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RESEARCH ARTICLE

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Urine inter-alpha-trypsin inhibitor family-related proteins may serve as biomarkers for disease activity of lupus

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Abstract

Background: Systemic lupus erythematosus (SLE) is a chronic inflammatory disease involving multiple tissues. Inter-Alpha-Trypsin Inhibitor (ITI) family proteins have a role in maintaining tissue homeostasis, but their possible clinical significance in the SLE patients has not been reported. The aim of this study was to analyze and verify the expression of ITI-related proteins in the urine of SLE patients, further explore the features of these proteins in disease activity.

Methods: Based on label-free proteomics technology and bioinformatics technology, we analyzed the expression of ITI family-related proteins in the urine of lupus. Subsequently, Western-blot and targeted proteomics were used to qualitatively and quantitatively verify the expression of these proteins, respectively.

Results: A total of seven ITI family-related proteins were screened and identified; and six of these proteins were differentially expressed in the urine of SLE patients. Further quantitative analysis showed that the expressions of ITIH2, ECM1, and ITIH5 in urine between active SLE group and stable SLE group were consistent with the preliminary screening results. The expression of ITIH2 and ECM1 in the renal damage group were also consistent with the screening results. Moreover, ITIH2 and ECM1 have a good correlation with disease activity and have a certain correlation with renal damage.

Conclusions: In this exploratory study, we evaluated the expression of ITI family-related proteins in the urine of SLE and found that urine ITIH2 and ECM1 were closely related to SLE activity, especially kidney damage, providing an experimental basis for further exploration of the potential roles in monitoring lupus and lupus nephritis activity.

KEYWORDS
disease activity, inter-alpha-trypsin inhibitor family, proteomics, systemic lupus erythematosus, urine

1 | INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease with diverse clinical manifestations. It usually accumulates extensively in multiple organs, leading to serious complications and increasing the risk of death.[1,2] Therefore, dynamic monitoring of SLE disease activity, early detection, timely intervention, and personalized treatment could reduce organ damage, improving quality of life and reducing mortality in SLE patients.[3,4] At present, the evaluation of SLE disease activity largely depends on the clinical manifestations of damaged tissues and organs, and the application of routine laboratory indicators is limited. Most indicators used to monitor SLE activity were panels of serum biomarkers. However, repeated venipuncture in SLE patients brought certain pain and inconvenience during monitoring.

In recent years, with the development of proteomics, urinary proteomics has been widely used in various diseases including neoplastic diseases and non-neoplastic diseases, and significant progress has been made in identifying clinical biomarkers and new therapeutic targets.[5,6] More and more studies on the biomarkers of urine protein in the diagnosis and activity judgment of SLE have aroused extensive attention.[7] In the early stage, we used label-free technology to study the urine proteins between active SLE group (SLEDAI-2 K ≥ 5) and stable SLE group (SLEDAI-2 K < 5), and found many differentially expressed proteins. Among the differentially expressed proteins, Inter-alpha-trypsin inhibitor (ITI) family-related proteins change significantly in SLE patients. In particular, the expression of ITIH3 and ITIH2 had the largest difference between active SLE group and stable SLE group. These have aroused our interest.

Inter-alpha-trypsin inhibitor (ITI) family proteins are a group of plasma serine protease inhibitors, containing a light chain (bikunin) and five homologous heavy chains (HC1, HC2, HC3, HC4, HC5).[8] There are usually two major forms of ITI existing in plasma: Inter-alpha Inhibitor (IαI), in which bikunin is linked to HC1 and HC2, and Pre-alpha Inhibitor (PαI), in which bikunin is linked to HC3.[9,10] IαI family members interact with extracellular matrix molecules (mainly hyaluronic acid) and this interaction may play a key role in maintaining the homeostasis of various tissues (liver, kidney, lung, connective tissue, and central nervous system, etc.).[11] In addition, some studies have found that increased expression of ITI family proteins are associated with a variety of pathological conditions, including tissue damage, tissue repair, and inflammation.[12–15] However, their expression and possible clinical value in SLE patients have not been reported. Current studies have reported that ITI family proteins can inhibit complement and provide cell regulation function, which is a key factor of innate immunity.[16,17] IαI contained the von-Willebrand type A (vWA) domains that bound to immune complexes and attenuated complement activation and complement-induced tissue damage.[18] We speculated that the changes of ITI family proteins in patients with SLE might reflect disease activity.

Therefore, this study intends to analyze the differential expression of ITI-related proteins in the SLE group by mass spectrometry and verify the changes of these related urine proteins in the disease activity. Then, we further explore the role of urinary ITI family-related proteins in SLE disease activity and the relationship with the development of SLE, so as to explore the potential value as non-invasive markers for predicting lupus activity.

2 | MATERIALS AND METHODS

2.1 | Study subjects

All participants gave informed consent, which was in accordance with the provisions of the Helsinki Declaration. This study was approved by the ethics committee of Beijing Shijitan Hospital, Capital Medical University. Then, patients with SLE and health controls were recruited at Beijing Shijitan Hospital between August 2020 and August 2021 excluding acute or chronic infections, tumors, severe kidney dysfunction caused by other diseases and other autoimmune diseases. All SLE patients were defined through the American College of Rheumatology (ACR) classification criteria in 1997.

In the screening cohort, the Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2 K)[19] was used to assess global disease activity in SLE patients and divided into two groups: active SLE group (Active) with SLEDAI-2 K ≥ 5; stable SLE group (Stable) with SLEDAI-2 K < 5. All lupus nephritis (LN) patients were proven by renal biopsy according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) in 2003 classification system.[20] Renal SLE disease activity index (rSLEDAI) were used to assess kidney disease activity, and further divided lupus nephritis patients into active LN group (aLN) with rSLEDAI ≥4 and inactive LN group (naLN) with rSLEDAI = 0. The rSLEDAI consists of four parameters: proteinuria, hematuria, pyuria, and urinary casts, each accounting for four scores, range 0–16.[21] In the validation cohort, the definition of active SLE group (Active, n = 12) and stable SLE group (Stable, n = 12) were the same as that of the screening cohort. We defined SLE patients with 24-h urine protein ≥0.5 g/24 h or active urine sediment (hematuria or cellular casts) as the Urine-Positive group (U-Pos, n = 8), considering kidney damage. Otherwise, SLE patients with normal urine results were defined as Urine-negative group (U-Neg, n = 16). Healthy controls (HC, n = 12) were chosen from those who have passed physical examinations, and their age and sex were matched with SLE patients. The basic clinical characteristics of SLE patients are shown in Table 1.

2.2 | Urine samples collection

All samples were collected in the first morning, before adjusting the medication regimen. Urine samples were collected into sterile polypropylene tubes, obtained from the midstream clean urine, and were transferred to the laboratory within 2 h. Then, urine samples were centrifuged at 400*g* for 5 min to remove cell debris and casts. The supernatants were divided in aliquots and frozen at −80°C until use.

