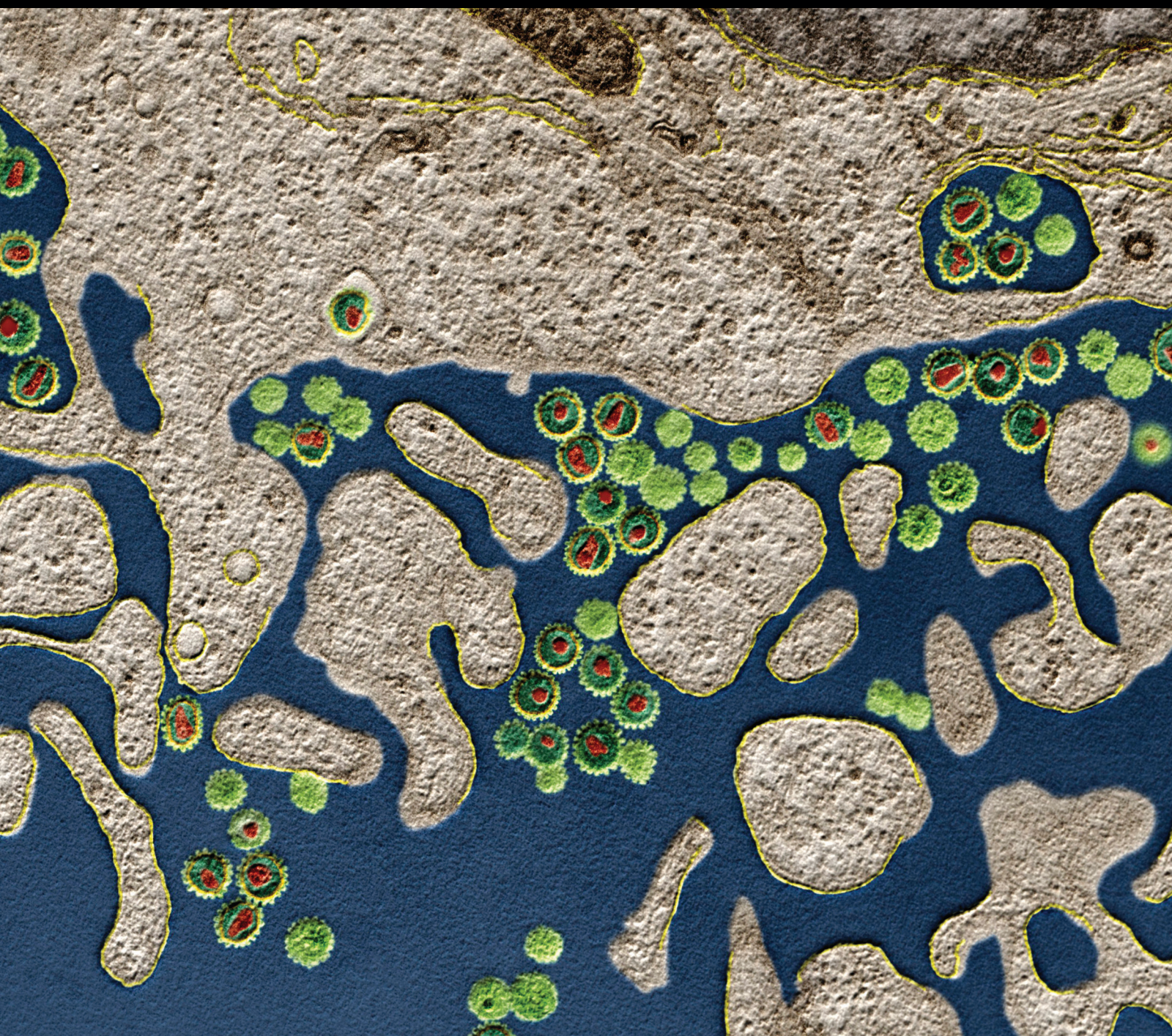
Journal of Immunology Research

New Biomarkers in Autoimmune Disease

Guest Editors: Guixiu Shi, Zhixin Zhang, and Quanzhen Li



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Editorial Board

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Journal of Immunology Research
Volume 2017, Article ID 8702425, 2 pages
https://doi.org/10.1155/2017/8702425

Editorial

New Biomarkers in Autoimmune Disease

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Received 21 March 2017; Accepted 21 March 2017; Published 12 April 2017

Autoimmune diseases are a group of diverse diseases with a common etiology in which the immune system responds to self-antigens leading to damage or dysfunction of tissues. Biomarkers are those measurable substances in the body which associate with the intensity of a disease or other physiological states. Biomarkers can be used in many fields of clinical practice such as disease diagnosis, disease activity evaluation, and disease pathogenesis study. Biomarker research is a rapidly advancing area in autoimmune disease. A variety of researches on discovering new biomarkers including autoantibodies in autoimmune diseases have been conducted in recent years.

Based on this background, this special issue presents recent advances for new biomarkers in autoimmune diseases. From 17 submissions, 8 papers are published covering these autoimmune diseases including systemic lupus erythematosus (SLE), lupus nephritis (LN), polymyositis, and ankylosing spondylitis (AS).

SLE is one of the classical autoimmune diseases characterized by a wide profile of autoantibodies. Z. Da et al. in their article "CXCL13 Promotes Proliferation of Mesangial Cells by Combination with CXCR5 in SLE" found CXCL13 was overexpressed in SLE especially in lupus nephritis patients, it promoted the proliferation of a human renal mesangial cell, and the effect was weakened after the silence of CXCR5. CXCL13-CXCR5 axis is suggested as a new therapeutic target in LN. In another research, De N. He et al. in their article "Association of Serum CXCL13 with Intrarenal Ectopic Lymphoid Tissue Formation in Lupus Nephritis" discovered increased serum levels of CXCL13 play an important role in the formation of renal ectopic lymphoid tissue and the pathological renal impairment process in LN and suggested CXCL13 and the formation of ELT in renal tissues might be an important therapy target for LN. J. Xue et al. in their article "Dickkopf-1 Is a Biomarker for Systemic Lupus Erythematosus and Active Lupus Nephritis" compared the concentrations of Wnt-3A, Frizzled-8, and Dickkopf-1 (DKK-1) of Wnt signaling in SLE patients (with or without LN) and healthy cohorts. The result demonstrated that the serum DKK-1 was considered a better positive biomarker for identification of LN in SLE patients. Therefore, the serum and/or urine DKK-1 may be a valuable and independent biomarker for identification of SLE patients with active LN. R. Bai et al. in their article "Depressive and Anxiety Disorders in Systemic Lupus Erythematosus Patients without Major Neuropsychiatric Manifestations" observed high prevalence of depression and anxiety in SLE patients without major neuropsychiatric manifestations, and anxiety was associated with anti-P0 antibody. S. Li et al. in their article "Link-Polymorphism of 5-HTT Promoter Region Is Associated with Autoantibodies in Patients with Systemic Lupus Erythematosus" found the frequency of SS genotype and S allele of serotonin transporter-linked polymorphic region (5-HTTLPR) in SLE patients with positive anti-Sm antibody and anti-U1RNP antibody significantly higher than the other genotypes and allele and speculated 5-HTTLPR affecting production of some autoantibodies, especially anti-Sm and anti-U1RNP antibody in SLE.

Ankylosing spondylitis is the prototype of immune-mediated inflammatory rheumatic arthritis. C. Wang et al. in their article "Serum HMGB1 Serves as a Novel Laboratory Indicator Reflecting Disease Activity and Treatment Response

in Ankylosing Spondylitis Patients" analyzed the association between serum levels of HMGB1 and clinical features of AS patients before and during treatment. Serum level of HMGB1 was higher in AS patients. It reflects the disease activity of AS and serves as a laboratory indicator for the therapeutic response.

The dysfunction of a peripheral blood mononuclear cell (PBMC) is involved in autoimmune diseases. Q. Xie et al. in their article "DEPTOR-mTOR Signaling Is Critical for Lipid Metabolism and Inflammation Homeostasis of Lymphocytes in Human PBMC Culture" reported combination of DEP domain-containing mTOR-interacting protein (DEPTOR) overexpression and mTORC2/AKT inhibitors effectively inhibits lipogenesis and inflammation in lymphocytes of PBMC culture, implying DEPTOR-mTOR in lymphocytes of PBMC as a biomarker for detecting and treating autoimmune diseases. G. Yin et al. in their article "Identification of Palmitoleic Acid Controlled by mTOR Signaling as a Biomarker of Polymyositis" confirmed the increased palmitoleic acid level in PBMC of patients with polymyositis, and it bears the potential to be a new marker of abnormal PBMC in polymyositis.

These papers above represent some interesting, novel advance in a biomarker for autoimmune diseases. We hope this special issue would provide some new ideas for diagnosis and treatment.

Acknowledgments

We would like to express our great appreciation to all the authors, reviewers, and editors for the support that makes this special issue possible.

Guixiu Shi
Zhixin Zhang
Quanzhen Li

Hindawi
Journal of Immunology Research
Volume 2017, Article ID 6861575, 13 pages
https://doi.org/10.1155/2017/6861575

Research Article

Dickkopf-1 Is a Biomarker for Systemic Lupus Erythematosus and Active Lupus Nephritis

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Received 1 July 2016; Accepted 8 December 2016; Published 8 March 2017

Academic Editor: Quanzhen Li

An early diagnosis of lupus nephritis (LN) has an important clinical implication in guiding treatments of systemic lupus erythematosus (SLE) in clinical settings. In this study, the concentrations of Wnt-3A, Frizzled-8 (FZD-8), and Dickkopf-1 (DKK-1) of Wnt signaling, as well as their diagnostic values for accessing LN, were evaluated by ELISA in sera and urine of 111 SLE patients (31 with LN and 80 without LN) and 70 healthy cohorts. Significantly more abundances of DKK-1 protein were determined in both of sera and urine of SLE patients compared to healthy cohorts ($p < 0.0001$); in particular the serum DKK-1 concentration was even higher in LN-SLE patients relative to non-LN SLE subjects ($p < 0.0001$). Intriguingly, concentrations of above examined proteins in SLE patients showed no correlation between serum and urine. Moreover, a combination of DKK-1 with anti-dsDNA and/or levels of complement C3 and C4 could not increase the specificity and/or sensitivity for identification of patients with LN diseases, but both ROC curve and multiple-factor nonconditional logistic regression analysis showed that serum DKK-1 was considered better positive biomarker for identification of LN in SLE patients. These results imply that serum and/or urine DKK-1 may be a valuable and independent biomarker for identification of SLE patients with LN.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that can be characterized by producing various autoantibodies against self-antigens (autoantigens) [1]. The process of SLE pathogenesis can affect multiple systems and major organs, among which lupus nephritis (LN) is one of the most common major organ manifestations and the main cause of morbidity and mortality in SLE patients [2]. In this regard, LN may affect up to 40–80% of SLE patients, partially owing to adverse effects (AEs) of an immunosuppressive treatment for LN on kidney, which may result in chronic renal failure and sequentially increase the morbidity and mortality in SLE patients [1]. This suggests that an involvement of renal disease activity is one of the most important prognostic factors for patients with SLE, and the identification of LN in SLE patients thus has an important clinical implication in guiding the treatment of SLE, which may avoid an immunosuppressive overtreatment in clinical settings [3].

In general, SLE is recognized as a disease that is primarily attributed to autoantibodies, cytokines, and immune complex deposition, and a compelling body of study has demonstrated cytokines such as soluble interleukin-7 receptor (sIL-7R) and autoantibodies to complement C1q, histone, chromatin, and nuclear and double-strand DNA (dsDNA) alone or in combination with anti-C1q, anti-dsDNA, and/or antibodies and/or nucleosome were strongly correlated with renal diseases and could be used for prognosis of patients with LN [2, 4–7]. However, antibodies to dsDNA and the reduction of complements were also found in non-LN patients and clinically inactive SLE patients with a relatively high percentage [8].

Such a lack of specificity of anti-dsDNA antibodies for renal flare was also observed in other conventional parameters such as antinuclear antibody (ANA), levels of complements C3 and C4, proteinuria, and urine sediment [9], which thus led to search other reliable biomarkers for identifying SLE patients with active nephritis [10].

The Wnt signaling has been demonstrated to play crucial roles in several biological aspects, including cellular proliferation, embryonic development, tissue homeostasis, development of immune system, and other systemic effects [11]. In addition to its dispensable roles in the development of T cells and the immune system, mounting evidence has recently suggested that it is involved in the pathogenesis of many types of autoimmune diseases, including rheumatoid arthritis (RA), SLE, spondyloarthritis (PsA), and ankylosing spondylitis (AS) [12–17]. In SLE patients, aberrant Wnt/β-catenin signaling was observed in both peripheral B and T cell fractions [18]. Based on the dependence of β-catenin, Wnt signaling pathways can be thus further characterized by Wnt/β-catenin or canonical pathway and several "non-canonical pathways" (β-catenin independent). The latter includes the planer cell polarity (PCP), c-Jun N-terminal protein kinases (JNK), protein kinase C/calcium (PCK/Ca^{2+}) pathway, receptor-like tyrosine kinase (RYK), and receptor tyrosine kinase-like orphan receptor (Ror) pathways [11]. Among these, the Wnt/β-catenin signaling pathway is the most investigated and the best characterized Wnt signaling pathway.

Activation of Wnt/β-catenin signaling can be triggered by the binding of Wnt ligand to its coreceptor low-density lipoprotein receptor-related protein 5 (LRP-5) or LRP6 and frizzled (FZD) family of proteins [19]. Intriguingly, Wnt signaling can also be regulated by extracellular antagonists such as Wise (Sostdc1), secreted frizzled-related protein (SFRP), the Wnt inhibitory factor 1 (WIF1), Cerberus, and the Dickkopf (DKK) family of secreted proteins [20]. Among them, the DKK family of Wnt antagonists has recently spurred increased interests. The DKK family comprises four members of proteins, DKK-1, DKK2, DKK3, and DKK4, which are synthesized as precursor proteins activated by a proteolytic cleavage [21]. The DKK-1 are the most studied members of this family, which can inhibit the Wnt signaling by binding to LRP-5/6 and then degrading the coreceptor and thus have been considered as potential targets in diseases with an aberrant Wnt signaling activity [22, 23].

In RA patients, serum DKK-1 levels were correlated with parathyroid hormone, bone erosions, and bone mineral density [24], and DKK-1 polymorphisms of DKK-1 were associated with the RA structural severity and expression of DKK-1 protein [25]. Activation of DKK-1 and the TNF-alpha-stimulated integrin-related FAK signaling could induce the dissociation of beta-catenin/E-cadherin, which in turn promoted RA fibroblast-like synoviocytes (FLS) migration [26], and such dysregulated DKK-1 pathway could be involved in the pathogenesis and perpetuation of the inflammatory response in early clinically apparent stages of RA [27]. Similarly, in patients with SLE, an aberrant expression of Wnt/β-catenin signaling related genes HIG2, TCF7, KHSRP, WWP1, SMAD3, TLK2, AES, CCNI, and PIM2 was observed in the peripheral blood $CD4^+$ T cells in patients with SLE [18, 28]. These genes have been demonstrated to play an important role in the regulation of T cell proliferation and differentiation [29]. Both human and LN MRL/lpr mouse studies indicated that the DKK-1 protein was significantly higher in the sera of SLE patients compared with control subjects, and the LN MRL/lpr mice exhibited a phenotype with an enhanced Wnt/β-catenin activity, accompanied by an increased level of DKK-1 in the renal tissues and sera and an increased frequency of apoptotic cells of the renal tubular and renal interstitial tissues [30]. Notably, the beta-catenin transcriptional activity in leukocytes of lupus-prone mice and SLE patients was diminished, particularly in myeloid cells [31]. Such an activated Wnt signaling was further evidenced in human renal tissues of patients with LN by accessing β-catenin at both transcriptional and translational levels using assays including immunohistochemistry staining, qRT-PCR, and western blotting, suggesting that a dysregulated Wnt/β-catenin signaling was related to the pathogenesis of LN and might play a role in the renal fibrosis [15].

Recently, an increasing number of studies have demonstrated the potentially diagnostic and/or prognostic values of DKK-1 in varied cancers, such as lung cancer [32–34], gastrointestinal cancers [23], pancreatic cancer [35], and hepatocellular carcinoma [36–38], as well as rheumatic disorders, including RA [24] and AS [39, 40]. Together with aforementioned pathogenic roles of Wnt signaling in LN development, these studies clearly imply that Wnt signaling, in particular the DKK-1, may be a novel biomarker for identification of LN for patients with SLE. To this end, we evaluated concentrations of Wnt-3A, a ligand of Wnt/β-catenin signaling, FZD-8, a receptor of the signaling, and DKK-1 in the sera and urine of 111 SLE patients in a single center and investigated the clinical significance of such antibodies alone or in combination with anti-dsDNA antibodies and/or serum levels of C3 and C4 for accessing active nephritis in SLE patients in the present study. Our results showed that only DKK-1 protein was increased in both sera and urine of SLE patients, but only the serum DKK-1 exhibited a statistical difference between LN-SLE patients and non-LN-SLE subjects. However, a combination of DKK-1 with anti-dsDNA and/or levels of complements C3 and C4 could not increase the specificity and/or sensitivity for identification of patients with LN diseases. These data suggest that the serum and/or urine DKK-1 may be a valuable and independent biomarker for identification of SLE patients with active LN.

2. Materials and Methods

2.1. Ethics Statement. Human blood and urine samples were collected with a protocol approved by the Ethic Committee for the Conduct of Human Research at Ningxia Medical University (NXMU-E2012-102p). Written consent was obtained from every individual according to the Ethic Committee for the Conduct of Human Research protocol. For the participants younger than 18 years old, written informed consent was obtained from their guardians or parents on behalf of

the children. No special informed consent was required for Chinese Hui, Man, and Mongolian minorities in this study. All participants provided a written informed consent for the publication of the data. The PI of this study maintains human research records, including signed and dated consent documents, for ten (10) years after the age of majority. The Ethic Committee the Conduct of Human Research at Ningxia Medical University approved the consent procedure for this study (NXMU-2012-102e).

2.2. Blood and Urine Samples. Blood and urine samples of 111 consecutive SLE patients (103 females and 8 males) were collected from the outpatient rheumatology clinics of the General Hospital of Ningxia Medical University from January to December 2015. The mean ± SD age for the SLE patients at the time of the sample drawn was 38.23 ± 11.17 years old (range 17 to 76), with an average duration of diseases of 6.14 ± 4.24 (0.3 to 14 years). The American College of Rheumatology (ACR) criteria were used to diagnose a patient with SLE [41, 42], and the disease activity was defined according to SLE Disease Activity Index (SLEDAI) criteria [43, 44]. A patient with SLEDAI ≥ 10 was defined as active SLE. Renal involvement was defined based on clinical and laboratory manifestations. An active LN was defined as urine protein excretion ≥500 mg/day or cellular casts [41]. Sera and urine of 70 gender and age-matched healthy individuals (7 males and 63 females) were also collected. These healthy control cohorts were recruited from those who had undergone comprehensive medical screening at the General Hospital of Ningxia Medical University and who had no history of chronic diseases, no family history of autoimmune diseases. The demographics of individuals involved in this study were outlined in Table 1. All sera were treated with heparin. Both serum and urine samples were frozen in 100 μL aliquots at −80°C until analyzed. The ethnic populations of subjects in this study included Chinese Han, Chinese Hui, Chinese Man, and Chinese Mongolian Mongolian. Ethnic populations were determined based on criteria of purely Chinese Han, Hui, Man, or Mongolian descents for at least three generations (Table 1). There was no genetic relationship among these individuals. All the samples were collected under informed consent.

2.3. Detection of Wnt-3A, FZD-8, and DKK-1 by Enzyme-Linked Immunosorbent Assay (ELISA). Concentrations of Wnt signaling Wnt-3A, FZD-8, and DKK-1 proteins in serum and urine were measured by ELISA using commercially available kits according to the manufacturer's instructions. The ELISA Kits for human Wnt-3A and FZD-8 were products of R&D Systems China Co., Ltd. (Shanghai, China); the ELISA Kit for DKK-1 was a product of BosterBio Inc. (Wuhan, China). For measurement of Wnt-3A and FZD-8, both urine and serum were diluted with dilution buffer by 5. For detection of DKK-1 protein, the serum and urine were diluted with dilution buffer by 20 and 10, respectively. Briefly, diluted samples were added to each well; the wells were then washed with high ionic strength buffer after being incubated at room temperature for 1 h. Then horseradish peroxidase-conjugated anti-human IgG supplied with the kit was used as the secondary antibody. After 30 min incubation, the wells were extensively washed for three times, followed by the addition of 100 μl trimethylbenzene solution and incubation for 30 min before an 100 μl of stopping solution was added to each well. The optical density was then measured at 450 nm. The absorbance ($OD_{450\,nm}$) was then converted into a concentration (ng/mL) through standard curve. Other laboratory data, including urinalysis, serum levels of complements C3 and C4 and hemoglobin, antinuclear antibodies (ANA), anti-dsDNA antibodies, antiribonucleoprotein, perinuclear antineutrophil cytoplasmic antibody (pANCA), antibodies to Sjogren's syndrome A (SSA) and B (SSB), and anti-Smith (Sm) were also recorded, respectively. The sensitivity, specificity, and predictive values were calculated using formula described in a previous report [45].

2.4. Statistical Analysis. All laboratory data were entered into and extracted from PRISM (version 5) (GraphPad Software, La Jolla, CA, USA) and/or SPSS for Windows (version 17.0) (SPSS Inc., Chicago, IL, USA). Statistical evaluation of the data was performed by one-way ANOVA when more than two groups were compared with a single control and t-test for comparison of differences between the two groups. ROC (receiver operator characteristic) curve was used to find out the best cut-off value and validity of certain variable. The multiple-factor nonconditional logistic regression analysis was employed with SPSS Software. The association between qualitative variables was evaluated by Spearman correlation. Data was presented as the mean standard error of mean (SEM) or mean ± standard deviation (SD). A p value of less than 0.05 was considered statistically significant. $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.0001$.

3. Results

3.1. SLE Demographics Data. The unselected SLE population studied in this study included 103 (92.8%) females and 8 males (7.2%) with a mean age of 38.23 ± 11.17 years old (range 17 to 76), and the average duration of diseases was 6.14 ± 4.24 (0.3 to 14 years) at the time of the sample collection (mean ± SD). The mean of SLEDAI score of SLE was 8.55 ± 5.14 (range 0 to 27). The majority of distribution of ethnic population was 80.2% of Chinese Han (Table 1). The demographic data of LN-SLE and non-SLE patients, as well as control healthy cohorts, were presented in Table 1. Laboratory parameters between active and inactive SLE, with and without renal involvement were also listed in Table 2.

3.2. Concentrations of Wnt-3A, FZD-8, and DKK-1 Proteins in Sera of SLE Patients. In order to determine whether Wnt signaling was correlated with SLE activity, serum concentrations of Wnt-3A, FZD-8, and DKK-1 were evaluated in SLE patients with and without renal flare and healthy subjects. Serum concentrations of respective Wnt-3A, FZD-8, and DKK-1 were 45.54 ± 2.24, 4.96 ± 0.22, and 7.32 ± 0.33 ng/mL for healthy subjects; 44.73 ± 1.86, 4.67 ± 0.20, and 14.28 ± 0.53 ng/mL for SLE patients; 53.54 ± 3.44, 5.49 ± 0.25, and 17.02 ± 0.72 ng/mL for LN-SLE patients; and 41.55 ± 2.20,

Table 1: Demographics of patients with systemic lupus erythematosus (SLE) without lupus nephritis (LN) (non-LN-SLE) ($N = 80$), SLE with LN (LN-SLE) ($N = 31$), and healthy control cohorts.

Demographics	Non-LN-SLE ($n = 80$)	LN-SLE ($n = 31$)	Healthy ($n = 70$)
Ethnics (Chinese Han/Hui/Man/Mongolian)	80 (66/12/1/1)	31 (23/8/0/0)	70 (43/27/0/0)
Age (mean ± SD) (range, years old)	40.14 ± 11.90 (18–76)	33.30 ± 9.29 (17–52)	30.14 ± 8.43 (20–65)
Gender (male/female) (% female)	4/76 (95.6)	4/27 (87.1)	7/63 (90.0)
Disease duration (mean ± SD) (range, years)	6.23 ± 4.21 (0.3–12)	5.89 ± 4.32 (0.6–14)	NA
SLEDAI score (range)	7.31 ± 4.83 (0–19)	11.74 ± 5.93 (4–27)	NA

Data represented the mean ± SD analyzed by Student's t-test using SPSS. NA: unrelated.

5.16 ± 0.22, and 12.22 ± 0.55 ng/mL for non-LN patients (Table 3 and Figure 1). Surprisingly, no significant difference of abundance of serum Wnt-3A protein was found between SLE patients and healthy subjects, although a statistical higher level of Wnt-3A was determined in LN-SLE patients relative to non-LN-SLE patients (Figure 1(a)). Interestingly, there was no significant difference of serum FZD-8 protein detected between healthy individuals and SLE patients and between SLE patients with LN and without LN (Figure 1(b)). Notably, a significantly more abundant serum DKK-1 protein was determined in SLE patients compared to healthy cohorts ($p < 0.0001$) (Figure 1(c)). More importantly, a strikingly higher level of serum DKK-1 was found in SLE patients with renal involvement in comparison with those without renal flare ($p < 0.0001$) (Figure 1(c)).

3.3. Concentrations of Wnt-3A, FZD-8, and DKK-1 Proteins in Urine of SLE Patients. Since an activated Wnt signaling was reported in kidney of SLE patients with renal involvement, we next evaluated levels of Wnt-3A, FZD-8, and DKK-1 in urine of SLE patients. Urine levels of respective Wnt-3A, FZD-8, and DKK-1 were 71.71 ± 1.31, 5.96 ± 0.20, and 2.07 ± 0.10 ng/mL for healthy individuals; 64.51 ± 1.01, 5.85 ± 0.16, and 2.68 ± 0.11 ng/mL for SLE patients; 64.69 ± 1.73, 6.41 ± 0.21, and 2.87 ± 0.22 ng/mL for LN-SLE patients; and 64.43 ± 1.25, 5.64 ± 0.20, and 2.58 ± 0.13 ng/mL for non-LN patients (Table 3 and Figure 2). Of note, a significantly less abundant Wnt-3A protein could be detected in urine of SLE patients relative to control subjects ($p < 0.0001$), but no difference was found between SLE patients with and without renal flare ($p = 0.9805$) (Figure 2(a)). Similar to serum FZD-8, there was no statistical difference found in urine FZD-8 between healthy individuals and SLE patients, despite a moderately more abundance of urine FZD-8 protein was determined in SLE patients with LN relative to those without renal involvement ($p < 0.05$) (Figure 2(b)). Of importance, a significantly higher level of urine DKK-1 protein was determined in SLE patients relative to healthy individuals ($p = 0.0003$) (Figure 2(c)), but unlike what is seen in serum, no difference of the abundance of urine DKK-1 was detected between SLE patients with renal involvement and those without LN ($p = 0.2633$) (Figure 2(c)).

3.4. Correlations of Wnt-3A, FZD-8, and DKK-1 Concentrations in Sera and Those in Urine of SLE Patients. Above data showed DKK-1 protein was more abundant in both serum and urine of SLE patients compared with those of healthy subjects; the correlation of above examined proteins in serum and urine was analyzed. Unexpectedly, there was no association between serum and urine in SLE patients determined for Wnt-3A ($r = 0.159$, $p = 0.0955$, and $N = 111$), FZD-8 ($r = -0.0892$, $p = 0.3518$, and $N = 111$), and DKK-1 ($r = -0.0246$, $p = 0.7976$, and $N = 111$) (Figure 3). In addition, association analysis serum or urine DKK-1 and clinical SLEDAI score or other serological biomarkers including anti-C1q, anti-dsDNA, ANA, and C3 and C4 levels also showed no significant correlation between DKK-1 and aforementioned serological biomarkers or SLEDAI score (data not shown).

3.5. Significance of DKK-1 for the Identification of Patients with LN. Higher levels of serum and urine DKK-1 protein were detected in SLE patients compared with healthy subjects; in particular serum DKK-1 was even more abundant in patients with LN-SLE in comparison with non-LN-SLE patients (Table 3, Figures 1 and 2). In order to evaluate the significance of serum DKK-1 in clinical settings, we analyzed the sensitivities and specificities of serum DKK-1, anti-dsDNA antibodies, and levels of C3 and C4 alone or in a combination for the identification of patients with LN (Table 4). DKK-1 alone displayed a superior sensitivity for identifying patients with LN to serum levels of C3 and C4 but inferior to anti-dsDNA antibodies (Table 4). Furthermore, a combination of serum DKK-1 and anti-dsDNA antibodies or serum levels of C3 and C4 could not increase specificities and sensitivities in identification of patients with LN in comparison with these serological markers alone (Table 4). Of interest, the multiple-factor nonconditional logistic regression analysis of impacts on LN-SLE suggested that serum DKK-1 was a factor with clinical significance for LN-SLE with an odd ratio (OR) (95% CI) of 1.271 ($p = 0.045$) (Table 5). The ROC curve also showed that DKK-1 (Figure 4), particularly the serum DKK-1, was considered better positive biomarker than negative in LN with higher sensitivity (Figure 4). The area under curve (AUC) for serum DKK-1 was 0.783 (SE: 0.053; range: 0.678–0.888) (Figure 4(a)); and the AUC for urine DKK-1 was 0.516 (SE: 0.077; range: 0.365–0.667). These results may imply that the serum DKK-1 protein may be an independent biomarker for identification of LN in SLE patients.

4. Discussion

With increasing appreciation for findings that dysregulated Wnt/β-catenin signaling is involved in the development

Table 2: Association of the presence of laboratory parameters between active and inactive SLE, with and without renal involvement (mean ± SD).

	Activity of SLE			Renal involvement of SLE		
	Active SLE (n = 20)	Inactive SLE (n = 91)	p	LN (n = 31)	Non-LN (n = 80)	p
CANCA (+) number (%)	0/20 (0)	0/91 (0)	NA	0/31 (0)	0/80 (0)	NA
ANA (+) number (%)	17/20 (85.0)	55/91 (86.0)	NA	28/31 (90.3)	44/80 (55.0)	NA
Anti-Rib-P (+) number (%)	2/20 (10.0)	COPD11/91 (12.1)	NA	4/31 (12.9)	9/80 (11.2)	NA
Anti-SSA Ab (+) number (%)	8/20 (40.0)	30/91 (33.0)	NA	15/31 (48.4)	23/80 (28.8)	NA
Anti-SSB Ab (+) number (%)	4/20 (20.0)	11/91 (12.1)	NA	6/31 (19.4)	9/80 (11.3)	NA
pANCA (+) number (%)	1/20 (5.0)	1/91 (1.1)	NA	0/31 (0)	2/80 (2.5)	NA
U1RNP (+) number (%)	9/20 (45.0)	18/91 (19.8)	NA	10/31 (32.3)	17/80 (21.2)	NA
Anti-Smith (+) number (%)	6/20 (30.0)	6/91 (7.0)	NA	4/31 (12.9)	8/80 (10.0)	NA
ACA (+) number (%)	15/20 (75.0)	30/91 (32.4)	NA	20/31 (64.5)	25/80 (31.3)	NA
C3 (μg/mL)	0.48 ± 0.18	0.60 ± 0.19	0.000	0.43 ± 0.18	0.58 ± 0.21	0.0067
C4 (μg/mL)	0.054 ± 0.023	0.57 ± 0.022	0.146	0.055 ± 0.021	0.088 ± 0.026	0.0913
Anti-dsNDA (IU/mL)	214.45 ± 103.76	90.30 ± 70.17	0.000	229.70 ± 111.05	156.91 ± 102.30	0.045

A SLEDAI score not less than 10 was defined as active SLE. Data represented the mean ± SD; variance was analyzed by independent-samples t-test using GraphPad Prism5. Ab: antibody; ACL: anticardiolipin; ANA: antinuclear antibody; LN: lupus nephritis; pANCA: perinuclear antineutrophil cytoplasmic antibody; Rib-P: ribosomal P-proteins; RNP: ribonucleoprotein; SSA: Sjogren's syndrome A; and SSB: anti-Sjogren's syndrome B.

