Interleukin-35 as a New Biomarker of Renal Involvement in Lupus Nephritis Patients

Di He, Min Liu, and Bo Liu

1Department of Rheumatology and Immunology, China-Japan Union Hospital of Jilin University, Changchun, Jilin, China

Systemic lupus erythematosus (SLE) is an autoimmune connective tissue disease with a wide range of clinical presentations. Lupus nephritis (LN) is the most serious manifestation of SLE. Interleukin-35 (IL-35), a member of the interleukin-12 family, has been identified as a novel anti-inflammatory cytokine. In the past ten years, the role of IL-35 in inflammatory and autoimmune diseases has been studied extensively. Serum IL-35 levels, however, have not been studied in LN patients. The aim of the study was to determine serum IL-35 levels in SLE patients with and without nephritis, and their clinical values. The study was carried out on 120 SLE patients, which comprised 80 LN patients and 40 SLE patients without nephritis. SLE disease activity was measured according to the Systemic Lupus Erythematosus Disease Activity Index-2k (SLEDAI-2k). Statistical evaluation was based on Mann-Whitney U-test, t-test, chi-square test, Spearman rank correlation test and Pearson’s correlation test. The result showed that active SLE patients (n = 65) have lower serum IL-35 levels, compared to inactive SLE patients (n = 55, P < 0.001). Furthermore, serum IL-35 levels were significantly lower in LN patients (n = 80) than SLE patients without nephritis (n = 40, P = 0.013). Serum IL-35 levels had significant correlations with SLEDAI-2k (r = –0.626, P < 0.001) in SLE patients and estimated glomerular filtration rate (eGFR) (r = 0.348, P = 0.002) in LN patients. These results indicate that IL-35 is a potential biomarker of renal involvement in LN patients.

Keywords: biomarker; cytokines; interleukin-35; lupus nephritis; systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is a chronic, systemic disease of connective tissues that can affect multiple major organs, but very often injures the kidney. The pathogenesis for lupus nephritis (LN) is complex and involves complicated interaction among genetic and environmental factors, and the innate and adaptive immune systems (Koutsokeras and Healy 2014). LN is currently diagnosed by histopathology of the specimen obtained by percutaneous renal biopsy and is associated with increased morbidity and mortality (Weening et al. 2004; Goilav et al. 2015). The kidney biopsy allows for some risk stratification, and provides a snapshot of the degree of scarring and irreversible damage (Goilav et al. 2015). The procedure of the kidney biopsy, however, requires the involvement of several medical teams and prolonged observation post procedure, and is also associated with a risk of bleeding, infection and allergic reactions to anesthetics and sedatives (Goilav et al. 2015). It has thus become clear that there is a real need for a better understanding of the pathogenesis of SLE and investigating surrogate markers to predict and monitor renal involvement in LN.

Production of autoantibody and immune complex deposition with subsequent infiltration of inflammatory cells, and perturbed cytokine activities are central to both onset and progression of renal pathology (Waldman and Madaio 2005; Rahman and Isenberg 2008; Munroe et al. 2017). Furthermore, it has been suggested by several studies that regulatory T cells (Treg), as well as its associated cytokines such as interleukin 35 (IL-35), play a pivotal role in the progression of SLE (Valencia et al. 2007; Bonelli et al. 2008; Tselios et al. 2014; Cai et al. 2015a, b).

IL-35 is a newly identified heterodimeric cytokine belonging to the interleukin-12 (IL-12) family including IL-12, interleukin-23 (IL-23), and interleukin-27 (IL-27), and is surprisingly different from its siblings in several ways. IL-35 consists of IL-12A (p35) and Epstein-Barr virus induced 3 (EBI3) (Collison et al. 2010, 2012; Sawant et al. 2015). The receptor for IL-35 is a unique IL-12Rβ2:gp130 heterodimer or homodimers (Collison et al. 2012; Egwuagu et al. 2015; Sawant et al. 2015).
2015; Guan et al. 2017). Increasing evidence has shown that IL-35 is involved in the pathogenesis of a wide range of inflammatory and autoimmune diseases, including chronic periodontitis (Okada et al. 2017), septic acute kidney injury (AKI) (Hu et al. 2017), rheumatoid arthritis (RA) (Niedbala et al. 2007; Kochetkova et al. 2010), dermatomyositis (Yin et al. 2016), systemic sclerosis (Tomcik et al. 2015) and SLE (Qiu et al. 2013; Cai et al. 2015a, b). However, little is known about the function of IL-35 in LN patients.

