IFN-γ, CXCL16, uPAR: potential biomarkers for systemic lupus erythematosus

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Abstract

Objective

IFN-γ, CXCL16 and uPAR have recently been regarded as potential biomarkers in systemic lupus erythematosus (SLE). However, few researches have focused on the comparison of these three markers in SLE. We conducted this study to evaluate their role as biomarkers of disease activity and renal damage.

Methods

We enrolled 50 SLE patients with or without lupus nephritis (LN) and 15 healthy control subjects. The levels of IFN-γ, CXCL16, uPAR in serum, urine and renal tissues were detected by ELISA or immunohistochemistry. Relevant clinical and laboratory features were recorded.

Results

Serum and urine IFN-γ, CXCL16 and suPAR levels in SLE patients were significantly higher than that in healthy controls. Moreover, LN patients had higher levels than non-LN patients. A positive correlation was observed between these markers, and disease activity and suPAR had a stronger association with disease activity. The expression of these biomarkers in renal tissues was significantly higher in LN patients and was also associated with the activity of pathological lesions.

Conclusion

IFN-γ, CXCL16 and uPAR are promising as effective biomarkers of disease activity, renal damage, and the activity of pathological lesions in SLE.

Key words

systemic lupus erythematosus, lupus nephritis, urokinase plasminogen activator receptor, chemokine (C-X-C motif) ligand 16, interferon-γ

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Introduction

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease with multiple organs involvement, characterised by periods of disease activity, interspersed with periods of remission (1). Renal involvement is common in SLE often with the end point of end-stage kidney disease. The aetiopathogenesis of lupus nephritis (LN) is through autoantigen exposure, leading to multiple autoantibodies production and formation of immune complexes, triggering the complement system activation at the same time (2-4). If inefficient clearance is present, it could further promote inflammation reaction and tissue damage (5). However, limited reliable diagnostic biomarkers have been confirmed currently. At present, renal biopsy is still the "gold standard" for the diagnosis of lupus nephritis, but this is not easily accepted by some patients because of its invasive nature (6). There is a need for new convenient biomarkers of SLE to accurately reflect disease activity and organ damage, and to improve diagnosis and prognosis.

Interferons (IFNs) are a group of signalling molecules, whose major functions are to defend against viral infections and regulate the immune system (7). IFN-γ, the only member of Type II IFN, is mainly secreted by Th1-type T cells and NK cells (8). Several lines of evidence have proved the role of IFN-γ in the pathogenesis of autoimmune diseases, such as multiple sclerosis (9), rheumatoid arthritis (10), and SLE (8). The chemokine (C-X-C motif) ligand 16 (CXCL16) is one of the chemotactic cytokines involved in different inflammatory conditions. The elevated CXCL16 level has recently been identified in rheumatoid arthritis (RA) (11), Behçet's disease (12) and SLE (13) .

Urokinase plasminogen activator (uPA) is part of the family of the urokinase plasminogen activator system, which includes a proteinase (uPA), a receptor (uPAR) and inhibitors (PAI), is a glycosylphosphatidylinositol (GPI)-anchored membrane protein. It can be cleaved of the cytomembrane to the soluble form of the uPAR (suPAR). Elevated suPAR level was observed in cancer and infectious diseases in previous studies (14-16), and at present it has emerged as potential serum biomarker of inflammation and organ damage in SLE (17).

It has been reported that the IFN-γ, CXCL16 and suPAR levels in serum are significantly elevated in lupus. This prompts us to further investigate the level of IFN-γ, CXCL16 and uPAR in urine and kidney tissues, and whether they can effectively evaluate the disease activity and renal damage in Chinese SLE patients.

Methods

Patients and samples

We recruited a total of 50 patients with SLE (6 men, 44 women; mean age, 39.2±17.3 years; age range, 18–68 years) into the study, diagnosed between March 2013 and December 2013 in the Renal Division of the Second Xiangya Hospital, Central South University. All patients meet the 1997 American College of Rheumatology (ACR) revised criteria for the classification of SLE(18), except patients with cancer, infectious diseases and other rheumatic diseases. The patients were divided into two groups: 35 SLE patients with positive urinalysis (proteinuria or haematuria or casts) and biopsy-proven lupus nephritis were classified into the LN group (2 patients with class II, 4 with class III, 18 with class IV, 3 with class V, 3 with class III+V, 3 with class VI+V, 2 with class VI LN, basing on ISN/RPS 2003 classification of lupus nephritis) (19). In this group, 33 patients were in the active phase and 2 patients were in the remission phase. The other 15 SLE patients with negative urinalysis and normal renal function were classified into the non-LN group (2 patients in active phase, 13 patients in remission phase). The general characteristics of the patients at baseline are shown in Table I. Fifteen healthy individuals randomly selected from the Physical Examination Centre in the same hospital were taken as healthy controls. The normal renal tissues of 5 patients who underwent kidney cancer surgery with negative urinalysis and normal renal function were obtained as normal controls for immunohistochemistry.