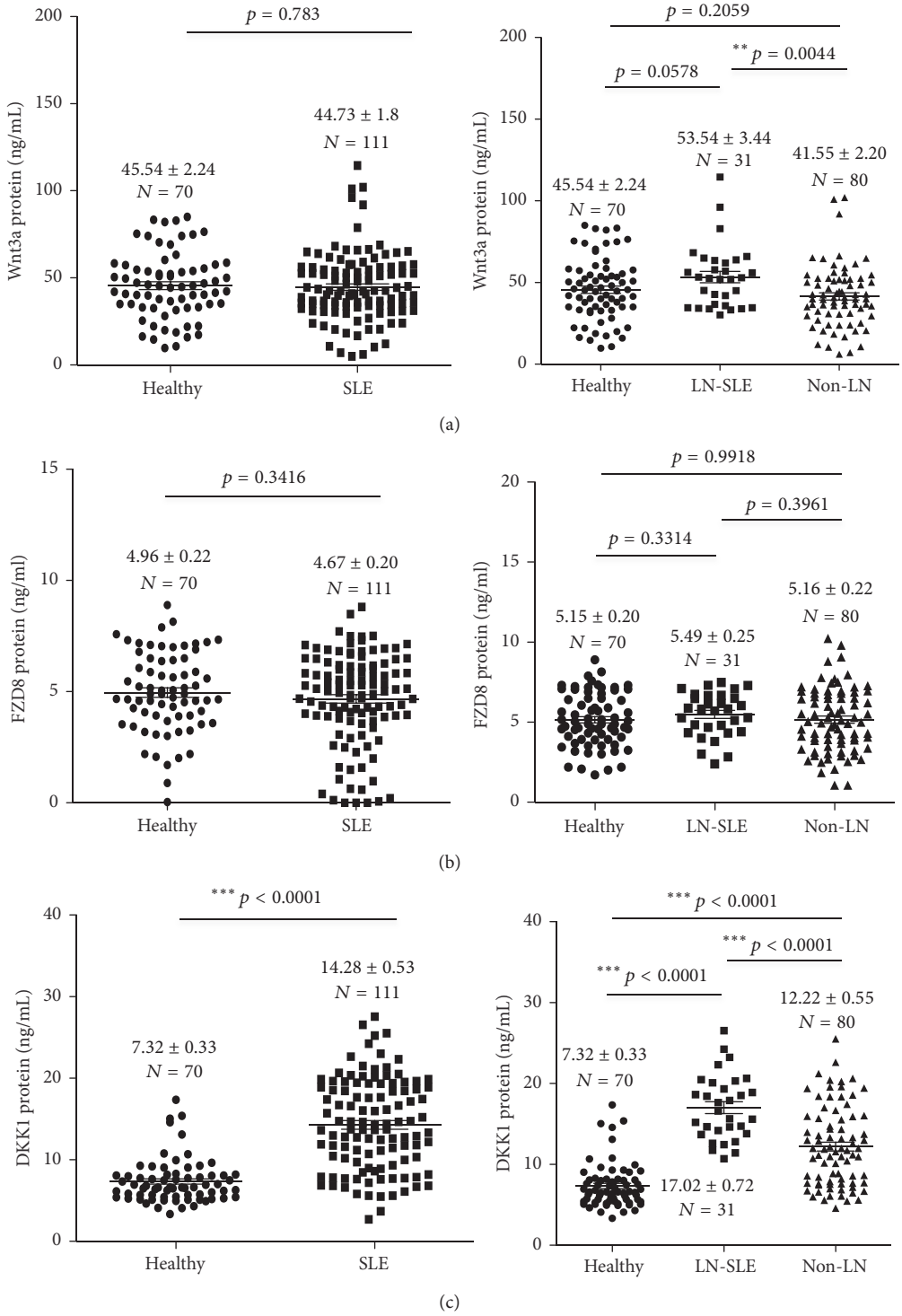

Figure 1: The serum concentrations of Wnt-3A, FZD-8, and DKK-1 proteins in healthy individuals and SLE patients. (a) The concentration of serum Wnt-3A protein in healthy individuals and SLE patients. No statistical difference was determined between healthy individuals and SLE patients (left panel), but more abundant Wnt-3A protein was found in sera of SLE patients with renal involvement (LN-SLE) compared with non-LN-SLE patients (right panel, $p = 0.0044$). (b) The concentration of serum FZD-8 protein in healthy individuals and SLE patients. No statistical difference was determined between healthy individuals and SLE patients (left panel), and LN-SLE and non-LN-SLE patients (right panel). (c) The concentration of serum DKK-1 protein in healthy individuals and SLE patients. Statistical differences were found between healthy individuals and SLE patients (left panel, $p < 0.0001$), LN-SLE ($p < 0.0001$) and non-LN-SLE patients (right panel, $p < 0.0001$). More abundant serum DKK-1 protein was detected in LN-SLE patients relative to healthy individuals and SLE patients without renal flare, and the highest level of serum DKK-1 protein was determined in LN-SLE patients. Bars indicate the average levels of indicated proteins in each group. Compared with the respective healthy and non-LN groups, $^{**}p < 0.01$ and $^{***}p < 0.001$. Data presents as the mean ± SEM in each groups.

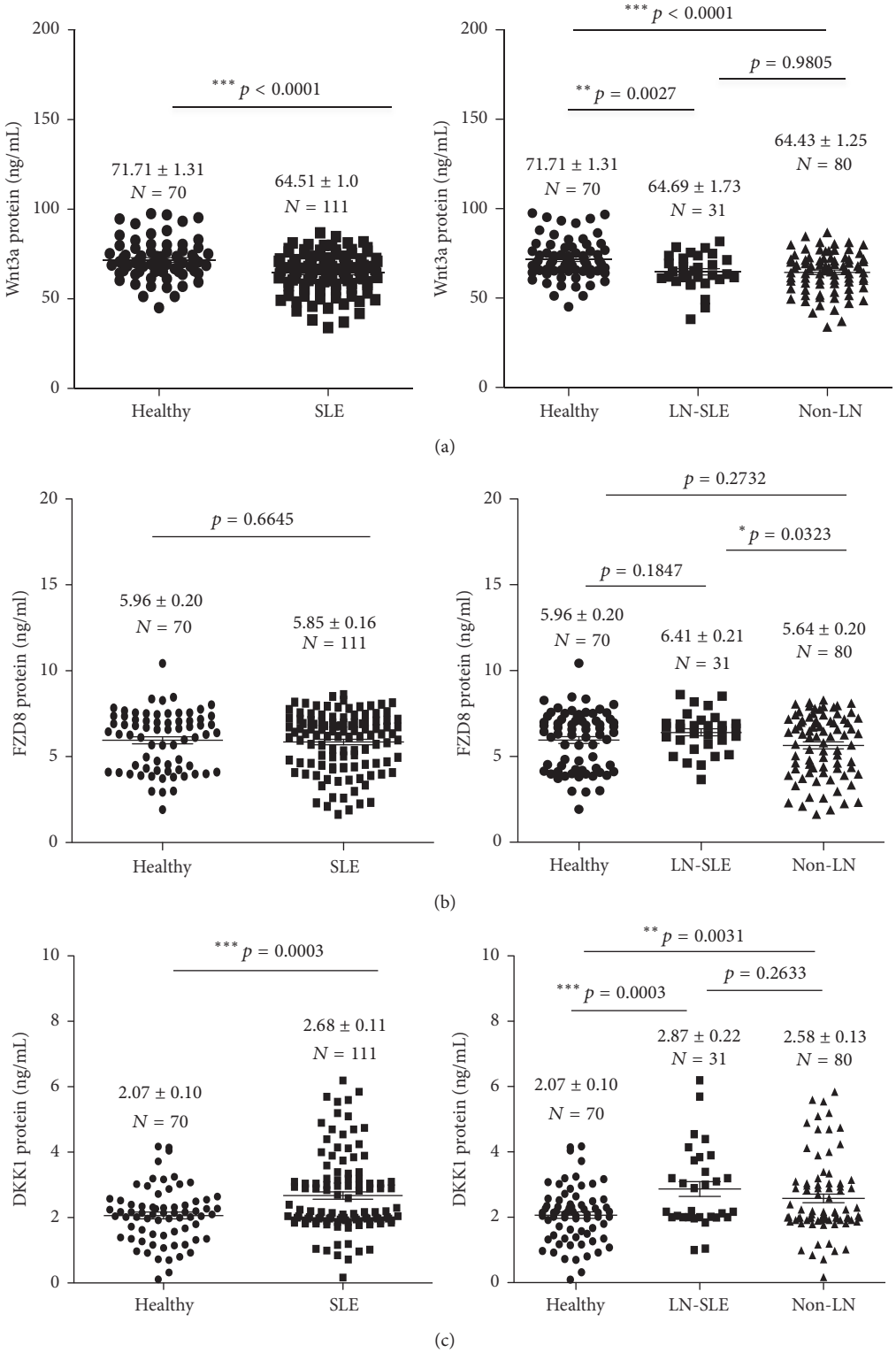

Figure 2: The concentrations of Wnt-3A, FZD-8, and DKK-1 proteins in urine of healthy individuals and SLE patients. (a) The concentration of urine Wnt-3A protein in healthy subjects and SLE patients. No statistical difference was determined between healthy individuals and SLE patients (left panel) and LN-SLE and non-LN-SLE patients as well (right panel). (b) The concentration of urine FZD-8 protein in healthy individuals and SLE patients. No statistical difference was determined between healthy individuals and SLE patients (left panel), but a moderately more abundant FZD-8 protein was found in the urine of LN-SLE patients compared with SLE patients without renal involvement (right panel, $p < 0.05$). (c) The concentration of urine DKK-1 protein in healthy individuals and SLE patients. A statistical difference was found between healthy individuals and SLE patients (left panel, $p < 0.0001$) but not between LN-SLE and non-LN-SLE patients (right panel, $p = 0.2633$). Bars indicate the average levels of indicated proteins in each group. Compared with respective healthy and non-LN groups, $^{*}p < 0.05$, $^{**}p < 0.01$ $^{***}p < 0.001$. Data presents as the mean ± SD in each groups.

Table 3: The abundances of Wnt-3A, FZD-8, and DKK-1 proteins in the sera and urine of patients with SLE.

	Serum SLE			Urine SLE		
	Non-LN-SLE ($n = 80$)	LN-SLE ($n = 31$)	p	Non-LN-SLE ($n = 80$)	LN-SLE ($n = 31$)	p
Wnt-3A	41.55 ± 2.20 (ng/mL)	53.54 ± 3.44	0.0044**	64.43 ± 1.25	64.69 ± 1.25	0.9805
FZD-8	5.16 ± 0.22 (ng/mL)	5.49 ± 0.25	0.3961	5.64 ± 0.20	6.41 ± 0.21	0.0323*
DKK-1	12.22 ± 0.55 (ng/mL)	17.02 ± 0.72	0.000***	2.58 ± 0.13	2.87 ± 0.22	0.2633

Data represented the mean ± SEM; variance was analyzed by independent-samples t-test using GraphPad Prism5. * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

Table 4: Significant difference in levels of serum DKK-1 and anti-dsDNA antibodies, C3, and C4 in patients with LN-SLE compared to non-LN-SLE individuals.

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
DKK-1 (serum)	24/31 (77.4)	34/80 (42.5)	24/70 (34.3)	46/53 (86.8)
Anti-dsDNA Ab	25/31 (80.6)	45/80 (62.5)	25/60 (41.7)	35/41 (85.4)
Levels of C3 and C4	15/31 (48.4)	40/80 (50.0)	15/55 (27.3)	40/56 (71.4)
Anti-dsDNA Ab, levels of C3 and C4	13/31 (41.9)	39/80 (48.8)	13/54 (24.1)	41/59 (69.5)
DKK-1 (serum) and dsDNA	12/31 (38.7)	33/80 (41.2)	12/59 (20.3)	47/66 (66.7)
DKK-1 (serum), levels of C3 and C4	11/31 (35.5)	33/80 (41.3)	11/58 (19.0)	47/67 (70.1)
DKK-1 (serum) and dsDNA, levels of C3 and C4	11/31 (35.5)	32/80 (40.0)	11/59 (18.6)	48/68 (70.6)

and pathogenesis of many disease types including cancers and autoimmune disorders, such as the SLE [16, 46, 47], several key molecules in Wnt/β-catenin signaling cascade have been investigated as biomarkers for disease diagnosis and/or prognosis [48–53], among them Wnt signaling inhibitor DKK-1 has spurred an increased interest in both cancers and autoimmune diseases [24, 27, 30, 34, 37, 40, 54–56]. In this report, we examined concentrations of Wnt-3A, FZD-8, and DKK-1 in the serum and urine of SLE patients and analyzed their diagnostic value for identification of SLE patients with renal flare. The results showed that significant more abundances of DKK-1 protein were determined in both sera and urine of SLE patients compared with healthy cohorts ($p < 0.0001$); in particular the serum DKK-1 concentration was even higher in LN-SLE patients relative to non-LN-SLE subjects ($p < 0.0001$). Consistently, less abundant Wnt-3A was also determined in urine of SLE patients relative to healthy cohorts, although there was no difference of Wnt-3A observed between sera of these two groups. In contrast, there was no significant difference of FZD-8 found in neither sera nor urine in this study. Intriguingly, the concentrations of above examined proteins in sera and urine showed no correlation. Moreover, a combination of DKK-1 with anti-dsDNA and/or levels of complements C3 and C4 could not increase the specificity and/or sensitivity for identification of patients with LN diseases, but both the ROC curve and the multiple-factor nonconditional logistic regression analysis showed that the serum DKK-1 was considered better positive biomarker for identification of LN in SLE patients. These results imply that the serum and/or urine DKK-1 may be valuable and independent biomarker for identification of SLE patients with active LN. These data are in line with previous findings of DKK-1 and Wnt signaling in SLE patients [15, 30, 56].

Several lines of evidences have demonstrated that aberrant canonical Wnt/β-catenin signaling is involved in autoimmune disorders. For instance, Wnt signaling has been recognized to play a central role in the bone development and homeostasis in adulthood, and a dysregulation of this signaling is associated with bone pathologies [24, 34]. In this context, Dickkopf-1 (DKK-1) is required for embryonic head development, which has been implicated in osteoclast dysregulation in RA [56, 57]. A blockade of DKK-1 may thus serve to restore the osteoblast-osteoclast balance and repair bone erosion in RA joints. Indeed, an evoked serumDKK-1 level was determined in RA patients as compared with healthy individuals, which was also found to correlate with parathyroid hormone, bone erosions, and osteoporosis [24]. In addition, several preclinical studies further showed that a neutralizing DKK-1 and/or enhancing Wnt/β-catenin signaling could be an effective therapeutic option in treatment of bone pathologies [24, 57–59]. Similarly, Wnt signaling inhibitors sclerostin and DKK-1 have been investigated as biomarkers for disease activity in ankylosing spondylitis (AS), and a lower level of serum DKK-1 was observed in AS patients [39, 60]. Interestingly, the amount of DKK-1 protein was found not to be consistently correlated to its capacity of binding to the LRP coreceptor in sera of AS patients, in which the DKK-1 in the sera was less able to suppress β-catenin translocation to the nucleus than control sera, implying that the DKK-1 might be dysfunctional in AS patients [61]. Mechanistically, aberrant TNF was suggested to contribute to inducing the evoked expression of DKK-1 and sclerostin in RA, and a neutralization of DKK-1 with antibodies led a reversed phenotype of erosions in several inflammatory arthritis murine models and altered the phenotype from bony erosion to proliferation [62]. Such an elevated serum DKK-1 level was also reported in SLE patients with bone erosion [30]. Furthermore, the blockade of DKK-1 with this antibody could promote the fusion of the sacroiliac joints in TNF-engineered RA mouse model [63]. Mechanistically, the TNF-induced release of DKK-1 might be able to inhibit Wnt signaling, which in turn diminished osteoprotegerin (OPG) expression and osteoblastogenesis and

Table 5: Multiple-factor nonconditional logistic regression analysis of the impact of serological factors on LN-SLE.

	DKK-1	SLEDAI	Anti-dsDNA	C3	C4	Sm	ANCA
WALD	4.016	1.672	1.818	1.379	0.43	3.332	0.723
p	0.045*	0.196	0.178	0.24	0.837	0.523	0.465
OR	1.271	1.166	1.008	0.019	0.1	0.1	0.16

* $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

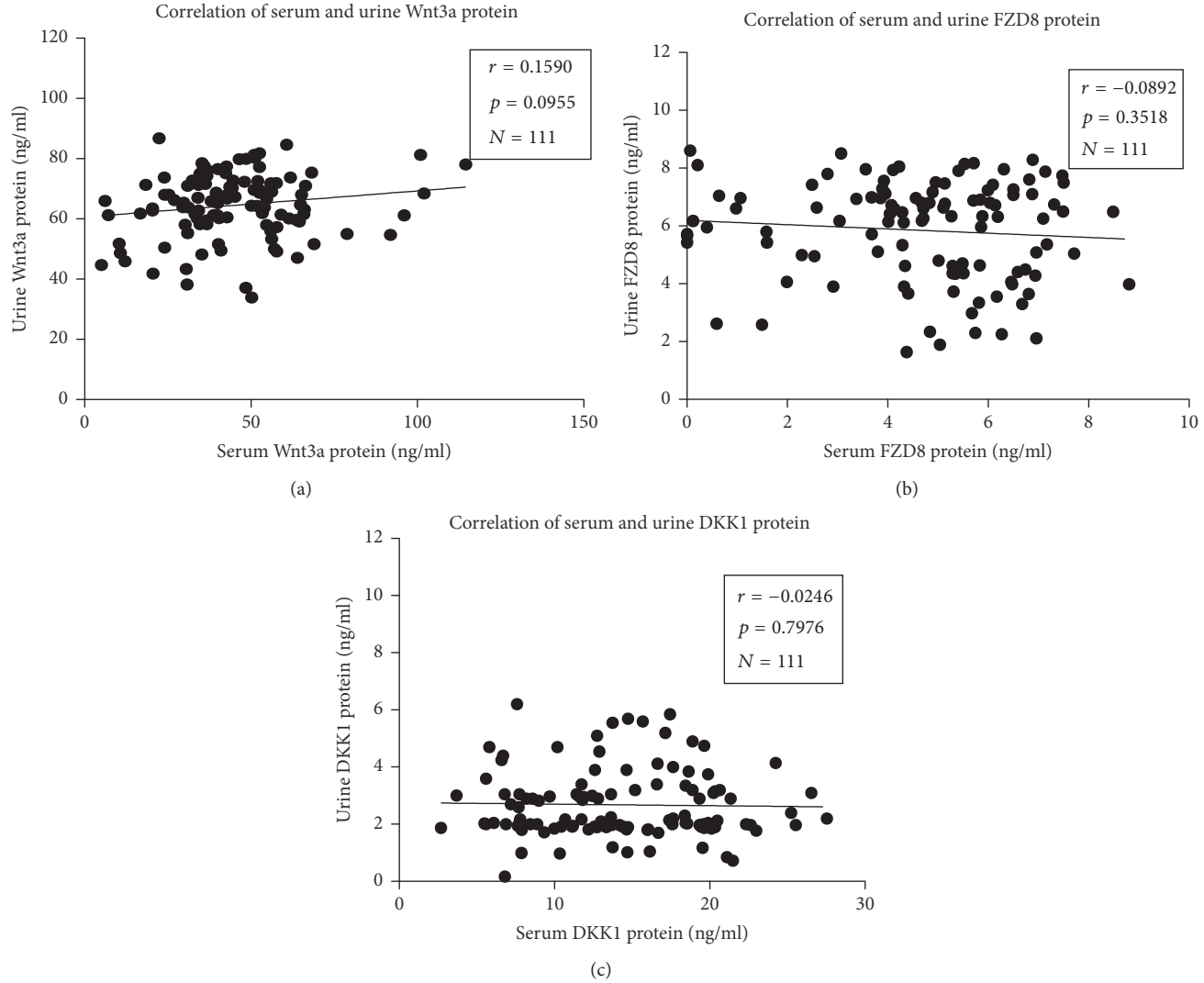

Figure 3: Correlations between serum and urine concentrations of Wnt-3A, FZD-8, and DKK-1. (a) Correlation between Wnt-3A protein levels in serum and urine. (b) Correlation between FZD-8 protein levels in serum and urine. (c) Correlation between DKK-1 protein levels in serum and urine. No significant correlation was determined between the serum and urine for all the three tested proteins. Spearman r and p values are displayed on each graph. A p value was determined by the two-tailed Pearson correlation test.

increased osteoclast activity and erosion [62]. This notion was in accordance with the finding of a decreased serum DKK-1 in RA patients but not in the AS patients treated with TNK inhibitors [56, 62]. These findings suggest that Wnt signaling, particularly the DKK-1, is a biomarker for autoimmune disease diagnosis and a target for disease treatment. Indeed, a recent meta-analysis of seven case-control trials with a total of 300 AS patients, 136 RA patients, and 232 healthy controls found that serum levels of DKK-1 were significantly higher in AS patients relative to normal controls, although there was significant difference in DKK-1 serum levels observed between RA patients and healthy controls [56].

With respect to SLE, dysregulated Wnt signaling activity was first determined in sera and kidneys of mice during lupus development by gene expression analysis [13]. In this report, an increased canonical Wnt/β-catenin signaling activity was

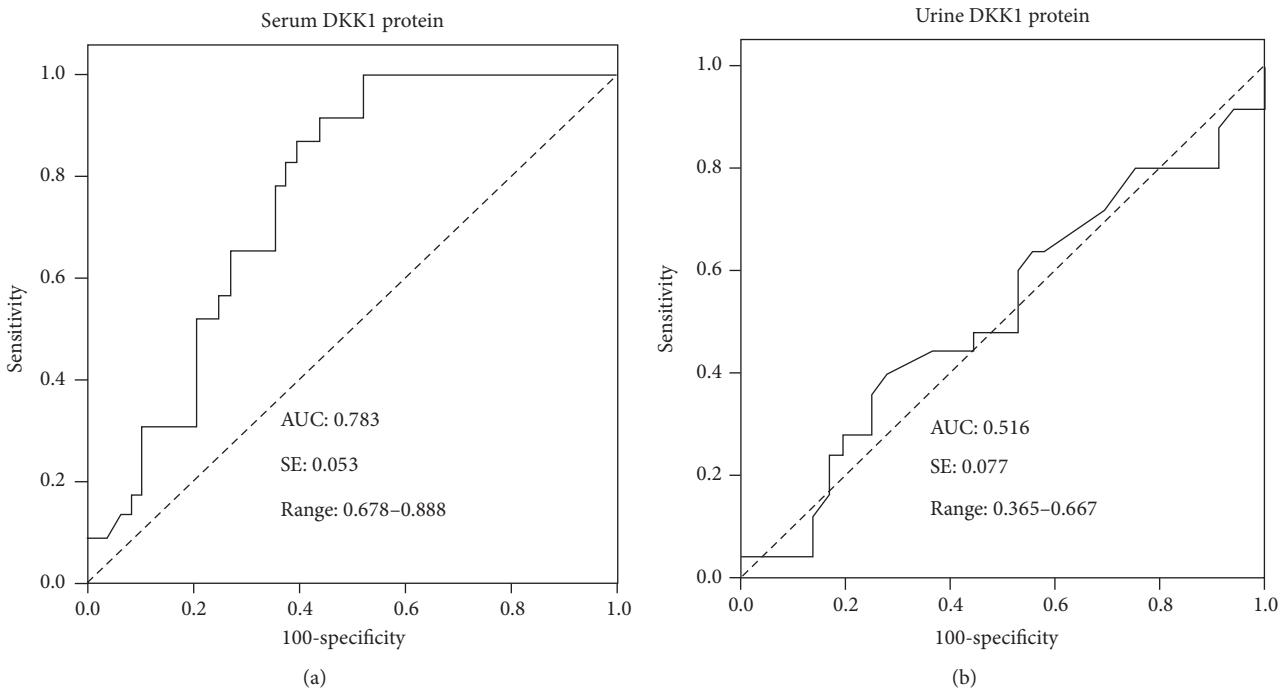

Figure 4: ROC curve for DKK-1 in active lupus nephritis. (a) ROC curve for serum DKK-1 in active lupus nephritis. (b) ROC curve for urine DKK-1 in active lupus nephritis.

determined in kidneys of (NZB × NZW) F1 mice during progression of lupus nephritis, which was paralleled by an increase in renal and serum levels of DKK-1. Notably, sera collected from mice with proteinuric stage of LN showed strong Wnt inhibitory effects, and the concentration of DKK-1 was comparable to those observed in lupus-prone mice induced apoptosis in tubular and mesangial cells in vitro [13]. This study thus indicated that Wnt signaling activity was enhanced in the kidney with LN, which was accompanied by increased renal and serum DKK-1 levels, suggesting that the canonical Wnt signaling was involved in the turnover of extracellular matrix constituents and represents a potential mediator of the morphologic changes that occur within the glomerulus during the development of nephritis, and the DKK-1 might be pivotal element in the development and progression of systemic and end-organ disease manifestations in SLE [13]. Consistent with findings in this SLE mouse model, Wang et al. recently reported enhanced activation of Wnt/β-catenin signaling in SLE patients with LN [15]. In this study, the authors evaluated the expressions of β-catenin, DKK-1, and AXIN-2 mRNAs and proteins in the renal biopsy of patients with LN-SLE by a quantitative RT-PCR and immunohistochemistry assay, respectively; the concentration of plasma DDK1 was also measured by ELISA. The immunohistochemistry and western blotting showed an increased expression of β-catenin in the renal tissues of patients with LN-SLE compared with control samples, and more abundant β-catenin and AXIN-2 transcripts were also detected in the LN renal tissues relative to controls. Of note, such an increased abundance of β-catenin transcript was positively correlated with the creatinine clearance rate (Ccr) and negatively correlated with chronicity indices of renal tissue injury [15]. More importantly, an evoked concentration of DKK-1 protein was found in the plasma of LN patients in comparison with controls, which was negatively correlated with anti-dsDNA antibody level and positively with serum C3 concentration [15].

In agreement with above findings, an increased concentration of DKK-1 was determined in the serum of SLE patients compared with healthy subjects. Even more notably, a significantly higher level of serum DKK-1 was detected in LN-SLE patients relative to those with non-LN-SLE. However, no statistical difference between SLE patients and control individuals was determined for Wnt-3A and FZD-8 in sera. As a manifestation with renal involvement in LN patients, concentrations of Wnt-3A, FZD-8, and DKK-1 were also examined in our study. Interestingly, a statistically less abundant Wnt-3A and more abundant DKK-1 were found in urine of SLE patients as compared with healthy subjects, although no significant difference of urine DKK-1 level was determined between patients with LN-SLE and non-LN-SLE. Of note, no correlation for respective concentrations of Wnt-3A, FZD-8, and DKK-1 was determined between sera and urine in SLE patients. In disagreement with results reported by Wang and colleagues [15], no correlation between concentration of serum or urine DKK-1 and other serological biomarkers, such as anti-dsDNA antibody and C3 and C4 levels, was determined. In our study, serum sample but not plasma was employed for examinations, and the ELISA Kit for DKK-1 measurement was also different from the study by Wang et al. [15]. These variations may be part of the reason that caused the discrepancy between these two studies, which required further investigation. Of importance,

both the ROC curve and the multiple-factor nonconditional logistic regression analysis showed that the serum DKK-1 was considered better positive biomarker for identification of LN in SLE patients. These data may imply that DKK-1 is an independent biomarker for identification of LN-SLE patients.

5. Conclusion

In conclusion, this study in 111 SLE patients confirms previous findings that circulating DKK-1 is a valuable biomarker for identification of SLE patients with LN. Intriguingly, an increased DKK-1 concentration along with a decreased Wnt-3A level was observed in urine of SLE patients relative to healthy cohorts. This finding further supports the notion that Wnt/β-catenin signaling pathway plays a key role in the initiation and progression of LN. However, no clinical significance was observed for serum Wnt-3A between SLE patients and control subjects. Interestingly, no correlation between sera and urine of SLE patients was determined for respective Wnt-3A, FZD-8, and DKK-1. In addition, a combination of DKK-1 with anti-dsDNA and/or levels of complements C3 and C4 could not increase the specificity and/or sensitivity for identification of patients with LN, although both the ROC curve and a logistic regression analysis demonstrated that the serum DKK-1 could be considered better positive biomarker. These results thus imply that DKK-1 is an independent biomarker for identification of LN in SLE patients, which warrants for further investigation in clinical settings. Limitations of this study include that only a small number of 111 SLE samples were studied, follow-up data were also lacking, and the LN activity was mainly determined by laboratory parameters and clinical manifestations rather than by pathogenic analysis in renal biopsies. Therefore, this finding deserves confirmation in a larger and more selected population in future.

Abbreviations

ACR: American College of Rheumatology
ANA: Antinuclear antibody
AS: Ankylosing spondylitis
AUC: Area under the curve
BD: Behcet's disease
BM-MSCT: Bone marrow-mesenchymal stem cell transplantation
C1q: Complement lq
CD: Crohn's disease
CIA: Collagen-induced arthritis
CTL: Cytotoxic T lymphocytes
DKK-1: Dickkopf-1
DM: Dermatomyositis
dsDNA: Double stranded DNA
ILs: Interleukins
LN: Lupus nephritis
JRA: Juvenile idiopathic arthritis
MCTD: Mixed connective tissue disease
MS: Multiple sclerosis
LRP: Low-density lipoprotein receptor-related protein
MSCs: Mesenchymal stem cells
NF-κB: Nuclear factor-kappa B
pANCA: Perinuclear antineutrophil cytoplasmic antibody
pSS: Primary Sjogren's syndrome
RA: Rheumatoid arthritis
RANKL: Receptor activator of nuclear factor-kappa B ligand
RhoA: Ras homolog gene family, member A
Rib-P: Ribosomal P-proteins
RMG: Retinal Müller glial cells
RNP: Ribonucleoprotein
ROC: Receiver operating characteristic
sIL-7R: Soluble interleukin-7 receptor
SFMCs: Synovial fluid mononuclear cells
SFRP4: Frizzled-related protein 4
SLE: Systemic lupus erythematosus
SLEDAI: SLE Disease Activity Index
SSA: Sjogren's syndrome A
SSB: Sjogren's syndrome B
SSc: Systemic sclerosis
TGF-β: Transforming growth factor β
T1DM: Type 1 diabetes mellitus
TNF-α: Tumor necrosis factor alpha
WISP3: Wnt1-inducible signaling pathway protein 3.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Shuhong Chi and Xiaoming Liu conceived and designed the experiments; Jing Xue and Jiali Yang analyzed the data and drafted the manuscript; Jing Xue, Jiali Yang, Lijuan Yang, and Shaolan Zhou performed experiments and acquired data; Jing Xue, Chen Ji, Xuemei Wang, and Nan Yu collected samples; Xiaoming Liu and Shuhong Chi interpreted data and critically revised the manuscript. All authors read and approved the final version of the manuscript.

Acknowledgments

This work was supported by a grant from Natural Science Foundation of Ningxia (NZ1213) to Shuhong Chi.

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 5252840, 9 pages
https://doi.org/10.1155/2017/5252840

Research Article

DEPTOR-mTOR Signaling Is Critical for Lipid Metabolism and Inflammation Homeostasis of Lymphocytes in Human PBMC Culture

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Received 30 June 2016; Accepted 2 February 2017; Published 27 February 2017

Academic Editor: Quanzhen Li

Abnormal immune response of the body against substances and tissues causes autoimmune diseases, such as polymyositis, dermatomyositis, and rheumatoid arthritis. Irregular lipid metabolism and inflammation may be a significant cause of autoimmune diseases. Although much progress has been made, mechanisms of initiation and proceeding of metabolic and inflammatory regulation in autoimmune disease have not been well-defined. And novel markers for the detection and therapy of autoimmune disease are urgent. mTOR signaling is a central regulator of extracellular metabolic and inflammatory processes, while DEP domain-containing mTOR-interacting protein (DEPTOR) is a natural inhibitor of mTOR. Here, we report that overexpression of DEPTOR reduces mTORC1 activity in lymphocytes of human peripheral blood mononuclear cells (PBMCs). Combination of DEPTOR overexpression and mTORC2/AKT inhibitors effectively inhibits lipogenesis and inflammation in lymphocytes of PBMC culture. Moreover, DEPTOR knockdown activates mTORC1 and increases lipogenesis and inflammations. Our findings provide a deep insight into the relationship between lipid metabolism and inflammations via DEPTOR-mTOR pathway and imply that DEPTOR-mTOR in lymphocytes of PBMC culture has the potential to be as biomarkers for the detection and therapies of autoimmune diseases.

1. Introduction

Autoimmune diseases, which are defined by abnormal immune response of the body against substances and tissues normally present in the body, increase the risk of developing multiple disorders [1–3]. Polymyositis [4, 5], dermatomyositis [6, 7], and rheumatoid arthritis [3, 8] are typical autoimmune diseases in modern society. For example, polymyositis is a chronic illness featuring progressive muscle weakness with periods of increased symptoms, including inflammation of the muscles or associated tissues [9, 10]. So far, the major understanding of pathophysiology in autoimmune diseases has been the irregular immunity and inflammation of immune cells [11, 12]. Based on this point, suppressive drugs are necessary to decrease the immune response and inflammation in the treatment of autoimmune diseases. Therefore, it is important to elucidate mechanisms of initiation and proceeding of inflammatory regulation in immune cells for autoimmune disease treatment.

Notably, mTOR signaling senses extracellular stimulations and regulates many biological processes including inflammations [13, 14]. The mechanistic target of rapamycin (mTOR) is a phosphatidylinositol 3-kinase- (PI3K-) like serine/threonine protein kinase that is evolutionarily conserved in all eukaryotes [15, 16]. Deregulation of mTOR signaling has been shown that it is closely associated with cancers and metabolic diseases as well as autoimmune diseases. mTOR resides in two distinct complexes referred to as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [17]. mTORC1 comprises mTOR, Raptor, DEPTOR, mLST8, and PRAS40, while mTORC2 comprises mTOR with Rictor, mSIN1, DEPTOR, mLST8, and Protor [13]. Interestingly, it is identified that DEP domain-containing mTOR-interacting protein (DEPTOR) directly interacts with both mTORC1 and

mTORC2 complexes [18]. Ectopic high DEPTOR expression decreases mTORC1 activity and S6K1-mediated feedback loop on PI3K/AKT to regulate cell metabolism and survival [18]. Thus, DEPTOR is even accepted as a natural endogenous mTORC1 inhibitor.

All through the regulation of inflammations, mTOR signaling modulates levels of inflammatory cytokines produced by immune cells, whereas mTOR (especially for mTORC1) is a master regulator of cell metabolism, such as protein synthesis, lipid biosynthesis (lipogenesis), and glucose oxidation [19]. Importantly, the SREBPs are key factors transcriptionally regulated by mTORC1, which stimulates the expression of genes encoding nearly all of these lipogenic enzymes [20]. Nowadays, it is appreciated that mTORC1 controls lipid homeostasis both physiologically and pathologically. On the other hand, synthesized free fatty acids (FFAs) are well-characterized factor for causing production of inflammatory factors [21, 22]. Hence, it is proposed that mTORC1 signaling may control inflammatory reactions via metabolic alternations.