TABLE 1 The basic clinical characteristics of SLE patients

Parameters	Total SLE (n = 24)	Active (n = 12)	Stable (n = 12)	HC (n = 12)	*p* Value
Age (year)	39.46 ± 14.58	39.75 ± 15.43	39.17 ± 14.36	38.75 ± 12.01	0.925
Gender Female, n (%)	24 (100%)	12 (100%)	12 (100%)	12 (100%)	—
Duration (year)	2.0 (0.69–8.00)	1.4 (0.08–13.75)	3.5 (1.06–8.00)	NA	0.340
SLEDAI-2 K (Score)	8.29 ± 8.38	14.58 ± 7.55	2.00 ± 1.91	NA	<0.001
Clinical manifestations					
Cutaneous, n (%)	5 (20.83%)	3 (25%)	2 (16.67%)	NA	0.615
Serositis, n (%)	7 (29.17%)	6 (50%)	1 (8.33%)	NA	0.069
Arthritis, n (%)	2 (8.33%)	1 (8.33%)	1 (8.33%)	NA	—
Renal damage, n (%)	8 (33.33%)	8 (66.67%)	0 (0.00%)	NA	0.001
Neurological symptoms, n (%)	2 (8.33%)	2 (16.67%)	0 (0.00%)	NA	0.478
Hematologic disorders, n (%)	7 (29.17%)	6 (50%)	1 (8.33%)	NA	0.069
Laboratory tests					
CRP (mg/L)	1.9 (1.00–3.70)	1.9 (1.38–4.37)	1.8 (0.78–3.70)	NA	0.603
ESR (mm/h)	30.25 ± 31.76	39.42 ± 40.54	21.08 ± 16.80	NA	0.169
C3 (g/L)	0.69 ± 0.28	0.52 ± 0.25	0.87 ± 0.18	NA	0.001
C4 (g/L)	0.1 (0.10–0.19)	0.1 (0.03–0.17)	0.1 (0.11–0.21)	NA	0.104
Proteinuria	6 (25%)	6 (50%)	0 (0.00%)	NA	0.014
Hematuria	6 (25%)	6 (50%)	0 (0.00%)	NA	0.014
Urinary casts	5 (20.83%)	5 (41.67%)	0 (0.00%)	NA	0.037
ANA	24 (100%)	12 (100%)	12 (100%)	NA	—
Anti-dsDNA Ab	9 (37.50%)	6 (50%)	3 (25%)	NA	0.400
Anti-Sm Ab	5 (20.83%)	3 (25%)	2 (16.67%)	NA	0.615
Treatment information					
Glucocorticoids (GC), mg/day	10.5 (4.00–22.50)	13.8 (1.25–40.00)	10.0 (4.00–14.38)	NA	0.506
Hydroxychloroquine (HCQ), n (%)	15 (62.50%)	5 (41.67%)	10 (83.33%)	NA	0.089
Immunosuppressant, n (%)	9 (37.5%)	3 (25%)	6 (50%)	NA	0.400

Note: Comparison of Active and Stable was performed using Student's *t*-test or Mann–Whitney *U*-test. *p*-Values <0.05 between Active and Stable are indicated as significant.

Abbreviations: Active, Active SLE group; ANA, Antinuclear antibody; Anti-dsDNA Ab, Anti-double-stranded DNA antibody; CRP, C-reactive protein; ESR, Erythrocyte Sedimentation Rate; HC, healthy controls; Stable, Stable SLE group.

2.3 | Mass spectrometry

For label-free proteomic analysis, Nano-upgraded reversed-phase liquid chromatography–tandem mass spectrometry (NanoRPLC-MS/MS) was performed in the urinary samples among active SLE (Active), stable SLE (Stable), active LN (aLN), and inactive LN (naLN). The experiment was repeated three times. The separated peptides were processed by a Q-Exactive mass spectrometer (Thermo Scientific) with ion source of EASY-Spray. The original mass spectrometry data were searched by MaxQuant, using the UniProt Homo sapiens database (https://www.uniprot.org/).

The heatmap was drawn to show the differential expression of selected proteins using Graphpad prism 9.0 software. Protein–protein interactions (PPI) were performed by using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database v11.0 (http://www.string-db.org/), a search tool for known and predicted associations between proteins, including both physical interactions as well as functional associations.[22] The functional enrichment of selected proteins were analyzed by Metascape (http://metascape.org/), a web tool designed to provide a comprehensive gene list annotation and analysis resource.[23]

2.4 | Western blot

To further verify the expression changes of ITI family-related proteins, we selected three proteins (ITIH3, ITIH2, and ECM1) with the largest difference for Western blot analysis. Urine samples were

collected by 12 active SLE, 12 stable SLE, and 12 healthy controls in the validation cohort. Then, these samples were added to 10 kD ultrafiltration devices (Millipore) and concentrated at 6000 *g* for 15 min. Seven micrograms of processed urine proteins were separated on the 12% Tris–HCL SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes by Trans-Blot Turbo (Bio-Rad). The PVDF membranes were incubated overnight at 1:2000 anti-ITIH3 antibody, 1:500 anti-ITIH2 antibody, and 1:1000 anti-ECM1 antibody, respectively. The 1×TBST buffer was used to wash the membranes three times, each 20 min, 1: 3000 second antibody was added and incubated at room temperature for 90 min. Finally, the PVDF membranes were washed by 1×TBST buffer and detected by enhanced chemiluminescence (Millipore).

2.5 | Parallel reaction monitoring and data analysis

A total of 24 SLE patients and 12 healthy controls were enrolled to the independent cohort for this experiment. Parallel Reaction Monitoring (PRM) assay was used to measure abundance of these selected ITI family related proteins in urine between active SLE and stable SLE. In addition, we further analyzed the expression of ITI family-related proteins in the urine of patients with and without renal injury. The urine samples were centrifuged at 2000 *g* for 10 min at 4°C. Then, the supernatant was denatured by shaking with 8 M urea at room temperature and concentrated by 10 k ultrafiltration tubes. To alkylate the reduced proteins, 1 M DTT (5 μl) and 1 M iodoacetamide (IAA) (20 μl) were added to each sample. The reaction mixture was incubated in darkness for 1 h at room temperature.

The proteins were added with Trypsin Gold (Promega) at 1:50 ratio and digested at 37°C for 16 h. After digestion, the mix-sample were fractionated using a C18 column (Waters BEH C18 4.6×250 mm, 5 μm) on a Rigol L 3000 HPLC operating at 0.7 ml/min. Buffer A (0.1% FA in ddH2O) and Buffer B (0.1% formic acid in ACN) were used to develop a gradient elution. The separation gradient is shown in Table 2.

For transition library construction, shotgun proteomics analyses were performed using an Q Exactive HF-X mass spectrometer (Thermo Fisher) operating in the data-dependent acquisition (DDA)

TABLE 2 Parameter settings for Skyline search

Parameters	Value
Enzyme	Trypsin
Static modification	Carbamidomethyl (C)
Dynamic modification	M Oxidation (15.995 Da)
	Acetyl (Protein N-terminal);
	Label:13C(6)15 N(2) (C-term K)
	Label:13C(6)15 N(4) (C-term R)
Precursor ion mass tolerance	±15 ppm
Fragment ion mass tolerance	±0.02 Da
Max missed cleavages	2

TABLE 3 The identified proteins of Inter-alpha-trypsin inhibitor (ITI) family for SLE disease activity and lupus nephritis (LN)

			Active/Stable		aLN/naLN		aLN/HC		naLN/HC	
Uniprot-ID	Protein name	Gene name	FC	Form of expression	FC	Form of expression	FC	Form of expression	FC	Form of expression
A0A087WW43	Inter-alpha-trypsin inhibitor heavy chain H3	ITIH3	560.18	Up	22.69	Up	17.88	Up	0.79	ns
Q5T985	Inter-alpha-trypsin inhibitor heavy chain H2	ITIH2	75.36	Up	1.86	Up	30.78	Up	16.51	up
P19827	Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1	30.28	Up	2.47	Up	1.87	Up	0.76	ns
Q16610	Extracellular matrix protein 1	ECM1	0.02	Down	0.45	ns	0.09	Down	0.19	Down
Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4	0.44	Down	0.90	ns	1.88	Up	2.10	Up
P02760	Protein AMBP	AMBP	0.48	Down	3.26	Up	2.12	Up	0.65	Down
A0A096LP62	Inter-alpha-trypsin inhibitor heavy chain H5	ITIH5	0.33	ns	0.97	ns	0.05	Down	0.05	Down

Note: Protein peptides were arranged in descending order of FC (Fold Change) value between Active and Stable. FC value >1.2 or <1/1.2, and p value <0.05 was considered a significant difference.
Abbreviations: aLN, active lupus nephritis group; Down, Protein expression was down-regulated; HC, healthy controls; ns, no significant difference; naLN, inactive lupus nephritis group; Up, Protein expression was up-regulated.

mode. The spray voltage of the ion source was set to 2.4 kV. In MS1, the precursor scan was acquired at 120,000 resolution with the 3×10^6 maximum C-trap capacity and 80-ms maximum injection time. The MS2 spectra were acquired 15,000 resolution, with the 5×10^4 maximum C-trap capacity and 54-ms maximum injection time. Standardized collision energy was set to 27%. The dynamic exclusion range was set to 16 s. The raw PRM data were analyzed by Skyline 21.1 software and peak lists were searched against protein database. The parameter settings for searching are shown in Table 1. The false discovery rate was set to 1% for proteins and peptides. Differential protein identifying conditions as follows: Fold Change > 1.2 or Fold Change < 1/1.2, p value <0.05.