Table I. Baseline characteristics of patients.

Characteristics	LN group (n=35)	Non-LN group (n=15)
Female (%)	88.6	86.7
Age (years)	41.8 ± 16.4	36.3 ± 12.9
SLEDAI	16.4 ± 5.4	5.6 ± 3.3
Rash (%)	34.3	66.7
Oral ulcer (%)	5.7	13.3
Alopecia (%)	25.7	26.7
Arthritis (%)	37.1	53.3
Serositis (%)	17.1	13.3
Neurological disorder (%)	5.7	6.7
Haematologic disorder (%)	22.9	40.0
Serum creatinine (mg/dl)	1.91 ± 2.46	0.74 ± 0.22
Serum albumin (g/l)	29.4 ± 6.8	40.3 ± 4.1
24-hour proteinuria (mg/d)	2327.2 ± 1244.9	59.4 ± 27.6
C3 (g/L)	0.41 ± 0.23	0.68 ± 0.30
ESR (mm/h)	86.1 ± 35.7	57.2 ± 35.4
Anti-dsDAN antibody (+) (%)	91.4	53.3

Results are expressed as mean±SD or percent.
C3: complement C3; ESR: erythrocyte sedimentation rate.

After obtaining informed consent, peripheral blood and morning urine were taken from each individual at the baseline stored at -80°C. The renal tissue was kindly provided by the Pathology Department. Approval was obtained from the local ethics committee for this study.

Clinical and laboratory data

We gathered and analysed the following clinical data: gender, age, fever, rash, alopecia, oral ulcer, arthritis, serositis, kidney manifestations, neurological manifestations and haematologic abnormalities. Disease activity was evaluated according to the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (20, 21).