Previous studies have reported that TNF-α, IL-6, and NF-κB are increased in nutrition-enriched conditions, and this increase is correlated with lipid-metabolic disorders [23–25]. Although these studies demonstrate lipid metabolism may correlate with inflammation, clear mechanisms of how lipid metabolism is coupled with inflammation in immune cells are not well defined. Here, we report that mTOR signaling is a central regulator of lipid metabolism-mediated inflammation in lymphocytes of human PBMC culture. Overexpression of mTORC1 inhibitor DEPTOR partly reduces mTORC1 activity but does not decrease lipogenesis and inflammation. We assume this may be due to activated mTORC2 pathway. Addition of AKT inhibitors decreases lipogenesis and inflammation in DEPTOR overexpressed cells. Moreover, DEPTOR knockdown activates mTORC1 and increases lipogenesis and inflammation. Our findings will help to provide a deep insight into lipid metabolism and inflammations coupling via DEPTOR-mTOR pathway and imply that DEPTOR-mTOR in lymphocytes of PBMC culture has the potential to be biomarkers for the detection and therapies of autoimmune diseases.

2. Materials and Methods

2.1. Chemicals and Reagents. RPMI-1640 and Fetal Bovine Serum (FBS) for PBMC culture were purchased from GIBCO Invitrogen (Carlsbad, CA, USA). The DEPTOR, mTOR, pp70S6K (Thr389), p70S6K, p4EBP1 (Thr37/46), 4EBP1, pAKT (Ser473), AKT, and beta-actin antibodies were all from Cell Signaling Technology (Danvers, MA, USA). To knockdown endogenous DEPTOR, shRNA targeting sequence of DEPTOR gene and a scramble shRNA (NC) were synthesized. shRNA-encoding plasmids were cotransfected with the Delta VPR envelope and CMV VSV-G packaging plasmids into growing lymphocytes of PBMC culture. Lentiviral shRNAs to human DEPTOR were previously described, which are named with the numbers found at the TRC public website: human DEPTOR_1 shRNA: TRCN0000110157; NM_145470.1-1164s1c1; human DEPTOR_2 shRNA: TRCN0000110159; NM_145470.1-1165s1c1 [18]. For DEPTOR overexpression, the myc-tagged DEPTOR construct was generated by subcloning the PCR-amplified human DEPTOR coding sequence into pRK5-myc vectors for further lentiviral transfections. Other chemicals were of the highest purity available.

2.2. Ethical Approval and Cell Cultures. Healthy volunteers for PBMC cutlure were provided written informed consent approved by the Ethics Administration Office of West China Hospital, Sichuan University. For the preparation of PBMC culture, cells were extracted from heparinized whole blood by differential centrifugation by Histopaque 1077 (Sigma) as instructions. For Western blots and real-time PCR experiments, cells were plated in 6-well plates at 1.0×10^6 cells/mL. The cells were incubated in RPMI-1640 medium containing 10% FBS plus antibiotics for 24 hours in 5% CO_2 at 37°C. After culturing, the cells were harvested for subsequent examinations.

2.3. Lysates Preparation and Western Blots. For Western blots, prepared cells were trypsinized and harvested, washed with PBS once, and resuspended in cell lysis buffer (PBS with 1% Triton X-100 and protease inhibitors). After brief sonication, cell lysates were centrifuged at 13,000 rpm for 5 min. Protein concentration was determined so that equivalent amounts of lysate based on protein concentration were added to an equal volume of 2X Laemmli buffer and boiled for 10 min. For Western blot analysis, the procedure was according to standard protocols. Finally, proteins were detected by Super Signal® enhanced chemiluminescence development (ECL) (Thermo Scientific Pierce) reagent and exposed to films (Kodak). The protein level quantification was carried out by ImageJ.

2.4. Quantitative Real-Time PCR. Total RNA was extracted from tissues using Trizol reagent (Invitrogen). RNA was subjected to reverse transcription with reverse transcriptase as per manufacturer's instructions (Fermentas). Quantitative real-time PCR was performed using the Bio-Rad iQ5 system, and the relative gene expression was normalized to internal control as beta-actin. Primer sequences for SYBR Green probes of target genes are as shown in Table 1.

2.5. Statistical Analysis. Data represent the mean and standard error of the mean (SEM). ANOVA tests for comparisons were performed for all statistical significance analysis using GraphPad Prism software. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3. Results

3.1. Overexpressed DEPTOR Decreases mTORC1 and Increases mTORC2 Activity. DEPTOR is a natural inhibitor of mTOR via directly binding to both mTORC1 and mTORC2 (Figure 1(a)). Previous studies identify that DEPTOR depletion activates mTORC1 and mTORC2 signaling in several cell and animal models [18, 26]. Moreover, overexpression of

Table 1: Primer sequences for SYBR Green probes of target genes.

Acl	F: 5′-GAAGCTGACCTTGCTGAACC-3′
	R: 5′-CTGCCTCCAATGATGAGGAT-3′
Acc1	F: 5′-TCTTTTCCTCGGAGCATGACA-3′
	R: 5′-GACCTCTCTACTCACTTCTCCAG-3′
Fasn	F: 5′-GGAGGTGGTGATAGCCGGTAT-3′
	R: 5′-TGGGTAATCCATAGAGCCCAG-3′
Scd1	F: 5′-TTTCGAAGACGTCAGAGTGC-3′
	R: 5′-TGCGACTGTAGGTCTGGTTC-3′
Il-1β	F: 5′-CTGGTGTGTGACGTTCCCATTA-3′
	R: 5′-CCGACAGCACGAGGCTTT-3′
Il-6	F: 5′-TTCCATCCAGTTGCCTTCTTG-3′
	R: 5′-TGGGAGTGGTATCCTCTGTGA-3′
Tnf-α	F: 5′-CATCTTCTCAAAATTCGAGTGACA-3′
	R: 5′-TGGGAGTAGACAAGGTACAACCC-3′
β-Actin	F: 5′-TCCATCATGAAGTGTGACGT-3′
	R: 5′-TACTCCTGCTTGCTGATCCAC-3′

DEPTOR inhibits mTORC1 and further activates PI3K/AKT signaling [18]. However, how DEPTOR regulates lymphocyte mTOR activity is not well defined. Thus, overexpression of DEPTOR and mTORC1/2 activity in lymphocytes of PBMC culture were firstly analyzed. Biochemical results showed that protein levels of markers of mTORC1 pathway (pp70S6K and p4EBP1) [27] were both decreased by DEPTOR overexpression (Figures 1(b) and 1(c)). On the other hand, it is noted that mTORC2 activity, indicated by phospho-AKT, is increased by DEPTOR overexpression in lymphocytes of PBMC culture (Figures 1(b) and 1(c)). Therefore, our results suggest that overexpressed DEPTOR decreases mTORC1 but increases mTORC2 activity, which may affect downstream lipid metabolism inflammations.

3.2. Overexpressed DEPTOR Does Not Inhibit Lipogenesis and Inflammation in Lymphocytes of PBMC Culture. Considering that mTOR pathway is a central regulator of lipid metabolism and inflammatory homeostasis [13], we further investigated whether DEPTOR overexpression would like to alter lipogenesis and inflammation in lymphocytes of PBMC culture. The expression of lipogenesis enzymes is controlled by the SREBP1 transcription factor, which is tightly controlled by mTORC1 [20] (Figure 2(a)). By real-time PCR assays, we found that the expression of lipid metabolism genes, in particular Acl, Acc1, Fasn, and Scd1, was not dramatically reduced by DEPTOR overexpression (Figure 2(b)). Since lipids are well-characterized factor for causing production of inflammatory factors, we further examined the expression of inflammation factors, including IL1-β, IL-6, and TNF-α [28]. The results of quantitative real-time PCR showed that the expressions of all these three inflammation factors are consistently not dramatically altered by DEPTOR overexpression (Figure 2(c)). Taken together, all these results indicate that inhibition of mTORC1 activity via DEPTOR may not inhibit lipogenesis and inflammation in lymphocytes of PBMC culture.

3.3. AKT Inhibitors Downregulate Lipogenesis and Inflammation in DEPTOR Overexpressed Cells. Our results support the fact that mTORC1 inactivation by DEPTOR may not inhibit lipogenesis and inflammation in lymphocytes of PBMC culture. Thus, we propose that increased mTORC2 may be responsible for the maintained lipid and inflammation homeostasis [29]. We examined the lipogenesis and inflammation factors in AKT-inhibited DEPTOR overexpressed cells. MK-2206 is a well-defined AKT inhibitor and inhibits autophosphorylation of AKT at T308 and S473 sites [30]. We applied MK-2206 to DEPTOR overexpressed cells and examined downstream signaling. Biochemical results showed that, as MK-2206 treatment, the mTORC1 and AKT activities were decreased, indicated by pp70S6K, p4EBP1, and pAKT (Figure 3(a)).

Accordingly, we further examined the lipogenesis and inflammation in these AKT-inhibited and DEPTOR overexpressed cells. As expected, real-time PCR results showed that expressions of lipogenesis genes, Acl, Acc1, Fasn, and Scd1, were dramatically reduced by the overexpression of DEPTOR in lymphocytes of PBMC culture (Figure 3(b)). Consistently, we found that expressions of inflammation factors, including IL1-β, IL-6, and TNF-α, were also reduced by MK-2206 treatment (Figure 3(c)). Moreover, we found that the protein level of TNF-α was consistently decreased by MK-2206 (Figure 3(d)). All these facts suggest that AKT inhibitors decrease mTORC1/2 signaling and lipogenesis and inflammation in DEPTOR overexpressed lymphocytes of PBMC culture.

3.4. DEPTOR Knockdown Increases mTORC1 Activity, Lipogenesis, and Inflammation in Lymphocytes of PBMC Culture. Since overexpression of DEPTOR has been found to inactivate mTORC1 pathway, we proposed that knockdown DEPTOR activity may activate mTORC1 and downstream lipogenesis and inflammations. To test this hypothesis, we carried out DEPTOR RNAi to inhibit DEPTOR expression and investigated the mTORC1 signaling alternations. Biochemical results showed that DEPTOR knockdown could increase mTORC1 activity (indicated by p70S6K and 4EBP1 phosphorylations) and slightly decrease mTORC2/AKT activity (Figure 4(a)). Based on this point, we further examined the lipogenesis and inflammation in DEPTOR knockdown cells. Results showed that expressions of lipogenesis genes, Acl, Acc1, Fasn, and Scd1, were increased by DEPTOR knockdown in lymphocytes of PBMC culture (Figure 4(b)). Moreover, inflammation factors, including IL1-β, IL-6, and TNF-α, were also increased by DEPTOR knockdown (Figure 4(c)). Moreover, we found that the protein level of TNF-α was consistently increased by DEPTOR knockdown (Figure 4(d)). Collectively, these data indicate that DEPTOR knockdown increases mTORC1 activity, lipogenesis, and inflammation.

4. Discussion

The balance of metabolic and inflammatory events is critical for lymphocyte homeostasis. However, the irregular lipid

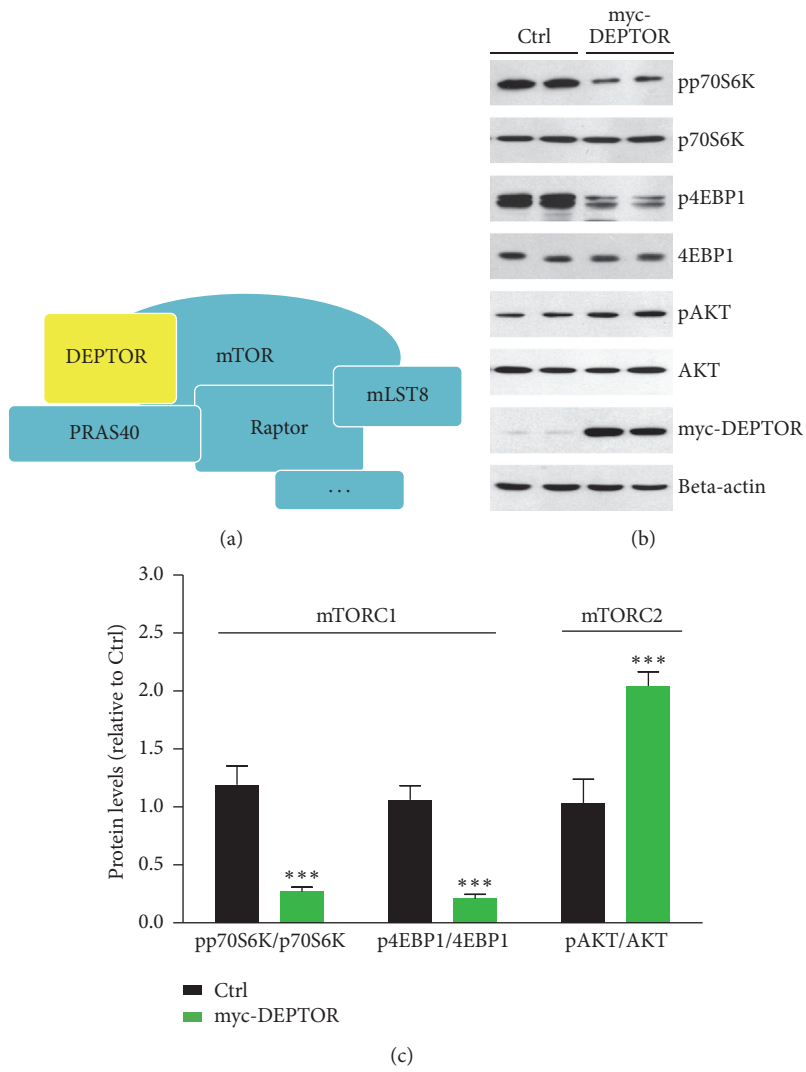

Figure 1: Overexpressed DEPTOR decreases mTORC1 and increases mTORC2 activity. (a) A schematic model showed the components of mTORC1 complexes. Raptor is the characterized adaptor protein of mTORC1, and mTOR works as the kinase to phosphorylate downstream substrates. DEPTOR is the natural inhibitor of mTORC1. (b-c) Western blots and quantifications showed that mTORC1 activity, indicated by phosphorylations of p70S6K and 4EBP1, was downregulated by myc-DEPTOR overexpression in lymphocytes of PBMC culture. Results are averages of three independent experiments. Data represent mean ± SEM. $^{***}P < 0.001$.

metabolism and ectopic inflammation may be a direct cause of autoimmune diseases, such as polymyositis, dermatomyositis, and rheumatoid arthritis [1, 6, 12]. In the present study, we find that DEPTOR, a natural inhibitor of mTOR, may regulate lipid homeostasis and inflammation in lymphocytes of PBMC culture. We show that overexpression of DEPTOR may partly reduce mTORC1 activity but may not decrease lipogenesis and inflammation. This is due to activated mTORC2 pathway and addition of AKT inhibitors may decrease lipogenesis and inflammations. Oppositely, DEPTOR knockdown activates mTORC1 and increases lipogenesis and inflammation in lymphocytes of PBMC culture. Our findings indicate that DEPTOR-mTOR signaling is critical for homeostasis of metabolic and inflammations and suggest that DEPTOR-mTOR signaling to be markers of autoimmune diseases (Figure 5).

Nowadays, it has been widely accepted that DEPTOR interacts with mTORC1 [18]. And DEPTOR regulates downstream metabolic events via mTORC1/2 signaling. For example, DEPTOR acts as a tumor suppressor by blocking mTORC1 activity to inhibit protein synthesis and cell proliferation [31]. DEPTOR expression can reduce mTORC1 activity and S6K1-mediated feedback inhibition of PI3K/AKT pathways, which contributes to the cell survival [32]. Therefore, the expression of intracellular DEPTOR must be precisely regulated. In our studies, we noted that the overexpression of DEPTOR indeed inhibits mTORC1 activity but failed to block lipogenesis and inflammation as our expectations. We demonstrate that increased mTORC2/AKT pathway may be responsible for this "resistance." Addition of AKT inhibitor MK-2206 may enhance the effect of DEPTOR on inactivation of lipogenesis and inflammation in lymphocytes of PBMC

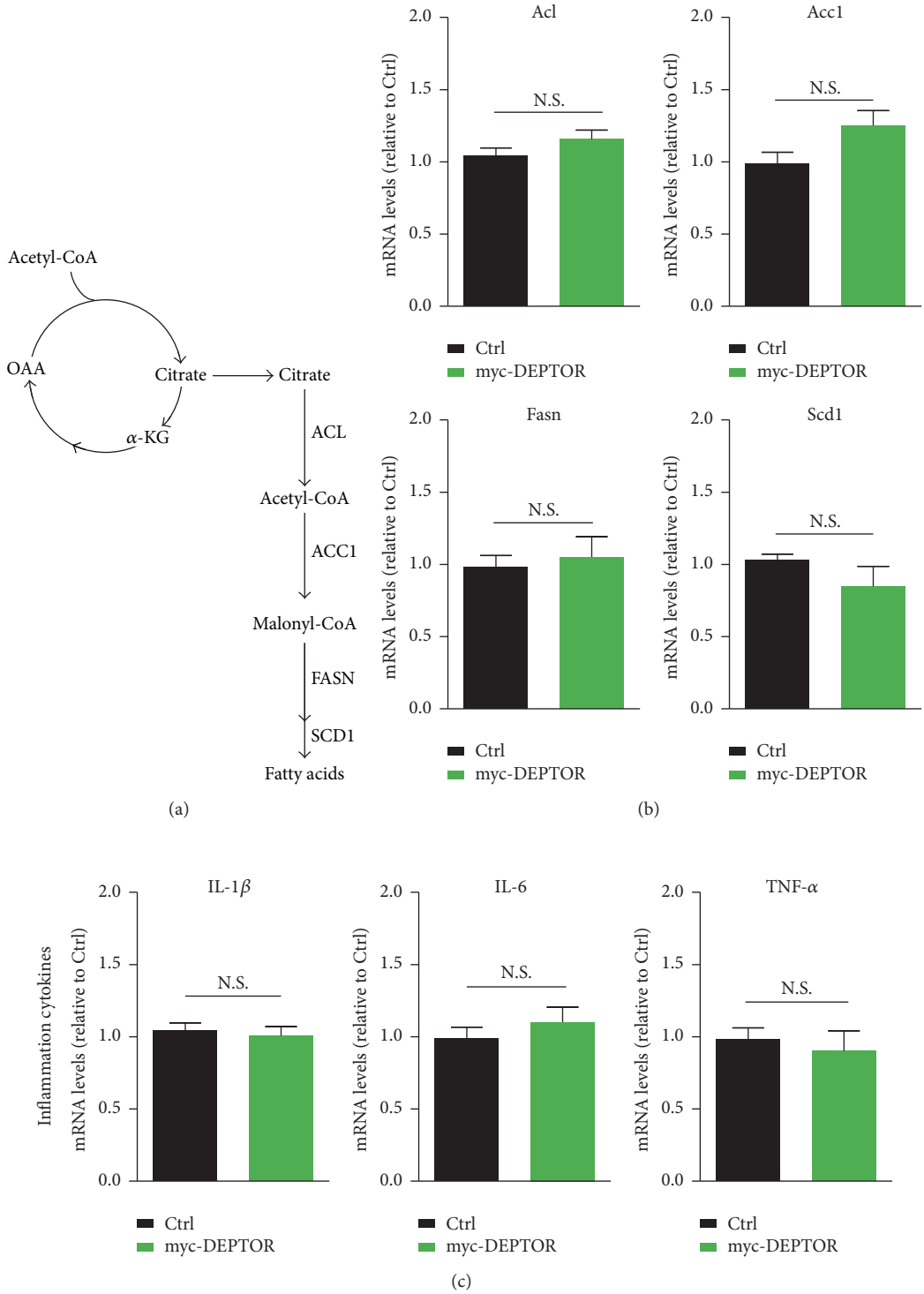

Figure 2: Overexpressed DEPTOR does not inhibit lipogenesis and inflammation. (a) A schematic model showed the signaling of mTORC1-controlled lipogenesis. ACL, ACC1, FASN, and SCD1 were downstream enzymes in fatty acid synthesis. (b) Real-time RCR results showed that mRNA levels of lipogenesis genes, including Acl, Acc1, Fasn, and Scd1, were not reduced in DEPTOR overexpressed cells. Results are averages of three independent experiments. Data represent mean ± SEM. N.S.: no statistical significance. (c) Real-time RCR results showed that mRNA levels of inflammation genes, including IL1-β, IL-6, and TNF-α, were not reduced in DEPTOR overexpressed cells. Results are averages of three independent experiments. Data represent mean ± SEM. N.S.: no statistical significance.

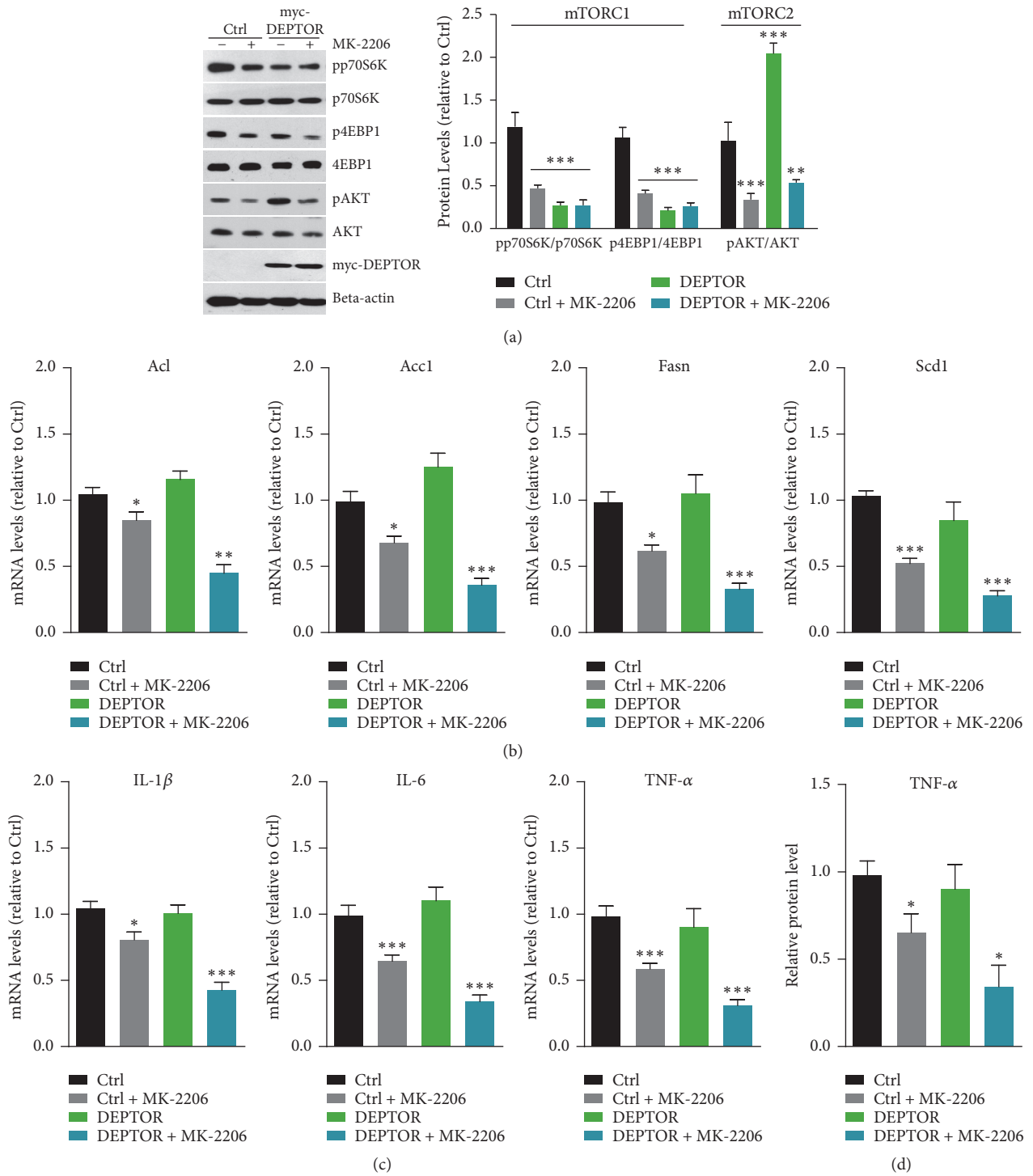

Figure 3: AKT inhibitors downregulate lipogenesis and inflammation in DEPTOR overexpressed cells. (a) Western blots and quantifications showed that mTORC1 activity, indicated by phosphorylations of p70S6K and 4EBP1, was downregulated by myc-DEPTOR overexpression and MK-2206 (1 μM for 4 hours, same as follows) conditions. However, mTORC2 activity (indicated by pAKT/AKT) was blocked by MK-2206 treatment in lymphocytes of PBMC culture. Results are averages of three independent experiments. Data represent mean ± SEM. Data represent mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$. (b) Real-time RCR results showed that mRNA levels of lipogenesis genes, including Acl, Acc1, Fasn, and Scd1, were dramatically reduced in MK-2206 treated DEPTOR overexpressed cells. Results are averages of three independent experiments. Data represent mean ± SEM. Data represent mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. (c) Real-time RCR results showed that mRNA levels of inflammation genes, including IL1-β, IL-6, and TNF-α, were dramatically reduced in MK-2206 treated DEPTOR overexpressed cells. Results are averages of three independent experiments. Data represent mean ± SEM. $^{*}P < 0.05$ and $^{***}P < 0.001$. (d) ELISA results showed that protein levels of TNF-α were decreased in MK-2206 treated DEPTOR overexpressed cells. Results are averages of three independent experiments. Data represent mean ± SEM. $^{*}P < 0.05$.

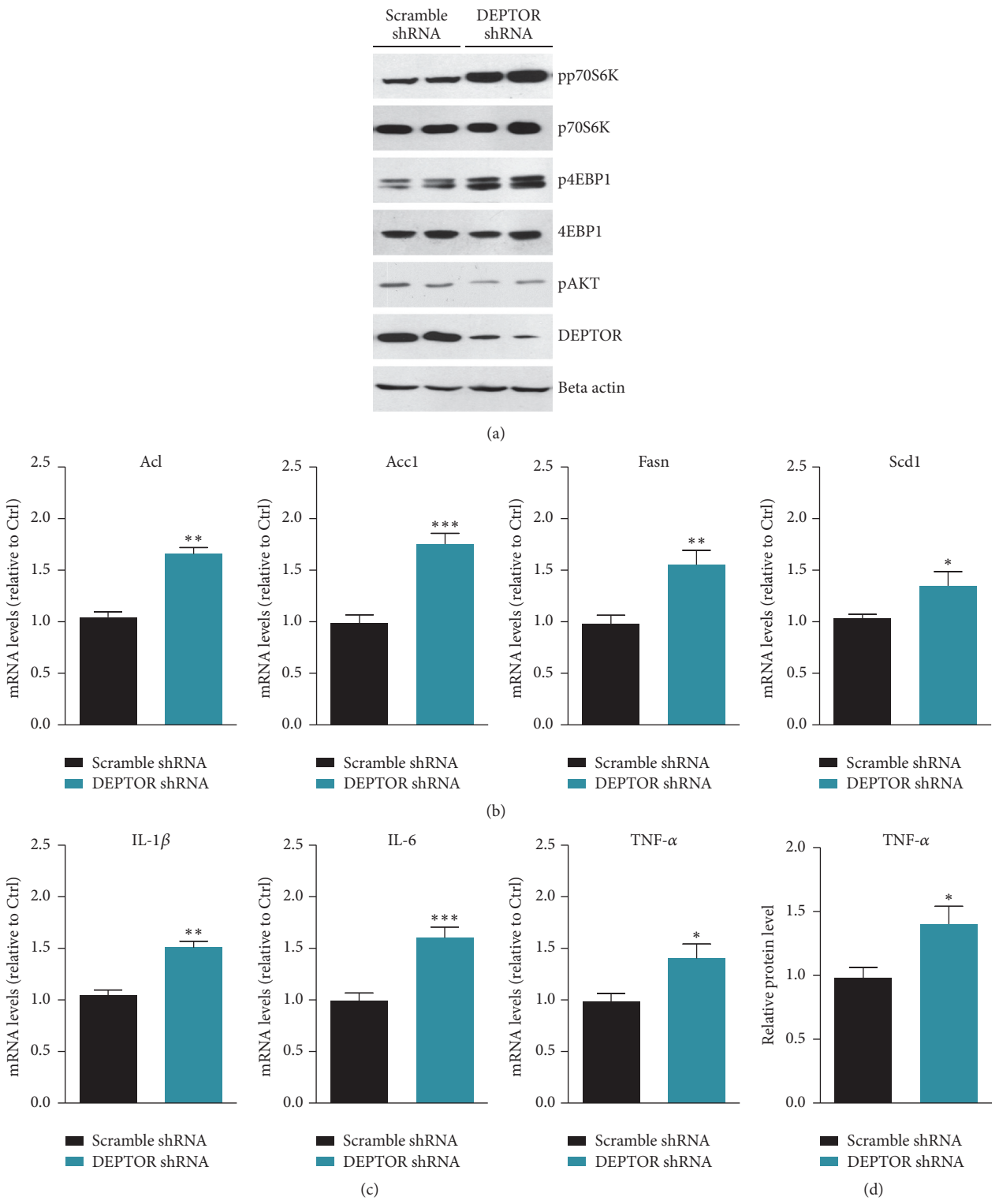

Figure 4: DEPTOR knockdown increases mTORC1 activity, lipogenesis, and inflammation in lymphocytes of PBMC culture. (a) Western blots showed that mTORC1 activity, indicated by phosphorylations of p70S6K and 4EBP1, was upregulated by DEPTOR shRNA in lymphocytes of PBMC culture. Noted that pAKT was slightly downregulated by DEPTOR knockdown. (b) Real-time RCR results showed that mRNA levels of lipogenesis genes, including Acl, Acc1, Fasn, and Scd1, were increased in DEPTOR knockdown cells. Results are averages of three independent experiments. Data represent mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. (c) Real-time RCR results showed that mRNA levels of inflammation genes, including IL1-β, IL-6, and TNF-α, were increased in DEPTOR knockdown cells. Results are averages of three independent experiments. Data represent mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. (d) ELISA results showed that protein level of TNF-α was increased in DEPTOR knockdown cells. Results are averages of three independent experiments. Data represent mean ± SEM. $^{*}P < 0.05$.

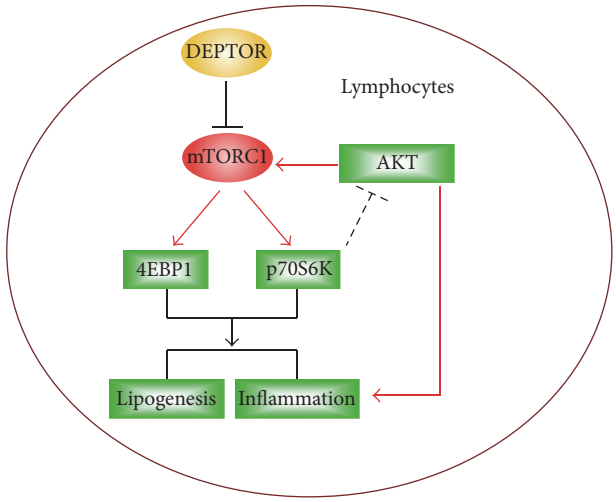

Figure 5: Schematic representation explaining the network of DEPTOR, mTORC1, mTORC2, and lipogenesis/inflammation networks. DEPTOR is a natural inhibitor of mTORC1 pathway. Overexpression of mTORC1 inhibitor DEPTOR may partly reduce mTORC1 activity but may not decrease lipogenesis and inflammation. Addition of AKT inhibitors may decrease lipogenesis and inflammation in DEPTOR overexpressed cells. Moreover, DEPTOR knockdown may activate mTORC1 and increase lipogenesis and inflammation.

culture. Therefore, DEPTOR seems to act as a key factor for the balance of mTORC1 and mTORC2 pathways, especially in metabolic and inflammatory regulations.

mTOR pathway is a master regulator of cell metabolism and inflammations [13]. In 2008, a hallmark paper demonstrates that mTORC1 inhibition blocks expressions of genes involved in lipogenesis and impairs the nuclear accumulation of the SREBPs [33]. Subsequent studies confirm this finding and figure out that mTORC2/AKT may regulate lipogenesis in mTORC1 dependent and independent manners [29]. Recently, it is reported that mTORC1 phosphorylates CRTC2 and attenuates its inhibitory effect on COPII-dependent SREBP1 maturation and downstream lipogenesis [34]. All these studies clearly show how mTORC1/2 pathway modulates lipid metabolism. However, the biological functions of mTOR-lipid metabolism in inflammatory reactions and immune systems have been not well defined. In previous studies, we demonstrate that mTORC1 pathway is critical for the initiation of inflammatory reactions in synovial cells [35]. Moreover, lipid peroxidation-mediated inflammation promotes cell apoptosis through activation of NF-κB pathway in rheumatoid arthritis synovial cells [28]. In the present study, we intended to study upstream regulators of mTOR pathway and its metabolic function in inflammatory reactions. Our data define DEPTOR, a well-known mTOR inhibitor, that is a critical mediator of lipogenesis and inflammation in lymphocytes of PBMC culture. The next question would be to clarify pathological functions of DEPTOR in autoimmune diseases and to design targeted drugs.

5. Conclusion

In conclusion, the present findings supported the fact that DEPTOR-mTOR signaling is a central regulator of lipid metabolism-mediated inflammation in lymphocytes of PBMC culture. Combination of DEPTOR overexpression and mTORC2/AKT inhibitors may effectively inhibit lipogenesis and inflammation. Moreover, DEPTOR knockdown may activate mTORC1 and increase lipogenesis and inflammations. Our findings provide a deep insight into lipid metabolism and inflammations coupling via DEPTOR-mTOR pathway and imply that DEPTOR-mTOR of lymphocytes of PBMC culture has potential to be markers for the detection and therapies of autoimmune diseases.

Competing Interests

The authors have declared that no competing interests exist.

Acknowledgments

This research was supported by National Natural Science Foundation of China (Grant no. 30901339 and Grant no. 81172869) and the Application Foundation Project in Sichuan Province (no. 2016JY0021 and no. 2017JY0025).