Our present study was the first to explore the potential role of IL-35 in the pathogenesis of LN. Yu et al. (2015) have found serum IL-35 levels were 76.07 ± 15.47 pg/mL in healthy subject (n = 50). We hypothesize that IL-35 may have a beneficial effect on the pathogenesis of LN. Thus, correlations between serum IL-35 levels, and SLE disease activity and renal damage were analyzed.

Materials and Methods

Patients and blood samples

A total of 120 patients with SLE were recruited from the Department of Rheumatology and Immunology in China-Japan Union Hospital of Jilin University after informed consent. The diagnosis of SLE was established according to the 1997 American College of Rheumatology (ACR) revised criteria for classification of SLE (Hochberg 1997). Individuals with other rheumatic or autoimmune diseases, renal diseases other than lupus nephritis, infections, malignant tumors or any other co-morbidities were excluded from this study. Moreover, patients with prior treatment with a monoclonal antibody or other biologic agents were excluded. Disease activity was evaluated by the Systemic Lupus Erythematosus Disease Activity Index-2 k (SLEDAI-2 k) (Gladman et al. 2002). Active SLE was defined as a SLEDAI-2 k score ≥ 10, and inactive SLE as SLEDAI-2 k < 10. 120 SLE patients were divided into 65 active and 55 inactive SLE patients. SLE patients were also divided into two groups: 80 patients with nephritis (LN group) comprising 45 active and 35 inactive SLE patients, and 40 patients without nephritis (SLE without nephritis group) comprising 20 active and 20 inactive SLE patients. LN patients were defined by persistent of proteinuria (≥ 0.5 g/24 h), or the presence of cellular casts or persistent hematuria. Glomerular filtration rate was estimated from serum creatinine using Cockcroft-Gault equations (eGFR) (Cockcroft and Gault 1976). Demographic data, clinical data and laboratory data were collected from hospital records or by questionnaire and reviewed by experienced physicians. The above protocol was approved by the ethics committee of China-Japan Union Hospital of Jilin University, and informed consent was obtained from all subjects according to the Declaration of Helsinki.

Serum immune globulin G (IgG) (P = 0.001), immunoglobulin A (IgA) (P = 0.009), immunoglobulin M (IgM) (P = 0.007) and ALB (P < 0.001) compared to SLE without nephritis group. Similarly, we found that LN group had notably higher serum levels of renal function indices compared with SLE without nephritis group, including BUN (P < 0.001), Scr (P < 0.001), BUA (P < 0.001), RBP (P = 0.001) and CysC (P = 0.003). Additionally, LN group had significantly lower levels of eGFR than SLE without nephritis group (P = 0.028). Nevertheless, there was no significant difference in
Table 1. Demographic, clinical and laboratory characteristics of systemic lupus erythematosus patients with and without nephritis.