The primary laboratory indicators of this study, including 24-hour proteinu-

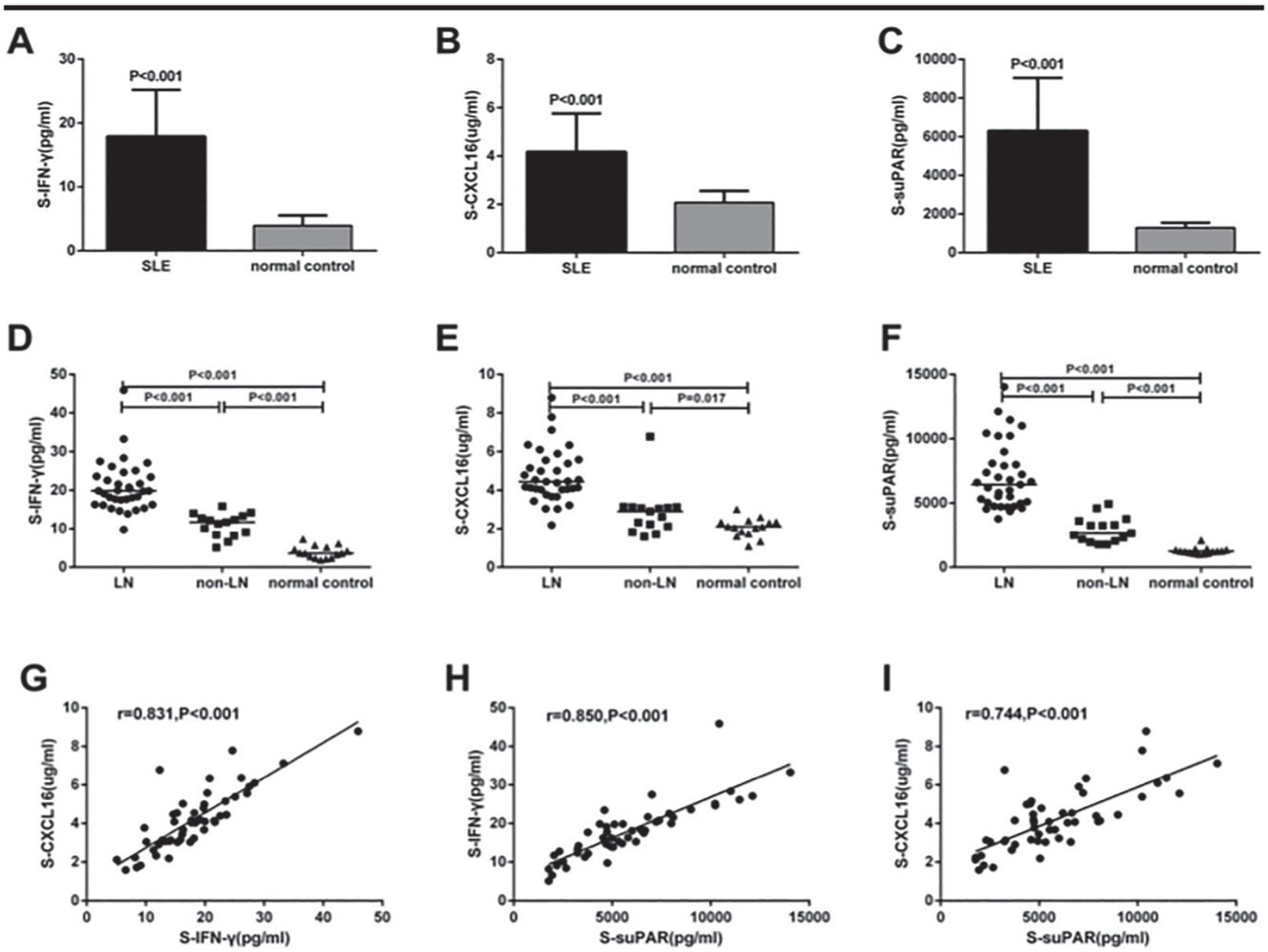

Fig. 1. Serum IFN-γ, CXCL16 and suPAR levels. Comparison of serum IFN-γ, CXCL16 and suPAR levels in patients with SLE and normal controls (A,B,C). Comparison of serum IFN-γ, CXCL16 and suPAR levels in SLE patients with and without LN, as well as normal controls (D,E,F). Correlation between serum IFN-γ and CXCL16 levels in SLE patients (G). Correlation between serum suPAR and IFN-γ levels (H). Correlation between serum suPAR and CXCL16 levels (I). S-IFN-γ: serum interferon-γ; S-CXCL16: serum chemokine (C-X-C motif) ligand 16; S-suPAR: serum soluble urokinase plasminogen activator receptor; SLE: systemic lupus erythematosus; LN: lupus nephritis.