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 3262384, 7 pages
https://doi.org/10.1155/2017/3262384

Research Article

Identification of Palmitoleic Acid Controlled by mTOR Signaling as a Biomarker of Polymyositis

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Received 30 June 2016; Revised 4 September 2016; Accepted 4 December 2016; Published 17 January 2017

Academic Editor: Kurt Blaser

Polymyositis (PM) is a chronic disease characterized by muscle pain, weakness, and increase in muscle-related enzymes, accompanied with inflammations in lymphocytes. However, it is not well understood how the molecular alternations in lymphocytes contribute to the development of polymyositis. The mechanistic target of rapamycin (mTOR) signaling is the central regulator of metabolism and inflammation in mammalian cells. Based on previous studies, we proposed that mTOR signaling may control inflammatory reactions *via* lipid metabolism. In this study, we aim to figure out the role of mTOR signaling in the development of polymyositis and identify novel biomarkers for the detection and therapy of polymyositis. After screening and validation, we found that palmitoleic acid, a monounsaturated fatty acid, is highly regulated by mTOR signaling. Inhibition of mTORC1 activity decreases palmitoleic acid level. Moreover, mTORC1 regulates the level of palmitoleic acid by controlling its de novo synthesis. Importantly, increased palmitoleic acid has been proven to be a marker of polymyositis. Our work identifies palmitoleic acid in peripheral blood mononuclear cells (PBMC) as a biomarker of polymyositis and offers new targets to the clinical therapy.

1. Introduction

Autoimmune diseases are defined as abnormal immune responses of the body against substances or cells normally present in the body, which are accompanied with multiple disorders [1–3]. Polymyositis (PM) [4, 5], dermatomyositis [6, 7], and rheumatoid arthritis [3, 8] are typical autoimmune diseases in modern society. Polymyositis is a chronic illness featuring progressive muscle weakness with periods of increased symptoms, including inflammation of the muscles and associated tissues [9, 10]. During the development of polymyositis, inflammatory reactions in lymphocytes play an important role. However, how inflammation in peripheral lymphocytes confers the development of polymyositis still needs to be elucidated.

Multiple signaling pathways are involved in the regulation of inflammations in lymphocytes, whereas mTOR signaling is a central regulator of the initiation and completion of inflammations. The mechanistic target of rapamycin (mTOR) is a phosphatidylinositol 3-kinase- (PI3K-) like serine/threonine protein kinase that is evolutionarily conserved in all eukaryotes [11, 12]. Dysregulation of mTOR signaling has been shown to be closely associated with cancers, metabolic diseases, and autoimmune diseases. mTOR resides in two distinct complexes referred to as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [13]. Previous studies have established the role of mTORC1 in inflammation [14–16]. For example, inhibition of mTORC1 in LPS-stimulated cells has been shown to attenuate the levels of phosphorylated STAT3 and thus decrease inflammatory activation [17].

Besides controlling the inflammations in lymphocytes, mTORC1 is a master regulator of metabolic network in mammalian cells. mTORC1 controls multiple metabolic events, such as protein synthesis, lipid biosynthesis (lipogenesis), and glucose oxidation [18]. To control lipid metabolism, mTORC1 transcriptionally regulates SREBPs, which stimulates the expression of genes encoding nearly all the lipogenic enzymes [19]. Therefore, mTORC1 controls lipid homeostasis

both physiologically and pathologically. On the other hand, synthesized free fatty acids (FFAs) are well-characterized factors for production of inflammations [20, 21]. Based on this point, we propose that mTORC1 signaling may control inflammatory reactions via lipid metabolism in the development of autoimmune diseases.

In previous studies, we identified the notion that mTORC1 pathway is critical for the initiation of inflammatory reactions in rheumatoid arthritis [22]. Now, we extend to study the role of mTORC1 in polymyositis. Here, we screened the altered metabolic substrates by mTORC1 inhibition in PBMC. After screening and validation, we found that several fatty acids, especially palmitoleic acid, were dramatically decreased after mTORC1 inhibition. Palmitoleic acid (C16:1) is an omega-7 monounsaturated fatty acid which has multiple biofunctions in vitro and in vivo [23, 24]. We examined and found that mTORC1 regulates the level of palmitoleic acid by controlling its de novo synthesis, termed lipogenesis. Finally, we confirmed the increase in the palmitoleic acid level in PBMC of patients with polymyositis. Our work identifies palmitoleic acid as a biomarker for the detection of polymyositis and offers new targets to clinical therapies of polymyositis.

2. Materials and Methods

2.1. Reagents and Antibodies. In western blot assays, all of the antibodies were of high quality and were validated. The pp70S6K, p70S6K, p4EBP1, 4EBP1, and beta-actin antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA), which were widely used in mTORC1 signaling study. For cell culture, RPMI-1640 and Fetal Bovine Serum (FBS) for PBMC culture were purchased from Gibco Invitrogen (Carlsbad, CA, USA). mTOR signaling inhibitors rapamycin and Torin1 were from Invitrogen (Carlsbad, CA, USA). Other chemicals were of the highest purity available.

2.2. Ethical Approval and Cell Cultures. Ethical approval was obtained before experiments. Participants, including healthy volunteers and polymyositis patients, provided written informed consent approved by the Ethics Administration Office of West China Hospital, Sichuan University. For the preparation of human peripheral blood mononuclear cells (PBMC), cells were extracted from heparinized whole blood by differential centrifugation by Histopaque 1077 (Sigma). Briefly, 5 mL whole blood collected from each subject was layered onto equivolume Histopaque 1077 (Sigma) in polypropylene tubes. Layered blood was then centrifuged at 4000 rpm for 30 min. Buffy coats were aspirated into new polypropylene tubes and washed twice with PBS and suspended in 0.5 mL of cold RPMI-1640 medium with 10% FBS plus antibiotics. Cell number was determined with a blood-cell counting chamber (Erma, Japan). The viability of PBMC was determined by trypan blue exclusion. To inhibit mTORC1 activity, rapamycin (100 nM) and Torin1 (200 nM) were applied to PBMC for 24 h. For western blots and real-time PCR experiments, cells were plated in 6-well plates at 1.0×10^6 cells/mL. After culturing, cells were harvested for subsequent examinations.

Table 1: Primer sequences for SYBR Green probes of target genes.

Gene	Primer sequence ($5' \rightarrow 3'$)
Srebp1c	
F	GGAGCCATGGATTGCACATT
R	GCTTCCAGAGAGGAGGCCAG
Acc1	
F	CCTCCGTCAGCTCAGATACA
R	TTTACTAGGTGCAAGCCAGA
Fasn	
F	TGGGTTCTAGCCAGCAGAGT
R	ACCACCAGAGACCGTTATGC
Scd1	
F	GGAGAGAATTTCATCTTCCA
R	CTTCCCAAAGCGGTGTGAGT
Beta-actin	
F	GAGACCTTCAACACCCCAGC
R	ATGTCACGCACGATTTCCC

2.3. Metabolic Screening by MS. Generally, metabolic screening of PBMC treated with rapamycin and Torin1 was carried out by LC-MS. Extracts were prepared and then analyzed to measure the intracellular levels of metabolites. Metabolites were extracted on dry ice with 5 mL 80% methanol (−80°C). The extract was dried under nitrogen and resuspended in 80 μL of water just prior to the LC-MS run according to standard procedure [25].

2.4. Lysates Preparation and Western Blots. To assay mTORC1 activity in human PBMC, total proteins were extracted from cell harvest. For western blots, prepared cells were trypsinized and harvested, washed with PBS once, and resuspended in cell lysis buffer (PBS with 1% Triton X-100 and protease/phosphatase inhibitors). After brief sonication, cell lysates were centrifuged at 13,000 rpm for 5 min. Protein concentration was determined so that equivalent amounts of lysate based on protein concentration were added to an equal volume of Laemmli buffer and boiled for 10 min. For western blot analysis, the procedure was carried out according to standard protocols. Finally, proteins were detected by Super Signal® enhanced chemiluminescence development (ECL) (Thermo Scientific Pierce) reagent and exposed to films (Kodak). Protein level quantification was carried out by ImageJ.

2.5. Quantitative Real-Time PCR. To assay gene expressions of lipogenesis in human PBMC, total RNA was extracted from tissues using TRIzol reagent (Invitrogen). RNA was subjected to reverse transcription with reverse transcriptase as per the manufacturer's instructions (Fermentas). Quantitative real-time PCR was performed using Bio-Rad iQ5 system, and relative gene expression was normalized to internal control as *beta-actin*. Primer sequences for SYBR Green probes of target genes are listed in Table 1.

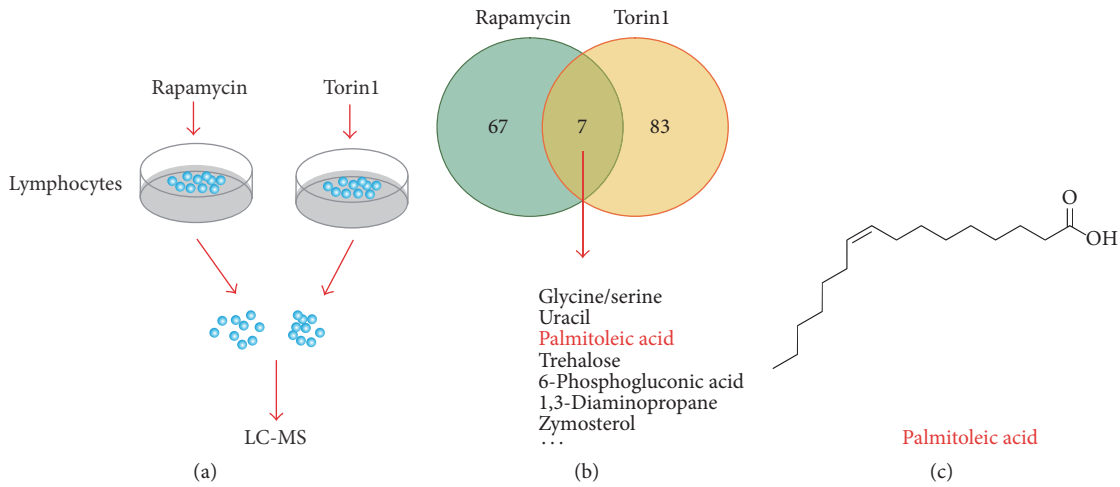

Figure 1: Screening of altered metabolites controlled by mTORC1 signaling. (a) A model showing the procedure of human PBMC cell culture and drug treatment. Rapamycin (100 nM) and Torin1 (200 nM) treatment time is 24 h. (b) A diagram showing the results of metabolites screening. Note that the crossing of the rapamycin and Torin1 groups contains about 7 substrates, including palmitoleic acid. (c) The molecular structure of palmitoleic acid.

2.6. Statistical Analysis. Data represent the mean and standard error of the mean (SEM). ANOVA tests for comparisons were performed for all the statistical significance analysis using GraphPad Prism software: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

3. Results

3.1. Screening of Altered Metabolites Controlled by mTORC1 Signaling. mTORC1 pathway is a master regulator of cell metabolism to modulate cell growth and death and inflammations in immune cells. Therefore, we focused on cell metabolism to reveal how mTORC1 regulates inflammations. To explore the metabolic atlas regulated by mTORC1, we screened metabolic alternations in human PBMC treated by either rapamycin or Torin1 (Figure 1(a)). Rapamycin is a specific mTORC1 inhibitor after a short time of treatment, whereas Torin1 inhibits both mTORC1 and mTORC2 activity [26]. Then, we used LC-MS for metabolic screening. We screened and got the altered metabolites by either rapamycin or Torin1 treatment (Figure 1(b)). Notably, we found that several one-carbon amino acids (e.g., serine and glycine), glucose intermediates, and several kinds of fatty acids (e.g., palmitoleic acid) (Figure 1(c)) were dramatically reduced after mTORC1 inactivation. Thus, our screening revealed novel metabolic targets of mTORC1 pathway on human PBMC.

3.2. Palmitoleic Acid Level Is Positively Regulated by mTORC1 Signaling. Palmitoleic acid (16:1ω-7, 16:1n-7) is a monounsaturated fatty acid (MUFA) that mainly originates from de novo lipogenesis in humans [23]. Palmitoleic acid has been proven to get involved in many metabolic regulation processes in vitro and in vivo. Firstly, we confirmed the alternation of palmitoleic acid by mTORC1 inhibition. Biochemical results showed that inhibition of mTORC1 activity by rapamycin indeed decreased the levels of palmitoleic acid in PBMC (Figure 2(a)), whereas the reduction of mTORC1 activity was confirmed by decreased phosphorylation of p70S6K1 and 4EBP1 (Figures 2(b) and 2(c)). Next, we wonder whether increased mTORC1 activity would induce palmitoleic acid contents. By knockdown of TSC1, mTORC1 signaling was dramatically activated and led to increased palmitoleic acid levels (Figures 2(d)–2(f)). Thus, our results indicate that palmitoleic acid level is positively regulated by mTORC1 signaling.

3.3. mTORC1 Signaling Regulates Palmitoleic Acid Level through Its De Novo Synthesis. Since mTORC1 signaling is critical for the homeostasis of palmitoleic acid, we next investigated how mTORC1 regulates palmitoleic acid levels. mTORC1 is a central regulator of lipid synthesis (lipogenesis) in mammalian cells at transcriptional level (Figure 3(a)). Lipogenesis is mediated by stearoyl-CoA desaturase 1 (SCD1), a key enzyme involved in the biosynthesis of MUFAs from SFAs [27]. Therefore, we examined whether mTORC1 signaling is required for the lipogenesis in PBMC. Real-time PCR results showed that inhibition of mTORC1 activity by rapamycin decreased expressions of lipogenesis enzymes, such as *Srebp1c, Acc1, Fasn*, and *Scd1* (Figure 3(b)), whereas induced mTORC1 activity by TSC1 knockdown reversely increased expressions of these lipogenesis enzymes (Figure 3(c)). Our results suggest that mTORC1 activity regulates palmitoleic acid by controlling its synthesis in PBMC.

3.4. Increased Palmitoleic Acid as a Biomarker of PBMC in Patients with Polymyositis. We have established the metabolic axis of mTORC1/palmitoleic acid in human PBMC and suggest that it is able to work as a biomarker of relative

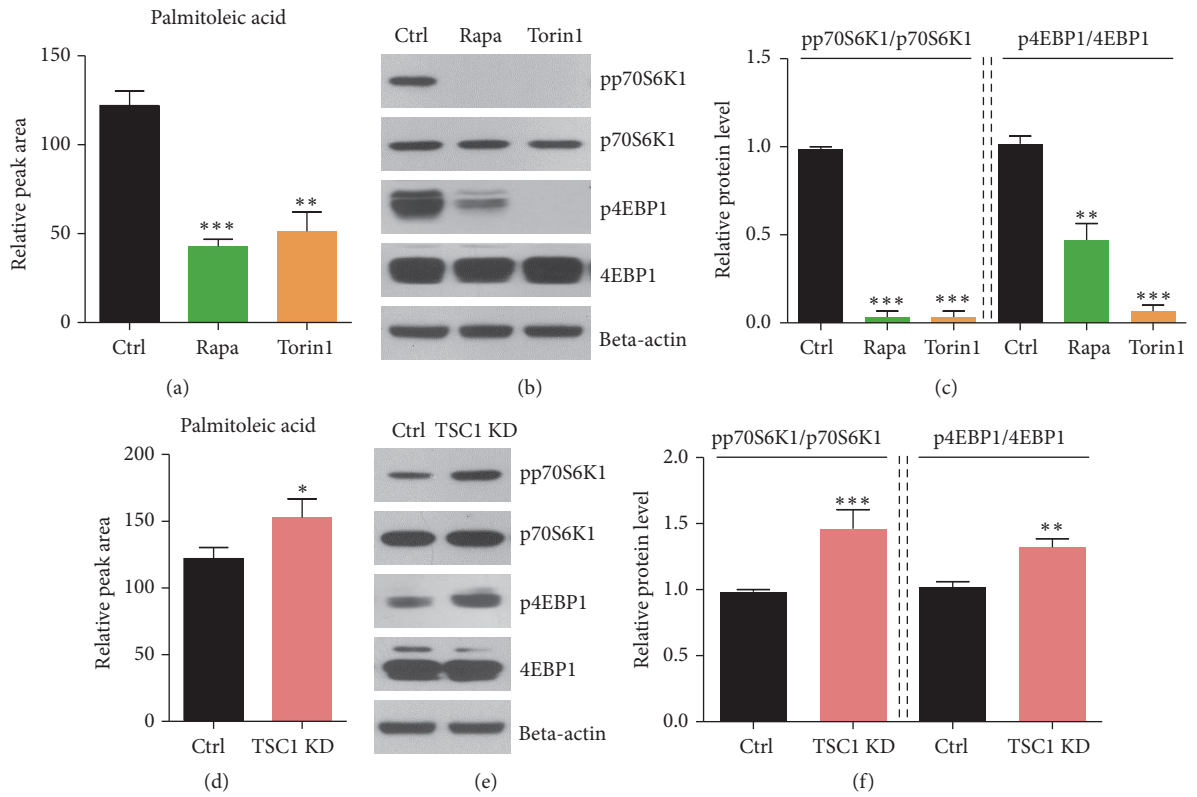

FIGURE 2: Palmitoleic acid level is positively regulated by mTORC1 signaling. (a) LC-MS results showed the reduction of palmitoleic acid level in both rapamycin (rapa) and Torin1 treated PBMC. Results are averages of five independent experiments. Data represent mean ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$. (b-c) Western blots and quantification showed that mTORC1 activity, indicated by pp70S6K1 and p4EBP1, was dramatically decreased by both rapamycin (rapa) and Torin1 treated cells. Results are averages of three independent experiments. Data represent mean ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$. (d) LC-MS results showed the increasing of palmitoleic acid level in TSC1 knockdown (KD) cells. Results are averages of five independent experiments. Data represent mean ± SEM. $^{*}p < 0.05$. (e and f) Western blots and quantification showed that mTORC1 activity was dramatically increased by TSC1 KD. Results are averages of three independent experiments. Data represent mean ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$.

diseases. Hence, we extend to study whether this metabolic axis could indicate the PBMC abnormality in polymyositis patients. We collected PBMC from patients with polymyositis and tested the levels of palmitoleic acid. Biochemical results showed that the level of palmitoleic acid was indeed increased in PBMC samples from patients with polymyositis (Figure 4(a)). In parallel, mTORC1 activity was also increased, consistent with palmitoleic acid levels (Figures 4(b) and 4(c)). Taken together, our results suggest that palmitoleic acid in PBMC has the potential to be a biomarker of polymyositis.

4. Discussion

Polymyositis is a chronic autoimmune disease. In this study, we aimed to screen the biomarker of PBMC in polymyositis for early detection and therapy. We found that palmitoleic acid, an important monounsaturated fatty acid, was positively regulated by mTORC1 signaling. mTORC1 regulated the levels of palmitoleic acid by controlling its de novo synthesis. Importantly, we found that palmitoleic acid level was increased in PBMC of patients with polymyositis. Our work identifies palmitoleic acid in PBMC as a potential biomarker of polymyositis.

Palmitoleic acid is a MUFA that mainly originates from de novo lipogenesis in humans. In the human body, palmitoleic acid of dietary origin is negligible, because most of it is oxidized shortly after absorption. In a recent study, palmitoleic acid from adipose tissue was found to promote insulin sensitivity in muscles and to suppress not only hepatosteatosis but also the expression of monocyte chemoattractant protein-1 and tumor necrosis factor-α in adipose tissue [28]. Subsequent animal studies also corroborate the favorable effects of palmitoleic acid on insulin action and lipid profile [29]. All these studies established the metabolic role of palmitoleic acid in adipose tissues. Here, we identified palmitoleic acid in PBMC to be a potential biomarker of polymyositis.

On the other hand, several studies have demonstrated the role of palmitoleic acid in inflammations. For example, palmitoleic acid reduces hepatic steatosis by inhibiting the expression of sterol regulatory element binding protein-1 (SREBP1), a transcription factor that is involved in the regulation of many enzymes involved in lipid synthesis [24].

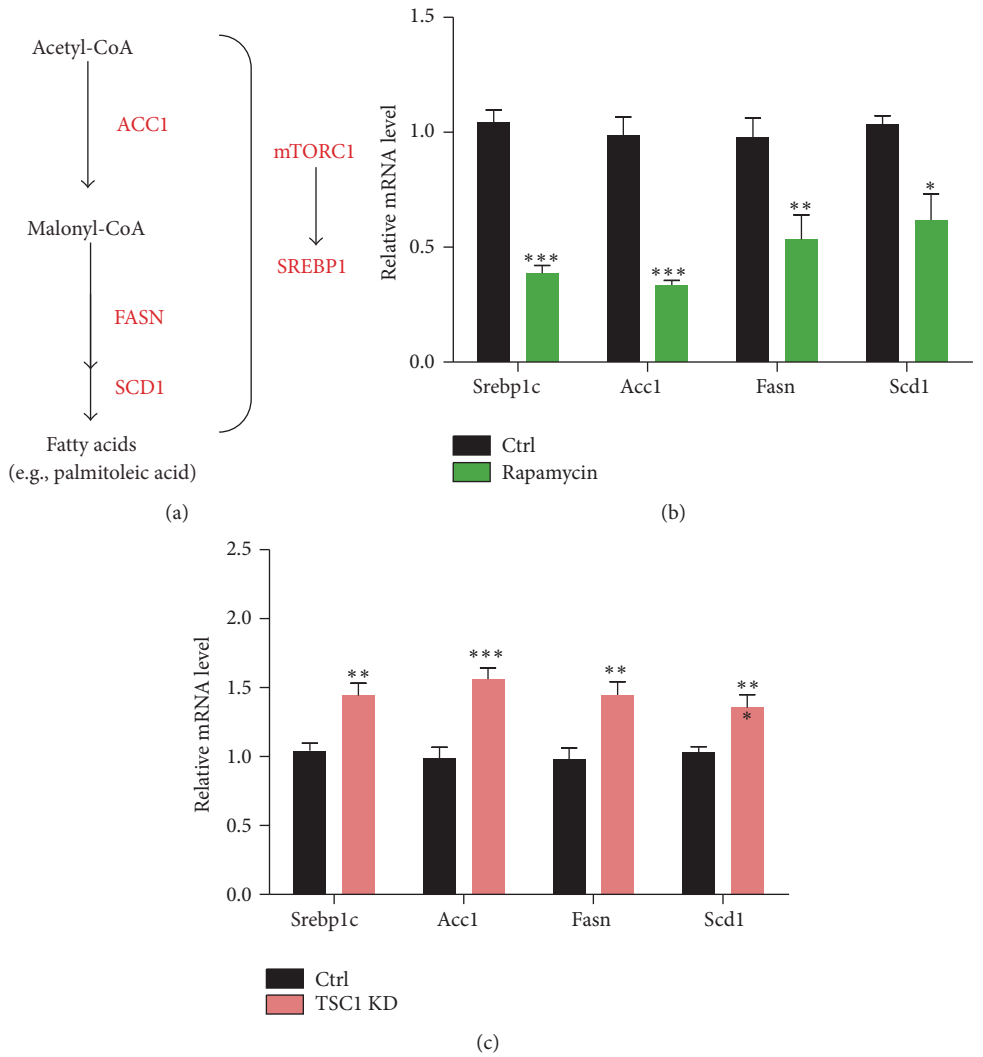

Figure 3: mTORC1 signaling regulates palmitoleic acid level through its de novo synthesis. (a) A model showing the de novo lipid synthesis in mammalian cells. mTORC1 mainly regulates gene transcriptions of *Srebp1c, Acc1, Fasn*, and *Scd1* by SREBP1. (b) Real-time PCR results showed the decreased gene expressions of *Srebp1c, Acc1, Fasn*, and *Scd1* in cells treated with rapamycin. Results are averages of three independent experiments. Data represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. (c) Real-time PCR results showed the increased gene expressions of *Srebp1c, Acc1, Fasn*, and *Scd1* in cells by TSC1 KD. Results are averages of three independent experiments. Data represent mean ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$.

Also, it was demonstrated that palmitoleic acid is a positive modulator of white adipose lipolysis through a mechanism that involves an increase in the content of the adipose triglyceride lipase (ATGL) and requires the activation of nuclear receptor PPARα [30]. All these metabolic functions suppress the expression of proinflammatory genes, primarily by inactivating the master proinflammatory transcription factor NF-κB and thus reducing the production of cytokines and tissue inflammation caused by ectopic fatty livers. However, all these functions were present in the peripheral adipose or livers. The further metabolic role of palmitoleic acid has not been well elucidated. In this study, we firstly demonstrate the role of palmitoleic acid in human PBMC. We identified palmitoleic acid in PBMC as a novel biomarker of polymyositis. Our work offers new targets to early detection and clinical therapies of polymyositis.

5. Conclusion

In conclusion, the present findings supported the fact that palmitoleic acid is highly regulated by mTOR signaling, and inhibition of mTORC1 activity decreases palmitoleic acid levels in human PBMC. Moreover, it is suggested that mTORC1 may regulate the level of palmitoleic acid by controlling its de novo synthesis. Increased palmitoleic acid level in PBMC has the potential to be a marker of abnormal PBMC in polymyositis. Our findings provide novel markers and targets for early detection and therapy of polymyositis.

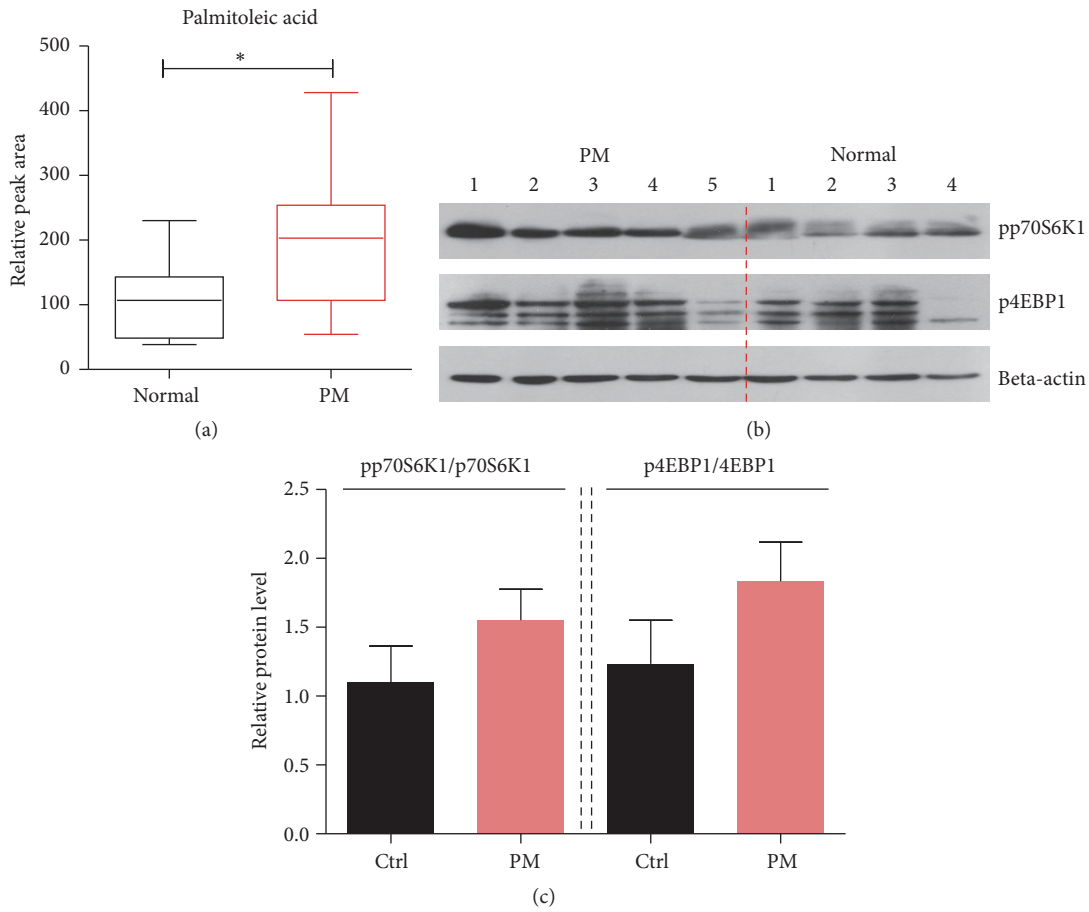

Figure 4: Increased palmitoleic acid as a biomarker of PBMC in patients with polymyositis. (a) LC-MS results showed the increasing of palmitoleic acid level in PBMC of polymyositis (PM) patients compared with normal ones. Results come from 12 normals and 16 PM patients. Data represent mean with min. to max. $^{*}p < 0.05$. (b and c) Western blots and quantification showed that mTORC1 activity, indicated by pp70S6K1 and p4EBP1, was increased in PBMC of polymyositis (PM) patients compared with normal ones. Results are averages of nine independent experiments. Data represent mean ± SEM.

Abbreviations

PM: Polymyositis
mTOR: Mechanistic target of rapamycin
PI3K: Phosphatidylinositol 3-kinase
PBMC: Peripheral blood mononuclear cells
MUFA: Monounsaturated fatty acid
SREBP1: Sterol regulatory element binding protein-1.

Competing Interests

The authors declared that no competing interests exist.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (Grant no. 30901339 and Grand no. 81172869) and the Application Foundation Project in Sichuan Province (no. 2016JY0021).

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 4832543, 8 pages
http://dx.doi.org/10.1155/2016/4832543

Research Article

Association of Serum CXCL13 with Intrarenal Ectopic Lymphoid Tissue Formation in Lupus Nephritis

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Received 30 June 2016; Revised 31 August 2016; Accepted 6 September 2016

Academic Editor: Guixiu Shi

Aims. To assess the concentrations of serum CXCL13 and intrarenal ectopic lymphoid tissue (ELT) profiles and their correlation in the patients with lupus nephritis (LN). *Methods.* Serum CXCL13 levels were measured using enzyme-linked immunosorbent assays (ELISA). The expression of CD3, CD20, and CD21 in renal biopsy specimens was tested using immunohistochemical methods. *Results.* Serum CXCL13 levels were significantly higher in the LN group than those in the SLE group without LN and also in the type III and IV LN patients than in type V LN patients. LN patients with positive CD20 expression (CD20+ LN) had a longer disease course and poorer response to combination therapy and higher serum CXCL13 levels than CD20− LN patients. Moreover, the serum CXCL13 level was positively correlated with the number of B cells/HP in the renal tissue of LN patients. The coexpression patterns of CD3, CD20, and CD21 in the renal tissue of LN patients with different WHO pathological types were significantly different. Serum CXCL13 levels were significantly higher in ELT-2 type LN patients than in 0 or 1 type LN patients. *Conclusions.* This study suggested that increased serum levels of CXCL13 might be involved in renal ELT formation and renal impairment process in LN.

1. Introduction

Lupus nephritis (LN) is characterized by immune complex deposition and inflammation in glomeruli and the tubulointerstitium, but its precise pathogenesis is not very much clear until now. Many studies have indicated that systemic loss of B-cell tolerance results in the local deposition of immune complexes [1, 2]. With the successful application of targeted B-cell therapy in the treatment of refractory LN, the role of B cells in the pathogenesis of LN has attracted increased consideration. It is known that B lymphocytes are involved in the pathogenesis of systemic lupus erythematosus (SLE) in autoantibody-dependent mechanisms. While some recent studies suggested that autoantibody-independent mechanisms might be more important, the specific mechanism is still unclear [3–5]. Investigations in the MRL/*lpr* mouse model of lupus nephritis have indicated that B cells exert a pathogenic role in the absence of soluble autoantibody production [6]. Local B-cell infiltration and related abnormal expression of ectopic lymphoid tissue (ELT) in the renal tissues of LN mice models was related to the severity of renal impairment [7]. These findings suggested that excessive intrarenal B-cell infiltration and related ELT formation might play an important role in LN occurrence and development. Similar results have been rarely reported in humans.

CXCL13 is a small-molecule cytokine and belongs to the chemokine family, which can regulate transport of various types of white blood cells by binding to its receptor, CXCR5. Because of the ability to attract B cells, it is also known as a B-lymphocyte chemokine (BLC). Research performed in NZB/W-F1 mice and LN patients [4] suggested that CXCL13 played an important role in the initiation and development of LN as a B-lymphocyte chemokine. Whether CXCL13 is related to the renal pathological damage observed in LN and

the formation of abnormal B-lymphocyte infiltration-related ELT have not been reported in detail [8, 9].

Thus, we speculated that an abnormally increased serum CXCL13 level could induce excessive chemotaxis of B cells, T cells, and dendritic cells into renal tissues of LN and subsequent excessive ELT formation, which promotes a vicious cycle of the expansion and perpetuation of inflammatory cell aggregates resulting in persistence and chronicity of the renal inflammation in LN patients. This study demonstrated intrarenal B-cell infiltration and related ELT formation in the LN patients with different WHO pathological classifications and showed serum CXCL13 levels pre- and posttreatment and their role in the process of intrarenal ELT formation. Moreover, we explored the role of intrarenal B-cell infiltration and related ELT formation, as well as an increase in serum CXCL13 levels in the pathogenesis, diagnosis, and treatment of LN; CXCL13 may function as a reliable and practical marker of serology and excessive ELT formation as a marker of histology for the clinical diagnosis of LN. They might provide a basis for more precise treatment of LN in the future.

2. Patients and Methods

2.1. Patients. A prospective study of 114 patients who attended the Department of Rheumatology and Immunology of Guangdong General Hospital was carried out. All patients fulfilled the American College of Rheumatology classification criteria for the diagnosis of SLE [10]. Clinical evidence of LN was obtained in all cases and LN diagnosis was confirmed by examination of renal biopsy specimens. All SLE patients included in this study are with age of no more than 70 years without hepatitis B-associated nephritis or the following complications including infection, a tumor, and severe cardiopulmonary dysfunction.