2.6 | Statistical analysis

Values were shown as mean ± standard deviation or median (Interquartile range) according to the nature and distribution of the variables. Student's t-test or Mann–Whitney U-test and ANOVA were performed to analyze differences among the continuous variables. Experimental results were statistically analyzed using SPSS 21.0 software (IBM Corp.), and Visual analysis drawn by GraphPad Prism 9.0 statistical software (GraphPad Software Inc.). The correlation of continuous variables was evaluated by the Spearman correlation coefficient. The definition of correlation strength[24]: strong ($r \geq 0.7$); good ($0.5 \leq r < 0.7$); moderate ($0.3 \leq r < 0.5$); weak ($r \leq 0.3$). Receiver operating characteristic (ROCs) curves were plotted for assessing the sensitivity and specificity. All p values were two-tailed, and p values less than 0.05 were considered statistically significant.

3 | RESULTS

3.1 | Profiling inter-alpha-trypsin inhibitor family-related proteins in the urine of lupus patients

Inter-alpha-trypsin inhibitor family-related proteins were identified by searching the MaxQuant human database with a false discovery rate (FDR) set at 0.01 (Table 3). Manual inspection of the data revealed ITI family-related proteins, namely Inter-alpha-trypsin inhibitor heavy chain H3 (ITIH3), Inter-alpha-trypsin inhibitor heavy chain H2 (ITIH2), Inter-alpha-trypsin inhibitor heavy chain H1 (ITIH1), Inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4), Inter-alpha-trypsin inhibitor heavy chain H5 (ITIH5), Protein AMBP (AMBP), and Extracellular matrix protein 1 (ECM1), showing differential expression in active SLE (Active) relative to stable SLE (Stable) or healthy controls (HC). The significantly different proteins met the following requirements: fold change >1.2 or <1/1.2, p-value <0.05, as shown in Figure 1A. The expressions of ITIH3, ITIH2, and ITIH1 were up-regulated, and the expressions of ITIH4, AMBP, and ECM1 were down-regulated. The relative abundance of these proteins among Active, Stable, and HC is shown in Figure 1B.

Moreover, compared with the inactive LN (naLN) or healthy controls (HC), the seven ITI family-related proteins also differed to varying degrees in active LN (aLN), as shown in Figure 1C. The expressions of ITIH3, ITIH2, ITIH1, and AMBP were up-regulated in aLN compared with naLN. In addition, the expression of ITIH2 was up-regulated in both aLN versus HC and naLN versus HC, while ECM1 and ITIH5 were down-regulated. The relative abundance of these proteins among aLN, naLN and HC is shown in Figure 1D.

3.2 | Functional analysis of potential urinary proteins

Protein–protein interactions network of these functional proteins were performed using the STRING database. Interaction analysis of seven selected proteins, namely ITIH3, ITIH2, ITIH1, ITIH4, ITIH5, AMBP, and ECM1, were shown in Figure 1E. Except for ITIH5, which had no significant difference between Active and Stable group, was not in the interaction network, the other six proteins had strong interactions (PPI confidence score is 0.4). Additionally, the functional enrichment analysis of these six differentially expressed proteins based on the Metascape platform showed that the main pathway involved in the negative regulation of peptidase activity, hyaluronan metabolic process, and platelet degranulation (Figure 1F). The interactions and biological functions of these proteins provide information for further understanding the mechanisms of SLE disease activity.

3.3 | Qualitative analysis of differential urine proteins

In order to preliminarily verify the results of mass spectrometry screening, we selected three proteins with the most obvious difference from these six ITI related proteins using Western-blot, namely ITIH3, ITIH2, and ECM1. The results showed that the expression of ITIH2 protein in the urine of most patients with Active was significantly increased compared with Stable and HC. However, ITIH3 and ECM1 did not change significantly among the three groups (Figure 2). Furthermore, the detection rate of proteins in the urine of individual SLE patients and healthy controls was low, maybe related to the limitation of the sensitivity of the Western blot method. The expression of differential proteins in the urine of these three groups could not be completely verified by this method.

3.4 | Quantitative verification of ITI family-related urine protein

Parallel reaction monitoring assay is a mass spectrometry-based targeted proteomics technology performed in high resolution and high mass accuracy mode on a mass spectrometer with high sensitivity, reproducibility as well as throughput, and has become an alternative