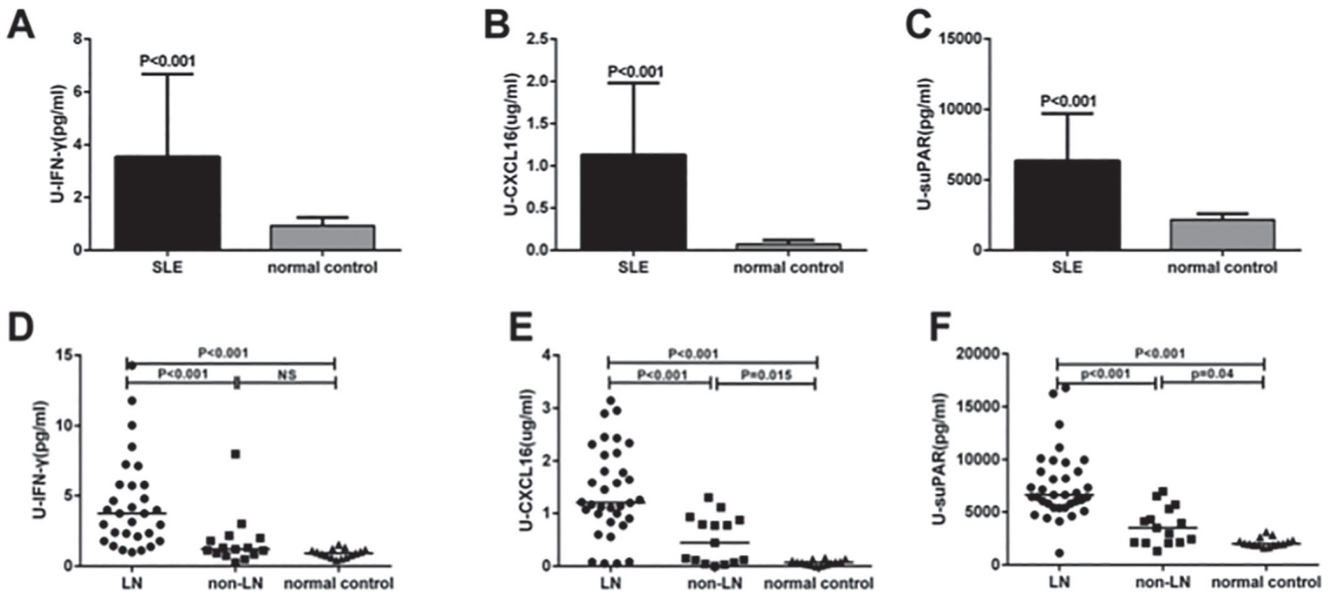

Fig. 2. Urine IFN-γ, CXCL16 and suPAR levels. Comparison of urine IFN-γ, CXCL16 and suPAR levels in patients with SLE and normal controls (A,B,C). Comparison of urine IFN-γ, CXCL16 and suPAR levels in SLE patients with and without LN, as well as normal controls (D,E,F).
U-IFN-γ: urine interferon-γ; U-CXCL16: urine chemokine (C-X-C motif) ligand 16; U-suPAR: urine soluble urokinase plasminogen activator receptor.

ria, serum albumin, serum creatinine (Scr), erythrocyte sedimentation rate (ESR), complement C3 and anti-dsDNA antibody, were performed at the Clinical Laboratory Department, Second Xiangya Hospital. *Crithidia luciliae* (Inova Diagnostics, USA) was used to detect anti-dsDNA antibody by indirect immunofluorescence. Immunofluorescence, light and electron microscopy were utilised to analyse renal biopsy specimens. As for light microscopy, specimens were stained with haematoxin eosin (HE), periodic acid-Schiff (PAS), periodic acid-silver methenamine (PASM) and Masson trichrome stains. Besides, the pathological activity of lupus nephritis was evaluated by active index (AI) and chronic index (CI) (22).

Detection of serum and urine IFN-γ, CXCL16, suPAR levels

Serum and urine IFN-γ, CXCL16, suPAR levels were detected in 50 SLE patients and 15 healthy control subjects. For the quantification of serum and urine IFN-γ concentration, the Human IFN-γ ELISA kit (Mabtech, Sweden) was used. As for quantification of CXCL16 and suPAR concentration, the Human uPAR Quantikine ELISA Kit and the Human CXCL16 Quantikine ELISA Kit (R&D Systems, USA) were used following the manufacturer's instructions. The absorbance was acquired with Fluorescence Reader (Molecule Devices, USA).

Detection of IFN-γ, CXCL16 and uPAR levels in renal pathology

We detected the expression of IFN-γ, CXCL16 and uPAR in renal tissues of 35 lupus nephritis patients and 5 normal controls by immunohistochemical staining using IFN-γ antibody (Santa Cruz Biotechnology, USA), CXCL16 antibody (GeneTex, USA) and uPAR antibody (abcam, UK). All the images were acquired using fluorescence microscope (Olympus, Japan). Image-Pro Plus v. 6.0 software was used to test the average integrated optical density (IOD) value of positive staining (23, 24).

Statistic analysis

Measurement data were expressed as mean±SD, and the enumeration data were expressed as percent. We used SPSS 18.0 software for statistic analysis. The comparisons between two groups were conducted using the Student's *t*-test, and the comparisons among three groups were conducted using Kruskal-Wallis test and Mann-Whitney test. The correlations between the three biomarkers and laboratory indicators were evaluated using Pearson test. $p<0.05$ was considered statistically significant.

Results

General characteristics

Age and gender ratio were comparable among patients with lupus nephritis, SLE patients without lupus nephritis and healthy controls (41.8±16.4 *vs*. 36.3±12.9 *vs*. 31.6±6.5; 31/4 *vs*. 13/2 *vs*. 12/3; $p>0.05$).

Serum IFN-γ, CXCL16 and s uPAR levels

Levels of serum IFN-γ, CXCL16 and suPAR in the SLE patients were significantly higher than that in the healthy subjects (17.92±7.26pg/ml *vs*. 3.94±1.60 pg/ml, $p<0.001$; 4.18±1.59 ug/ml *vs*. 2.07±0.49 ug/ml, $p<0.001$; 5805.24±2909.58 pg/ml *vs*. 1286.61 ±257.78 pg/ml, $p<0.001$; respectively). We also compared the difference between LN group, non-LN group and normal control group. The serum IFN-γ, CXCL16 and suPAR levels in LN group were also significantly higher compared with that in non-LN group ($p<0.001$), see Fig. 1.