The following demographic, clinical, and serologic data were collected at the time of renal biopsy: sex, age, duration of SLE and LN, systemic lupus erythematosus disease activity index (SLEDAI), 24 h proteinuria, levels of blood urea nitrogen, serum creatinine, serum C3, C4, antinuclear antibodies (ANA), anti-Sm, anti-ribonucleoprotein (anti-RNP), anti-double-stranded DNA (anti-dsDNA), and antinucleosome antibodies were determined. SLEDAI was used to estimate global disease activity.

This study was performed according to the principles of the Declaration of Helsinki and each participant completed written informed consent before measurements. This study was also approved by the Local Ethics Committee (Institutional Review Board of Guangdong General Hospital).

All LN patients underwent combination therapy with glucocorticoids (GC) and immunosuppressants (IS). IS mainly referred to cyclophosphamide (CTX), mycophenolate mofetil (MMF), and cyclosporine A (CSA). After 6 months of treatment, responses in LN patients were evaluated that included complete remission (CR) and no CR. CR criterion referred to the international standards for evaluating the therapeutic effects in LN [11].

2.2. Main Reagents and Instruments. A CXCL13 kit was bought from R&D Company in the United States. Other reagents included Clone PS1, Novacastra as a CD3 antibody (T-cell surface marker); Clone L26, DAKO Company as a CD20 antibody (B-cell surface marker); and Clone 2G9, Gene Company as a CD21 antibody [follicular dendritic cell (FDC) surface marker]. DAKO EnVision plus kit was used for immunohistochemical test. MN1616, German BARD Company, was used as a percutaneous automatic biopsy gun.

2.3. Histological Observation of Sampled Renal Specimen. All enrolled clinically diagnosed LN patients undertaken renal biopsy with B-mode ultrasound-guidance. Native kidney biopsies will need to be divided into three parts for conventional light microscopy, electron microscopy, and immunofluorescence studies. Two-thirds of renal biopsy samples were stereotyped in 10% Neutral Buffered Formalin Fixative, dewatered gradually, and soaked in paraffin. Serial sample sections were marked with alternating hematoxylin and eosin (HE), periodic acid-Schiff (PAS), Masson's trichrome, and periodic acid-silver methenamine. One-third of fresh renal tissue was quickly frozen, and 4 μm unfixed and frozen sections were stained with fluorescein isothiocyanate- (FITC-) conjugated rabbit antisera against human IgG, IgA, IgM, C1q, and C3 (DAKO, Denmark), and the direct immunofluorescence of these sections was observed. The pathological diagnosis for biopsy specimens was established using the international standards for pathological classification of LN [12].

2.4. Immunohistochemical Staining Examination of CD3, CD20, and CD21 Coexpression in the Kidney. Renal biopsy was performed for all included LN patients. A routine pathological examination was carried out, and CD3, CD20, and CD21 coexpression were detected using immunohistochemical staining kits (DAKO EnVision plus). Serial paraffin-fixed tissue sample sections were cut to a thickness of 3 μm and mounted on positively charged glass slides and kept in a stove at 55°C overnight. Slides were dewaxed and hydrated through a series of washes with xylene and graded alcohols. Endogenous peroxidase was quenched by treatment with 3% H_2O_2 for 10 min. Antigen retrieval was carried out by dipping the slides into antigen repair solution (pH 9.0 Tris-EDTA) and heating at 100°C in a microwave at 650 W for 25 min. First mouse monoclonal anti-human CD20, CD3, and CD21 antibodies were added to the slides at dilution of 1 : 800, 1 : 100, and 1 : 200, respectively, in 1% bovine serum albumin in phosphate buffered saline (BSA/PBS) and subsequently stored them 70 min at room temperature. Slides were rinsed with PBS (pH 7.4) between each step (2 times for 5 min). Slides were then mounted with a secondary goat anti-mouse IgG antibody (EnVision/HRP; DAKO) for 40 min at room temperature. Slides were rinsed in PBS (pH 7.4) between each step (2 times for 5 min), then incubated in DAB solution for 1–3 minutes, and rinsed in distilled water. Finally, sections were counterstained with Mayer's hematoxylin, air-dried, cleared in xylene, and coverslipped with mounting medium. Tonsil tissue was stained as a positive control, and primary antibodies were replaced with PBS as a negative control. Positive results manifest with brown particles localized at

cytomembrane or cytoplasm of CD3 expression, or cytoplasm of CD20 and CD21 expression.

Based on the CD20, CD3, and CD21 coexpression patterns in the kidney, the LN patients could be divided into 4 categories of ELTs [13]: class 0, class 1, class 2, and class 3. Class 0 had scattered CD3+ cells infiltration only, but without CD20+ cells and CD21+ cells expression; class 1 had scattered CD20+ cells and CD3+ cells infiltration, but without CD21+ cells expression; and class 2 had a nodular aggregation of CD20+ cells and CD3+ cells without CD21+ cells expression. Class 3 demonstrated a nodular aggregation of CD20+ cells and CD3+ cells with CD21+ cells expression.

2.5. Determination of Serum CXCL13 Concentration. 2 mL venous blood samples were drawn from an enrolled individual in the morning before breakfast and then centrifuged at 3000 r/min for 5 minutes to obtain serum (stored at −80°C until analysis). CXCL13 was tested using an ELISA kit, and quantification was accomplished according to the manufacturer's protocol.

2.6. Statistical Analysis. All statistical analyses were performed using the SPSS 13.0 package program. All measurement data with normal distribution are expressed as mean ± standard deviation ($\overline{X} \pm S$) and analyzed by t-test between two groups or by ANVOA and SNK test between multiple groups. The measurement data with abnormal distribution such as serum CXCL13, 24-hour urine protein, and anti-dsDNA quantitation are presented as medians (quartile) and were compared using the Mann–Whitney-Wilcoxon test between 2 groups and using nonparametric Kruskal Wallis test among multiple groups. Correlation analyses were performed using the Spearman method. Categorical data are presented as percentages and were compared using Crosstabs and Chi-square test among multiple groups. A P value of <0.05 was considered significant.

3. Results

3.1. Demographic, Clinical Characteristics, and Laboratory Results of LN Patients. Firstly, in this prospective study, 114 SLE patients (89 LN patients and 25 SLE patients without LN) and 21 healthy individuals were enrolled. 89 LN patients (78 women and 11 men; mean age ± SD: 35 ± 12.8 years) were separated into 2 groups according to the renal CD20 expression: LN with intrarenal B cells (LN-B group) and LN without intrarenal B cells (LN non-B group). The LN-B group comprised 69 (77.5%) patients (61 women and 8 men, 30.6 ± 12.2 years) and the LN non-B group comprised 20 (22.5%) patients (17 women and 3 men, 41.5 ± 12.9 years). No significant difference was detected between the two groups in terms of age or gender ($P > 0.05$). When compared with the LN-non-B-cell group, the LN-B-cell group had a longer disease course (21.8 ± 9.9 months versus 9.8 ± 6.2 months, resp.; $t = 2.185$, $P = 0.045$) and a poor complete remission (CR) rate after 6 months of treatment (63.8% versus 90%, resp.; $\chi^2 = 2.384$, $P = 0.047$). Other clinical characteristics (age, sex, SLEDAI, urine protein quantitation (mg/24 h), serum creatinine, C3, C4, and anti-dsDNA level) between both groups demonstrated no differences. See Table 1.

3.2. Histopathology, B-Lymphocyte Infiltration, and ELT Distribution Characteristics in the LN Patients. Of the enrolled 114 SLE patients, 89 LN patients were diagnosed clinically and confirmed by light microscopy, immunofluorescence, and electron microscopy. According to the ISN/RPS classification, 21 patients were diagnosed with type III (23.6%), 53 patients with type IV (59.6%), and 15 patients with type V (16.8%). No type I, II, or VI was found. Lymphocytes infiltration was mainly distributed in the renal interstitium of 77 patients (86.5%), around the renal tubules of 9 patients, and around the glomeruli of 3 patients. Lymphocytes aggregation was regionally distributed in 51 patients (57.3%), while no typical lymph follicles as in tonsil tissue were found. The activity index (AI) score for the renal pathology was 10.7 ± 4.8 (3–19), and the chronicity index (CI) score was 3 ± 1 (1–4). There was no significant difference at the distribution of LN pathological classifications, AI score, and CI score between the LN-B-cell group and the LN-non-B-cell group. See Table 1.

Based on the CD20, CD3, and CD21 coexpression patterns in the kidney, the 89 LN patients could be divided into 3 categories: class 0, class 1, and class 2. No formation of class 3 ELT was found in the LN patients enrolled in our study. See Figure 1.

The disease duration between the three ELT classes groups was different. The ELT class 2 group showed significantly longer disease duration than the ELT class 0 and the ELT class 1 ($P = 0.017$ and 0.039, resp.), while there was no significant difference ($P = 0.280$) between the ELT class 0 and the ELT class 1. The difference was significant in the AI score between the ELT class 2 and the ELT class 0 ($P = 0.047$), while it was not significant between the ELT class 1 and the ELT class 2 ($P = 0.056$). Other clinical parameters (SLEDAI score, CI score, C3, C4, 24-h urine protein, and anti-dsDNA antibody) in the 3 ELT classes groups demonstrated no differences. See Figure 1 and Table 2.

We noted that the LN kidney tissues for different WHO LN pathological types had different proportions of the three classifications of ELT. See Table 3. The proportions of ELT classes composition in type III, IV, and V LN group were compared using a χ^2 test, and the differences were significant ($\chi^2 = 28.04$, $P < 0.0001$). The proportions of ELT classes involving types III and IV were significant ($\chi^2 = 10.78$, $P = 0.0046 < 0.0167$ [0.05/3]), as were those with types IV and V ($\chi^2 = 28.29$, $P < 0.0001 < 0.0167$ [0.05/3]). The proportions of ELT classes involving types III and V demonstrated no differences ($\chi^2 = 5.07$, $P = 0.0793 > 0.0167$ [0.05/3]).

3.3. Changes in Serum CXCL13 Levels in LN Patients. According to the presence of renal involvement, SLE patients ($n = 114$) were divided into the LN group ($n = 89$) and the SLE group without renal involvement (SLE-no-LN) ($n = 25$). Serum CXCL13 levels in SLE patients (230.65 [145.92–365.40] pg/mL) were significantly higher than those of healthy individuals (85.13 [62.23–131.58] pg/mL) ($Z = 4.42$, $P < 0.001$). Moreover, serum CXCL13 levels in the

Table 1: Clinical data of the B-cell group and the non-B-cell group in LN patients.

	LN-B-cell ($n = 69$)	LN-non-B-cell ($n = 20$)	$\chi^2/t/H$ value	P value
Male*	8 (11.6)	3 (15.0)	0.786	0.294
Age (years)	30.6 ± 12.2	41.5 ± 12.9	−1.542	0.144
Duration (months)	21.8 ± 9.9	9.8 ± 6.2	2.185	0.045
Urine protein# (mg/24 h)	3 689.5 (1 568.7~8 686.5)	1785.6 (965.8~3 516.5)	−0.679	0.497
Serum Cr (umol/L)	107.2 ± 58.5	98.3 ± 44.9	0.277	0.785
C3 (mg/L)	476.9 ± 261.8	345.8 ± 89.7	0.966	0.350
C4 (mg/L)	151.6 ± 136.7	84.4 ± 29.8	0.956	0.354
Anti-dsDNA# (Iu/mL)	282.5 (115.6~418.6)	312.5 (135.6~462.5)	−0.341	0.733
WHO classification*			0.263	0.877
LN (III)	16 (23.2)	5 (25.0)		
LN (IV)	42 (60.9)	11 (55.0)		
LN (V)	11 (15.9)	4 (20.0)		
CI score	3.1 ± 1.6	2.2 ± 1.0	0.559	0.584
AI score	10.3 ± 4.7	10.9 ± 5.1	0.569	0.578
SLEDAI score	17.3 ± 7.9	15.3 ± 3.0	0.082	0.624
CR at 6 months*	44 (63.8%)	18 (90%)	2.384	0.047
TR at 6 months*	59 (85.5%)	19 (95%)	1.26	0.212

Note: when compared with the LN-non-B-cell group, there was a significant difference only at the disease duration and the CR for 6-month treatment in the LN-B-cell group. CR: complete remission; TR: total remission; dsDNA: double strand DNA; LN: lupus nephritis; AI: activity index; CI: chronicity index; SLEDAI: SLE disease activity index; *expressed in n (%); #expressed in median (interquartile range) and nonparameter test.

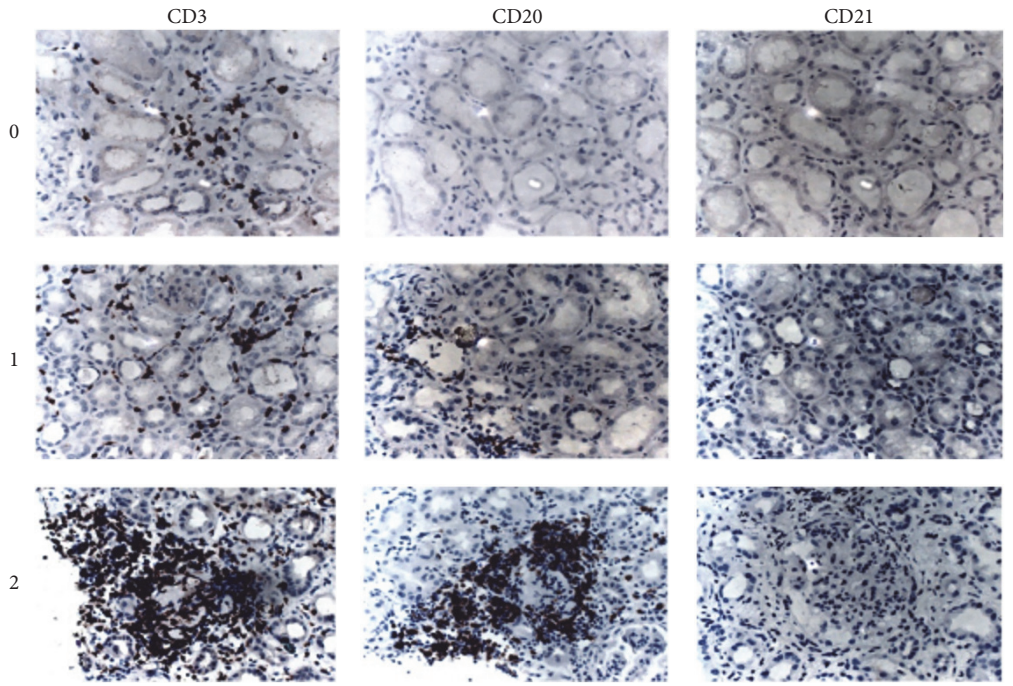

Figure 1: Ectopic lymphoid tissue classifications according to CD20, CD3, and CD21 immunohistochemical features in LN patients (×400). CD20, CD30, and CD21 were stained in serial sections. Class 0 had no CD20+ cell expression, with scattered CD3+ cells only, but without CD21+ cell expression; class 1 had scattered CD20+ cells and CD3+ cells infiltration, but without CD21+ cells infiltration; and class 2 had a nodular aggregation of CD20+ cells and CD3+ cells without CD21+ cell expression.

Table 2: The clinical data of patients with different ELT classes of renal tissues.

	Class 0 ($n = 20$)	Class 1 ($n = 18$)	Class 2 ($n = 51$)	F/χ^2 value	P value
Age (years)	41.5 ± 12.9	31.4 ± 13.3	30.1 ± 12.4	1.127	0.352
Duration (months)	9.8 ± 6.2	16.4 ± 7.8	24.4 ± 10.2	3.899	0.035
Urine protein (mg/24 h)	1 546.8 (856.9~2 578.6)	2031.6 (956.6~3 956.5)	2365.5 (755.6~9 560.6)	1.123	0.353*
Serum creatinine (μmol/L)	98.3 ± 44.9	100.5 ± 42.6	111.4 ± 69.2	0.091	0.914
C3 (mg/L)	345.8 ± 89.7	440.8 ± 278.8	499.5 ± 267.6	0.529	0.600
C4	84.4 ± 29.8	216.9 ± 127.0	110.9 ± 50.8	1.763	0.208
Anti-dsDNA (IU/mL)	196.9 (79.6~796.8)	115.9 (72.6~685.6)	215.6 (115.5~565.7)	0.133	0.936*
CI score (points)	5.3 ± 2.2	6.2 ± 1.3	5.6 ± 2.0	0.442	0.652
AI score (points)	5.8 ± 2.6	8.3 ± 4.1	12.5 ± 5.1	2.986	0.041
SLEDAI score (points)	15.3 ± 3.0	14.6 ± 10.0	19.0 ± 6.5	1.707	0.310

Note: *nonparametric test; in all clinical characteristics, there was a significant difference only in the disease duration and the renal pathological activity index (AI) among the class 0, 1, and 2 ELT group.

Table 3: ELT formation patterns in renal tissues comprising different LN pathological types.

Pathological types	ELT class		
	Class 0	Class 1	Class 2
Type III ($n = 21$)	7	2	12
Type IV ($n = 53$)	3	14	36
Type V ($n = 15$)	10	2	3

Note: the proportions of ELT classes composition in type III, IV, and V LN group were compared using a χ^2 test, and the differences were significant ($\chi^2 = 28.04$, $P < 0.0001$). The proportions of ELT classes involving types III and IV were significant ($\chi^2 = 10.78$, $P = 0.0046$), as were those with types IV and V ($\chi^2 = 28.29$, $P < 0.0001$). The proportions of ELT classes involving types III and V demonstrated no differences ($\chi^2 = 5.07$, $P = 0.0793$).

LN patients (258.86 [185.50–392.18] pg/mL) were higher than those of the SLE-no-LN group (136.39 [104.23–257.50] pg/mL) ($Z = 2.53$, $P = 0.01$) (Figure 2(a)). After 6 months of treatment, serum CXCL13 levels and SLEDAI were significantly decreased in the LN group (CXCL13: 258.86 [185.50–392.18] pg/mL versus 195.54 [165.98–233.01] pg/mL, resp.; $Z = -2.59$, $P = 0.009$; SLEDAI: 10 [8.75–12] versus 6 [4–6.5], $Z = -2.56$; $P = 0.01$).

After 6 months of immunosuppressive therapy, The LN patients were divided into a complete remission group ($n = 56$) and noncomplete remission group ($n = 33$). Serum CXCL13 levels for the LN group in complete remission (189.46 [114.23–216.50] pg/mL) were significantly lower than those of the LN group with noncomplete remission (286.75 [181.40–386.56] pg/mL) ($Z = 2.48$, $P = 0.048$).

The correlation of serum CXCL13 with SLEDAI in SLE patients was analyzed. Serum CXCL13 levels in SLE patients were positively correlated with SLEDAI ($r = 0.55$, $P < 0.001$) (Figure 2(b)) and negatively correlated with C3 ($r = -0.39$, $P < 0.001$) (Figure 2(c)), and there was no significant correlation with the anti-dsDNA antibody titer ($r = 0.037$, $P = 0.72$).

3.4. Serum CXCL13 Levels in LN Patients with Different Pathologic Classifications. Serum CXCL13 levels for type III and IV LN group were higher than those of type V LN group (type III: 287.58 [198.25–355.44] pg/mL; type IV: 271.96 [189.71–434.99] pg/mL; type V: 169.71 [101.09–252.69] pg/mL; type III versus type V, $P = 0.013$; type IV versus type V, $P = 0.002$), while serum CXCL13 levels in type III and IV LN patients demonstrated no difference ($P = 0.39$) (Figure 2(d)).

3.5. ELT Patterns in the Renal Tissue of LN Patients and Its Correlation with Changes in Serum CXCL13 Levels. Serum CXCL13 levels in the LN patients with class 2 ELT were significantly higher than those of the LN patients with class 0 and 1 ELT (class 0: 180.96 [101.93–218.88] pg/mL; class 1: 216.91 [158.47–327.15] pg/mL; class 2: 351.80 [251.13–470.36] pg/mL; class 2 versus class 0: $Z = 4.79$, $P < 0.001$; class 2 versus class 1: $Z = 2.32$, $P = 0.02$) (Figure 2(f)). Further, serum CXCL13 levels in the patients with class 1 ELT were higher than those of the patients with class 0 ELT ($Z = 2.13$, $P = 0.033$). Moreover, serum CXCL13 levels were positively correlated with the number of B cells/HP in the renal tissue of LN patients ($r = 0.41$, $P = 0.048$).

4. Discussion

The role of long-lived memory plasma cells and B-cell hyperactivity has been recently elucidated in the pathogenesis of SLE [14, 15]. A high prevalence of intrarenal B cells has been noted in immune-mediated diseases, such as renal transplant rejection and glomerulonephritis [16–18] thus indicating that local B-cell infiltrates play a role in tissue injury such as tissue fibrosis, neolymphangiogenesis, and ectopic lymphogenesis [19]. It is hypothesized that intrarenal B cells associated ELTs form part of a local autoimmune system with pivotal involvement in the pathogenesis of lupus nephritis. Our study suggested that the abnormal aggregation of B lymphocytes and related ELT formation in the renal tissue of LN patients might play a unique role in promoting LN persistent progression. Elevated serum CXCL13 level might take part in this process.

In this study, 77.5% of LN patients had excessive B-lymphocyte infiltration in renal tissues. These patients, especially those with focal accumulation of B lymphocytes and T lymphocytes (type 2 ELT), had a significantly longer

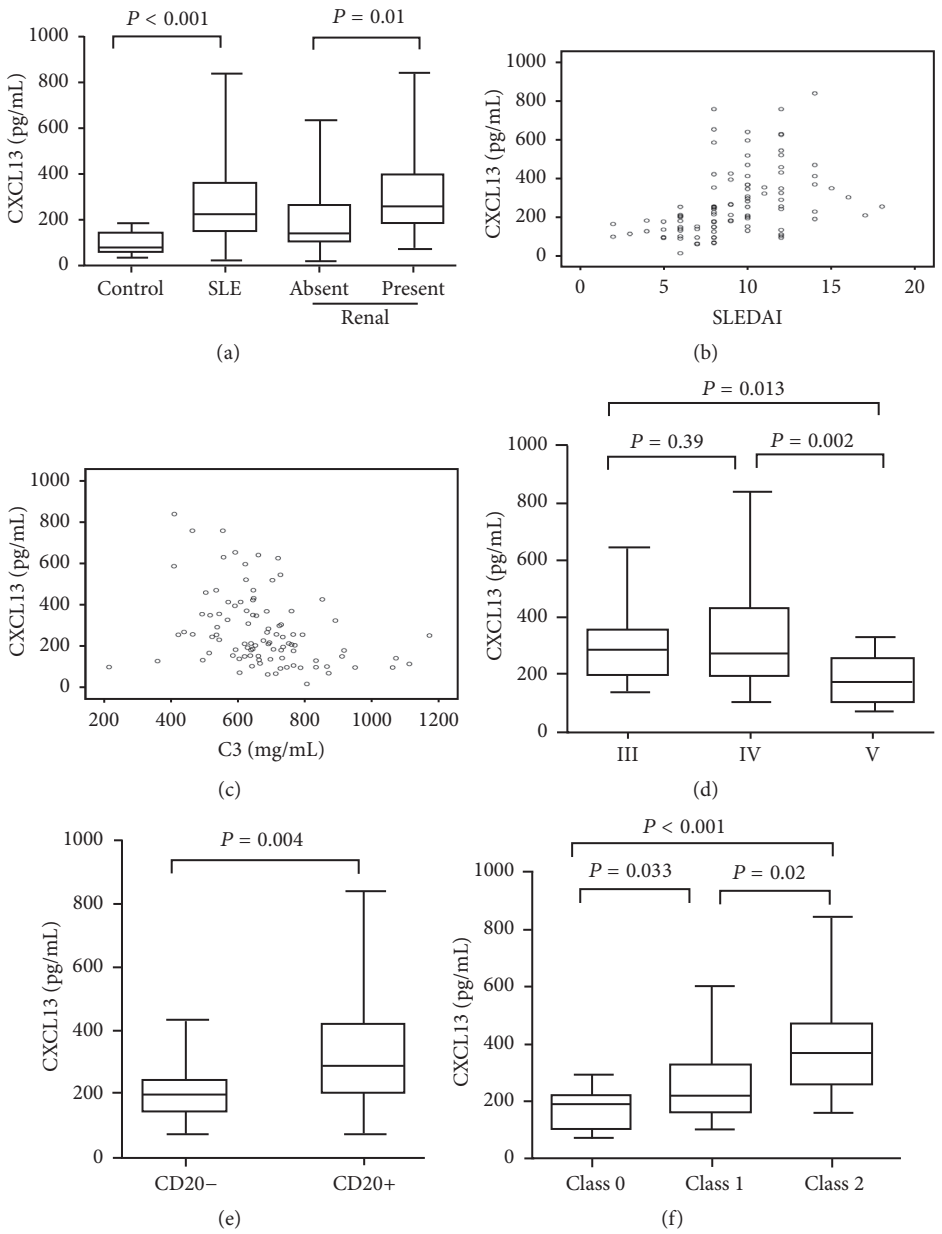

Figure 2: Changes in serum CXCL13 levels in LN patients. CXCL13 levels were increased in LN patients (a). Serum CXCL13 levels correlated with SLEDAI (b) and complement C3 (c). CXCL13 levels were different in LN patients with different WHO pathologic classifications (d), different CD20 expression (e), and different ELT class (f). Horizontal bars indicate median values and the boxes encompass the 25th percentile and 75th percentile, and whiskers indicate maximum and minimum.

disease course than those LN patients without B-lymphocyte infiltration. Moreover, LN patients with regional B-cell accumulation had a poor response to combination therapy with GC and IS, suggesting that the abnormal intrarenal B-cell accumulation was related to the longer disease course and poor treatment response in LN. Steinmetz and colleagues [13] observed the renal biopsy specimens of 32 patients with LN and 16 patients with ANCA-associated vasculitis and found that traditional treatment failed to eliminate B-lymphocyte accumulations in the renal tissue of LN patients. In patients with active or refractory LN, more B lymphocytes accumulated in renal tissues and interacted with T lymphocytes and FDC to promote ELT formation, thus leading to chronic renal injury and LN recurrence. Blocking or removing abnormally accumulated B lymphocytes in renal tissues as early as possible might be important to the effective treatment of refractory LN and might be the basis for the therapeutic effect of CD20 monoclonal antibodies in refractory LN [20–22].

This study showed that type 2 ELT was mostly seen in LN patients (n = 51, 57.3%), but no typical lymphoid follicles

were found. Lymphocyte infiltration was mostly found in the renal interstitium ($n = 77$, 86.5%) that was consistent with a study by Shen et al. [23]. The study enrolled 192 patients with LN and found no CD21+ cell expression in renal tissues. Chang and colleagues [24] conducted a study that enrolled 64 LN patients and found CD20+ B-lymphocyte and CD3+ CD4+ T-lymphocyte infiltration in the renal interstitium and lymphatic germinal center-like structures composed of CD138+ plasma cells and CD21+ dendrite cells. Steinmetz and colleagues [13] observed the renal biopsy specimens of 32 patients with LN and 16 patients with ANCA-associated vasculitis and also found the expression of CD21+ follicular dendrite cells. The diverse ELT patterns demonstrated in various studies may be related to different regions, ethnicities, and inclusion criteria. All the above-mentioned points indicate that preventing ELT formation in the renal tissue of LN patients is an important therapeutic target. Since the renal ELTs in LN patients are obviously different from the secondary lymph node structure such as tonsil, the types of their core immune cell components are different, and their interaction modes may be different, and the roles and abilities of these intrarenal ELTs during LN onset and development may differ from typical secondary lymph tissue, thus necessitating more studies to elucidate.

This study also revealed different ELT distribution characteristics in various LN pathological types. As compared with type V LN, type III and IV LN showed more class 2 ELT distribution. This result is consistent with a study conducted by Shen et al. [23].

Today, the role of CXCL13 in the pathogenesis of SLE has attracted wide attention [25]. We observed changes in serum CXCL13 levels in different WHO classifications of LN and different ELT classes to determine the role of CXCL13 in the pathogenesis of LN and the formation of B-cell-infiltration-related ELT. The results showed that LN patients with WHO types III and IV (especially WHO-IV) and class 1 and class 2 ELT (especially class 2) had significantly higher serum CXCL13 levels. Moreover, serum CXCL13 levels were positively correlated with SLEDAI, the degree of B-cell infiltration in renal tissue, and AI but were negatively correlated with the C3 level, suggesting a possible correlation between abnormally increased serum CXCL13 levels and ELT formation and renal impairment in LN patients. How blood CXCL13 induces locating of B cells mainly in the renal tubular interstitial region and thus forming ELT deserves further study.

In conclusion, this study suggested that abnormal B-cell infiltration and related ELT formation in renal tissues might play an important role in LN occurrence and persistent development, and increased serum CXCL13 levels might take part in ELT formation and the pathological renal impairment process in LN patients. Blocking the biological effects of CXCL13 and the formation of ELT in renal tissues might be important targets in the medical treatment of LN in the future.

Disclosure

De Ning He and Wen Li Chen are co-first authors.

Competing Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Authors' Contributions

Guang Fu Dong designed and performed research, analyzed and interpreted data, and wrote the paper; De Ning He, Wen Li Chen, and Kang Xia Long performed research, interpreted data, and wrote the paper; Xiao Zhang designed research and revised the paper.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 3042726, 6 pages
http://dx.doi.org/10.1155/2016/3042726

Research Article

Link-Polymorphism of 5-HTT Promoter Region Is Associated with Autoantibodies in Patients with Systemic Lupus Erythematosus

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Received 28 June 2016; Revised 6 September 2016; Accepted 18 September 2016

Academic Editor: Guixiu Shi

Serotonin transporter linked polymorphic region (5-HTTLPR) was reported to associate with depression in systemic lupus erythematosus (SLE) patients by our team. To explore whether 5-HTTLPR plays a role in the pathogenesis of SLE, we tested 138 SLE patients and 138 age and sex matched health controls (HCs) for 5-HTTLPR by polymerase chain reaction (PCR) and agarose gel electrophoresis. Interestingly, the results suggest that the frequencies of SS genotype and S allele in SLE patients with positive anti-Sm antibody and anti-U1RNP antibody were both significantly higher than the other genotypes and alleles. However, the frequencies of 5-HTTLPR genotypes and alleles were of no significant difference between SLE patients and HCs. This suggested that 5-HTTLPR was not a high-risk susceptible gene in SLE but might relate to SLE by affecting production of some autoantibodies, especially anti-Sm and anti-U1RNP antibody.

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease mediated by multiple genetic and environmental factors. Complicated interactions of genetic and molecular factors are involved in the pathogenesis of SLE. SLE is featured by a variety of autoantibodies produced by B cells derived from abnormal activation and differentiation. B cells can attract and present autoantigens to T cells through special immunoglobulins on their surfaces. Abnormalities like dysfunction of T and B cells, proliferation of B cells, and dominance of T helper (Th) 2 cells are involved in the pathogenesis of SLE. Meanwhile, multiple cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-10 (IL-10), IL-4, and chemokines appear and cause inflammation in tissues [1].

Serotonin (5-HT) plays an important regulatory role in all kinds of immune responses mediated by T cells, B cells, and macrophages through direct and indirect ways. It participates in autoimmune reaction by mediating the activation of T and B cells in several pathways [2]. Serotonin transporter (5-HTT or SERT) locates on the presynaptic membrane and regulates the reuptake of 5-HT in synaptic cleft. The serotonin transporter linked polymorphic region (5-HTTLPR) modifies 5-HTT function in both transcription and translation levels and affects the ability of taking in and releasing 5-HT procedures of 5-HTT; thus, it regulates the concentration of 5-HT eventually. 5-HT combines the corresponding serotonin receptor (5-HTR) and affects the activity of 5-HTT in return. This study was designed to find out the relationship between serotonin system and SLE.

2. Material and Methods

2.1. Subjects. 138 SLE patients were recruited from April 2011 to May 2012 from inpatient and outpatient centers

of the Department of Rheumatology and Immunology of First Affiliated Hospital of Kunming Medical University, Kunming, Yunnan, China, a member unit of Chinese SLE Treatment and Research Group (CSTAR). They were all diagnosed with SLE according to the 1997 revised American College of Rheumatology (ACR) criteria for the classification of lupus [3].

The inclusion criteria included the following factors: (1) diagnosis of SLE according to the 1997 revised ACR criteria for the classification of lupus; (2) capability of reading and writing; (3) being between the ages of 15 and 60; (4) voluntary writing consent of this study from patients or statutory guardians.

The exclusion criteria included the following factors: (1) patients with diagnosis of rheumatoid arthritis (RA), systemic sclerosis (SSc), Sjogren's syndrome (SS) (primary or secondary), other connective tissue diseases (CTD), or drug induced SLE; (2) patients with severe disorders of major organs such as heart, liver, or kidney; (3) patients with present or previous diagnosis of neurological or psychiatric disease; (4) patients with kidney failure or other pathologic conditions which may induce encephalatrophy (e.g., stroke, hypertension, diabetes mellitus, and addiction to alcohol or drugs); (5) patients with present or previous diagnosis of epilepsy; febrile convulsion in childhood is not included.

138 health controls (HCs) were recruited in the Physical Examination Center from the same hospital. All HC group members received thorough physical examinations to exclude SLE and other major diseases excluded in the SLE group.

Prior to entry into the study, each participant provided written informed consent after receiving a complete description of the study. All the participants were Chinese Han population. This research was approved by the Institutional Review Board of Kunming Medical University, Yunnan Province, China (ClinicalTrials.gov: NCT00703742).

2.2. Collection of Population Statistics and Clinical Data. Demographic and clinical data of both SLE patients and HCs were collected, including name, sex, age, marriage status, education background, profession, family history, and medical history. Disease status like symptoms, physical signs, and durations of disease was also recorded in SLE patients.