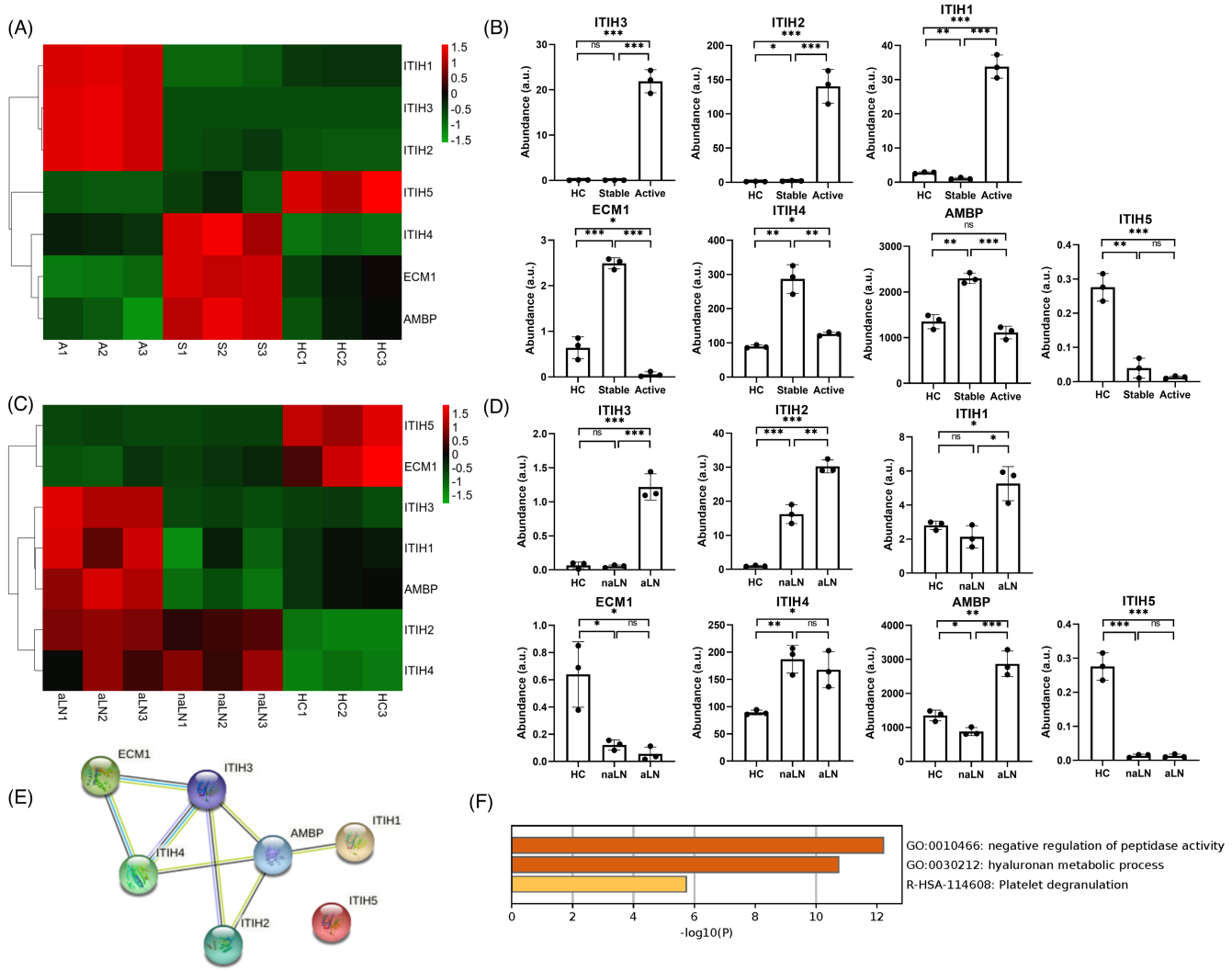

FIGURE 1 Label-free analysis of ITI family-related proteins. (A) The clustering heatmap shows distinction of active SLE (Active), stable SLE (Stable), and healthy controls (HC) in specifically proteomic features. (B) Abundance of ITI family-related proteins in arbitrary units among Active, Stable, and HC. All results are mean ± SEM and significance was set at $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$, ns: not significant. (C) The clustering heatmap shows distinction of active LN (aLN), inactive LN (naLN), and healthy controls (HC) in specifically proteomic features. (D) Abundance of ITI family-related proteins in arbitrary units among aLN, naLN, and HC.(E) PPI network of seven identified proteins of ITI family. (F) Pathway analysis of the six differentially expressed proteins using Metascape web-based platform. ITI, Inter-Alpha-Trypsin Inhibitor; SLE, Systemic lupus erythematosus.

targeted quantitative method.[25,26] Therefore, we used a PRM assay to further quantitatively validate the six proteins related to the ITI family. Unfortunately, AMBP was difficult to identified because its peptide spectrum matches (PSM) is lower from DDA database (Only the best scoring peptide to spectrum match for each LC/MS spectrum is considered as the potential peptide identification and is taken to the subsequent statistical validation).[27]The results of PRM analysis are shown in Table 4.

In the comparative analysis of these six proteins between Active and Stable, there were significant differences in the intensity of ITIH2 and ECM1 ($p < 0.05$), while there was no significant difference in the intensity of ITIH5 ($p > 0.05$). The PRM results exhibited the expression of ITIH2, ECM1, and ITIH5 among the Active, Stable, and HC (Figure 3A). The levels of ITIH2 were elevated and ECM1 was decreased in Active compared with the Stable. The results indicated the expression of these three proteins between Active and Stable were consistent with the previous screening protein profile, suggesting the well specificity and reproducibility of these candidate proteins.

In addition, we further analyzed the levels of these three proteins in the urine of patients with and without renal damage. The results displayed that the expression of ITIH2 was increased and ECM1 was decreased in the renal injury group (U-Pos) compared with the Urine-negative group (U-Neg). There was no significant difference in ITIH5 between the two groups as shown in Figure 3B. The expression of ITIH2 and ECM1 in U-Pos were consistent with the preliminary screening results, indicating that these two proteins were significantly expressed in SLE activity, and exhibited the same trend in renal injury.

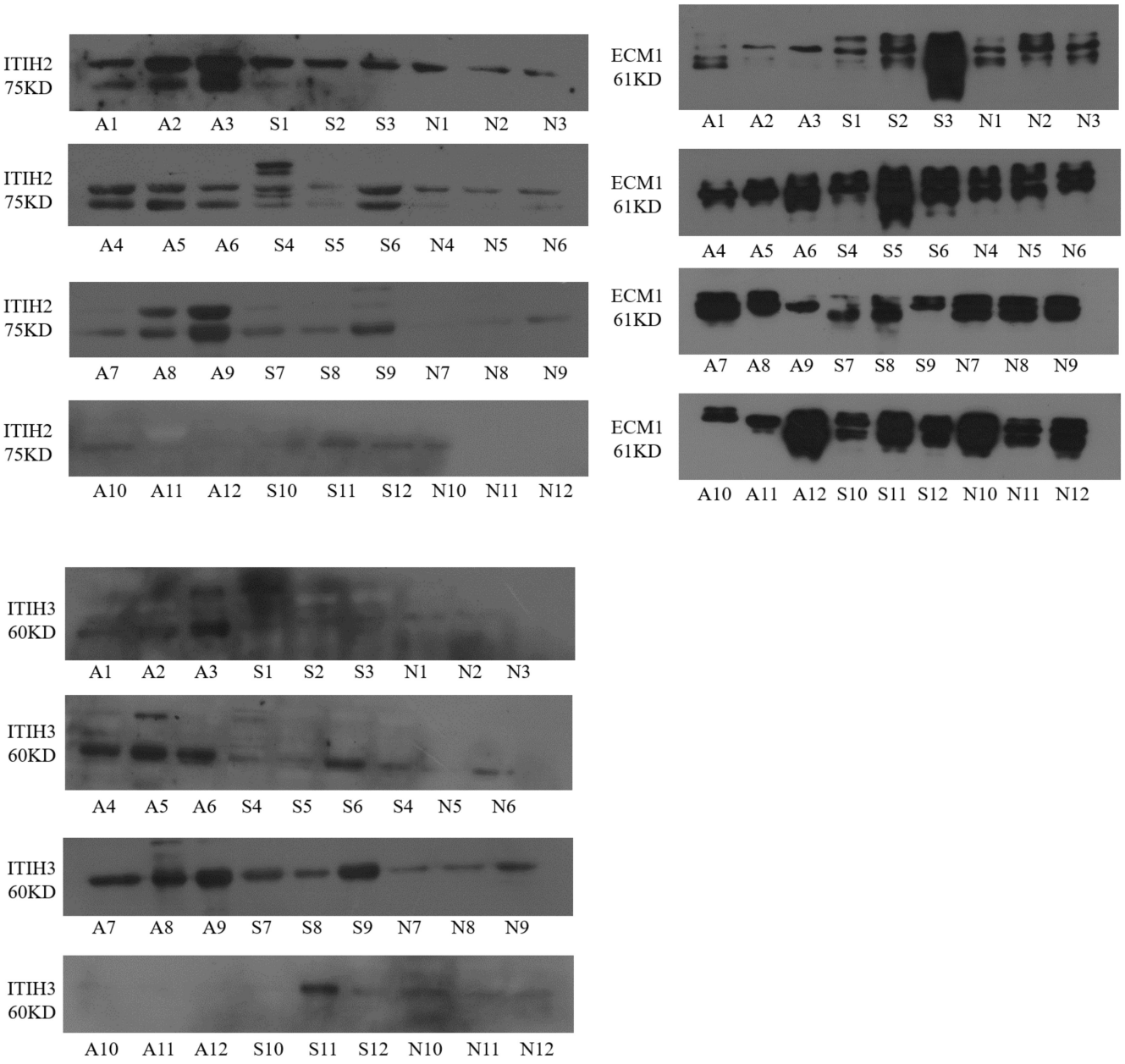

FIGURE 2 The expression changes of urine ITIH2, ITIH3, and ECM1 determined using Western blotting in different groups. The total protein was enriched from the urine of each subject. A1–A12: Western blot detection in urinary samples from 12 active SLE patients; S1–S12: Western blot detection in urinary samples from 12 stable SLE patients; N1–N12: Western blot detection in urinary samples from 12 healthy controls.

