP (> 1.90 ng/mgCr)	2.265	6.545	9.627 (1.698-54.571)	0.011

VDBP, vitamin D binding protein; RBP4, retinol binding protein 4; PTGDS, prostaglandin D synthase

In multivariate analysis, adjusted covariates were age, sex, previous history of active LN, disease duration of SLE, hypertension, diabetes, renal insufficiency with eGFR < 60 mL/min/1.73 m^2, serologic biomarkers (anti-dsDNA titer and serum complements), additional immunosuppressive treatments (cyclophosphamide, mycophenolate mofetil, cyclosporine, tacrolimus, or azathioprine), use of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, and hydroxychloroquine treatment. Cut-off values of urine biomarkers were obtained from ROC curve analyses. Variables presenting *P*-value < 0.05 in Cox regression analysis were shown in table.

Figure 1

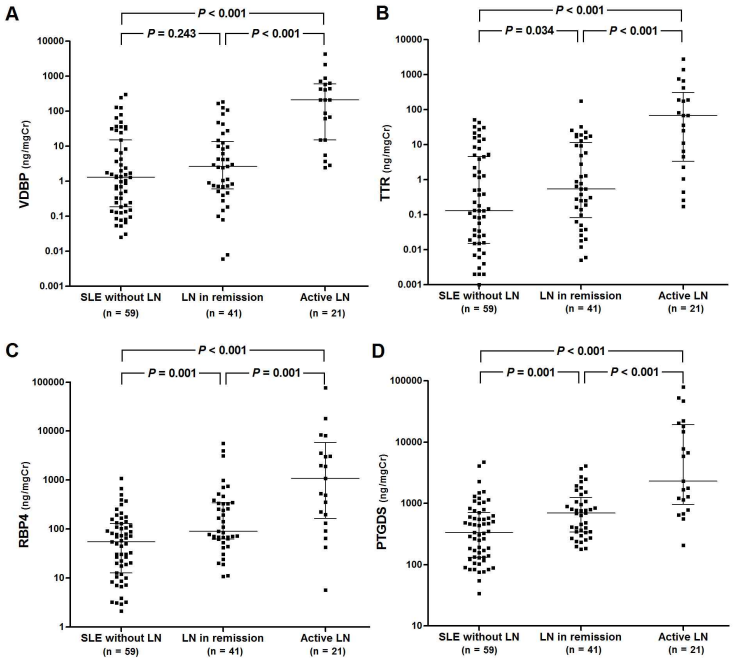

Figure 1. Levels of urinary biomarker candidates according to LN status in SLE patients. Urine levels of all proteins (VDBP, TTR, RBP4, and PTGDS) were significantly higher in SLE patients with active LN (n = 21) than in those with inactive LN (n = 41) and without LN (n = 59). Urinary TTR, RBP4, and PTGDS in SLE with inactive LN were significantly elevated compared to those in SLE without LN. Graphs represent median levels with the interquartile range (25th and 75th percentiles). Note that the y axis is on the log scale because of the skewness. Active LN was defined as renal SLEDAI ≥ 4 with UPCR ≥ 0.5 mg/mg or 24h urine protein ≥ 500 mg. Remaining SLE patients were divided according to history of previous renal involvement. Urine concentration of each protein was normalized to urine creatinine concentrations. VDBP, vitamin D binding protein; TTR, transthyretin; RBP4, retinol binding protein 4; PTGDS, prostaglandin D synthase