2.3. Test of Autoantibodies. Anti-nuclear antibody (ANA) was tested by indirect immunofluorescence assay (IFA) method with ANA (Hep-2) IFA kit (Xinsaier Technology Co., Ltd., Kunming, China). ANA spectrum was detected by line immunoassay (LIA) method with ANA spectrum linear immunoassay kit (IMTEC Company, Berlin, German).

2.4. Test of 5-HTTLPR. (1) DNA extraction: extract DNA from 5 mL peripheral venous blood anticoagulated by 2% EDTA with the TaKaRa MiniBEST Universal Genomic DNA Extraction Kit Ver.4.0 D824A (TaKaRa Biotechnology Co., Ltd., Dalian, China); (2) polymerase chain reaction (PCR) amplification of target genes: sense primer (5′- ATG CCA GCA CCT AAC CCC TAA TGT -3′) and antisense primer (5′- GG ACC GCA AGG TGG GCG GGA -3′) were synthesized by Sangon Biotech Co., Ltd., Shanghai, China. In a 50 μL nuclease-free microcentrifuge tube, the following were combined according to the manufacturer's instructions (TaKaRa LA Taq DRR002A, TaKaRa Biotechnology Co., Ltd., Dalian, China): template DNA (2.5 ng), dNTP mixture (8 μL), PCR Buffer II (Mg^{2+} Plus) (5 μL), sense primer (1 μL), antisense primer (1 μL), and sterilized saline in a 50 μL reaction volume. Touchdown PCR procedures were carried out in the following manner: initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 1 minute; 26 cycles were performed; the temperature was then held at 72°C for 10 minutes. The PCR products were compared with DL2000 DNA Marker as reference. (3) Genotype reading: the PCR products were separated by 2% agarose gel electrophoresis (voltage 180 V, 20 min) and were observed under ultraviolet (UV) gel imaging system Gel DocEQ (Bio-Rad Laboratories, Inc., CA, USA). Imaging analysis detected gene segments of 419 bp and 375 bp. The 375 bp was designated as the "S" or "short" variant, while the 419 bp was designated as the "L" or "long" variant. Thus, the three possible genotypes were "SS" homozygote, "LS" heterozygote, and "LL" homozygote.

2.5. Statistical Analysis. Statistical analysis was conducted with SPSS 17.0 (SPSS Inc., 1989–2004). Descriptive statistics and chi-square test were used to describe and compare the differences of genotype and allele frequency between SLE patients and HCs and within the SLE patients. Odds ratio (OR) and confidence interval (CI) were calculated and the results were statistically significant when $p < 0.05$. All statistical tests were two-sided.

3. Results

3.1. Demographical and Clinical Data. This research involved 138 SLE patients in which 18 were males and 120 were females; they were from 16 to 52 years old, on average 30.51 ± 8.58 years old; their education varied from 5 to 19 years, on average 11.65 ± 3.47 years. 138 HCs were matched by age and sex, and their education varied from 6 to 20 years, on average 15.13 ± 2.75 years. Their education level was comparable ($p > 0.05$).

3.2. Autoantibody Levels in SLE Patients. Several autoantibodies were detected in SLE patients. All patients were ANA positive. There were 75 patients (54.3%) with positive anti-double stranded (ds) DNA antibody; 80 patients (57.9%) with positive anti-Sm antibody; 68 patients (49.2%) with positive anti-nucleosome antibody; 41 patients (29.0%) with positive anti-U1 ribonucleoprotein (RNP) antibody; 79 patients (57.2%) with positive anti-histone antibody; 59 patients (42.7%) with positive anti-ribosomal P0 antibody; 62 patients (44.9%) with positive anti-SSA 52 kD antibody; 89 patients (64.5%) with positive anti-SSA 60 kD antibody; 39 patients (28.2%) with positive anti-SSB antibody.

3.3. Hardy-Weinberg Genetic Equilibrium Test. The 5-HTTLPR genotype frequency of both the SLE patients and HCs was consistent with the Hardy-Weinberg Equilibrium.

3.4. 5-HTTLPR Genotypes in SLE Patients and HCs. The frequency of SS genotype was 58.7% in SLE patients and 49.3% in HCs, while the frequency of LL was 7.2% in SLE patients and 10.1% in HCs; thus, the frequency of LS was 34.1% and 40.6% in each group ($\chi^2 = 2.587$, $p = 0.274$) (see Figure 1(a)). The frequency of S allele was 75.7% and 69.6% in SLE patients and HCs, respectively, while the L allele was 24.3% and 30.4% in corresponding groups ($\chi^2 = 2.635$, $p = 0.105$) (see Figure 1(b)). Both genotypes and alleles were not significantly different between the SLE patients and HCs ($p > 0.05$).

In SLE patients with positive anti-Sm antibody, the frequency of SS genotype was significantly higher than LL/LS genotype ($p = 0.014$, OR = 2.385, 95% CI 1.188–4.785) (see Figure 1(c)); the same result was also found in SLE patients with positive anti-U1RNP antibody ($p = 0.025$, OR = 2.46, 95% CI 1.108–5.461) (see Figure 1(d)). In patients with all the other antibodies, no significant differences were found.

In SLE patients with positive anti-U1RNP antibody, the frequency of S allele was significantly higher than L allele ($p = 0.015$, OR = 2.308, 95% CI 1.161–4.591) (see Figure 1(e)). In patients with all the other antibodies, no significant differences were found.

In SLE patients with positive anti-Sm antibody, the frequency of SS genotype was significantly higher than the other 2 genotypes ($p = 0.028$) (see Figure 1(f)). In patients with all the other antibodies, no significant differences were found.

4. Discussion

5-HT is usually considered as a typical neurotransmitter. It also plays a regulatory role in the immune system. 95% of 5-HT in peripheral circulation are synthesized by pheochromocytes in the gastrointestinal tract and then released to blood. 5-HTTs of platelets take in and store 5-HT in platelet dense granules, which composes the main pool of 5-HT. There are several studies which revealed that 5-HTTLPR could regulate the expression of 5-HTT and affect the 5-HT of synaptic cleft afterwards. 5-HTTLPR is the main regulatory region of 5-HTT expression and modifies its function at transcription and translation levels [4]. The S allele-dominant variant downregulates the expression of 5-HTT and leads to low levels of uptake and release of 5-HT, while the L allele-dominant variant acts the opposite way. The transcriptional activity of L allele-dominant variant is 3 times that of S allele [5]. S allele limits the transcriptional activity of 5-HTTLPR and leads to low expression of 5-HTT. Lesch and Merschdorf found that the transport activity of 5-HT of L allele was higher than S allele [6]. 5-HT can induce the upregulation of 5-HT1A receptor and proliferation of T and B cells by activating mitogen. 5-HT is also associated with increase of translocation of nuclear factor-kappa B (NF-κB) in cell nucleus [7]. Müller et al. found that 5-HT could increase the production of proinflammatory factors, such as IL-6, IL-4, and IL-10, and decrease the levels of Th1 cell-related cytokines and IL-12p70 in dendrite cells (DCs). Meanwhile, 5-HT upregulates the secretion of Th2 chemoattractant CCL22 and downregulates the Th1 chemoattractant CXCL10 in DCs by binding to 5-HTR4 and 5-HTR7. DCs induce Th2 dominance in $CD4^+$ T cell through 5-HT [8]. Lood et al. found that SLE patients had decreased 5-HT levels in serum and platelets compared with HCs. This decrease was related to severe disease subtypes such as lupus nephritis (LN), and it might be mediated by type I interferon [9]. Our team has found the significant hypomethylation of promoter region of 5-HTR1A (PR-HTR1A) and higher expression of 5-HTR1A mRNA in SLE patients [10]. Birmingham et al. found that self-perceived stress of patients with LN carrying the 5-HTR1A-1019 G allele could increase the risk of lupus flares, although 5-HTTLPR was not associated with lupus flares [11]. These studies suggested that the regulation of 5-HT system might involve the pathogenesis of SLE.

There are several studies about 5-HTTLPR and other autoimmune diseases. One study indicated that depressed women with SS genotype had higher levels of thyroid stimulating hormone (TSH) and worse antidepressant response compared to LL/LS genotypes [12]. A Japanese clinical trial showed that, in women with diabetes mellitus, hypercholesterolemia, and hypertension, SS genotype showed a larger increase in blood glucose after meals than fasting blood glucose (FBG) [13]. A recent study showed that, in patients with type 2 diabetes mellitus (T2DM), the frequencies of SS and LS genotypes were higher than HCs. S allele might play a role in the pathogenesis of T2DM [14]. An RA animal study showed that, with no intervention, monkeys with S allele had significantly higher neutrophil-to-lymphocyte ratios. 5-HTTLPR might be a unique marker of autoimmune inflammation [15]. Ferreira et al. found that although 5-HTTLPR was not associated with osteoporosis, another gene polymorphism serotonin transporter with a variable number of tandem repeats (5-HTTVNTR) was associated with osteoporosis [16]. Sikander et al. found that the LL genotype of 5-HTTLPR was associated with microscopic colitis and ulcerative colitis and serotonin levels were significantly higher in those patients compared to HCs [17]. Colucci et al. found no association of 5-HTTLPR with irritable bowel syndrome (IBS), while the LS and SS genotypes were significantly correlated with IBS symptom severity [18]. Yamakawa et al. found that S allele of the 5-HTTLPR was associated with stress reactivity like increased heart rate, secretion of IL-1β, and cortisol levels in multilevel stress-related biological systems [19]. The studies above indicated that 5-HTTLPR was involved in the pathogenesis of several autoimmune diseases. There is no research about 5-HTTLPR and the pathogenesis of SLE yet.

Our study showed that the frequencies of 5-HTTLPR genotypes and alleles were of no significant difference in SLE patients and HCs of our center, which might suggest that 5-HTTLPR is not a high-risk susceptible gene of SLE and more researches are needed.

During the progression of SLE, B lymphocytes proliferate and produce large amounts of autoantibodies which target different parts of cells such as nucleus, cell membrane, and cell

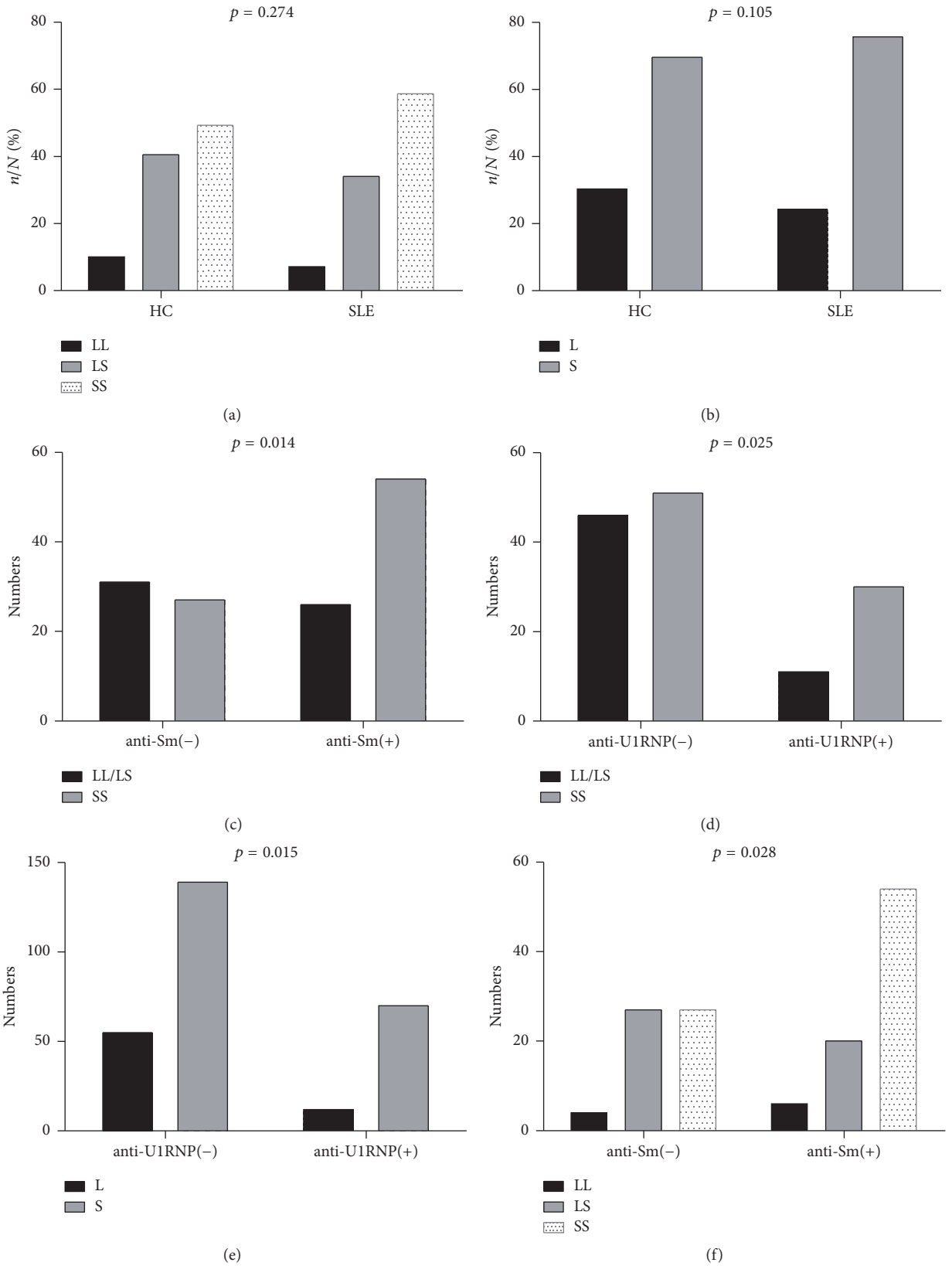

Figure 1: 5-HTTLPR genotypes in SLE patients and HCs. (a) The frequency of 5-HTTLPR genotypes in HCs and SLE patients; (b) the frequency of 5-HTTLPR alleles in HCs and SLE patients. (c) The frequency of SS genotype was significantly higher than LL/LS genotype in SLE patients with positive anti-Sm antibody; (d) the frequency of SS genotype was significantly higher than LL/LS genotype in SLE patients with positive anti-U1RNP antibody; (e) the frequency of S allele was significantly higher in SLE patients with positive anti-U1RNP antibody; (f) the frequency of SS genotype was significantly higher than the other 2 genotypes in SLE patients with positive anti-Sm antibody.

plasma. These autoantibodies, including ANA, anti-dsDNA, anti-Sm, and anti-U1RNP, not only are the consequence of disorders of immune system but also are aggravating the disease. In 1979, Lerner and Steitz confirmed that protein Sm and RNP were both located at small nuclear ribonucleoprotein (snRNP) [20]. In snRNP, Sm is located at U1, U2, U4, and U5 snRNP. The most commonly used anti-RNP antibody usually only binds to U1 part, so we call it anti-U1RNP antibody. Since they share the same antigen epitopes, anti-U1RNP antibodies are sometimes concurrent with anti-Sm antibodies [21]. In SLE patients, anti-Sm antibody is highly specific and associated with disease activity and certain subtypes such as LN and neuropsychiatric SLE (NPSLE). Some researchers believed that anti-Sm antibody was more related to nephropathy than anti-dsDNA antibody [22–24]. Anti-U1RNP antibody can be positive in several autoimmune diseases, but it is considered to be a symbolic autoantibody in mixed connective tissue disease (MCTD) [25]. In MCTD patients, 95% are anti-U1RNP antibody positive in high titers with anti-Sm antibody negative. In other autoimmune diseases such as SLE, only 20–40% patients are anti-U1RNP antibody positive with anti-Sm antibody positive. It is still controversial whether anti-U1RNP antibody is associated with SLE disease activity. Asian and African populations have higher prevalence of anti-U1RNP antibody. Some studies showed that SLE patients with NPSLE, LN, pulmonary fibrosis, or pericarditis were usually with positive anti-Sm/U1RNP antibodies. Anti-U1RNP antibody is also believed to relate to Raynaud's phenomenon and dermal involvement [26–31].

This study showed that the frequencies of SS genotype and S allele in SLE patients with positive anti-Sm and anti-U1RNP antibody were significantly higher. The specific mechanism is not clear and it may suggest that SS homozygote and S allele are associated with the production of anti-Sm and anti-U1RNP antibody. More related studies, including tests of serotonin and B cell numbers and functions, are warranted.

Based on the researches above, we conclude that 5-HTTLPR might not be a high-risk susceptible gene in SLE. However, it may relate to SLE by affecting the autoantibodies generation, especially anti-Sm antibody and anti-U1RNP antibody.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Authors' Contributions

Shu Li, Shuang Liu, and Fan Chen equally contributed to this study.

Acknowledgments

This work was supported by grants from National Natural Science Foundation of China (81160379, 81460256, 81560233, and 81501406), the Ministry of Science and Technology of Yunnan Province (2012FB158), the Funding of Yunnan Provincial Health Science and Technology Plan (2014NS171, 2014NS172), the Funding of Yunnan Provincial Public Health for Academic Leader (D-201218 and D-201239), and Yunnan Provincial Fund for Preparatory Young Leaders in Academia and Technology (2015HB071).

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 6537248, 8 pages
http://dx.doi.org/10.1155/2016/6537248

Research Article

Serum HMGB1 Serves as a Novel Laboratory Indicator Reflecting Disease Activity and Treatment Response in Ankylosing Spondylitis Patients

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Received 28 April 2016; Accepted 10 July 2016

Academic Editor: Quanzhen Li

Objective. High mobility group box 1 (HMGB1) is a late inflammatory factor participating in the pathogenesis of various autoimmune and inflammatory diseases. In the current study, we analyzed the association between serum levels of HMGB1 and clinical features of AS patients before and during treatment. *Methods*. Serum HMGB1 was detected in 147 AS patients and 61 healthy controls using ELISA. We evaluated the association between HMGB1 and extra-articular manifestations as well as disease severity indices. Among these AS patients, 41 patients received close follow-up at 1, 3, and 6 months after treatment. This group comprised 25 patients treated with anti-TNF-α biologics and 16 patients receiving oral NSAIDs plus sulfasalazine. *Results*. The serum HMGB1 of AS patients was significantly higher than in healthy controls and positively correlated with BASDAI, BASFI, ASDAS-ESR, ASDAS-CRP, ESR, and CRP, but not with HLA-B27, anterior uveitis, and recurrent diarrhea. There was no significant difference between patients with radiographic damage of hip joints and those without. We observed that serum HMGB1 paralleled disease activity after treatment. *Conclusion*. Serum level of HMGB1 is higher in AS patients, and to some extent, HMGB1 can reflect the activity of AS and be used as a laboratory indicator to reflect the therapeutic response.

1. Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory disease, characterized by the inflammation of sacroiliac joints and the spine, which mainly affects young males including disability and decreased quality of life [1]. Apart from the involvement of axial joints, extra-articular manifestations such as inflammatory bowel disease (IBD) and anterior uveitis are relatively common. Although the pathogenesis of AS is not completely unclear, it is widely accepted as the interaction between environmental factors and genetic risk factors, which has a strong association with human leukocyte antigen-B27 (HLA-B27) [2, 3]. However, recent genome-wide association studies (GWASs) have identified that some single nucleotide polymorphisms (SNPs) were associated with AS. Many of these gene loci assemble in dissimilar immunoregulatory pathways [4], which are related to NF-κB signaling, IL-23 pathway, and T cell phenotype as well as antigen presentation [5, 6]. Inflammation and new bone formation are considered as the critical factors of AS, within the genetically susceptible individuals. Abnormal inflammation and immunity are also considered as major etiological factors, involving the upregulation of the inflammatory cytokines including tumor necrosis factor-alpha (TNF-α), IL-23, and IL-17 [7], and anti-IL17 agents showed similar therapeutic efficacy to TNF blockers in clinical trials [8]. DAMP (damage-associated

molecular pattern) formed by exogenous microbial PAMPs (pathogen-associated molecular patterns) and endogenous alarmins play important roles in inflammatory processes. Previous studies have revealed that PAMPs, such as intra-articular microbial antigens, may be involved in the development of syndesmophytes and bamboo spine in AS [9]. As a typical alarmin, high mobility group box 1 (HMGB1) acts as an inflammatory mediator to participate in the pathogenesis of many kinds of autoimmune and inflammatory diseases. However, there exists scarce research pertaining to HMGB1 and AS, which prompted us to investigate the association between HMGB1 and AS.

HMGB1 is a highly conserved nuclear nonhistone protein, which has been previously characterized as DAMP involved in inflammatory processes [10]. HMGB1 is distributed in the nuclei of almost all eukaryotic cells and can shuttle from nucleus to cytoplasm depending on the acetylation of lysine residues within the nuclear localization signal domains (NLS) [11]. AS a typical alarmin, HMGB1 acts as a proinflammatory cytokine when released to the extracellular environment actively or passively and binds to receptors such as TLR2, TLR4, and RAGE, which is short for receptor for advanced glycation end products. HMGB1-mediated signaling pathway can promote the production of TNF-α, IL-1β, and IL-6, which are all involved in the pathology of AS [12, 13]. Furthermore, HMGB1 can be chemotactic to mesenchymal stromal cells, monocytes, and osteoblasts, which leads to ectopic enchondral bone formation [9]. When released by apoptotic osteoblasts in vitro, HMGB1 plays a role as a bone-active cytokine, which can promote the activation and differentiation of osteoclasts leading to bone destruction [14, 15]. Taken together these observations indicate that HMGB1 may play a role in the pathological progression of AS.

However, little research has focused on the association between HMGB1 and AS. Results from Oktayoglu et al. [16] and Chen et al. [17] indicated that high expression of HMGB1 may be involved in the pathogenesis of AS, but all consequent studies involved limited numbers of cases, lacking in consistent observations including the changes of HMGB1 during disease progression and different treatment strategies in AS patients. In our current study, we detected the serum levels of HMGB1 in 147 AS patients compared with 61 healthy controls and analyzed the relationship between HMGB1 and disease activity. Furthermore, we examined the trend of HMGB1 in AS patients during six-month follow-up, including 25 patients who were treated by anti-TNF-α block therapy and 16 patients treated with NSAIDs and SASP.

2. Materials and Methods

2.1. Patient Characteristics and Samples. 147 AS patients (23 women, 124 men; age range, 15~56 y; mean age, 30.28 ± 9.85 y) were recruited from outpatients of the Department of Rheumatology at Tongji Hospital of Tongji Medical College, Huazhong University of Science & Technology (HUST), between January 2015 and September 2015. The enrolled patients fulfilled the modified New York criteria [18, 19] and were without treatment during the previous 3 months. The exclusion criteria of this study were patients with a history of cancer, recurrent infections, or other types of rheumatic diseases. All patients filled in a questionnaire, which was used to record the clinical information as well as disease severity index, including name, age, sex, Bath AS Disease Activity Index (BASDAI), AS Disease Activity Score (ASDAS), and Bath AS Functional Index (BASFI). We also obtained laboratory assessments of all participants, including C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR). The detection of ESR used the Westergren method, while CRP was measured by immunonephelometry using CRP reagents (BioSystems SA, Spain). The normal range of CRP was defined as 0~10 mg/dL in Tongji Hospital. 135 enrolled AS patients were tested with HLA-B27, while 101 patients received CT or MRI of sacroiliac joints as well as hip joints in the last 2 months. Among all AS patients, only 41 patients received close observation and follow-up for six months. These patients were divided into 2 groups, including an anti-TNF-α therapy group composed of 25 patients who were given adalimumab ($n = 12$), etanercept ($n = 8$), and infliximab ($n = 5$) for a total period of 6 months. The other group was treated with nonsteroidal anti-inflammatory drugs (NSAIDs) for one month followed by sulfasalazine (SASP) 2-3 g/d for the remainder of the study. Moreover, we also recruited 61 age- and sex-matched healthy volunteers as controls, without a history of cancers, recurrent episodes of infections, or family history of AS. This study was approved by the ethics committee of Tongji Hospital of Tongji Medical College, HUST (IRB ID: TJ-C20141213), and all patients registered their informed consent to participate in this study.

2.2. Samples and Determination. Peripheral blood was obtained from all enrolled outpatients and the follow-up patients as well as the healthy volunteers. The blood samples were centrifuged at 4000 rpm for 5 minutes. Serum was stored at −80°C. Serum levels of HMGB1 were measured with the commercially available enzyme linked immunosorbent assay (ELISA) kit (Uscn Life Science Inc., Wuhan, China) according to the instruction manual.

2.3. Statistical Analysis. Database management and statistical analyses were performed using SPSS 19.0 (SPSS, Chicago, IL, USA). The results of baseline and follow-up from different groups were compared with independent samples using Student's t-test and expressed as mean value ± standard deviation (SD). Correlations between variables were conducted using Spearman's rank correlation test and P values < 0.05 were set as a statistically significant difference.

3. Results

Table 1 shows baseline characteristics of 147 AS patients and 61 healthy controls in our study. The level of serum HMGB1 in AS patients was significantly higher than the healthy controls (HMGB1: 106.81 ± 30.87 ng/mL versus 27.68 ± 17.95 ng/mL, $P < 0.001$). We assessed the correlation between HMGB1 and other indices during this study, including the baseline and the follow-up time point. The HMGB1 level was positively correlated with BASDAI ($r = 0.304$), BASFI ($r = 0.184$),

Table 1: Baseline characteristics of 147 ankylosing spondylitis patients.

	AS	HC
Age (years)	30.28 ± 9.85	29.36 ± 5.85
Sex (male/female)	124/23	45/16
HMGB1 (ng/mL)**	106.81 ± 30.87	27.68 ± 17.95
BASDAI	3.59 ± 1.71	
BASFI	2.48 ± 1.83	
ASDAS-ESR	2.89 ± 1.29	
ASDAS-CRP	2.97 ± 1.33	
ESR (mm/h)	32.93 ± 27.82	
CRP (mg/L)	24.97 ± 32.41	

AS: ankylosing spondylitis, HC: healthy control, BASDAI: Bath AS Disease Activity Index, BASFI: Bath AS Functional Index, ASDAS: AS Disease Activity Score, ESR: erythrocyte sedimentation rate, and CRP: C-reactive protein. Values are shown as mean ± SD. $^{**}P < 0.01$.

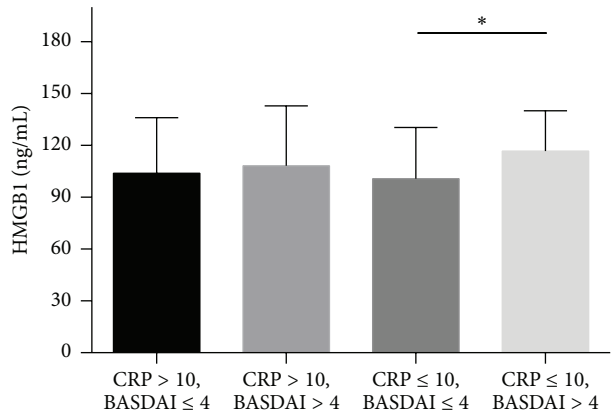

Figure 1: The comparison of serum HMGB1 in 4 groups of 147 AS patients, which were divided according to the value of CRP and BASDAI. Data include serum HMGB1 levels of 147 AS patients which were divided into 4 groups, defined as group 1 (CRP > 10 mg/dL + BASDAI ≤ 4, $n = 36$), group 2 (CRP > 10 mg/dL + BASDAI > 4, $n = 40$), group 3 (CRP ≤ 10 mg/dL + BASDAI ≤ 4, $n = 51$), and group 4 (CRP ≤ 10 mg/dL + BASDAI > 4, $n = 20$). BASDAI: Bath AS Disease Activity Index; CRP: C-reactive protein. $^{*}P < 0.05$.

ASDAS-ESR ($r = 0.275$), ASDAS-CRP ($r = 0.251$), CRP ($r = 0.132$), and ESR ($r = 0.162$); results are shown in Table 2.

The 101 AS patients receiving CT or MRI of sacroiliac joints as well as hip joints in the last 2 months were divided into two groups based on whether hip joints were involved. Pathological changes of hip joints were identified according to CT or MRI diagnosis and the minimum inclusion criterion was bone erosion. Serum HMGB1 showed no statistically significant difference between the two groups (HMGB1 109.40 ± 36.23 ng/mL versus 99.94 ± 25.31 ng/mL), while the BASDAI, BASFI, ASDAS-ESR, and ASDAS-CRP scores in the AS patients with hip joint involvement were all significantly higher than those without pathological changes (BASDAI: 3.85 ± 1.54 versus 3.13 ± 1.56, $P < 0.05$; BASFI: 2.88 ± 1.88 versus 2.01 ± 1.50, $P < 0.05$; ASDAS-ESR: 3.15 ± 1.48 versus 2.55 ± 0.99, $P < 0.05$; ASDAS-CRP: 3.23 ± 1.47 versus 2.64 ± 1.09, $P < 0.05$).

In 135 AS patients HLA-B27 was detected (107 positive versus 28 negative), and there was no significant statistical difference between the positive and the negative group (108.71±30.65 ng/mL versus 98.08±28.92 ng/mL). The serum HMGB1 of the enrolled AS patients who had a history of recurrent episodes of anterior uveitis (13 positive versus 134 negative) or diarrhea (13 positive versus 134 negative) was not significantly higher than in those without these symptoms (103.77 ± 44.36 ng/mL versus 107.11 ± 29.46 ng/mL, 93.14 ± 22.31 ng/mL versus 108.14 ± 31.32 ng/mL). Additionally, there were no statistically significant differences in the other indexes (BASDAI, BASFI, and ASDAS) between these two groups (data not shown).

In this study, the normal range of CRP was 0~10 mg/dL, while the patients with active disease were defined as a BASDAI of 4 [20–22]. 147 AS patients were divided into 4 groups defined as follows: CRP > 10 mg/dL + BASDAI ≤ 4 ($n = 36$), CRP > 10 mg/dL + BASDAI > 4 ($n = 40$), CRP ≤ 10 mg/dL + BASDAI ≤ 4 ($n = 51$), and CRP ≤ 10 mg/dL + BASDAI > 4 ($n = 20$). As reflected in Figure 1, despite the normal CRP value at baseline (CRP ≤ 10 mg/dL), serum HMGB1 of the BASDAI > 4 group was significantly higher than in the group with BASDAI ≤ 4 (116.80 ± 23.29 ng/mL versus 98.74 ± 33.16 ng/mL) as well as BASFI (2.73 ± 1.76 versus 1.32±1.09), ASDAS-ESR (2.83±0.66 versus 1.90±0.59), and ASDAS-CRP (2.61 ± 0.73 versus 1.77 ± 0.64), which is not shown in Figure 1. Nevertheless, there was no statistical difference in HMGB1 between the other two groups with CRP > 10 mg/dL at baseline, regardless of whether BASDAI > 4.

In addition to this cross-sectional study, 41 enrolled patients were measured regularly during conducting a regular follow-up for a period of six months in our study. These AS patients were divided into two groups based on the therapeutic approach. Anti-TNF biologics treatment group included 25 patients and did not take oral medication during the six-month treatment period. The other group of 16 patients received accepted NSAIDs and SASP according to the recommended dosage during this period research. As shown in Table 3, the characteristics, including age, ESR, CRP, BASDAI, BASFI, ASDAS-CRP, and ASDAS-ESR, of the two treatment groups at baseline were very similar and had no statistically significant differences in the level of $P = 0.05$.

Compared with the oral medication group, HMGB1 level and other indexes of the biologic group all decreased significantly relative to the baseline at three follow-up time points (Table 4 and Figure 2). It is worth noting that all indicators as well as HMGB1 decreased significantly in the first month and maintained a relatively steady downward trend for the next period of the study. Small fluctuations did not affect the downward trend throughout the 6 months of treatment. At the time point of 6 months during the treatment, HMGB1 of oral medicine group was significantly higher than biological group (96.39 ± 23.51 ng/mL versus 73.68 ± 24.20 ng/mL, $P < 0.01$). There also exist significantly statistical differences of the other indices at the three time points between the two groups, except for the BASFI (Figure 2(c)). And moreover, HMGB1 decreased by 20.57 ng/mL (20.21%, $P < 0.05$ versus

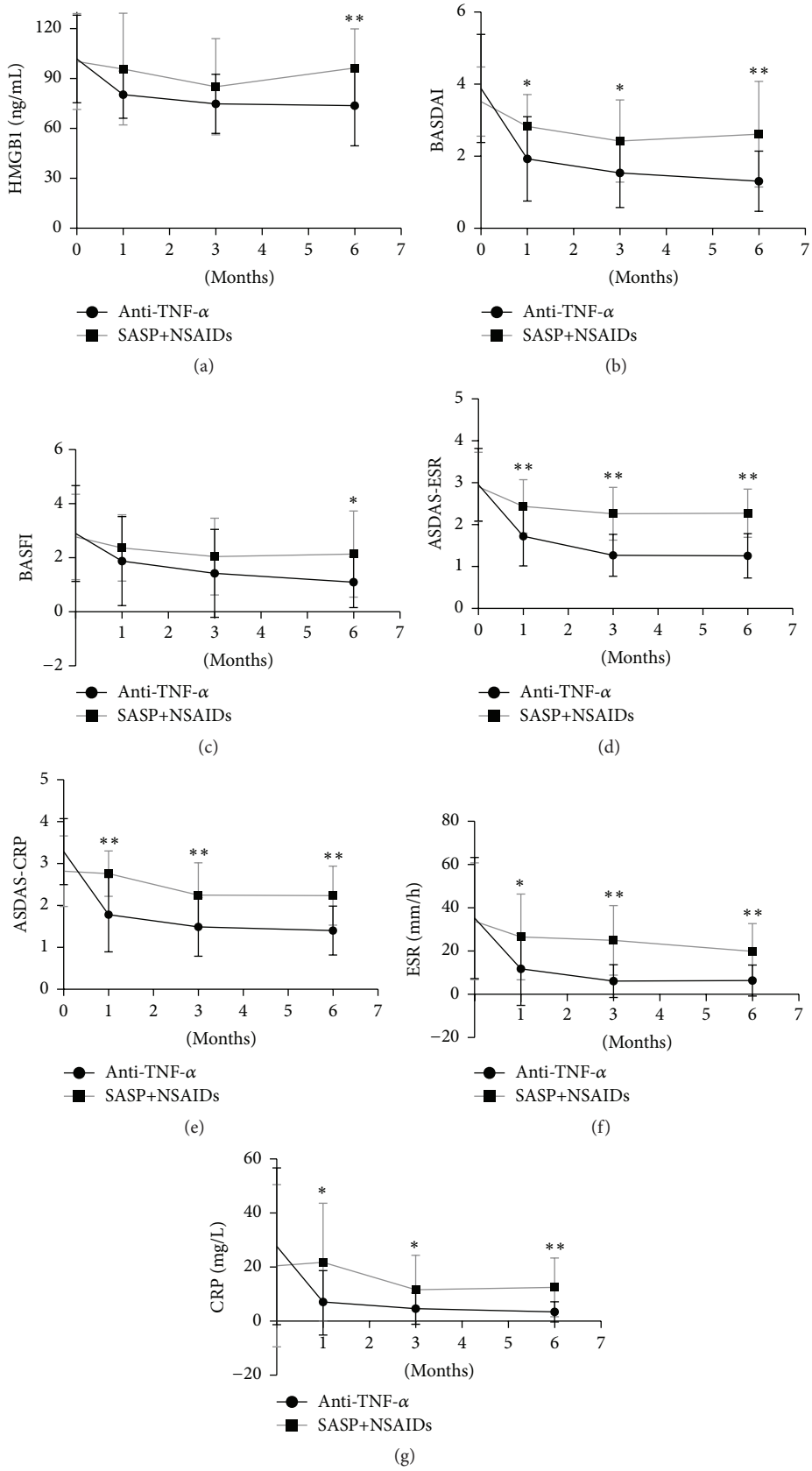

Figure 2: Changes in HMGB1 level and disease severity indices during follow-up. The black lines represent above indexes of AS patients treated with anti-TNF-a biologics and the gray lines represent patients treated with SASP and NSAIDs, during follow-up period. $*$ indicates $P < 0.05$; $**$ indicates $P < 0.01$.