3.5 | Trend analysis and correlation analysis

Trend analysis was performed between SLEDAI-2 K and PRM signal intensity of ITIH2 and ECM1 (Figure 3C). The results showed that the signal intensity of ITIH2 correlated well with the trend of SLEDAI-2 K. As the SLEDAI-2 K score increased, the signal intensity of ITIH2 increased, while the signal intensity of ECM1 decreased. The relationship between candidate proteins and SLEDAI-2 K, renal injury were analyzed by Spearman correlation analysis. The signal intensity of ITIH2 was positively correlated with SLEDAI-2 K ($r = 0.685$, $p < 0.05$) and renal injury ($r = 0.626$, $p < 0.05$) in SLE patients, while the signal intensity of ECM1 was negatively correlated with SLEDAI-2 K ($r = -0.626$, $p < 0.05$) and renal injury ($r = -0.472$, $p < 0.05$) in SLE patients. The correlation between the signal intensity of ECM1 and renal injury was moderate, and the other correlations were good.

3.6 | ROC curves of urine ITIH2 and ECM1 for predicting disease activity and renal damage in SLE patients

The ROC curves were established based on the signal intensity values of urine ITIH2 and ECM1 between 12 active SLE patients and 12 stable SLE patients, as shown in Figure 3D. The areas under

TABLE 4 The selected proteins to be verified by Parallel reaction monitoring (PRM) analysis

				Active/Stable			U-Pos/U-Neg		
Uniprot-ID	Peptide	Gene Name	M/Z	FC	*p*-Value	Form of expression	FC	*p*-Value	Form of expression
Q5T985	FLHVPDTFEGHFDGVPVISK	ITIH2	747.72	2.30	0.013	Up	3.00	0.003	Up
Q16610	LLPAQLPAEK	ECM1	540.33	0.43	0.015	Down	0.44	0.023	Down
A0A096LP62	DHLISVTPDSIR	ITIH5	676.86	0.84	0.002	ns	0.84	0.003	Down

Note: FC value >1.2 or <1/1.2, and *p* value <0.05 was considered a significant difference.

Abbreviations: Down, Protein expression was down-regulated; M/Z, mass charge ratio; ns, no significant difference; U-Neg, Urine-negative group; Up, Protein expression was up-regulated; U-Pos, Urine-Positive group.

the curves (AUCs) of urine ITIH2 and ECM1 were 0.799 (95% CI = 0.616–0.982) and 0.792 (95% CI = 0.606–0.977), respectively. ITIH2 had better diagnostic value for predicting the disease activity of SLE, which provided an 83.33% sensitivity and a 66.67% specificity. ECM1 similarly had high discriminatory capacity for disease activity, with 75.00% sensitivity and 75.00% specificity.

According to SLE patients were combined with or without renal damage, the ROC curves were established based on the signal intensity values of urine ITIH2 and ECM1 between eight patients with renal injury (U-Pos) and 16 patients without renal injury (U-Neg), as shown in Figure 3E. Urine ITIH2 had the best predictive value for renal damage, with AUCs of 0.883 (95% CI = 0.738–1.000), and its sensitivity and specificity were 87.50% and 81.25%, respectively. Comparably, the AUCs of urine ECM1 was 0.789 (95% CI = 0.564–1.000), with sensitivity of 100% and specificity of 62.50% in SLE patients. Urine ITIH2 and ECM1 both had good discriminatory capacity for SLE disease activity or renal damage.

4 | DISCUSSION

Systemic lupus erythematosus involved multiple systems and organs, with diverse clinical manifestations and complex pathogenesis. At present, the comprehensive assessment of SLE disease activity mainly relies on clinical manifestations and few laboratory indicators. It is time consuming and inconvenient for the patients during the evaluation.[28] In order to find fast and reliable markers of disease activity, we conducted proteomic studies on the urine peptides of SLE patients. Our previous studies found that the expression of ITIH2 and ITIH3 was significantly increased in the urine of active SLE patients. However, the relationship between ITI family members and SLE has not yet been reported. In this study, we focus on exploring the relationship between ITI family-related proteins and SLE, and these proteins may have clinical value in disease activity. Based on bioinformatics, we first analyzed the expression of ITI family-related proteins in the urine of SLE patients, studied the function of these proteins, and explored the relationship between these proteins and global disease activity, renal damage in SLE. Then, the ITI family-related proteins were further verified using PRM targeted proteomics technology. This study is the first to explore ITI family-related proteins in the urine of SLE patients. Our results indicated that ITIH2 and ECM1 both have a better discriminatory capacity for disease activity and renal damage in SLE patients.

Inter-alpha-trypsin inhibitor family proteins are composed of light chains and many highly homologous heavy chains. These proteins existed in various organs and tissues and were initially found in plasma and urine.[29,30] Many studies have found that ITI family proteins play an important biological role in regulating the integrity of cells and matrix.[31,32] The current research on the ITI family is mostly centered on inflammation. These studies have found that increased the complex of the heavy chain and hyaluronic acid in the tissues and body fluids of patients with arthritis, asthma, sepsis, and skin injuries.[33–36] Recently, several studies have reported that the gene expression of ITI family members was reduced in multiple solid tumors (breast, lung, kidney), which might have a potential inhibitory effect in malignancies and tumor metastasis.[37,38] Furthermore, some studies have shown that the genetic variation of inter-alpha-trypsin inhibitor heavy chains were relevant in psychiatric disorders.[39] The research of ITI family proteins in rheumatic immune diseases has attracted more and more attention, and there are few studies in SLE patients.

ITIH1, ITIH2, and ITIH3 are the inter-alpha-trypsin inhibitor heavy chains HC1, HC2, and HC3. They act as the carrier of hyaluronic acid or the binding protein of hyaluronic acid and other matrix proteins in serum to regulate the localization, synthesis, and degradation of hyaluronic acid, and stabilize the extracellular matrix (ECM).[40] In our study, only the levels of ITIH2 were consistent with the previous mass spectrometry results, and the expression was up-regulated in the urine of Active compared to the urine of Stable. Likewise, the expression of ITIH2 was elevated in active LN as well as inactive LN compared to HC, with the highest levels in active LN. The results of PRM verification showed that the expression of ITIH2 was significantly higher in the Urine-Positive group (U-Pos) than in the Urine-negative group (U-Neg). However, ITIH1 showed the opposite result that the expression was up-regulated in the urine protein profile of Active SLE and down-regulated in the PRM verification. Inter-α-inhibitor (IαI) is usually composed of heavy chains HC1, HC2 and light chains. Some studies have found that IαI is a substrate of extracellular matrix proteases and HCs are transferred from IαI to HA, forming HC–HA complex to stabilize ECM in the inflammatory state.[41] Moreover, HC2 was cleaved and released during inflammation, and regulated leukocyte infiltration and complement