<table>
<thead>
<tr>
<th></th>
<th>LN group (n = 80)</th>
<th>SLE without nephritis group (n = 40)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (female/male)</td>
<td>75/5</td>
<td>38/2</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Age (year)</td>
<td>34.9 ± 8.5</td>
<td>35.45 ± 10.0</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>SLEDAI-2 k</td>
<td>10.0 (8.0-12.0)</td>
<td>9.0 (7.0-11.75)</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Disease duration, (month)</td>
<td>29.50 (9.25-64.50)</td>
<td>15.00 (2.25-60.00)</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>anti-dsDNA (+/–)</td>
<td>63/17</td>
<td>34/6</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>anti-dsDNA (IU/mL)</td>
<td>378.00 (258.00-794.00)</td>
<td>437.50 (194.25-878.75)</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>C3 (g/L)</td>
<td>0.48 (0.32-0.67)</td>
<td>0.52 (0.36-0.64)</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>C4 (g/L)</td>
<td>0.08 (0.05-0.13)</td>
<td>0.06 (0.03-0.11)</td>
<td>P = 0.043</td>
</tr>
<tr>
<td>ESR (mm/1 h)</td>
<td>26.00 (16.00-36.00)</td>
<td>32.00 (20.00-55.75)</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>4.16 (1.83-7.65)</td>
<td>3.76 (1.38-9.72)</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>IgG (g/L)</td>
<td>12.70 (6.57-19.95)</td>
<td>17.55 (15.03-21.30)</td>
<td>P = 0.001</td>
</tr>
<tr>
<td>IgA (g/L)</td>
<td>2.53 (1.08-3.19)</td>
<td>2.72 (1.96-4.03)</td>
<td>P = 0.009</td>
</tr>
<tr>
<td>IgM (g/L)</td>
<td>0.93 ± 0.42</td>
<td>1.17 ± 0.53</td>
<td>P = 0.007</td>
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<tr>
<td>ALB (g/L)</td>
<td>28.97 ± 5.89</td>
<td>37.04 ± 5.05</td>
<td>P &lt; 0.001</td>
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<tr>
<td>BUN (mg/dL)</td>
<td>15.84 (12.03-22.57)</td>
<td>11.46 (8.74-15.63)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Scr (mg/dL)</td>
<td>0.79 (0.67-1.03)</td>
<td>0.70 (0.61-0.78)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>BUA (μmol/L)</td>
<td>328.76 (268.78-416.96)</td>
<td>262.51 (217.17-308.82)</td>
<td>P &lt; 0.001</td>
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<td>RBP (mg/L)</td>
<td>47.80 ± 15.85</td>
<td>37.60 ± 15.28</td>
<td>P = 0.001</td>
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<tr>
<td>CysC (mg/L)</td>
<td>1.39 (1.01-1.95)</td>
<td>1.17 (1.00-1.30)</td>
<td>P = 0.003</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73 m²)</td>
<td>88.57 (66.11-103.17)</td>
<td>96.09 (84.40-119.06)</td>
<td>P = 0.028</td>
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</table>

Normally distributed data are shown as the mean ± SD, other continuous variables are shown as the median (IQR). LN group: systemic lupus erythematosus (SLE) patients with nephritis; SLE without nephritis group: SLE patients without nephritis; SLEDAI-2 k: Systemic Lupus Erythematosus Disease Activity Index-2 k; Serum anti-dsDNA levels which were no less than 100 IU/mL were analyzed statistically by Mann-Whitney test. eGFR: estimated glomerular filtration rate from serum creatinine using Cockcroft-Gault Equation.
the erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) or complement C3 (C3) between LN group and SLE without nephritis group (all P > 0.05).

**Correlation between serum IL-35 levels and indexes related disease activity in SLE patients**

Active SLE patients (n = 65) have lower serum IL-35 levels, compared to inactive SLE patients (n = 55) (60.25 ± 21.50 pg/mL vs. 91.58 ± 22.88 pg/mL, P < 0.001). As shown in Fig. 1A, a significant correlation was observed between serum IL-35 levels and SLEDAI-2k scores (r = −0.626, P < 0.001) in SLE patients. IL-35 was significantly associated with serum anti-dsDNA levels in anti-dsDNA-positive SLE patients (r = −0.343, P = 0.001, Fig. 1B). We also found that serum IL-35 levels were significantly related to C3 (r = 0.443, P < 0.001) and ESR (r = −0.354, P < 0.001) in SLE patients (data not shown). There was no significant correlation between serum IL-35 levels and IgG (data not shown).

**Comparison serum IL-35 levels between SLE patients with and without nephritis**

As shown in Fig. 2A, the serum levels of IL-35 were significantly reduced in LN patients compared with SLE patients without nephritis (69.21 ± 22.94 pg/mL vs. 85.40 ± 31.43 pg/mL, P = 0.013).

**Correlation between serum IL-35 levels and clinical parameters of renal involvement in LN patients**

As shown in Table 2, the serum levels of IL-35 were significantly higher in LN patients with normal serum levels of BUN (75.49 ± 22.07 pg/mL vs. 54.57 ± 17.98 pg/mL, P < 0.001), Scr (72.97 ± 21.85 pg/mL vs. 55.29 ± 22.07 pg/mL, P = 0.004), or BUA (75.59 ± 21.67 pg/mL vs. 58.58 ± 21.28 pg/mL, P = 0.001) compared with LN patients with higher serum levels of corresponding factors. In addition, serum IL-35 levels were significantly lower in LN patients with low eGFR than those with normal eGFR (62.75 ± 20.65 pg/mL vs. 76.37 ± 22.47 pg/mL, P = 0.008), as well as in LN patients with high serum CysC levels than those with normal CysC levels (60.37 ± 20.64 pg/mL vs. 81.81 ± 20.21 pg/mL, P < 0.001) (data not shown).