We further investigated the correlations of serum IFN-γ, CXCL16 and suPAR levels in SLE patients finding that there was a significant positive correlation between IFN-γ and CXCL16

Table II. Correlations between IFN-γ, CXCL16, suPAR levels and SLEDAI scores, the level of 24-hour proteinuria, ALB, Scr, ESR, C3 in SLE patients.

r	SLEDAI scores	24-hour proteinuria	Scr	ESR	ALB	C3
S-IFN-γ	0.728*	0.733*	0.257	0.349*	-0.731*	-0.633*
S-CXCL16	0.630*	0.648*	0.236	0.349*	-0.614*	-0.563*
S-suPAR	0.752*	0.837*	0.290*	0.467*	-0.757*	-0.621*
U-IFN-γ	0.464*	0.498*	0.286	0.153*	-0.336*	-0.338*
U-CXCL16	0.523*	0.731*	0.417*	0.371*	-0.490*	-0.471*
U-suPAR	0.620*	0.710*	0.015	0.459*	-0.672*	-0.466*

*$p<0.05$.
r: correlation coefficient; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index; Scr: serum creatinine; ESR: erythrocyte sedimentation rate; ALB: serum albumin.

(r=0.831), as well as suPAR and IFN-γ (r=0.850), CXCL16 (r=0.744), $p<0.001$ (Fig 1).

Urine IFN-γ, CXCL16 and suPAR levels

The IFN-γ, CXCL16 and suPAR levels in urine were also detected. The urine IFN-γ, CXCL16 and suPAR levels of the SLE patients were significantly higher than that of the healthy subjects (3.54±3.14 pg/ml *vs*. 0.93±0.31 pg/ml, $p<0.001$; 1.13±0.85 ug/ml *vs*. 0.07±0.05 ug/ml, $p<0.001$; 6359.27±3343.10 pg/ml *vs*. 2158.83±418.43 pg/ml, $p<0.001$; respectively). In the comparison of them among LN group, non-LN group and normal control group, we got the same conclusion as in serum, except that the difference of IFN-γ level between non-LN group and control group was not significant ($p=0.089$) (Fig. 2).

Associations of serum and urine IFN-γ, CXCL16, suPAR levels with clinic and laboratory indicators

In the correlation analysis, we compared serum and urine IFN-γ, CXCL16, suPAR levels with SLEDAI scores and the level of 24-hour proteinuria, serum albumin, Scr, ESR, C3 in the SLE patients. As shown in Table II, IFN-γ, CXCL16 and suPAR were positively correlated with SLEDAI scores, 24-hour proteinuria, Scr and ESR levels and negatively correlated with serum albumin and C3 levels, whereas there was no significant correlation between serum creatinine and serum IFN-γ, CXCL16 as well as urine IFN-γ, suPAR. The relations between IFN-γ, CXCL16, suPAR levels and SLEDAI scores are shown in Figure 3. In addition to this, the patients with positive anti-dsDNA antibody presented significantly higher serum IFN-γ, CXCL16, suPAR and urine suPAR levels than those with negative anti-dsDNA, $p<0.05$.

IFN-γ, CXCL16 and uPAR levels in renal pathology

The IOD scores of IFN-γ, CXCL16 and uPAR in renal biopsy tissues of lupus nephritis patients increased significantly compared with that in normal renal tissues (30141.0±6362.8 *vs*. 10014.4±2052.0, $p<0.001$; 59033.6±16186.6 *vs*. 29123.8±11918.4, $p<0.001$; 53049.3±12937.3 *vs*. 29767.2±6409.6, $p<0.001$; respectively) (Fig. 4).

Associations of IFN-γ, CXCL16 and uPAR levels in renal pathology with disease activity

We analysed the association between the IOD scores of IFN-γ, CXCL16 and uPAR and disease activity based on AI/CI scores (Fig 5). IFN-γ, CXCL16 and uPAR IOD scores in renal tissues of lupus nephritis patients were positively associated with renal AI scores (r=0.569, 0.717, 0.574; $p<0.001$), and nega-