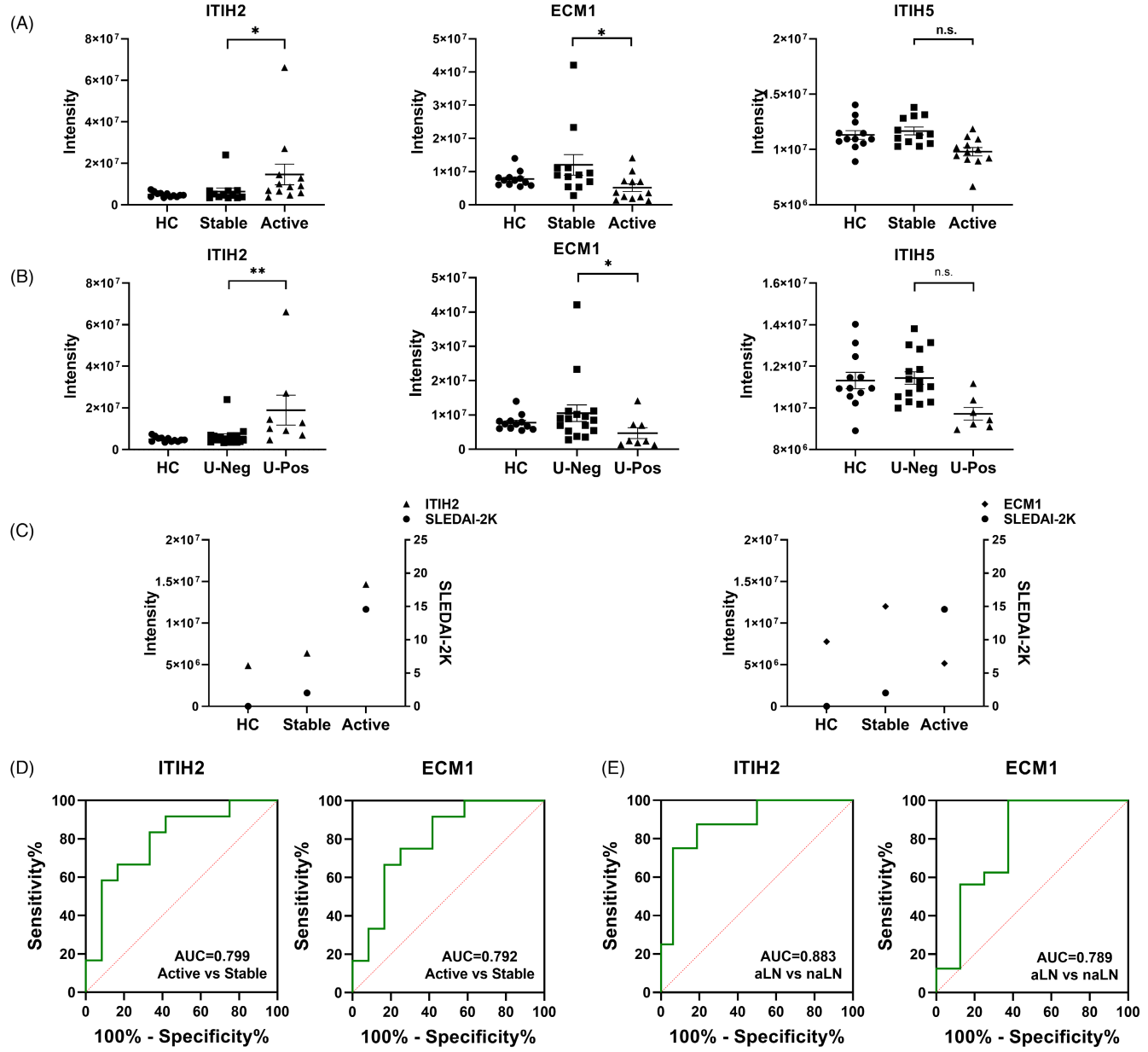

FIGURE 3 The comparison of selected protein expression between SLE and healthy controls (HC) by PRM analysis. (A) The expression of ITIH2, ECM1, and ITIH5 among the active SLE (Active), stable SLE (Stable), and HC. The ordinate is the group, and the abscissa is the intensity of protein level, an asterisk (*) indicated a significant change in protein abundance between the two groups ($P < 0.05$), Two asterisk (*) indicated a difference between the two groups with the P value <0.01, and 'n.s.' represented 'not significant difference'. Data were shown as mean ± SEM. (B) The expression of ITIH2, ECM1, and ITIH5 among the Urine-Positive group (U-Pos), Urine-negative group (U-Neg), and HC. (C) The correlation between SLEDAI-2 K and signal intensity of ITIH2 and ECM1 was determined by Spearman correlation analysis. (D) Receiver operating characteristic (ROC) curve analysis of ITIH2 and ECM1 to distinguish SLE disease activity from stable. (E) ROC curve analysis of ITIH2 and ECM1 to distinguish SLE with renal injury (U-Pos) and SLE without renal injury (U-Neg). ITI, Inter-Alpha-Trypsin Inhibitor; SLE, Systemic lupus erythematosus.

activation.[42,43] In addition, some studies have reported that proteins containing HC2 promoted the formation of hyaluronic acid complexes, but HC1 or HC3 did not possess this activity and failed to promote the transfer of inflammation-related proteins.[44]

In our study, the expression of ITIH2 is elevated in urine of active SLE, especially during renal injury, and has a good correlation with SLEDAI-2 K and renal damage, which may be associated with inflammation and complement activation aggravated kidney injury during disease activity. In the analysis of the discriminatory capacity for disease activity, we found that urine ITIH2 had good discriminatory capacity, with an area under the curve of 0.799 (95% CI = 0.616–0.982), and a better sensitivity of 83.33%. In the ROC curve analysis of ITIH2 for SLE complicated with renal injury, we observed that ITIH2 also had a better predictive capacity, with an AUC and a good sensitivity of 0.883 and 87.50%. These results indicate that ITIH2 may be a potential urinary biomarker of SLE

disease activity, especially kidney injury. Furthermore, the expression of ITIH1 in the urine of Active was decreased, and ITIH3 was not significantly different in the urine of the two groups in our analysis. We speculated that these results may be related to the types of heavy chains and the different binding degree and stability of HC–HA.

ITIH5 is known as a tumor suppressor gene for various cancers (breast cancer, bladder cancer, pancreatic cancer, etc.).[45–47] In recent years, some studies have found that ITIH5 is the major ITIH family member expressed in human skin and is a natural stabilizer of hyaluronic acid.[48] The complex formed by ITIH5 and HA stabilized HA and promoted the formation of ECM structure, meanwhile regulating inflammation.[49] The current research on ITIH5 mainly focuses on the gene level of tissue cells, while we concentrate on the expression of proteins in urine. Our results showed that the expression of ITIH5 had no significant difference between Active and Stable, as well as between active LN and inactive LN, but the specific role and function of the ITIH5 in SLE need to examine the level of gene in skin tissue. ITIH4 exists as a free HC isoforms in the blood circulation without binding to Bikunin, and has important biological functions either independent of other protein family members.[50,51] Although there is increasing evidence that ITIH4 is associated with human diseases, the biological role of ITIH4 is still unknown.[52] However, we verified that there was no significant correlation between the expression of ITIH4 in the urine and disease activity.

ECM1 is a soluble protein involved in endochondral bone formation, angiogenesis, and tumor biology. It also interacts with a variety of extracellular and structural proteins, contributing to the maintenance of skin integrity and homeostasis, but its exact function is not yet completely understood.[53] The mouse model proved that ECM1 is a direct target molecule of parathyroid hormone-related peptide. Inhibition of ECM1 could enhance the differentiation and hypertrophy of chondrocytes in vitro and in vivo, and negatively regulate cartilage production and ossification of the inner cartilage.[54] Some studies have found there is selective dysregulation and disassembly of structural and extracellular matrix molecules in the absence of ECM1 expression in fibroblasts, which may lead to skin and mucosal lesions.[55] In our study, the expression of ECM1 is down-regulated in the urine of patients with active SLE and renal injury, which has a good correlation with disease activity and a moderate correlation with renal injury. On the one hand, ECM1 may be correlated with the secretion and ossification of chondrocytes, on the other hand, it is related to the structure and dysfunction of extracellular matrix and impaired skin integrity in the active stage of SLE. The relationship between ECM1 and cartilage, joint or skin damage in SLE needs to be verified by expanding the sample size and further discussing patients with different target organ damage. In the analysis of the discriminatory capacity for disease activity, urine ECM1 also showed good discrimination ability, with sensitivity and specificity of 75%. Similarly, ECM1 also showed a certain discriminative ability in predicting kidney injury. Urine ECM1 has potential value as a biomarker for monitoring lupus disease activity including renal damage according to our results.