A significant correlation was found between serum IL-35 levels and eGFR (r = 0.348, P = 0.002, Fig. 2B) in LN patients. Furthermore, serum IL-35 levels correlated significantly with serum CysC (r = −0.510, P < 0.001, Fig. 2C) and albumin (ALB) (r = 0.587, P < 0.001, Fig. 2D) in LN patients. The serum IL-35 levels also correlated significantly with BUN (r = −0.395, P < 0.001, Table 2), BUA (r = −0.378, P = 0.001, Table 2), the number of urinary red blood cells (RBC) (r = −0.390, P < 0.001) and urinary white blood cells (WBC) (r = −0.515, P < 0.001) in LN patients (data not shown). Moreover, a weak correlation was observed between IL-35 and Scr (r = −0.296, P = 0.008) in LN patients. No significant correlations were found between serum IL-35 levels, and RBP or 24-h proteinuria (data not shown).

**Discussion**

To the best of our knowledge, this is the first study to show the relationship between IL-35 and renal involvement in lupus nephritis patients. In this study, we focused on exploring the correlation between IL-35 and disease activity in all SLE subjects as defined by SLEDAI-2k, anti-dsDNA, ESR, and C3 (Sule et al. 2015). We showed distinctly
reduced serum levels of IL-35 in active SLE patients compared to inactive SLE patients. Simultaneously, our data also proved that serum IL-35 levels correlated negatively with SLEDAI-2k, which was consistent with other studies (Qiu et al. 2013). Previous studies have reported that SLE patients had lower serum IL-35 levels compared with healthy individuals, and the levels were inversely associated with disease activity (Ouyang et al. 2014; Yin et al. 2016). Recently, Cai et al. (2015a) have found the remission of SLE together with elevated concentrations of serum IL-35 in mouse model upon recombinant IL-35 treatment.

In addition, the results revealed that elevated anti-dsDNA and ESR showed inverse correlations with serum IL-35 levels in SLE patients ($r = -0.343$, $P = 0.001$, $r = -0.354$, $P < 0.001$, respectively). In contrast, decreased serum concentrations of C3 were positively correlated with IL-35 in SLE patients ($r = 0.443$, $P < 0.001$). These findings raised the possibility that higher disease activity was linked with lower expression levels of IL-35 as a predictive marker.

It has been confirmed that the majority of IL-35 is secreted by Treg (Collison et al. 2007; Vignali et al. 2008), which largely resides in the fraction of CD4+CD25+T cells.
that express the highest density of CD25 in human (Lin et al. 2008). The quality or quantity of CD4+CD25 high Tregs from peripheral blood of patients with active SLE was significantly decreased as compared with normal donors and patients with inactive SLE (Valencia et al. 2007; Bonelli et al. 2009; Cai et al. 2015b). Moreover, Cai et al. (2015b) found that the expression of CD4+CD25 high Tregs was negatively correlated with SLEDAI. In contrast to our results, Qiu et al. (2013) reported that serum IL-35 concentrations were elevated in active SLE patients, and the levels decreased after the administration of large doses of anti-inflammatory prednisone. Cai et al. (2015b) showed that serum IL-35 levels were higher in active SLE patients than inactive SLE patients and healthy people, and there was no significant correlation of plasma concentrations of IL-35 with SLEDAI-2 k in SLE patients. The heterogeneity of SLE and different medical treatment may account for the difference.

Whether IL-35 plays a role in renal involvement in lupus nephritis remains to be determined. The serum levels of IL-35 were significantly lower in LN patients than SLE patients without nephritis. Previous investigators have reported that chemokines took part in inflammation via attracting T cells in the target organ (Cao et al. 2003; Liao et al. 2017). Moreover, increased levels of chemokines in SLE were well confirmed (Wong et al. 2008; Munroe et al. 2017), and might lead to the accumulation of IL-35 in renal tissue. This, in part, may explain the decrease in serum concentrations of IL-35 in LN patients.