Table 2: Spearman's correlation analysis between serum HMGB1 and clinical parameters of the 147 AS patients, including the baseline and the follow-up time points.

	HMGB1	BASDAI	BASFI	ASDAS-ESR	ASDAS-CRP	CRP	ESR
HMGB1		0.304**	0.184**	0.275**	0.251**	0.132*	0.162**
BASDAI			0.635**	0.668**	0.678**	0.364**	0.385**
BASFI				0.595**	0.607**	0.396**	0.418**
ASDAS-ESR					0.884**	0.668**	0.797**
ASDAS-CRP						0.738**	0.669**
CRP							0.761*

BASDAI: Bath AS Disease Activity Index, BASFI: Bath AS Functional Index, ASDAS: AS Disease Activity Score, ESR: erythrocyte sedimentation rate, and CRP: C-reactive protein. Values are shown as r. r is determined by Spearman's correlation analysis. $^{*}P < 0.05$. $^{**}P < 0.01$.

Table 3: Baseline characteristics of the two follow-up therapeutic AS groups.

	Anti-TNF	NSAIDs + SASP
N	25	16
Age (years)	28.48 ± 6.73	32.81 ± 9.40
HMGB1 (ng/mL)	101.76 ± 26.31	100.28 ± 28.83
BASDAI	4.07 ± 1.44	3.52 ± 0.96
BASFI	3.14 ± 1.94	2.77 ± 1.58
ASDAS-CRP	3.23 ± 0.72	2.82 ± 0.84
ASDAS-ESR	2.95 ± 0.79	2.90 ± 0.83
ESR (mm/h)	27.44 ± 29.22	28.13 ± 26.02
CRP (mg/L)	35.23 ± 28.10	26.22 ± 32.35

baseline) but increased by 1.52 ng/mL (1.51%, $P > 0.05$ versus baseline) after 1 month in the anti-TNF and SASP + NSAIDs groups. Further, HMGB1 decreased by 25.35 ng/mL (24.91%, $P < 0.05$ versus baseline) and 12.97 ng/mL (12.93%, $P < 0.05$ versus baseline) at 3 months, respectively. At the end of the study, HMGB1 decreased by 28.10 ng/mL (27.60%, $P < 0.05$ versus baseline) while there was almost no statistical change compared with baseline in the oral medication group at 1 month and 6 months (Figure 2(a)). There was also a significant decline in BASDAI, BASFI, ASDAS-CRP, ASDAS-ESR, CRP, and ESR during the 6-month treatment period. This is further characterized by a particularly deep decline for the first month and maintained a downward trend during the six months of treatment. Scores for BASDAI, BASFI, ASDAS-ESR, and ASDAS-CRP decreased by 1.87 (45.95%, $P < 0.05$ versus baseline), 1.17 (37.26%, $P < 0.05$ versus baseline), 1.30 (44.07%, $P < 0.05$ versus baseline), and 1.57 (48.61%, $P < 0.05$ versus baseline) in the first month, respectively. Unlike the biological therapy group, except for the statistical reducing of BASDAI (16.19%, $P < 0.05$ versus baseline) and ASDAS-ESR (21.03%, $P < 0.05$ versus baseline) and at 1 month, there was no statistical decreasing of other parameters of the oral medicine treatment group in the first month. With the exception of ESR, significant differences of others indicators between treatments were observed during the follow-up (Table 4).

Table 4: Absolute changes in HMGB1, BASDAI, BASFI, ASDAS-ESR, ASDAS-CRP, ESR, and CRP in the two follow-up therapeutic AS groups.

	Anti-TNF	SASP + NSAIDs
HMGB1 change, mean (SD), ng/mL		
1 month	−20.57 (22.25)[ab]	1.52 (24.99)
3 months	−25.35 (23.70)[a]	−12.97 (19.89)[a]
6 months	−28.10 (28.68)[ab]	−3.89 (25.30)
BASDAI change, mean (SD)		
1 month	−1.87 (1.51)[ab]	−0.57 (0.51)[a]
3 months	−2.29 (1.61)[ab]	−1.06 (0.77)[a]
6 months	−2.57 (1.51)[ab]	−0.90 (1.16)[a]
BASFI change, mean (SD)		
1 month	−1.17 (1.56)[ab]	−0.15 (0.61)
3 months	−1.23 (1.65)[a]	−0.87 (1.00)[a]
6 months	−1.80 (1.69)[ab]	−0.74 (1.17)[a]
ASDAS-ESR change, mean (SD)		
1 month	−1.30 (1.07)[a]	−0.61 (0.59)[a]
3 months	−1.67 (0.86)[ab]	−0.82 (0.62)[a]
6 months	−1.70 (0.89)[ab]	−0.63 (0.69)[a]
ASDAS-CRP change, mean (SD)		
1 month	−1.57 (0.79)[ab]	−0.12 (0.81)
3 months	−1.72 (0.83)[ab]	−0.76 (1.00)[a]
6 months	−1.88 (0.63)[ab]	−0.58 (0.96)[a]
ESR change, mean (SD), mm/h		
1 month	−24.67 (24.61)[a]	−12.82 (19.22)
3 months	−29.47 (25.91)[a]	−14.15 (18.18)[a]
6 months	−28.96 (26.69)[a]	−13.94 (22.47)[a]
CRP change, mean (SD), mg/L		
1 month	−20.55 (12.99)[ab]	−4.25 (18.54)
3 months	−19.70 (25.99)[a]	−12.76 (25.57)
6 months	−24.23 (28.41)[a]	−8.04 (26.32)

[a]$P < 0.05$ versus baseline. [b]$P < 0.05$ versus SASP + NSAIDs.

4. Discussion

Earlier diagnosis and treatment are urgently required and effective in reducing disease burden of AS patients while both of them are usually impeded due to the seronegative character and the absence of hallmark. Thus a number of emerging researches are focusing on the new biomarkers and antibodies, which can not only contribute to the diagnosis, but also reflect disease activity and curative effect [5, 23], while the currently new biomarkers are still elusive due to the inconsistent results of different researches as well as lacking of multicenter studies. Therefore, only HLA-B27 and inflammatory marker CRP are widely accepted as routinely clinical biomarkers of AS. Detecting HLA-B27 is useful only if AS is strongly suspected due to lack of specificity [5, 24]. Moreover, only half of the AS patients with the evaluated CRP and earlier studies indicated the poor predictive value of CRP and the poor correlation with BASDAI [25, 26]; however, a study in 2010 showed a significant correlation between CRP and disease activity [27]. Thus, AS poses significant challenge to the quest for biomarkers to assess disease activity and monitor treatment response.

HMGB1 was initially recognized as a DNA-binding protein widespread in the mammal eukaryotic cell nucleus until the discovery that HMGB1 serves as a late inflammatory cytokine in the progress of sepsis and endotoxaemia in 1999 [28]. Extracellular HMGB1 acts as a proinflammatory mediator participating in the pathological processes of acute lung inflammation, transplant rejection, and ischemia-reperfusion injury [29–31] in addition to rheumatoid diseases such as systemic lupus erythematosus, rheumatoid arthritis, and Sjogren's syndrome [32–34]. However, little research has focused on the association between HMGB1 and AS. The literature thus far is inconsistent with respect to a correlation between serum HMGB1 level and disease status. Although two studies demonstrated that the level of HMGB1 was significantly higher than healthy controls, Chen et al. [17] suggested that HMGB1 might be a good laboratory candidate for the evaluation of disease severity and activity. However, Oktayoglu et al. [16] adhered to the opposite viewpoint. The contrasting results of these two studies may be due to the different inclusion criteria of AS patients and the fact that the number of cases was insufficient. In addition, these two studies did not observe the effects of different treatment strategies on HMGB1, as well as the correlation between HMGB1 and other important cytokines involved in the pathogenesis of AS.

In our present study, we collected a relatively large sample composed of 147 AS patients and 61 healthy controls that did not receive any drugs or treatment during the previous 3 months, thus avoiding the influence of medicine on HMGB1 levels. We found that the serum HMGB1 level was significantly higher in AS patients than in healthy controls (HMGB1: 106.81 ± 30.87 ng/mL versus 27.68 ± 17.95 ng/mL, $P < 0.001$). HMGB1 was also positively correlated with BASDAI, BASFI, ASDAS-ESR, ASDAS-CRP, CRP, and ESR. However, no correlation was detected between the serum HMGB1 level and HLA-B27 or with respect to extra-articular manifestations, including recurrent anterior uveitis and diarrhea. In addition, there were no statistically significant differences in the serum HMGB1 level between the AS patients with pathological changes involving hip joints and those without. Although CRP is considered as a laboratory indicator to reflect inflammation levels and disease activity we found that there were 13.61% patients who have high disease activity (BASDAI > 4) and severe clinical symptoms but with normal CRP values. Among patients with normal CRP, serum HMGB1 of the BASDAI > 4 group was significantly higher than in those with BASDAI ≤ 4, which indicated that HMGB1 may be a better laboratory index reflecting ongoing inflammatory disease, particularly in those cases with CRP in the normal range (Figure 1).

It is noteworthy that HMGB1 has a chemotactic effect on osteoblasts, monocytes, and endothelial cells, as well as the recruitment of mesenchymal stromal cells [9]. TNF-α can stimulate the activated monocyte-macrophage cells to secrete HMGB1, which then interacts with multiple receptors including RAGE, TLR2, and TLR4. HMGB1-mediated signaling can lead to the activation of nuclear factor κB (NF-κB) and the production of various cytokines such as IL-6 and TNF-α, which are involved in the pathogenesis of AS [35]. While the HMGB1-involved signal pathways which can participate in the pathogenesis of AS are still rather ambiguous, further in-depth study is needed.

The present study is the first long-term controlled trial focusing on variation in the HMGB1 level in AS patients receiving different treatment. Although several clinical trials have shown a relatively good response to TNF-α blockers as therapy for AS, there are individual differences between patients [36]. More sensitive laboratory indicators are needed for clinicians to predict the therapeutic effect of biological treatments. We observed that the TNF-α blockade group quickly achieved remission of joint symptoms and demonstrated an improved quality of life and work. Within this group, HMGB1 significantly decreased in the first month of treatment (20.21%, $P < 0.05$ versus baseline) and then maintained a relatively steady downward trend during treatment. This pattern was reflected in the disease activity indexes such as BASDAI, BASFI, ASDAS, CRP, and ESR. However, compared with baseline, the HMGB1 level of the oral treatment group did not have a statistical difference, while the other indicators had only slight fluctuations without a clear downward trend (Table 4). Further, there also existed the significant correlation between HMGB1 and AS related indicators (BASDAI, BASFI, ASDAS-ESR, ASDAS-CRP, ESR, and CRP) during the 6-month follow-up period. Therefore, to a certain extent, we speculate that the HMGB1 may be considered as an indicator to monitor the therapeutic effect.

Taken together, our results revealed an increase of HMGB1 levels in AS patients, but HMGB1 had no significant correlation with HLA-B27 and extra-articular manifestations. Although HMGB1 was not correlated with the radiographic destruction severity of involved joints, HMGB1 can reflect the activity and severity of AS disease and is a relatively good biomarker of the therapeutic effect to a certain extent. Further studies must be performed to clarify the pathogenetic role of HMGB1 in AS.

Competing Interests

The authors have declared no competing interests.

Acknowledgments

This work was supported by Grants from National Natural Science Foundation of China (no. 31270965 to Lingli Dong) and New Century Excellent Talents Program Supported by Education Ministry (no. NCET-13-0223 to Lingli Dong).

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 2829018, 7 pages
http://dx.doi.org/10.1155/2016/2829018

Research Article

Depressive and Anxiety Disorders in Systemic Lupus Erythematosus Patients without Major Neuropsychiatric Manifestations

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Received 28 June 2016; Accepted 31 August 2016

Academic Editor: Guixiu Shi

Depressive and anxiety disorders are frequently observed in patients with Systemic Lupus Erythematosus (SLE). However, the underlying mechanisms are still unknown. We conducted this survey to understand the prevalence of depression and anxiety in SLE patients without major neuropsychiatric manifestations (non-NPSLE) and to explore the relationship between emotional disorders, symptoms, autoantibodies, disease activity, and treatments in SLE. 176 SLE patients were included, and SLE disease activity index (SLEDAI), Hamilton Depression Rating Scale (HAMD), and Hamilton Anxiety Rating Scale (HAMA) were recorded to evaluate their disease activity and emotional status. We found that depressive and anxiety disorders were common among SLE patients: 121 (68.8%) patients were in depression status while 14 (8.0%) patients could be diagnosed with depression. Accordingly, 101 (57.4%) were in anxiety status and 21 (11.9%) could be diagnosed with anxiety. Depression was associated with disease activity, and anxiety was associated with anti-P0 antibody, while both of them were associated with proteinuria. HAMA and HAMD scores were in strong positive correlation and they were independent risk factors of each other. We concluded that the high prevalence of depression and anxiety and the association between depression and SLE disease activity might reveal the covert damage of central nervous system in SLE. The role of anti-P0 antibody in SLE patients with emotional disorders warrants more researches.

1. Introduction

Systemic Lupus Erythematosus (SLE) is a typical connective tissue disease with multiple organs involved, including central nervous system (CNS), peripheral nervous system (PNS), and autonomic nervous system. Neuropsychiatric symptoms are common and serious manifestations and sometimes can cause disability or death. Major neurological and mental disorders like stroke or schizophrenia are not as common as subtle ones such as headaches, emotional disorders, and cognitive deficiencies. Clinical evaluations are the major diagnostic methods of neuropsychiatric Systemic Lupus Erythematosus (NPSLE), though it is often quite difficult to make a definite diagnosis, mostly only "hypothetical" ones [1]. Sometimes NPSLE can present as cognitive dysfunction and emotional disorders and affect patients' quality of life severely [2, 3]. Severe neuropsychiatric symptoms are reported to be associated with long-term progression of disease and could cause death in 7–19% cases [4]. Thus, it is important for clinical physicians to recognize signs and symptoms of NPSLE in early stage.

SLE is characterized by repeated flares and remissions of variable symptoms and signs, of which proteinuria, rashes, and arthritis are the most common ones. Besides those frustrating symptoms, social stress such as loss of working abilities, decreased incomings, and limitations in social activities are also a major problem. Altogether they may cause emotional disorders like depression and anxiety in SLE patients [5]. Some researchers believed that emotional disorders could be the initial symptoms of NPSLE as a result of

inflammation and consequences of certain antibodies [6–8]. However, emotional disorders cannot always be recognized in early phase by clinicians due to lack of awareness [9]. In SLE patients without "obvious" or major neuropsychological symptoms like seizures or mental disorders, the so-called "non-NPSLE" patients, the incidence and characteristics of emotional disorders are not completely studied. Several studies in the potential neurobiological mechanisms indicated autoantibody production, microvasculopathy, and proinflammatory cytokines might play essential roles [4]. Thus, it is crucial to identify specific autoantibodies and tests to help recognize emotional disorders. Our study included 176 non-NPSLE patients with normal conventional brain imaging and no history of neuropsychiatric disease and intended to explore the prevalence of depression and anxiety in these patients and understand the relationship between emotional disorders, symptoms, autoantibodies, disease activity, and treatments in SLE.

2. Materials and Methods

2.1. Subjects. 176 SLE patients were recruited from inpatient and outpatient centers from the Department of Rheumatology and Immunology of the First Affiliated Hospital of Kunming Medical University, Kunming, Yunnan, China, which is a member unit of Chinese SLE Treatment and Research Group (CSTAR). All patients were from Chinese Han population. All participants signed informed consents after a complete description of the study and experienced full physical examinations and neuropsychiatric scales to evaluate disease activity and neuropsychiatric status by a multidisciplinary team with rheumatologists, neurologists, and psychiatrists. The major scales included a self-made questionnaire, Hamilton Depression Rating Scale (HAMD), Hamilton Anxiety Rating Scale (HAMA), and Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) to get the general conditions, emotional status, and disease activity of patients [10–12]. All patients had routine brain magnetic resonance imaging (MRI) scans to rule out major CNS diseases. Full set of autoantibodies including antinuclear antibody (ANA), anti-ribosomal P0 antibody (anti P0 antibody), anti-double stranded deoxyribonucleic acid (dsDNA) antibody, anti-Sm antibody, anti-U1-ribonucleoprotein (U1-RNP) antibody, anti-SSA-52 kD antibody, anti-SSA-60 kD antibody, anti-SSB antibody, anti-histones antibody, anti-nucleosome antibody, anti-cardiolipin (aCL) antibody, and lupus anticoagulant complex (LAC) was tested. This research was approved by the Institutional Review Board of Kunming Medical University, Yunnan Province, China (ClinicalTrials.gov: NCT00703742).

The exclusion criteria included the following: (1) patients with rheumatoid arthritis (RA), systematic sclerosis (SSc), idiopathic or secondary Sjogren's syndrome (SS) or other connective tissue diseases (CTD), or drug-induced SLE; (2) patients with serious disorders of heart, liver, kidney, or other major organs; (3) patients with disorders of central or peripheral nervous system; (4) patients with conditions which could induce cerebral atrophy such as stroke, kidney failure, high blood pressure, diabetes, and drug or alcohol dependence; (5) patients with a history of epilepsy, except for infantile febrile convulsion.

2.2. Statistical Analysis. Statistical analysis was conducted with SPSS 17.0 (SPSS Inc., 1989–2004). Variables were tested to find whether they met normal distribution. Normally distributed variables were shown with mean and standard deviation (SD), while nonnormally distributed ones were shown with median and interquartile range (IQR). Univariate comparisons between categorical variables were performed by chi-square test, while Mann-Whitney test was performed to evaluate numerical variables. For correlation between two numerical variables, we used Pearson's or Spearman's correlation. Finally, we used binary logistic regressions to find possible risk factors of depression and anxiety. The results were considered significant when $p < 0.05$.

3. Results

3.1. General Conditions of Patients. This study included 176 SLE patients with 23 males and 153 females. Their age ranged within 13–52 years with a mean age of 30.5. 92 (52.3%) patients were newly diagnosed. Patients treated with glucocorticoids (GCs), cyclophosphamide (CTX), and hydroxychloroquine (HCQ) were 131 (74.4%), 37 (21.0%), and 82 (46.6%), respectively. The results of general conditions were shown in Table 1.

3.2. Emotional Disorder Conditions. Depression was evaluated via HAMD scores, with score less than 7 as normal, 7–17 as mild or probable depression, 18–24 as moderate or definite depression, and more than 24 as severe depression. 121 (68.7%) patients got scores defined as mild to severe depression, 107 (60.8%) as mild depression, 13 (7.4%) as moderate depression, and 1 (0.6%) as severe depression. Anxiety was evaluated through HAMA scale, with score less than 7 as normal, 7–14 as mild or probable anxiety, 15–21 as moderate or definite anxiety, and more than 21 as severe anxiety. Anxiety was present in 101 (57.4%) patients while 80 (45.5%) patients had mild anxiety, 19 (10.8%) had moderate anxiety, and 2 (1.1%) had severe anxiety. Patients with SLEDAI scores less than 9 were inactive while those with scores of 9 and above had active disease status.

3.3. Association between Emotional Disorders and Clinical Phenotypes of SLE. We considered patients with HAMD scores more than 17 as definite depression and others as nondepression. Chi-square analysis showed the prevalence of depression was higher in patients with proteinuria, pyuria, hematuria, and anxiety (14.3% versus 4.8%, $\chi^2 = 4.142$, $p = 0.042$; 13.8% versus 4.7%, $\chi^2 = 4.551$, $p = 0.033$; 16.1% versus 4.3%, $\chi^2 = 5.502$, $p = 0.019$; 42.9% versus 3.2%, $\chi^2 = 25.129$, $p = 0.000$, resp.), while the prevalence of anxiety was higher in patients with elder age, alopecia, proteinuria, negative ant-P0 antibody, and depression (17.9% versus 6.7%, $\chi^2 = 5.127$, $p = 0.024$; 24.4% versus 7.6%, $\chi^2 = 6.980$, $p = 0.008$; 19.6% versus 7.7%, $\chi^2 = 4.968$, $p = 0.026$; 16.7% versus 5.9%, $\chi^2 = 4.383$, $p = 0.036$; 64.3% versus 7.4%, $\chi^2 = 25.129$, $p = 0.000$, resp.) (see Figures 1(a) and 1(b)).

Table 1: General conditions of 176 SLE patients.

	Median, IQR
Age (year)	29.5 (24, 37)
Disease duration (month)	8 (1, 32)
Proteinuria (g/day)	0.295 (0.1, 1)
SLEDAI	11 (6, 17)
Cumulative dosage	
Prednisone (g)	0.93 (0.16, 9)
CTX (g)	0 (0, 0.2)
HCQ (g)	1 (0, 11.6)
SLEDAI	11 (6, 17.75)
HAMD	9 (6, 13)
HAMA	7 (4, 11)
	n (%)
Female	153 (86.9%)
Autoantibodies	
Antinuclear antibody (ANA)	176 (100.0%)
Anti-P0 antibody	68 (38.6%)
Anti-dsDNA antibody	92 (52.3%)
Anti-Sm antibody	100 (56.8%)
Anti-U1-RNP antibody	61 (34.7%)
Anti-SSA-52 kD antibody	79 (44.9%)
Anti-SSA-60 kD antibody	112 (63.6%)
Anti-SSB antibody	47 (26.7%)
Anti-histones antibody	96 (54.5%)
Anti-nucleosome antibody	86 (48.9%)
Anticardiolipin (aCL) antibody	35 (19.9%)
Lupus anticoagulant complex (LAC)	60 (34.1%)
Active disease activity (SLEDAI > 9)	95 (54.0%)
Arthritis	58 (33.0%)
Myositis	13 (7.4%)
Urinary casts	5 (2.8%)
Hematuria	56 (31.8%)
Proteinuria	59 (33.5%)
Pyuria	65 (36.9%)
New rash	57 (32.4%)
Alopecia	41 (23.3%)
Mucosal ulcers	16 (9.1%)
Pleurisy	21 (11.9%)
Pericarditis	17 (9.7%)
Low complement	143 (81.3%)
Fever	38 (21.6%)
Thrombocytopenia	30 (17.0%)
Leukopenia	49 (27.8%)
Lupus headache	6 (3.4%)
Vasculitis	10 (5.7%)
Visual disturbance	2 (1.1%)
Seizure, psychosis, organic brain syndrome, cranial nerve disorder, and cerebrovascular accidents	0 (0%)
Depression	14 (8.0%)
Anxiety	21 (11.9%)

SLEDAI: Systemic Lupus Erythematosus Disease Activity Index; CTX: cyclophosphamide; HCQ: hydroxychloroquine; HAMD: Hamilton Depression Rating Scale; HAMA: Hamilton Anxiety Rating Scale.

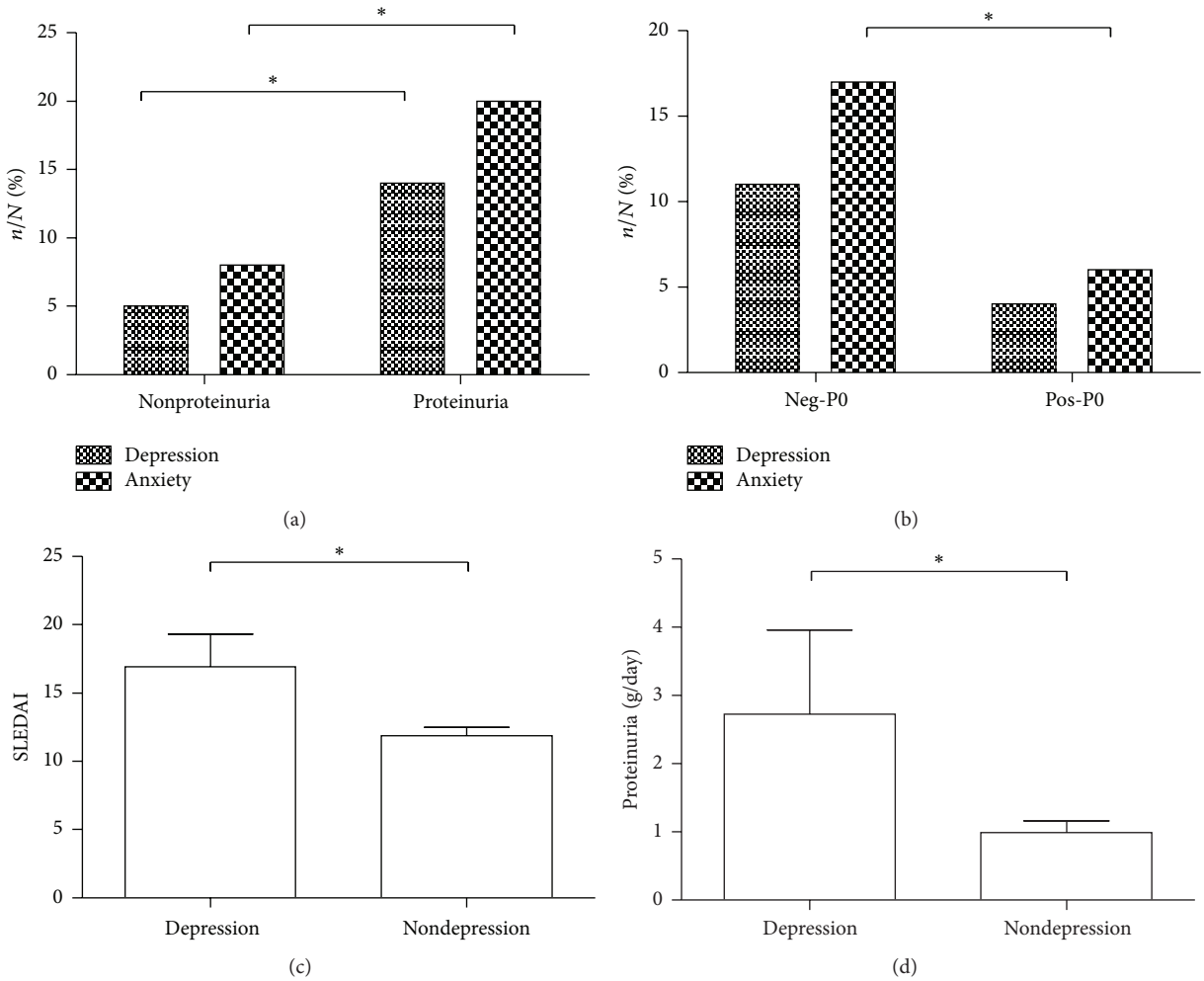

Figure 1: Association between emotional disorders and clinical phenotypes of SLE. (a) The prevalence of depression and anxiety was higher in patients with proteinuria; (b) the prevalence of anxiety was higher in patients with negative P0 antibody; (c) the SLEDAI score of depression patients was higher; (d) depression patients were with more proteinuria. $*$ showed $p < 0.05$.

Mann-Whitney analysis showed that the depression group had a higher score of SLEDAI and HAMA and higher proteinuria (16.93 versus 11.89, $p = 0.027$; 16.64 versus 7.23, $p = 0.000$; 2.73 g/day versus 0.99 g/day, $p = 0.010$, resp.) (see Figures 1(c) and 1(d)).

Spearman correlation tests showed that HAMA scores were in strong positive correlation with HAMD scores ($r = 0.82$, $p = 0.000$) (see Figure 2). The cumulative dosage of HCQ was in positive correlation with both HAMD and HAMA scores ($r = 0.173$, $p = 0.038$; $r = 0.243$, $p = 0.003$, resp.). The age and disease duration were also in positive correlation with HAMA scores ($r = 0.182$, $p = 0.016$; $r = 0.264$, $p = 0.001$, resp.).

However, when we analyzed the possible risk factors we got from the analysis above in binary logistic regression, we found that only pyuria, hematuria, and HAMA score were the risk factors of depression, and proteinuria, SLEDAI, and cumulative dosage of HCQ were not significantly relevant. As to anxiety, we found alopecia and HAMD score were the risk factors, and age had a trend, while disease duration, proteinuria, anti-P0 antibody, and cumulative dosage of HCQ were not significantly relevant with anxiety (see Tables 2(a) and 2(b)).

4. Discussion

Neuropsychiatric symptoms are major symptoms in SLE patients, and 19 of them are considered as NPSLE. NPSLE patients may have poorer prognosis and higher mortality [13]. Severe NPSLE like seizures, stroke, or mental disorders are well recognized in clinical situations. However, due to lack of awareness, subtle NPSLE syndromes like emotional disorders including depression and anxiety are not well recognized. Some doctors may even consider these symptoms as "non-NPSLE" when the patients have no history of "neuropsychiatric disorders" and normal conventional brain MRI scans, just like the patients we recruited. We can recognize patients with emotional disorders with thorough psychiatric evaluations in early phase. The prevalence of depression was reported to be 10.8–68%, while that of anxiety was 15.6–46.5% [6, 14–16]. Our study showed that, in the patients of

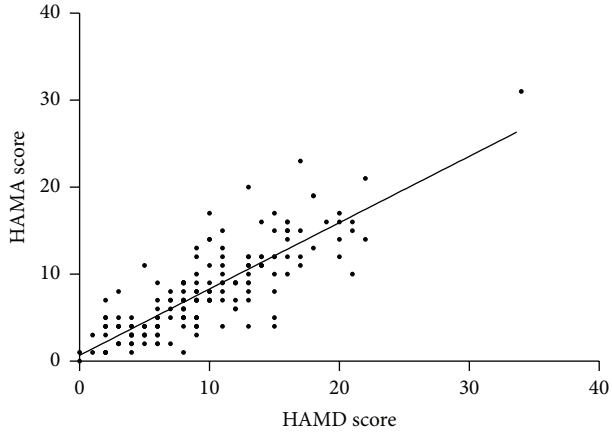

Figure 2: The correlation between HAMD and HAMA scores (r = 0.82, p = 0.000).

Table 2: (a) The risk factors of depression by binary logistic regression. (b) The risk factors of anxiety by binary logistic regression.

(a)

Independent variable	OR	95% CI	p
Pyuria	6.275	1.059–37.199	0.043
Hematuria	5.155	1.020–26.047	0.047
HAMA score	43.611	7.148–266.095	0.000

(b)

Independent variable	OR	95% CI	p
Alopecia	5.460	1.358–21.949	0.017
HAMD score	30.458	6.040–153.594	0.000
Age	4.451	0.916–21.632	0.064

our sample, depression and anxiety were quite common in non-NPSLE patients, with a proportion of 68.7% and 57.4%, respectively, which is consistent with prior studies.

The association between depressive and anxiety disorders and clinical symptoms was quite different in various studies. Factors like ethnicity, rashes, disease activity, and certain antibodies like anti-P0 antibody or aCL antibody were all involved [16–21]. In our study, we found that depression was associated with urinary symptoms and SLEDAI, and anxiety was associated with negative P0 and age, while proteinuria was associated with both of them and cumulative dosage of HCQ was in positive correlation with both of them. Depression and anxiety were the most important predictors of each other.

The relationship between disease activity and depression and anxiety was studied by several researchers before and the results were inconsistent. Julian et al., Nery et al., and Bachen et al. reported disease activity was relevant with depression and anxiety, while Huang et al. and Hanly et al. found no relevance in their studies [17–21]. Thus, Palagini et al. summarized that as lack of studies and methodological limitations; the relationship might remain contradictory before more studies [22]. Our study showed association between depression and disease activity. And both depression and anxiety were associated with proteinuria and higher cumulative dosage of HCQ, which might reveal higher disease activity. Thus, we assumed that emotional disorders were related to disease activity.