Figure 2

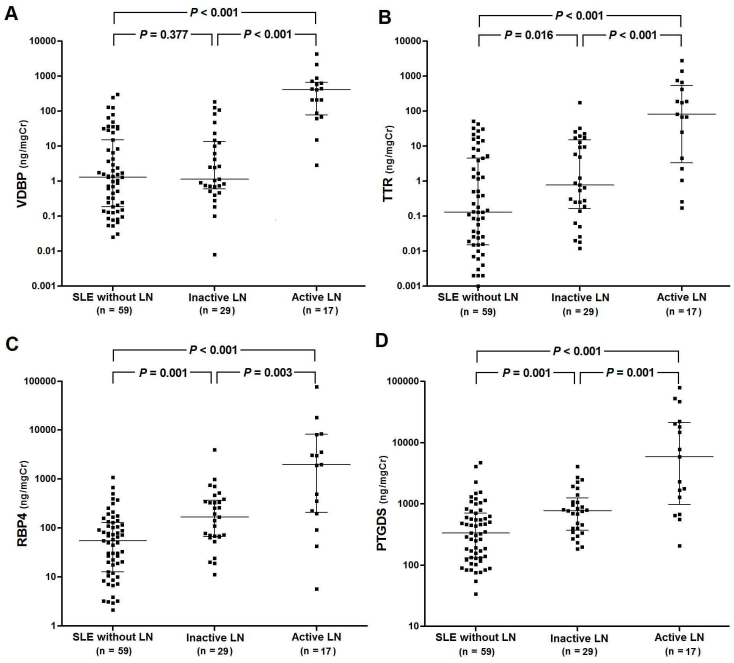

Figure 2. Levels of urinary biomarker candidates according to LN status in patients with biopsy-proven LN. When classification of LN was restricted to biopsy-proven LN, 46 patients were diagnosed with LN by kidney biopsy. Urine levels of all proteins (VDBP, TTR, RBP4, and PTGDS) were significantly elevated in SLE patients with active LN (n = 17). Urinary TTR, RBP4, and PTGDS in patients with inactive LN (n = 29) were higher than those in patients without LN (n = 59). VDBP, vitamin D binding protein; TTR, transthyretin; RBP4, retinol binding protein 4; PTGDS, prostaglandin D synthase

Figure 3

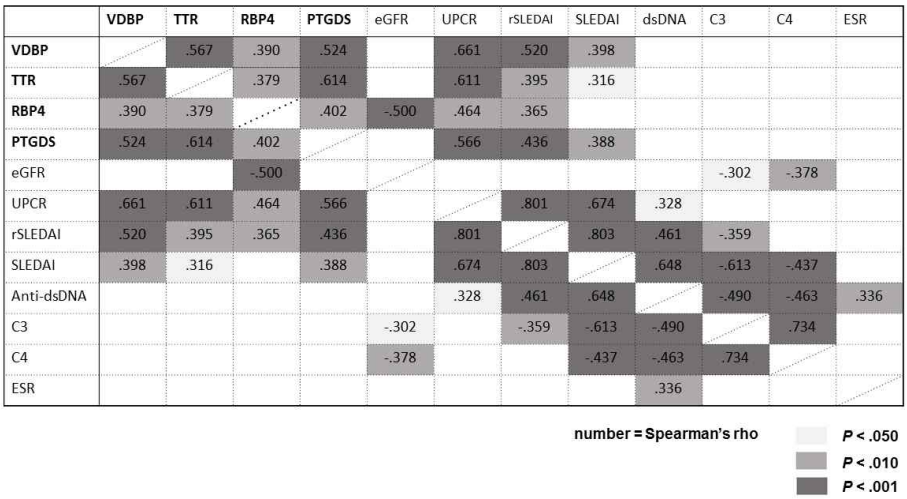

Figure 3. Correlations between urinary biomarker candidates and laboratory measures in SLE patients with LN (n = 62). All urinary biomarkers (VDBP, TTR, RBP4, and PTGDS) were correlated closely with disease activities of LN, such as UPCR and renal SLEDAI. RBP4 was inversely correlated with eGFR. Any of urinary biomarkers was not related to anti-dsDNA titer, serum complements, and ESR. VDBP, vitamin D binding protein; TTR, transthyretin; RBP4, retinol binding protein 4; PTGDS, prostaglandin D synthase