In addition, we used the PRM technology for verification, which is based on the quantitative results of the discovery of proteomics, and has been successfully applied to verify the relative abundance of proteins and post-translational modifications, providing a new strategy to find biomarkers of diseases.[56,57] In recent years, PRM technology has been widely applied in increasing diseases due to its high specificity and sensitivity.[58–60] The popular application of PRM is attributed to the simple and direct data acquisition method as well as high selectivity and specificity, because the complete MS/MS spectrum of each target ion needs to be collected with high resolution and high-quality accuracy.[61] Therefore, we hope to verify the relationship between ITI family proteins and SLE disease activity by examining the urine protein of SLE, and explore the potential value as biomarkers.

5 | CONCLUSIONS

In conclusion, we analyzed and verified the expression of ITI family urinary proteins in different activity states of SLE by PRM technology. We found that the expression of urine ITIH2 increased in active SLE, especially with renal injury, while the expression of urine ECM1 decreased. These two proteins were significantly associated with SLEDAI-2 K and might be used as exploratory urinary markers for lupus activity, particularly renal damage, laying the foundation for monitoring disease activity. The relationship between ITI family-related proteins and the study of SLE has not been reported. Moreover, urine ITIH2 and ECM1 had discriminatory capacity for lupus activity. Further large-scale samples are needed to analyze the changes of these proteins in different activity and damage organs, so as to provide deeper insights into the pathophysiological process involved in disease activity and the prediction of lupus activity.

AUTHOR CONTRIBUTIONS

Conceptualization, MZ and JJ; methodology, MZ, JJ and JZ; software, JJ and JZ; validation, MZ, JJ, and DL; formal analysis, JJ and DL; investigation, MZ and YHW; resources, MZ, JZ, and YHW; data curation, JJ, MZ, and YHW; writing – original draft preparation, JJ and MZ; writing – review and editing, JJ, MZ, and JZ; visualization, JJ; supervision, MZ and JZ; project administration, MZ; funding acquisition, MZ. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Kiriakidou M, Ching CL. Systemic lupus erythematosus. *Ann Intern Med*. 2020;172:C81-C96.
2. Chambers SA, Allen E, Rahman A, Isenberg D. Damage and mortality in a group of British patients with systemic lupus erythematosus followed up for over 10 years. *Rheumatology (Oxford)*. 2009;48:673-675.
3. Murimi-Worstell IB, Lin DH, Nab H, et al. Association between organ damage and mortality in systemic lupus erythematosus: a systematic review and meta-analysis. *BMJ Open*. 2020;10:e31850.
4. Gergianaki I, Bortoluzzi A, Bertsias G. Update on the epidemiology, risk factors, and disease outcomes of systemic lupus erythematosus. *Best Pract Res Clin Rheumatol*. 2018;32:188-205.
5. Wang W, Wu J, Liu P, et al. Urinary proteomics identifying novel biomarkers for the diagnosis and phenotyping of carotid artery stenosis. *Front Mol Biosci*. 2021;8:714706.
6. Iwan K, Clayton R, Mills P, et al. Urine proteomics analysis of patients with neuronal ceroid lipofuscinoses. *iScience*. 2021;24:102020.
7. Pejchinovski M, Siwy J, Mullen W, et al. Urine peptidomic biomarkers for diagnosis of patients with systematic lupus erythematosus. *Lupus*. 2018;27:6-16.
8. Salier JP, Rouet P, Raguenez G, Daveau M. The inter-alpha-inhibitor family: from structure to regulation. *Biochem J*. 1996;315(Pt 1):1-9.
9. Enghild JJ, Thogersen IB, Pizzo SV, Salvesen G. Analysis of inter-alpha-trypsin inhibitor and a novel trypsin inhibitor, pre-alpha-trypsin inhibitor, from human plasma. Polypeptide chain stoichiometry and assembly by glycan. *J Biol Chem*. 1989;264:15975-15981.
10. Odum L, Halkier T, Hojrup P, Schousboe I. Characterization of urinary proteinase inhibitors with segments of amino acids sequences identical to sequences of pancreatic secretory trypsin inhibitor. *Int J Biochem*. 1989;21:1319-1327.
11. Lord MS, Melrose J, Day AJ, Whitelock JM. The inter-alpha-trypsin inhibitor family: versatile molecules in biology and pathology. *J Histochem Cytochem*. 2020;68:907-927.
12. Kim B, De La Monte S, Hovanesian V, Patra A, et al. Ontogeny of inter-alpha inhibitor protein (IAIP) expression in human brain. *J Neurosci Res*. 2020;98:869-887.
13. Hennies HC. All is balanced: inter-alpha-trypsin inhibitors as unseen extracellular matrix proteins in epidermal morphology and differentiation. *Exp Dermatol*. 2015;24:661-662.
14. Seifert G, Kurzinger RP, Hopt UT, Wittel UA. Systemic differential gene regulation of the inter-alpha-trypsin inhibitor family in acute necrotizing pancreatitis in mice. *J Surg Res*. 2013;180:e83-e90.
15. Kanayama S, Yamada Y, Onogi A, et al. Bikunin suppresses expression of pro-inflammatory cytokines induced by lipopolysaccharide in neutrophils. *J Endotoxin Res*. 2007;13:369-376.
16. Kobayashi H. Endogenous anti-inflammatory substances, inter-alpha-inhibitor and bikunin. *Biol Chem*. 2006;387:1545-1549.
17. Fries E, Kaczmarczyk A. Inter-alpha-inhibitor, hyaluronan and inflammation. *Acta Biochim Pol*. 2003;50:735-742.
18. Garantziotis S, Hollingsworth JW, Ghanayem RB, et al. Inter-alpha-trypsin inhibitor attenuates complement activation and complement-induced lung injury. *J Immunol*. 2007;179:4187-4192.
19. Gladman DD, Ibanez D, Urowitz MB. Systemic lupus erythematosus disease activity index 2000. *J Rheumatol*. 2002;29:288-291.
20. Weening JJ, D'Agati VD, Schwartz MM, Seshan SV, et al. The classification of glomerulonephritis in systemic lupus erythematosus revisited. *Kidney Int*. 2004;65:521-530.
21. Zhu H, Wan H, Duan S, et al. Value of monitoring urine ammonia at time of biopsy in patients with lupus nephritis. *BMC Nephrol*. 2020;21:442.
22. Szklarczyk D, Gable AL, Nastou KC, et al. The STRING database in 2021: customizable protein-protein networks, and functional characterization of user-uploaded gene/measurement sets. *Nucleic Acids Res*. 2021;49:D605-D612.
23. Zhou Y, Zhou B, Pache L, et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nat Commun*. 2019;10:1523.
24. Hazra A, Gogtay N. Biostatistics series module 6: correlation and linear regression. *Indian J Dermatol*. 2016;61:593-601.
25. Rauniyar N. Parallel reaction monitoring: a targeted experiment performed using high resolution and high mass accuracy mass spectrometry. *Int J Mol Sci*. 2015;16:28566-28581.
26. Bourmaud A, Gallien S, Domon B. Parallel reaction monitoring using quadrupole-orbitrap mass spectrometer: principle and applications. *Proteomics*. 2016;16:2146-2159.
27. Chen G, Cheng J, Yu H, et al. Quantitative proteomics by iTRAQ-PRM based reveals the new characterization for gout. *Proteome Sci*. 2021;19:12.
28. Zucchi D, Elefante E, Calabresi E, Signorini V, Bortoluzzi A, Tani C. One year in review 2019: systemic lupus erythematosus. *Clin Exp Rheumatol*. 2019;37:715-722.
29. Shulman NR. A proteolytic inhibitor with anticoagulant activity separated from human urine and plasma. *J Biol Chem*. 1955;213:655-671.
30. Steinbuch M. The inter-alpha-trypsin inhibitor. *Methods Enzymol*. 1976;45:760-772.
31. Zhuo L, Hascall VC, Kimata K. Inter-alpha-trypsin inhibitor, a covalent protein-glycosaminoglycan-protein complex. *J Biol Chem*. 2004;279:38079-38082.
32. Cuvelier A, Muir JF, Martin JP, Sesboue R. Proteins of the inter-alpha trypsin inhibitor (ITI) family a major role in the biology of the extracellular matrix. *Rev Mal Respir*. 2000;17:437-446.
33. Petrey AC, de la Motte CA. Thrombin cleavage of inter-alpha-inhibitor heavy chain 1 regulates leukocyte binding to an inflammatory hyaluronan matrix. *J Biol Chem*. 2016;291:24324-24334.
34. Zhu L, Zhuo L, Kimata K, et al. Deficiency in the serum-derived hyaluronan-associated protein-hyaluronan complex enhances airway hyperresponsiveness in a murine model of asthma. *Int Arch Allergy Immunol*. 2010;153:223-233.
35. Stober VP, Lim YP, Opal S, Zhuo L, Kimata K, Garantziotis S. Inter-alpha-inhibitor ameliorates endothelial inflammation in sepsis. *Lung*. 2019;197:361-369.
36. Kuroda K, Fujimoto N, Tajima S. Abnormal accumulation of inter-alpha-trypsin inhibitor and hyaluronic acid in lichen sclerosus. *J Cutan Pathol*. 2005;32:137-140.
37. Hamm A, Veeck J, Bektas N, et al. Frequent expression loss of inter-alpha-trypsin inhibitor heavy chain (ITIH) genes in multiple human solid tumors: a systematic expression analysis. *BMC Cancer*. 2008;8:25.
38. Bayraktar E, Igci M, Erturhan S, et al. Reduced gene expression of bikunin as a prognostic marker for renal cell carcinoma. *Exp Oncol*. 2014;36:107-111.
39. Brandl EJ, Lett TA, Chowdhury NI, et al. The role of the ITIH3 rs2535629 variant in antipsychotic response. *Schizophr Res*. 2016;176:131-135.
40. Salier JP, Diarra-Mehrpour M, Sesboue R, et al. Isolation and characterization of cDNAs encoding the heavy chain of human inter-alpha-trypsin inhibitor (I alpha TI): unambiguous evidence for multipolypeptide chain structure of I alpha TI. *Proc Natl Acad Sci U S A*. 1987;84:8272-8276.
41. Milner CM, Tongsoongnoen W, Rugg MS, Day AJ. The molecular basis of inter-alpha-inhibitor heavy chain transfer on to hyaluronan. *Biochem Soc Trans*. 2007;35:672-676.
42. Scavenius C, Poulsen EC, Thogersen IB, Roebuck M, et al. Matrix-degrading protease ADAMTS-5 cleaves inter-alpha-inhibitor and