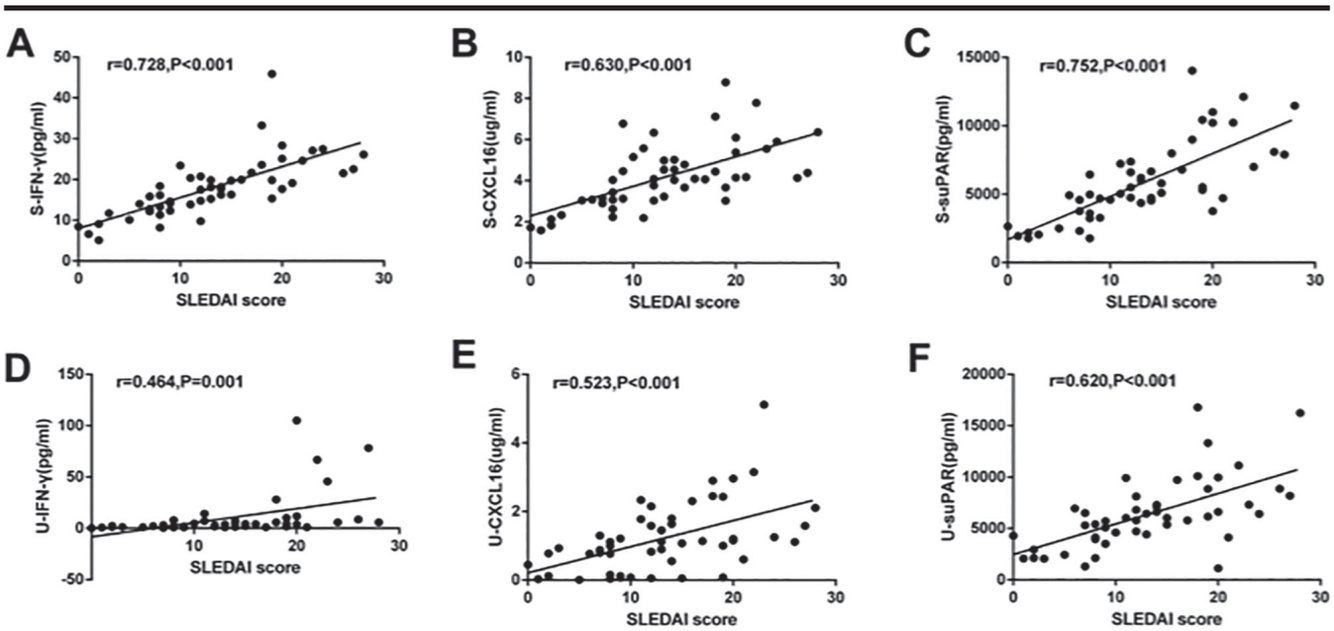

Fig. 3. Correlations between serum IFN-γ, CXCL16, suPAR levels and SLEDAI scores in SLE patients (A,B,C). Correlations between urine IFN-γ, CXCL16, suPAR levels and SLEDAI scores (D,E,F). SLEDAI: Systemic Lupus Erythematosus Disease Activity Index.

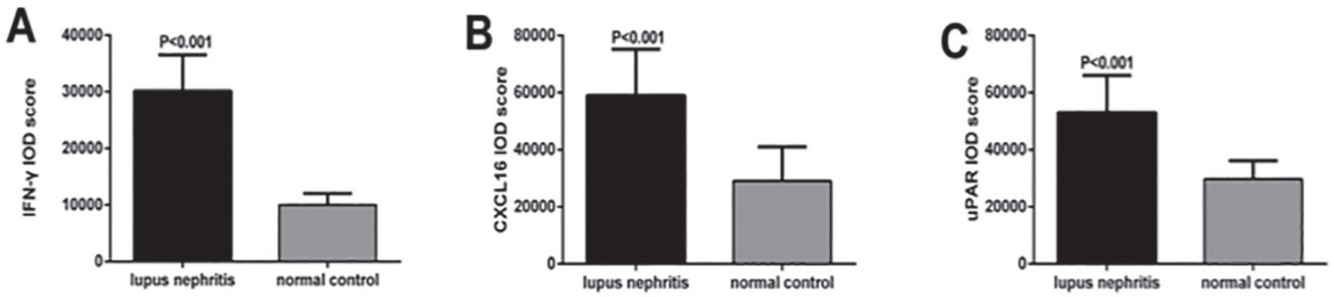

Fig. 4. IFN-γ, CXCL16 and uPAR levels in renal pathology. Comparison of renal IFN-γ, CXCL16, uPAR IOD scores in patients with lupus nephritis and normal controls. IOD: integral optical density.