Figure 4

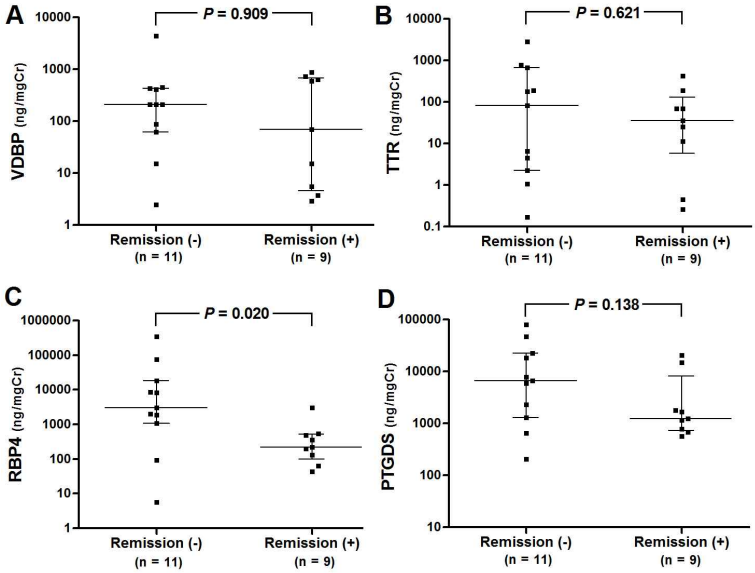

Figure 4. Levels of urinary biomarker (VDBP, TTR, RBP4, and PTGDS) in SLE patients with active LN, according to future achievement of remission. Among SLE patients (n = 21) with active LN at the time of urine sampling, excluding one patient lost to follow-up, 9 patients achieved remission of proteinuria (UPCR < 0.5 mg/mg). Urine RBP4 levels were lower in patients with remission. VDBP, vitamin D binding protein; TTR, transthyretin; RBP4, retinol binding protein 4; PTGDS, prostaglandin D synthase

Figure 5

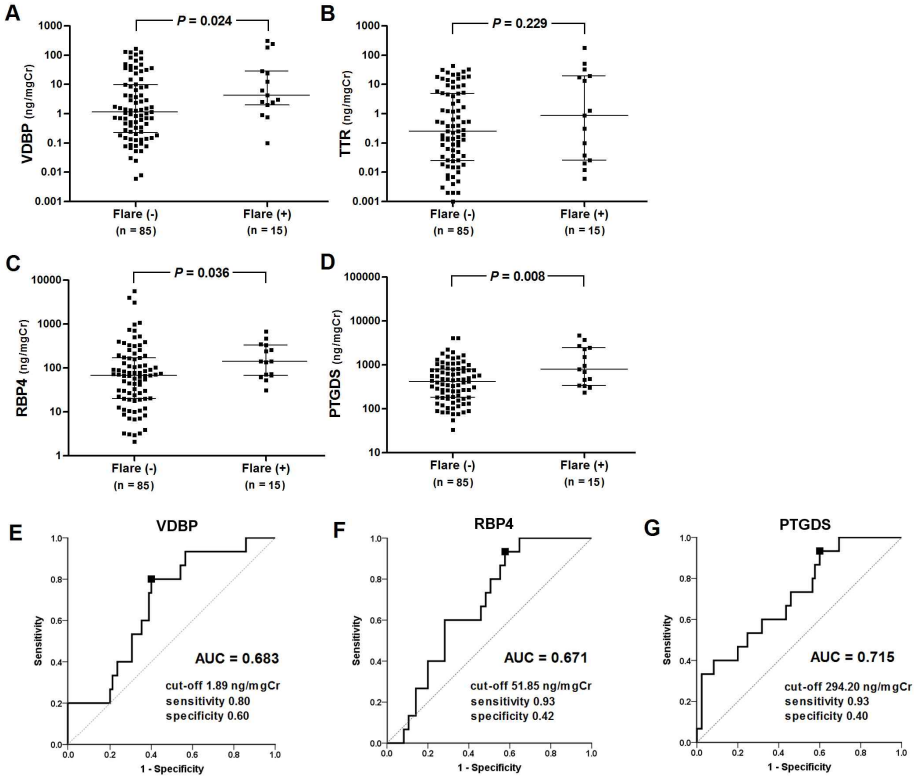

Figure 5. Levels of urinary biomarkers and ROC curves in SLE patients without proteinuria, according to future flare of proteinuria. Among SLE patients (n = 100) without definite proteinuria at the time of urine sampling, including those with inactive LN (n = 41), proteinuria with UPCR > 1.0 mg/mg have occurred in 15 patients. Urine levels of VDBP (A), RBP4 (C), and PTGDS (D) were significantly higher in patients with future proteinuric flare, but TTR levels (B) were not. ROC curve analysis of VDBP (E), RBP4 (F), and PTGDS (G) was performed to determine optimal cut-off values for prediction of future proteinuric flare. Cut-off values of urinary biomarkers were 1.89 ng/mgCr in VDBP, 51.85 ng/mgCr in RBP4, and 294.20 ng/mgCr in PTGDS. Graphs represent median levels with the interquartile range (25th and 75th percentiles).