Anti-ribosomal P (RP) antibody targets C-terminal region of ribosomal P protein, mainly ribosomal phosphoprotein P0, P1, and P2. Anti-RP antibody was considered as a specific antibody of SLE and one of the most relevant antibodies of NPSLE [23–27]. The prevalence of anti-RP antibody in SLE patients ranged from 6% to 42% and was supposed to be higher in Asian patients due to ethnic differences [25]. Several studies found that anti-RP antibody was associated with psychosis and depression [24–27]. Karimifar et al. found that the association occurred in early course of SLE patients and believed that anti-RP antibody could cause certain NPSLE symptoms [24]. The detection of anti-RP antibody in cerebrospinal fluid (CSF) was considered to be more meaningful than that in serum [28, 29]. And some researchers injected anti-RP antibodies directly into the brain ventricles of mice to induce depression-like behaviors, which could get improved by antidepressant drugs and blocking the antibodies [30–32]. However there were some studies that showed no relevance between anti-RP antibodies and neuropsychiatric symptoms [19, 33, 34]. Iseme et al. believed that anti-RP antibody could upregulate proinflammatory cytokines like interferon and could cause neuronal death via apoptosis, which was the underlying mechanism of neuropsychiatric symptoms [35]. Arnett et al. found that anti-RP antibody was strongly influenced by certain MHC class II alleles which might suggest the underlying genetic mechanism [36].

As to anxiety, Aldar et al. found that anti-RP antibodies were higher in anxious childhood-onset SLE patients [37], while most other studies showed no relation between anti-RP antibody and anxiety [6, 25, 38–40]. In our study, we found no association between anti-P0 antibody and depression. However, we found that patients with anti-P0 antibody had a lower chance of anxiety. The controversial results might be due to the heterogeneity of the disease. No association between anti-P0 antibody and anxiety was found in binary logistic regression; thus, this result requires further confirmation. Although the relationship between anti-RP antibody and NPSLE, especially depression, was quite certain, the variety of symptoms and classifications of NPSLE made it hard for us to understand the relationship between anti-RP antibody and specific NPSLE manifestations like anxiety. Whether it was significant needed more statistics. Another possible explanation might be that we chose "non-NPSLE" patients in this study, in which the proportion of positive anti-P0 antibody was low.

NPSLE could be the original manifestation of SLE. Depression and anxiety are major emotional disorders in SLE patients. However, lack of awareness and difficulties in early recognition make it hard to get early treatment for patients suffering from these conditions. Our study found that depression and anxiety were really common in SLE patients considered as "non-NPSLE," and they were strong risk factors of each other. Depression was associated with disease activity, while both depression and anxiety were

associated with proteinuria and higher cumulative dosage of HCQ, which might reveal the higher disease activity. This might suggest that emotional disorders could be early phase of SLE brain damage. Unexpectedly, anxiety was associated with negative anti-P0 antibody, which should be reexamined by more studies to find out the role of anti-P0 antibody in depression and anxiety.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Ru Bai, Shuang Liu, and Yueyin Zhao equally contributed to this study.

Acknowledgments

The authors thank all the volunteers who participated in this study. The authors also thank Daying Feng, Department of Rheumatology and Immunology of First Affiliated Hospital of Kunming Medical University, for recruiting the volunteers. This work was supported by Grants from the National Natural Science Foundation of China (81160379, 81460256, 81560233, and 81501406), the Ministry of Science and Technology of Yunnan Province (2012FB158), the Funding of Yunnan Provincial Health Science and Technology Plan (2014NS171, 2014NS172), the Funding of Yunnan Provincial Public Health for Academic Leader (D-201218, D-201239), and Yunnan Provincial Fund for Preparatory Young Leaders in Academia and Technology (2015HB071).

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 2063985, 8 pages
http://dx.doi.org/10.1155/2016/2063985

Research Article

CXCL13 Promotes Proliferation of Mesangial Cells by Combination with CXCR5 in SLE

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Received 29 January 2016; Revised 3 July 2016; Accepted 27 July 2016

Academic Editor: Quanzhen Li

As a CXC subtype member of the chemokine superfamily, CXCL13 is considered to be involved in systemic lupus erythematosus (SLE), especially in lupus nephritis (LN). To determine the effect of CXCL13 on SLE and explore the potential mechanisms, we tested serum concentrations of CXCL13 in patients and healthy individuals and found that CXCL13 expression was high in SLE patients especially in LN patients. When we treated human renal mesangial cells (HRMCs) *in vitro* with recombinant human CXCL13, the cell proliferation was accelerated, which was tested by Cell Counting Kit-8 assay and flow cytometry. Western blot and immunofluorescence assay revealed that CXCL13 would lead to phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2). However, the effect was weakened after the silence of CXCR5. The results of our study elaborated that high expression of CXCL13 could be involved in the pathogenesis of LN.

1. Introduction

Systemic lupus erythematosus (SLE) is a typical autoimmune disease with abundant production of autoantibodies [1]. Through the binding of antigen and antibody, the immune complexes develop and deposit in various blood vessels, leading to multiorgans or systems involvement [2, 3], which severely disturb the quality of life of patients [4, 5]. Although recent studies about the pathogenesis of SLE have achieved great progress, the exact mechanism remains ambiguous [6].

Lupus nephritis (LN) is the most common and serious complication in SLE patients characterized by proteinuria, hematuria, drop glomerular filtration rate, or renal dysfunction [7, 8]. It is often accompanied by pathological changes of podocytes [9–11], mesangial cells [12, 13], or renal interstitial [14]. Up to 20% of LN patients develop into end-stage renal disease, some even to death [15, 16]. Renal involvement deserves enough attention, because it is an important factor to assess the prognosis of SLE [17]. Therefore, it is necessary to explore the possible mechanisms of LN.

As the main segment in SLE, immunological abnormalities run through the whole process of this disease. In the past few years, B cell-attracting chemokine CXC ligand 13 protein, namely, CXCL13 or B lymphocyte chemoattractant (BLC), has been discovered in SLE [18, 19]. Researchers found that it was correlated with disease activity and renal involvement. The pathogenetic role of CXCL13 in podocytes has been found by Worthmann et al. [20]. However, there were few reports about the relationship between CXCL13 and mesangial cells.

In this study, we treated HRMCs *in vitro* with recombinant human CXCL13. The results revealed that CXCL13 promoted the proliferation of HRMCs by activating ERK1/2-MAPK signaling pathway. Interestingly, the effect became noteless when CXCR5, receptor of CXCL13, was silenced. Therefore, our data suggested that CXCL13 in association with CXCR5 played an important role in LN and might be a new target in LN treatment.

2. Materials and Methods

2.1. Patients and Controls. SLE patients were randomly recruited from Department of Rheumatology, Affiliated Hospital of Nantong University, from June 2013 to December

2014. All patients were diagnosed according to the American College of Rheumatology Criteria for the classification of SLE [21, 22]. Those with other autoimmune diseases, severe infections, or cancers were excluded. Healthy controls were from the Center of Health Examination of the same hospital. The study was approved by the Ethics Committees. Informed consent was obtained from all subjects.

2.2. Cells. Human renal mesangial cells (HRMCs) were purchased from JENNIO Biological Technology and cultured in RPMI-1640 medium (HyClone) with 10% fetal bovine serum (FBS, Gibco) at 37°C in 5% CO_2. Cells were treated with recombinant human CXCL13 (R&D Systems, Catalog Number 801-CX-025).

2.3. ELISA. The concentration of CXCL13 in the serum of SLE patients and healthy controls was measured by ELISA using a Human CXCL13/BLC/BCA-1 Immunoassay (R&D Systems, Catalog Number DCX130) according to the manufacturer's directions.

2.4. Transfection. Cells were seeded to a 6-well plate and incubated at 37°C, 5% CO_2 in the incubator before transfection. When the cells covered 50% to 70% of the plate, 250 μL base medium containing 100 pmol CXCR5 siRNA (Biomics Biotech, China) and 250 μL base medium containing 5 μL Lipofectamine® 2000 (Invitrogen by life technologies, USA) were mixed for 20 minutes. The transfection complex was added with another 1500 μL base medium to each well. After 4 to 6 hours, RPMI-1640 containing 10% FBS was replaced and transfection efficiency was detected within 48 to 72 hours by Western blot.

2.5. Cell Counting Kit-8 (CCK8) Assay. 100 μL cell suspension per well was prepared in 96-well plates and preincubated for 12 hours in an incubator (37°C, 5% CO_2). After treating with or without CXCR5 siRNA transfection, the cells were incubated with or without CXCL13 at 500 pg/mL for 4, 8, 12, or 24 hours. 10 μL CCK-8 solution (Sangon Biotech, Shanghai, China) and 90 μL medium were added to each well and incubated away from light for 2.5 hours. The optical density of each well was determined by a microplate reader set to 450 nm.

2.6. Flow Cytometry Analysis. To determine the difference of cell cycle in HRMCs with or without CXCL13 treatment, we used the flow cytometry analysis. Cells were trypsinized and centrifuged at 1000 rpm for 5 minutes, washed, and resuspended with cold PBS for three times. Then they were fixed with 70% ethanol in −20°C for 24 hours, followed by centrifuging, washing, resuspending in 500 μL PBS, and permeabilizing with PBS containing 1% Triton X-100. After 10 minutes, RNaseA was added and the cells were stained with 200 μL PI (0.05 mg/mL) for another 20 minutes in the dark. The fluorescence of PI was measured by Flow Cytometer. Cell proportion was calculated by MFLT32 Soft. Cell proliferation status was evaluated mainly by the ratio of S phase.

2.7. Western Blot. Cell proteins were extracted with RIPA Lysis Buffer (Beyotime Biotechnology, China) according to the manufacturer's instructions. With 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred to PVDF membranes. After blocking with 5% skimmed milk in TBS, membranes were incubated with primary antibodies for anti-CXCR5 (Abcam, EPR8837), phospho-ERK, total ERK (Santa Cruz, USA), and GAPDH overnight at 4°C. Followed by washing, blots were incubated with horseradish peroxidase-labelled goat anti-rabbit IgG (NeoBioscience, ANR02-1) as the secondary antibodies for one hour. The signal was detected by ECL.

2.8. Immunofluorescence. Cells were seated to the slides of cells in 24-well plates and incubated overnight before treatment. The supernatant was discarded. Cells were washed with ice-cold PBS and fixed with 4% paraformaldehyde for 40 minutes at room temperature, then blocked with Immunol Staining Blocking Buffer (Beyotime Biotechnology, China) followed by washing with PBS for three times, and incubated with anti-CXCR5 (Abcam, EPR8837) or anti-pERK antibody (Santa Cruz, CA, USA) as primary antibody overnight and Dylight 594, goat anti-rabbit IgG as secondary antibody for one hour in dark. Nuclei were stained with Hoechst (Beyotime Biotechnology, China). The slides were turned over with Antifade Mounting Medium (Beyotime Biotechnology, China). Images were acquired using a fluorescent microscope.

2.9. Statistical Analysis. All experiments were repeated three times at least. Normally distributed data were presented as mean ± standard deviation (SD) and skewed data as the median (interquartile range, IQR). Mann–Whitney rank sum test, Chi-square analysis, Student's t-test (2 groups), and one-way analysis of variance (ANOVA) (more than 2 groups) were used to indicate the differences with SPSS 20.0. Spearman's test was used to assess the correlation of CXCL13 with SLEDAI or renal SLEDAI score. The results were plotted with SigmaPlot 10.0. $P < 0.05$ was defined as being significant.

3. Results

3.1. The Expression of CXCL13 Was Increased in SLE Especially in LN. The clinical characteristics of subjects with SLE and controls were shown in Table 1. Of the 70 patients, there were 34 with renal involvement (LN) and 36 without (NLN). SLEDAI score and renal SLEDAI (rSLEDAI) score were assessed [23]. LN was defined as persistent proteinuria > 0.5 grams per day or more than 3+ on urine dipstick testing or cellular casts (maybe red cell, hemoglobin, granular, tubular, or mixed). These patients had active LN when we investigated. 32 healthy controls were matched by age and gender. Previous studies had reported that the expression of CXCL13 increased in SLE [19, 24]. Similarly, our results by ELISA indicated that the serum concentrations of CXCL13 in patients were higher than that of the healthy controls [376.92 (223.85–661.94) versus 82.46 (52.33–187.56) pg/mL, $P < 0.001$]. Particularly, the concentrations of patients with

Table 1: Clinical characteristics of patients with SLE and healthy controls.

	SLE	Controls	P	LN	NLN	P
Number	70	32	—	34	36	—
Sex (female/male)	63/7	29/3	0.8	33/1	30/6	0.1
Age (years)	36.8 ± 12.4	35.4 ± 7.9	0.5	38.8 ± 12.6	34.9 ± 12.1	0.2
SLEDAI score	13.00 ± 5.79	—	—	15.41 ± 5.32	11.03 ± 5.56	0.001

SLEDAI: systemic lupus erythematosus disease activity index.

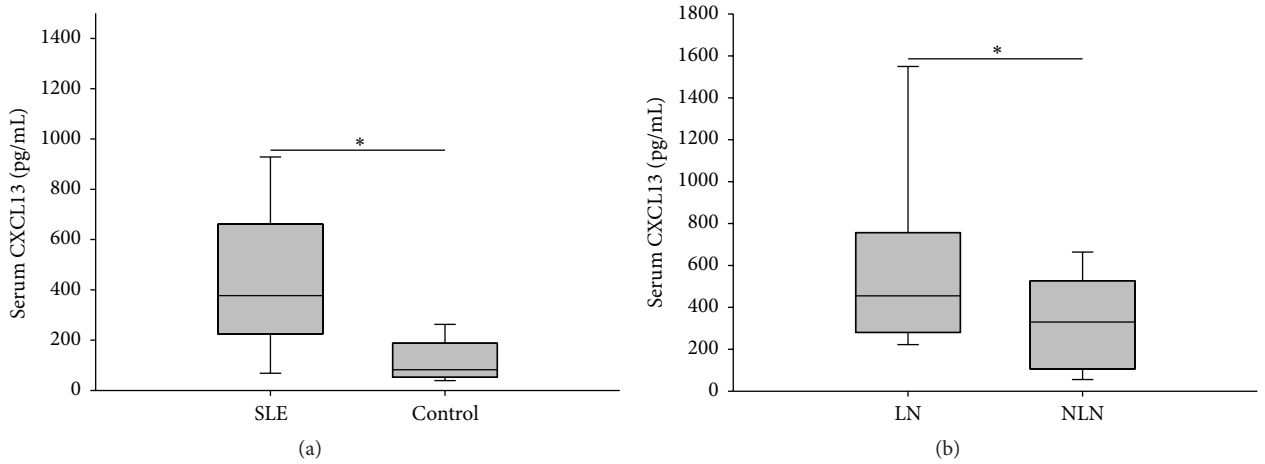

Figure 1: The expression of CXCL13 was increased in SLE especially in LN. (a) Serum CXCL13 of SLE patients and healthy controls. $^*P < 0.001$. (b) Serum CXCL13 of lupus nephritis (LN) and nonlupus nephritis (NLN). $^*P = 0.004$.

LN were even higher than those without [455.06 (280.04–756.84) versus 329.81 (105.42–526.98) pg/mL, $P = 0.004$] (Figure 1). Besides, serum CXCL13 concentration was positively correlated with SLEDAI score ($r = 0.258$, $P = 0.03$) and significantly positively correlated with rSLEDAI ($r = 0.748$, $P < 0.001$, data not shown).

3.2. CXCL13 Promoted the Proliferation of HRMCs. To investigate whether CXCL13 promoted the proliferation of HRMCs so as to be involved in SLE progression, we treated HRMCs with CXCL13 at 0.5 ng/mL, 10 ng/mL, and 20 ng/mL. Results were shown in Figure 2(a). The concentration 0.5 ng/mL (500 pg/mL) was optimal in our preliminary experiment which was also adopted by Worthmann et al. [20]. The proliferation ability of HRMCs was stimulated by 500 pg/mL CXCL13 determined by CCK8 (Figure 2(b)) and FCM (Figure 2(c)). Following CXCL13 treatment, the cell proliferation status was significantly improved. The ratio of cells in S phase in the treatment group was higher than that in the control group [(49.76 ± 1.11)% versus (34.96 ± 0.08)%, $P < 0.001$] and G1 phase decreased from (61.90 ± 0.29)% to (47.42 ± 0.71)%, $P < 0.001$.

3.3. HRMCs Responded Mildly to CXCL13 after the Silence of CXCR5. CXCR5 was reported to be the unique receptor of CXCL13 [25]. We found a positive expression of CXCR5 in HRMCs. After stimulation with CXCL13, the expression of CXCR5 increased (Figures 3(a) and 3(b)). Then we used transfection technology to silence CXCR5; Western blot indicated that siR4 was efficient (Figure 3(c)). To investigate whether CXCR5 was involved in proliferation of HRMCs, we repeated CCK8 and FCM to test cell proliferation status after the silence of CXCR5. Results presented slight proliferation of transfected cells treated with CXCL13 compared to normal cells without any treatment (Figures 2(b) and 2(c)). The quantified results of S phase from three independent experiments were shown in Table 2.

Table 2: Ratio of cells in S phase from different groups.

	S phase (mean ± SD)	P
Control%	34.96 ± 0.08	–
CXCL13%	49.76 ± 1.11	<0.001 (versus control%)
siR + CXCL13%	40.10 ± 0.46	<0.001 (versus CXCL13%)

Statistical analyses were performed by ANOVA followed by Dunnett's test.

3.4. CXCL13 Triggered ERK Tyrosine Phosphorylation. The extracellular signal-regulated kinase (ERK, including ERK1 and ERK2) was a crucial member of mitogen-activated protein kinase (MAPK) family [26]. ERK phosphorylation (pERK) was required for cell proliferation and differentiation [27, 28]. Previous research had reported the connection of ERK1/2 and cell proliferation [29]. We tried to find out

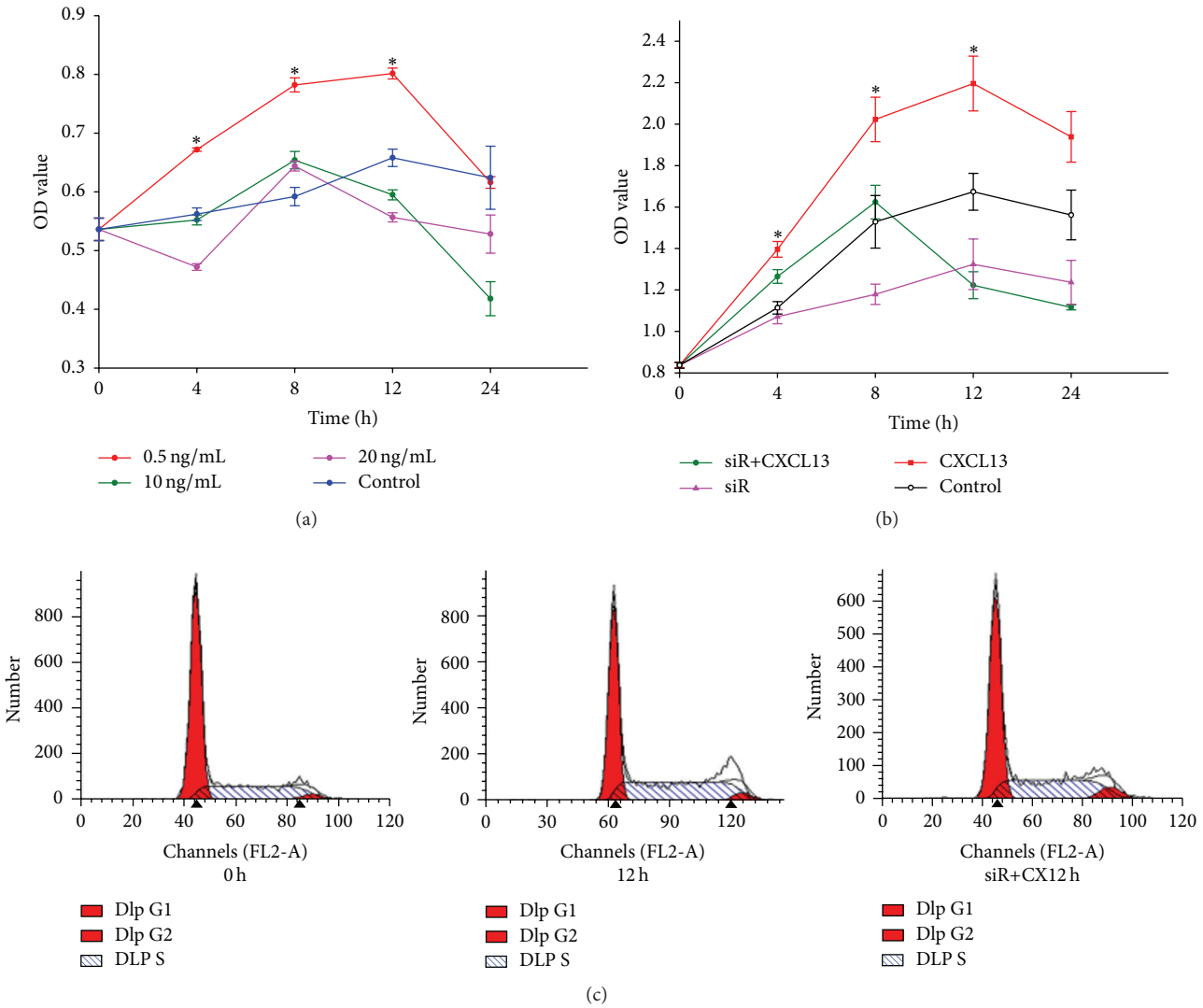

Figure 2: CXCL13 promoted the proliferation of HRMCs. (a) Cells were treated with CXCL13 at 0.5 ng/mL, 10 ng/mL, and 20 ng/mL. 0.5 ng/mL (500 pg/mL) was optimal concentration. (b) With 500 pg/mL CXCL13, the proliferation ability of HRMCs was shown. Both P values of CXCL13 versus control and siR + CXCL13 versus CXCL13 were less than 0.05. (c) Cell cycles of HRMCs with CXCL13 for 0 h and 12 h and transfected cells for 12 h were shown. Results indicated that CXCL13 promoted cells to enter S phase. However, the effect was weakened after the silence of CXCR5. $^{*}P < 0.001$.

possible mechanism involved in HRMCs. Our results indicated that CXCL13 triggered ERK tyrosine phosphorylation in HRMCs in comparison with the control group. In CXCR5 silenced cells, the phosphorylation was reversed. Western blot and immunofluorescence experiments were performed to recognize pERK1/2 (Figure 4). These data were in line with the results above.

4. Discussion

CXCL13 is a member of chemokine superfamily and the main function is effectively chemoattracting B cells. It is produced mainly by dendritic cells in secondary lymphoid organs and can induce generation of secondary lymphoid tissue in peripheral organs [19]. CXCL13 plays an important role in the formation of germinal centers, while germinal center is required in B lymphocytes development and maturation [30, 31]. As a result, it is an important element in autoimmune microenvironment. In 2001, Ishikawa et al. discovered high expression of CXCL13 in kidney of elderly BWF1 mice [24]. After that, Schiffer et al. demonstrated that serum levels of CXCL13 in SLE patients were higher than those of healthy controls and the levels of patients with LN were even higher than those without. They discovered CXCL13 in inflammatory infiltrates of nephritic NZB/W-F1 mice. *In vitro*, CXCL13 induced production of proinflammatory factors (CXCL1, CXCL12, MIF, and LIF) in human podocytes,

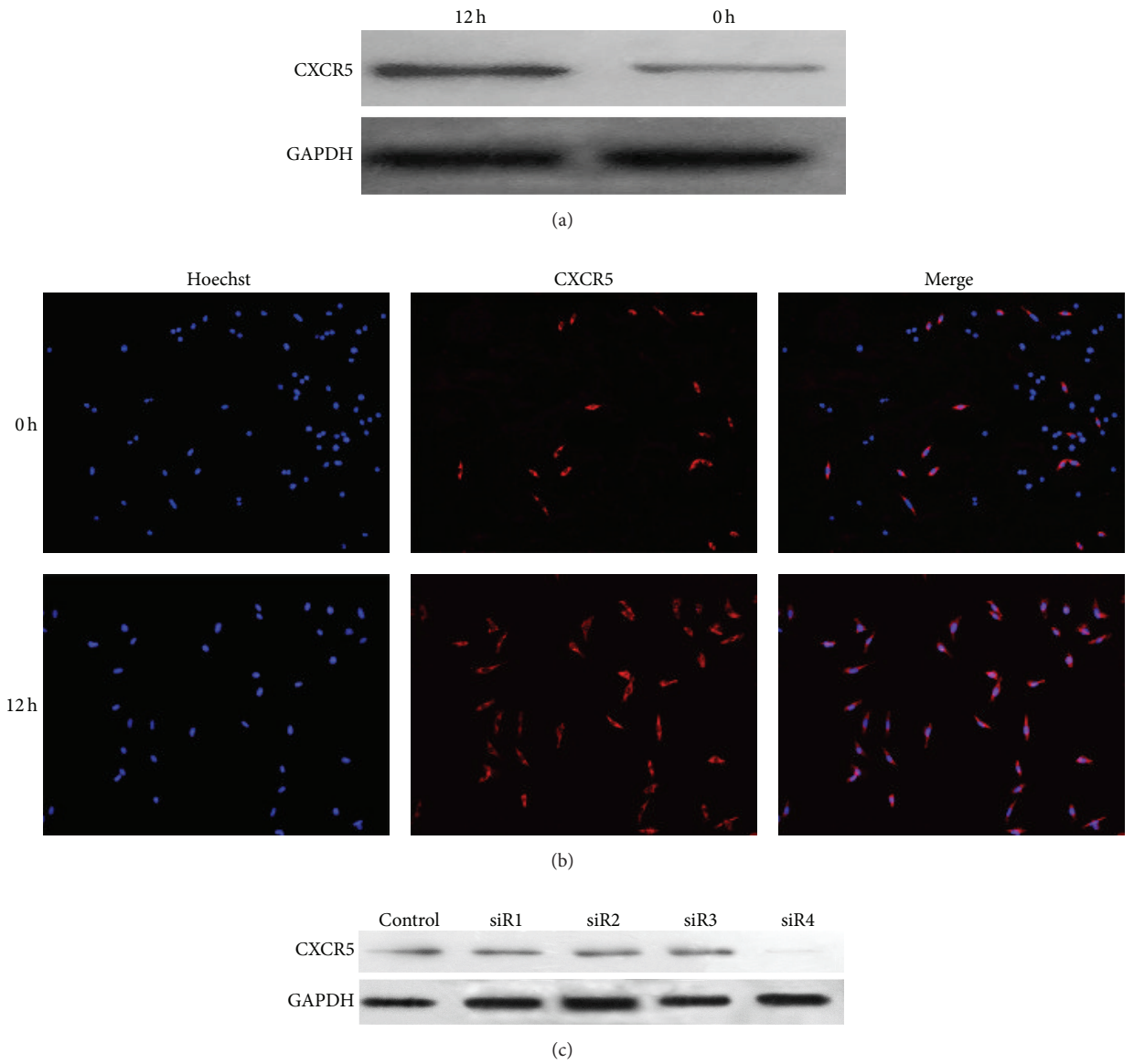

Figure 3: Positive expression of CXCR5 in HRMCs. (a and b) Western blot and immunofluorescence showed CXCR5 expression whether under stimulation of CXCL13 or not. (c) Transfection by siR4 was efficient.

which indicated that CXCL13 was closely related to renal inflammation [19, 20]. Recently, CXCL13 has been reported to participate in SLE-related autoimmune hemolytic anemia [32]. We also found that the expression of CXCL13 was high in Chinese SLE patients especially in LN patients and it was positively correlated with SLEDAI especially with rSLEDAI. According to these results, we hypothesize that CXCL13 may increase more when complications occur in SLE.

The receptor of CXCL13 is CXCR5, 7-transmembrane G protein-coupled receptors, mainly expressed in mature B cells and follicular helper T cells [25]. Research has reported that CXCL13-CXCR5 axis plays an important role in immune responses [33–35]. Moreover, it has been reported that CXCL13 mediated cell proliferation [36]. In our study, CXCL13 accelerated HRMCs proliferation, which was mainly presented by promoting cells to enter S phase followed by the activation of ERK1/2. Interestingly, when we silenced the expression of CXCR5, the proliferation was weakened. As HRMCs proliferation was involved in pathogenesis of LN [37, 38], our study highlighted the significant role of CXCL13-CXCR5 axis in HRMCs proliferation and further explained the importance of CXCL13 in LN.

In summary, our results point out that CXCL13 expression is high in LN. It may promote the proliferation of mesangial cells by combination with CXCR5 via ERK1/2 pathway to be involved in pathogenesis of LN. Blocking off CXCL13-CXCR5 axis is expected to become a new therapeutic strategy targeting LN.

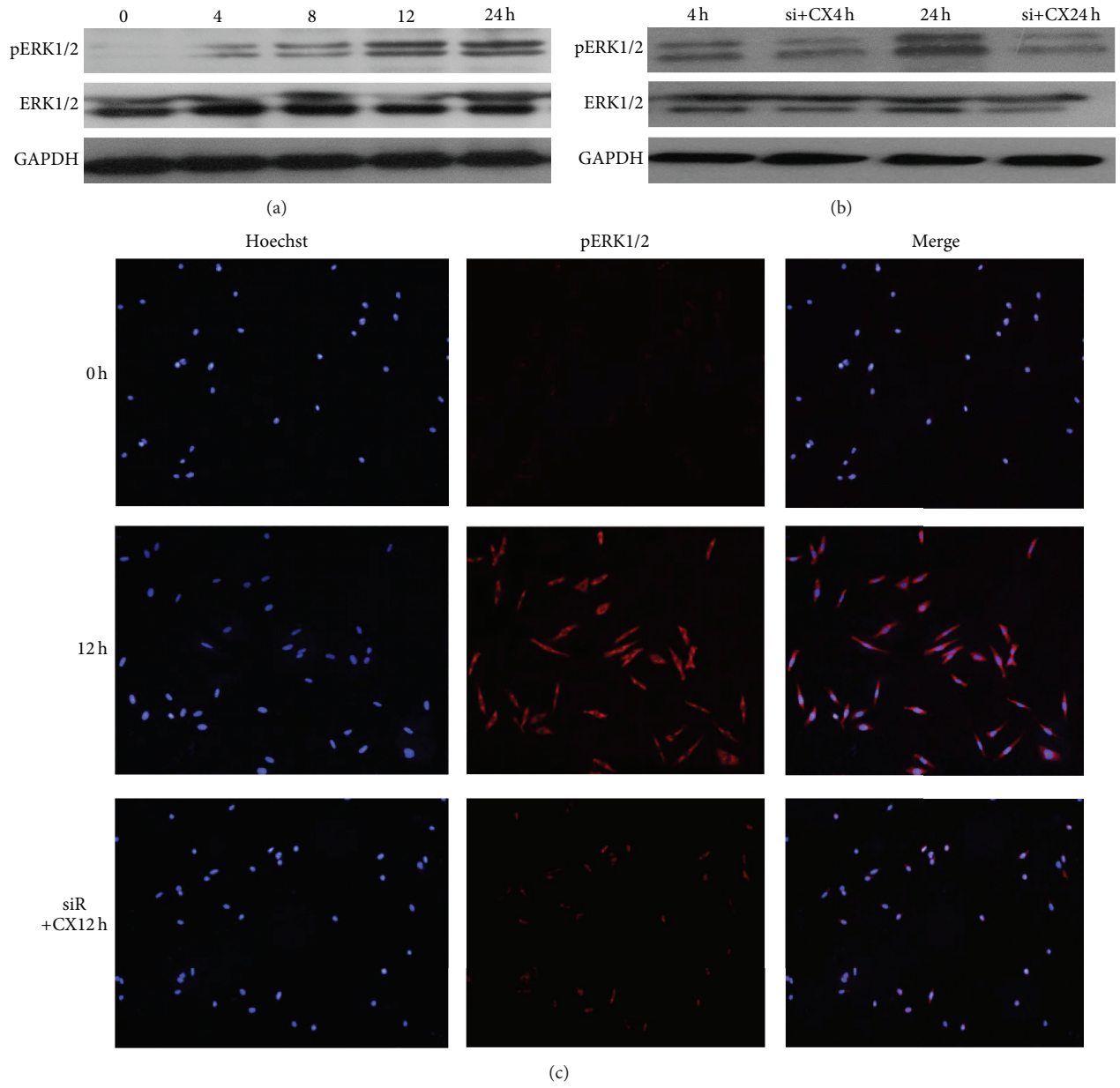

Figure 4: CXCL13 triggered ERK tyrosine phosphorylation. (a) Upon 4 hours, CXCL13 triggered ERK tyrosine phosphorylation of HRMCs as time went on. (b) ERK tyrosine phosphorylation decreased in CXCL13 treatment after the silence of CXCR5 compared to CXCL13 treatment alone. (c) The same result with (a) and (b) was found by immunofluorescence. The top row was detected at 0 hours and the middle at 12 hours after CXCL13 treatment. The bottom presented pERK of cells treating with CXCL13 for 12 hours after transfection.

Disclosure

This work is attributed to Department of Rheumatology, Affiliated Hospital of Nantong University, Nantong, Jiangsu Province, China.

Competing Interests

There are no commercial affiliations or competing interests to disclose.

Authors' Contributions

Zhanyun Da and Liuxia Li contributed equally to this work.

Acknowledgments

This work was supported by the "Top Six Types of Talents" Financial Assistance of Jiangsu Province (2011-WS-064); the project from Bureau of Public Health of Jiangsu Province (H201317); the Science and Technology Projects of Traditional Chinese Medicine of Jiangsu Province (LZ11098); the

Special Fund for Clinical Medical Science and Technology of Nantong (HS2014071); the National Natural Science Foundation of China (nos. 81172841 and 81471603); the Fourth "226 Project" Researcher Grants Programme of Nantong and the Colleges and Universities in Jiangsu Province Plans to Graduate Research and Innovation (YKC15082).

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