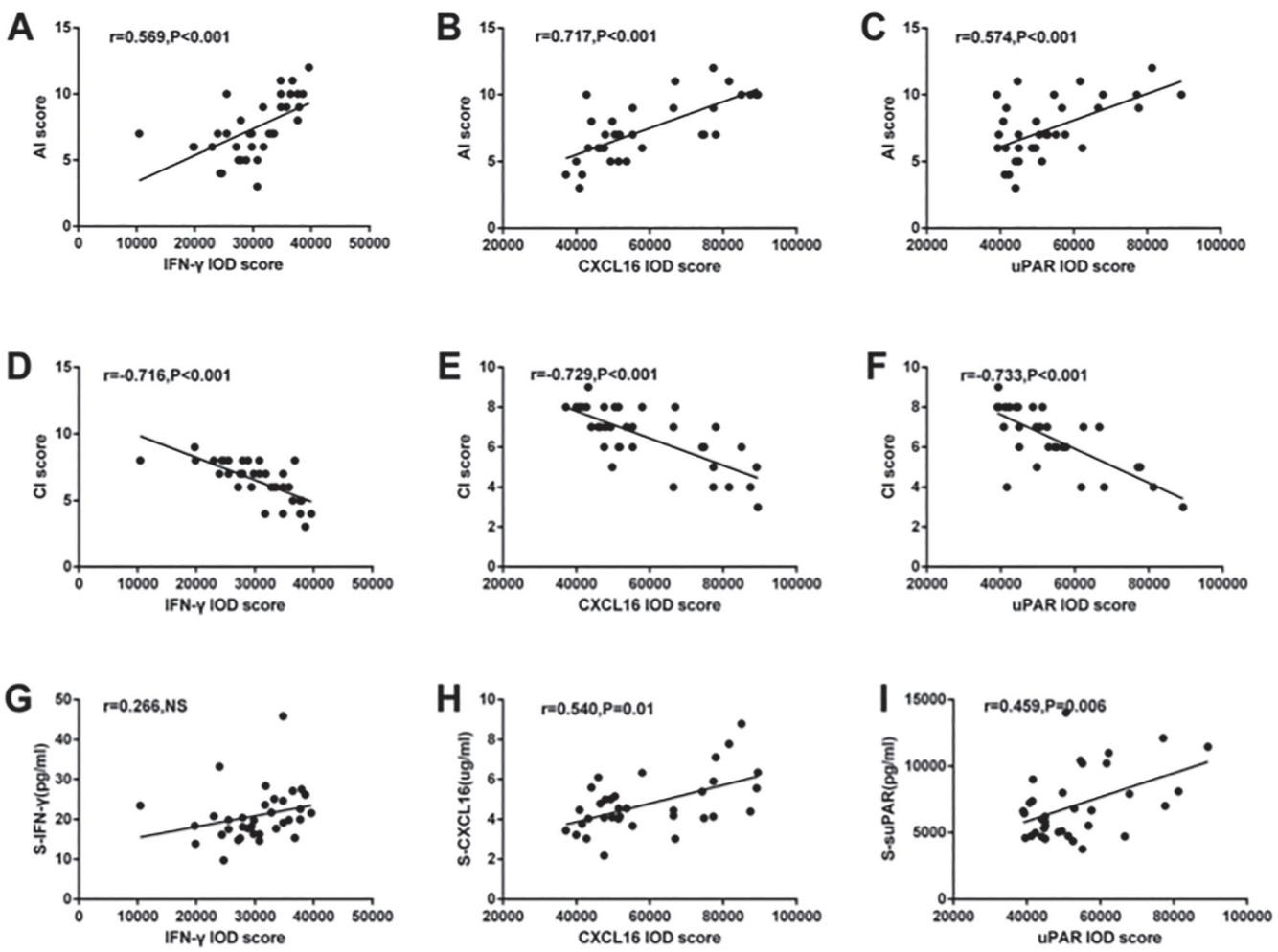

Fig. 5. Correlations between IFN-γ, CXCL16, uPAR levels in renal tissues and disease activity. Correlations of IFN-γ, CXCL16, uPAR in renal tissues and serum. Correlation between renal IFN-γ IOD score and AI score, CI score in LN patients (A,D). Correlation between renal CXCL16 IOD score and AI score, CI score (B,E). Correlation between renal uPAR IOD score and AI score, CI score (C,F). Correlation between renal IFN-γ IOD score and serum IFN-γ level in LN patients (G). Correlation between renal CXCL16 IOD score and serum CXCL16 level (H). Correlation between renal uPAR IOD score and serum suPAR level (I). uPAR: urokinase plasminogen activator receptor; AI: active index; CI: chronic index.

tively associated with renal CI scores (r= -0.716, -0.729, -0.733; $p<0.001$). Similarly, serum IFN-γ, CXCL16 and suPAR were also positively associated with renal AI scores (r= 0.459, 0.259, 0.440), and negatively associated with CI scores (r= -0.408, -0.369, -0.519). Only the correlation between CXCL16 and AI scores was not significant.