To further elucidate the potential role of IL-35 in renal involvement, we next investigated the correlation between IL-35 and clinical parameters of renal involvement, which included eGFR (Schetz et al. 2014), and levels of 24-h proteinuria, BUN, Scr, BUA (Stamenkovic et al. 1986), RBP (Aggarwal et al. 2017), CysC (Silva et al. 2011), ALB (Rahman et al. 2001), urinary erythrocytes and urinary leukocytes (Rahman et al. 2001) in LN patients. In such a pilot study, we observed that LN patients had significantly lower eGFR and strikingly higher levels of BUN, Scr, BUA, RBP and CysC than SLE patients without nephritis. Reduced serum levels of IL-35 in LN patients were closely related to decreased eGFR, elevated serum levels of BUN, Scr, BUA, CysC and increased urinary erythrocytes and leukocytes. The recent study by Hu et al. (2017) in China showed that IL-35 pretreatment could efficiently prevent lipopolysaccharide (LPS)-induced AKI via inhibiting nuclear factor kappa B (NF-κB) activation and reducing pro-inflammatory cytokine production. In addition, previous investigators have elucidated that changes in serum complement and anti-dsDNA levels are associated with the degree of kidney damage in LN patients (Sule et al. 2015). The correlations of IL-35 with anti-dsDNA and C3 have been described in the above paragraph. Therefore, the decreased IL-35 is likely to contribute to the renal involvement of LN patients. Serum IL-35 levels correlated positively with ALB (r = 0.587, P < 0.001), although we found no significant correlation between IL-35 and 24-h proteinuria, or serum RBP. This may be explained by the small sample size of the present study. Therefore, further studies are needed to confirm the role of IL-35 in lupus nephritis.

We therefore endeavor to clarify the association of IL-35 and 24-h proteinuria in order to further elucidate possible relations of IL-35 to the protection of charge barrier in glomerular basement membrane (GBM) and an effect to restore glomerular selective filtration function. We have presented the hypothesis that IL-35 might prevent kidney damage in chronic inflammation of lupus nephritis and further randomized studies are necessary to an in-depth study.

Several limitations should be noted in the study. Firstly, we evaluated a limited number of patients. Secondly, we investigated IL-35 only in peripheral blood, but not in renal tissue, which may weaken the specificity of IL-35 for LN. Thirdly, the medical treatments of SLE were not identical among the patients studied. This is the first

<table>
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<th>IL-35 (pg/mL)</th>
<th>r value</th>
<th>P value</th>
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<tbody>
<tr>
<td>BUN (N/H)</td>
<td>75.49 ± 22.07/54.57 ± 17.98</td>
<td>r = -0.395</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Scr (N/H)</td>
<td>72.97 ± 21.85/55.29 ± 22.07</td>
<td>r = -0.296</td>
<td>P = 0.008</td>
</tr>
<tr>
<td>BUA (N/H)</td>
<td>75.59 ± 21.67/58.58 ± 21.28</td>
<td>r = -0.378</td>
<td>P = 0.001</td>
</tr>
</tbody>
</table>

Data are shown as the mean ± SD; N/H: Normal serum level/High serum level; Normal BUN: lupus nephritis with blood urea nitrogen < 21.35 mg/dL; High BUN: lupus nephritis patients with blood urea nitrogen ≥ 21.35 mg/dL; Normal Scr: lupus nephritis patients with serum creatinine < 1.131 mg/dL; High Scr: lupus nephritis patients with serum creatinine ≥ 1.131 mg/dL; Normal BUA: lupus nephritis patients with blood uric acid < 357.00 μmol/L; High BUA: lupus nephritis patients with blood uric acid ≥ 357.00 μmol/L; P < 0.05 was considered statistically significant.
work that evaluates serum IL-35 concentrations, and the correlation between IL-35 and renal function in LN patient. Hence, further studies are needed to elucidate the exact effect and the signaling pathway of IL-35 in LN.

In conclusion, our results demonstrated the close relationship between IL-35 and disease activity in SLE patients, thereby providing the first evidence that IL-35 acts as a potential biomarker of renal involvement in LN patients. The expression of IL-35 was directly or indirectly inhibited by some mechanism during the development of lupus nephritis. Thus, IL-35 should be considered to have the advantageous effect on LN. Further studies are needed to elucidate the exact effect and the signaling pathway of IL-35 in LN, which may open a door to novel therapeutic options for LN.

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Conflict of Interest

The authors declare no conflict of interest.

References