Note that the y axis is on the log scale because of the skewness. Optimal cut-off value of ROC curve, represented as black square, was determined at the point for which (sensitivity plus specificity) is maximal according to Youden-index. Considering characteristics as the screening assay of urinary biomarker, higher sensitivity was favored for selection of cut-off value. VDBP, vitamin D binding protein; TTR, transthyretin; RBP4, retinol binding protein 4; PTGDS, prostaglandin D synthase

Figure 6

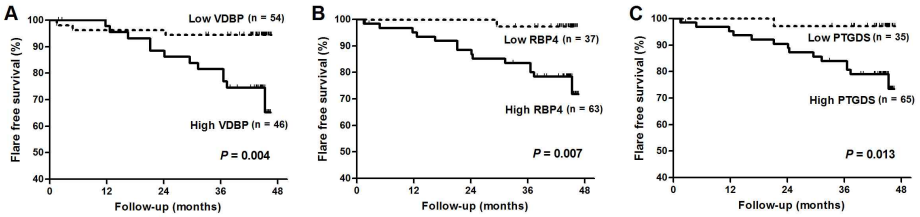

Figure 6. Development of proteinuric flare according to levels of baseline urine biomarkers in patients with SLE. SLE patients with higher levels, above the optimal ROC cut-off values, of VDBP, RBP4, and PTGDS, have developed proteinuric flare more frequently during follow-up. Mean duration of follow-up was 43.1 ± 6.9 months. Only three patients were lost to follow-up within 2 years. VDBP, vitamin D binding protein; RBP4, retinol binding protein 4; PTGDS, prostaglandin D synthase

요약 (국문 초록)

루푸스에서의 단백뇨 악화를 예측하는 새로운 소변 생체표지자로써의 비타민D 결합단백

서론: 루푸스신염은 전신홍반루푸스 환자의 절반 정도에서 나타나는 주요한 합병증으로 사망의 중요한 원인이다. 기존에 루푸스 환자에서 신질환의 활성도를 나타내기 위해 사용되고 있는 생체표지자들은 향후 예후를 예측하는데 있어서 불완전한 가치를 지니고 있다. 이번 연구의 목적은 루푸스신염의 질병 활성도와 예후를 반영할 수 있는 새로운 소변 생체표지자들을 조사하는 것이다.

방법: 루푸스신염을 구별하는 후보 단백질을 찾아내기 위해 액체 크로마토그래피 및 직렬식 질량 분석기를 사용한 소변 프로테오믹스 분석이 시행되었다. 이후 실제 임상에서의 소변 생체표지자의 유용성을 확인하기 위하여 루푸스 환자 121명에서 이들 단백질의 소변내 농도를 효소면역측정법을 통하여 측정하였다. 또한 이들 환자의 임상적 특징과 검사 결과들이 소변 생체표지자 농도와의 관련성을 파악하기 위해 조사되었다. 단백뇨를 포함한

신장 기능과 관련한 임상결과들이 4년간의 추적관찰 기간 동안 의무기록 정보를 이용하여 얻어졌다.

결과: 프로테오믹스 분석을 통하여 vitamin D binding protein (비타민D 결합단백), transthyretin, retinol binding protein 4 와 같이 루푸스신염의 활성 상태에서 높은 수준을 보였던 소변 단백질들이 정량적 측정을 위한 후보 단백질로 선택되었다. 이들 단백질들은 루푸스신염을 가진 루푸스 환자(62명), 특히 활동성 신염 환자 (21명)에서 루푸스신염이 없는 환자(59명)에 비하여 유의하게 상승되어 있었다. 소변 비타민D 결합단백은 단백뇨의 중증도 (상관계수 0.661, $P < 0.001$) 및 신장관련 루푸스 활성도 (상관계수 0.520, $P < 0.001$)와 밀접한 상관관계가 있었다. 4년의 추적 관찰 기간 동안 여러 교란 요인들을 보정한 후 소변 비타민D 결합단백은 단백뇨가 없던 루푸스 환자 (100명)에서의 단백뇨 악화에 대한 현저한 위험요인이었다 (위험도 9.627, 95% 신뢰구간 1.698-54.571, $P = 0.011$).

결론: 루푸스신염에 대한 새로운 생체표지자로써 소변 비타민D 결합단백은 단백뇨 및 신장 관련 루푸스 활성도와 상관관계가 있을 뿐 아니라 루푸스 환자에서의 단백뇨 악화를 예측하였다.

주요어: 루푸스신염, 비타민D 결합단백, 소변 생체표지자, 단백뇨 악화

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