Further analysis identified a positive correlation between CXCL16 IOD score and serum CXCL16 level (r=0.540, $p=0.01$) as well as uPAR IOD score and serum suPAR level (r=0.459, $p=0.006$), however, without significant association between IFN-γ IOD score and serum IFN-γ level (Fig. 5). The correlations between IFN-γ IOD and urinary IFN-γ, CXCL16 IOD and urinary CXCL16, uPAR IOD and urinary suPAR were found to be of no significance, $p>0.05$.

Discussion

In recent years, an increasing number of researches have focused on discovering fresh biomarkers of SLE. It will be of great importance for the diagnosis and judgement of disease activity of SLE. Even though many studies about these biomarkers have been reported, there is indeed a lack of research about their expression in renal tissues. Moreover, few researches have focused on the comparison of the three markers in SLE. Based on the results of this study, we confirmed their role of potential biomarkers in SLE, and that serum and urine IFN-γ, CXCL16, suPAR levels were correlated with disease activity and could reflect kidney damage. Moreover, we found IFN-γ, CXCL16 and uPAR levels elevated in renal biopsy tissues of lupus nephritis patients and related to the activity of renal pathological lesions.

Toldi *et al*. reported elevated plasma suPAR levels in patients with high disease activity (25). While the study by Qin *et al*. demonstrated a mild positive correlation between suPAR levels and SLEDAI scores (26). Similarly, Qin *et al*. recently reported that circulating CXCL16 level was correlated with SLE disease activity (13). Before that, a similar association between urine CXCL16 level and SLEDAI scores was shown by Wu *et al*. (27). In our study, we found serum and urine IFN-γ, CXCL16, suPAR levels increased in SLE patients. Further analysis on the SLEDAI scores of SLE patients showed positive correlations between their levels and disease activity. In addition, results on anti-dsDNA, complement C3 and ESR further confirmed their value as predictors of disease activity. These findings suggest that they might participate in the pathogenesis of SLE and can be regarded as markers of disease activity. Compared with the other markers, serum suPAR had stronger correlations with SLEDAI scores, 24-hour proteinuria.

Studies have demonstrated that the expression of CXCL16 was stimulated by IFN-γ (28). In this study, the correlation between serum IFN-γ and CXCL16 levels as well as suPAR and IFN-γ, CXCL16 levels were significant, suggesting that they possibly interact with each other in the pathogenesis of SLE.

Lupus nephritis (LN) is one of the severe complications of SLE, which is strongly associated with the mortality in SLE patients. We have confirmed the role of IFN-γ, CXCL16, suPAR as biomarkers of SLE, and continued to investigate their predictive value in renal damage. We found in LN patients an increased level of IFN-γ, CXCL16 and suPAR in both serum and urine compared with those in the non-LN group. In addition, our study revealed a positive correlation between the three biomarkers and 24-hour proteinuria level, which is the main manifestation of renal disorders. Enocsson *et al*. has reported a convincing association between suPAR level and the Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index (SDI) (29) of the renal domain, demonstrating an association between serum suPAR and irreversible renal damage in SLE (30). Elevated suPAR or CXCL16 levels in the synovial fluid of osteoarthritis (OA) patients, cerebrospinal fluid (CSF) of neurologic diseases patients, and synovium of rheumatoid arthritis (RA) patients have been reported (31-34). However, there are few studies on the detection of these biomarkers in renal tissues. We proved that the expression of IFN-γ, CXCL16 and uPAR in renal tissues were increased in LN patients. Even more important, based on the association between the IOD scores of these biomarkers and AI/CI scores, we found that the higher expression of IFN-γ, CXCL16 and uPAR are likely to manifest the more active pathological lesions and the less chronic pathological lesions in kidney. More than that, the levels of suPAR and CXCL16 in serum were in line with their expression in renal tissues. Further investigations are needed to evaluate the usefulness of these biomarkers in the follow-up of SLE patients.

It has been reported that membrane-anchored form CXCL16 are expressed on the surface of dendritic cells (DCs) and macrophages. In SLE patients, massive inflammatory cells including DCs migrate to target sites. From these results, we assume that IFN-γ, CXCL16 and uPAR might be involved in local inflammations, and express in the local tissues or organs in a high level, subsequently resulting in increased concentration in circulation including serum and urine to a measurable extent in SLE patients. The pathogenesis might be that a massive amount of inflammatory cells migrate to the inflammatory sites, followed by IFN-γ, CXCL16 and suPAR shedding from or secreted by immune cells (8, 13, 17).

Conclusion

Detection of serum and urine IFN-γ, CXCL16 and suPAR levels is promising as an effective way to evaluate disease activity and renal damage in SLE. IFN-γ, CXCL16 and uPAR in renal tissues and even in serum may become potential markers of the activity of renal pathological lesions in lupus nephritis.

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