releases active heavy chain 2 in synovial fluids from arthritic patients. *J Biol Chem*. 2019;294:15495-15504.
43. Okroj M, Holmquist E, Sjolander J, Corrales L, et al. Heavy chains of inter alpha inhibitor (Ialphal) inhibit the human complement system at early stages of the cascade. *J Biol Chem*. 2012;287:20100-20110.
44. Sanggaard KW, Sonne-Schmidt CS, Krogager TP, et al. The transfer of heavy chains from bikunin proteins to hyaluronan requires both TSG-6 and HC2. *J Biol Chem*. 2008;283:18530-18537.
45. Rose M, Kloten V, Noetzel E, et al. ITIH5 mediates epigenetic reprogramming of breast cancer cells. *Mol Cancer*. 2017;16:44.
46. Rose M, Noetzel E, Kistermann J, et al. The ECM modulator ITIH5 affects cell adhesion, motility and chemotherapeutic response of basal/squamous-like (BASQ) bladder cancer cells. *Cells*. 2021;10:1038.
47. Sasaki K, Kurahara H, Young ED, et al. Genome-wide in vivo RNAi screen identifies ITIH5 as a metastasis suppressor in pancreatic cancer. *Clin Exp Metastasis*. 2017;34:229-239.
48. Huth S, Heise R, Vetter-Kauczok CS, et al. Inter-alpha-trypsin inhibitor heavy chain 5 (ITIH5) is overexpressed in inflammatory skin diseases and affects epidermal morphology in constitutive knockout mice and murine 3D skin models. *Exp Dermatol*. 2015;24:663-668.
49. Huth S, Huth L, Marquardt Y, et al. Inter-alpha-trypsin inhibitor heavy chain 5 (ITIH5) is a natural stabilizer of hyaluronan that modulates biological processes in the skin. *Skin Pharmacol Physiol*. 2020;33:198-206.
50. Zhuo L, Kimata K. Structure and function of inter-alpha-trypsin inhibitor heavy chains. *Connect Tissue Res*. 2008;49:311-320.
51. Pu XP, Iwamoto A, Nishimura H, Nagasawa S. Purification and characterization of a novel substrate for plasma kallikrein (PK-120) in human plasma. *Biochim Biophys Acta*. 1994;1208:338-343.
52. Pihl R, Jensen RK, Poulsen EC, et al. ITIH4 acts as a protease inhibitor by a novel inhibitory mechanism. *Sci Adv*. 2021;7:eaba7381.
53. Chan I. The role of extracellular matrix protein 1 in human skin. *Clin Exp Dermatol*. 2004;29:52-56.
54. Kong L, Zhao YP, Tian QY, et al. Extracellular matrix protein 1, a direct targeting molecule of parathyroid hormone-related peptide, negatively regulates chondrogenesis and endochondral ossification via associating with progranulin growth factor. *FASEB J*. 2016;30:2741-2754.
55. Utsunomiya N, Utsunomiya A, Chino T, Hasegawa M, Oyama N. Gene silencing of extracellular matrix protein 1 (ECM1) results in phenotypic alterations of dermal fibroblasts reminiscent of clinical features of lichen sclerosus. *J Dermatol Sci*. 2020;100:99-109.
56. Kim YJ, Gallien S, El-Khoury V, Goswami P, et al. Quantification of SAA1 and SAA2 in lung cancer plasma using the isotype-specific PRM assays. *Proteomics*. 2015;15:3116-3125.
57. Khristenko NA, Larina IM, Domon B. Longitudinal urinary protein variability in participants of the space flight simulation program. *J Proteome Res*. 2016;15:114-124.
58. Kim BJ, Lueangsakulthai J, Sah B, Scottoline B, Dallas DC. Quantitative analysis of antibody survival across the infant digestive tract using mass spectrometry with parallel reaction monitoring. *Foods*. 2020;9:759.
59. Adeola HA, Calder B, Soares NC, Kaestner L, Blackburn JM, Zerbini LF. In silico verification and parallel reaction monitoring prevalidation of potential prostate cancer biomarkers. *Future Oncol*. 2016;12:43-57.
60. Ku X, Sun Q, Zhu L, et al. Deciphering tissue-based proteome signatures revealed novel subtyping and prognostic markers for thymic epithelial tumors. *Mol Oncol*. 2020;14:721-741.
61. Park J, Oh HJ, Han D, et al. Parallel reaction monitoring-mass spectrometry (PRM-MS)-based targeted proteomic surrogates for intrinsic subtypes in breast cancer: comparative analysis with immunohistochemical phenotypes. *J Proteome Res*. 2020;19:2643-2653.

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