Original Article
Severe vitamin D deficiency affects the expression of autophagy related genes in PBMCs and T-cell subsets in active systemic lupus erythematosus

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Abstract: We aimed to investigate whether vitamin D levels affect the expression of autophagy related genes (Atgs) and the counts of T-cell subsets in active systemic lupus erythematosus (SLE), as well as to assess the association between Atgs and T-cell subsets. Serum levels of 25(OH)D3, Atgs and T-cell subsets were measured in 50 patients with active SLE. Serum 25(OH)D3 levels <10 ng/ml and 10-30 ng/ml were defined as severe vitamin D deficiency and vitamin D insufficiency, respectively. Comparisons were made between values of severe vitamin D deficiency and vitamin D insufficiency patients, and the correlations between Atgs in PBMCs and T-cell subsets were carried out. mTOR mRNA levels were higher (p=0.036) and LC3 mRNA levels were lower (p<0.001) in severe vitamin D deficiency group compared to vitamin D insufficiency group. The counts of CD4+ T cells and the CD4/CD8 ratio were significantly higher in severe vitamin D deficiency group compared to vitamin D insufficiency group (p=0.001, p<0.001, respectively). LC3 mRNA levels correlated negatively with CD4+ T cells counts (r=-0.302, p=0.033), while correlated positively with CD8+ T cells counts (r=0.299, p=0.035). Serum 25(OH)D3 levels correlated negatively with the counts of CD4+ T cells (r=-0.423, p=0.002) and correlated positively with the counts of CD8+ T cells (r=0.318, p=0.024). Our results suggested that severe vitamin D deficiency affected the expression of Atgs in PBMCs and T-cell subsets in active SLE, indicating that vitamin D may affect T-cell subsets via regulating autophagy.

Keywords: Systemic lupus erythematosus, 25(OH)D3, vitamin D deficiency, autophagy related genes, T-cell subsets

Introduction

Systemic lupus erythematosus (SLE), one of the most important and classical autoimmune diseases, whose pathogenesis and etiology remains unclear, is still a challenge to the clinicians. It is generally accepted that vitamin D deficiency is highly prevalent among patients with SLE [1, 2]. Our previous study [3] found that severe vitamin D deficiency (25(OH)D3<10 ng/ml) increased the risk of moderate to severe disease activity in patients with SLE by 6.42 times.

Autophagy is involved in nearly all parts of the immune system, including pathogen recognition, antigen processing and presentation, immune cell development and function, and immunoregulation [4, 5]. mTOR, LC3, and p62 are primary autophagy related genes (Atgs) involved in the autophagy. mTOR is a major suppressor of autophagy induction [6], LC3 is used to monitor the number of autophagosomes as well as autophagic activity [7], and p62 is associated with the degradation of autophagosomes [8]. Evidences from genetic, cell biology and animal model studies suggest that autophagy plays a pivotal role in the occurrence and development of SLE [9, 10], but not yet fully elucidated. In pigs and IPEC-J2 cells, vitamin D3 was found alleviating rotavirus infection via regulating the autophagy signaling pathway [11]. Besides, in a random skin flap model, calcitriol was demonstrated to improve flap survival area by upregulating autophagy [12]. These observations indicate the role of vitamin D3 in stimulating autophagy.

Accumulating evidences indicated a crucial role of autophagy for homeostasis, activation, and
SLE and autophagy

Table 1. Baseline characteristics of SLE patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total</th>
<th>25(OH)D3 &lt;10 ng/ml</th>
<th>25(OH)D3 10-30 ng/ml</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female sex, n/N (%)</td>
<td>46/50 (92)</td>
<td>19/22 (86)</td>
<td>27/28 (96)</td>
<td>0.42</td>
</tr>
<tr>
<td>Age (years), mean ± SD</td>
<td>31.92±9.55</td>
<td>31.96±9.61</td>
<td>31.89±9.68</td>
<td>0.98</td>
</tr>
<tr>
<td>Leukopenia, n/N (%)</td>
<td>3/50 (6)</td>
<td>0</td>
<td>3/28 (11)</td>
<td>0.45</td>
</tr>
<tr>
<td>Lymphopenia, n/N (%)</td>
<td>9/50 (18)</td>
<td>2/22 (9)</td>
<td>7/28 (25)</td>
<td>0.23</td>
</tr>
<tr>
<td>Disease duration (months)</td>
<td>60.52±53.87</td>
<td>74.32±59.77</td>
<td>49.68±47.02</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Mean ± SD

Treatment, n (%)

- Glucocorticoids: 47/50 (94) | 20/22 (91) | 27/28 (96) | 0.42
- Hydroxychloroquine: 35/50 (70) | 17/22 (77) | 18/28 (64) | 0.33
- Leflunomide: 3/50 (6) | 1/50 (2) | 2/50 (4) | 0.7
- Mycophenolate mofetil: 7/(14) | 4/50 (8) | 3/50 (6) | 0.46
- Cyclophosphamide: 9/50 (18) | 3/50 (6) | 6/50 (12) | 0.48
- Tacrolimus: 4/50 (8) | 1/50 (2) | 3/50 (6) | 0.43
- Cyclosporine: 4/50 (8) | 2/50 (4) | 2/50 (4) | 0.8
- SLEDAI, mean ± SD: 10.64±4.99 | 11.68±5.47 | 9.82±4.50 | 0.12


Fifty patients with SLE were enrolled in this study. All participants met the revised criteria of the American College of Rheumatology (ACR) for classification of SLE [17] and with mild to severe disease activity (SLEDAI≥ 5). Patients were excluded if they met any of the following criteria: (1) age <18 years old or >60 years old; (2) pregnancy or lactation; (3) coexistence of other autoimmune diseases such as rheumatoid arthritis, systemic sclerosis, or chronic diseases affecting autophagy such as systemic infection, cancers, and neurodegeneration, etc.

Data regarding demographics and clinical parameters were collected. Disease activity was assessed in accordance with the systemic lupus erythematosus disease activity index 2000 (SLEDAI-2K) [18].

Measurement of serum vitamin D levels

Serum 25(OH)D3 levels were measured by electrochemiluminescence immunoassay on an automated analyzer (ELECSYS-2010) using kits supplied by Roche Diagnostics (Germany), according to the manufacturer’s instructions. Serum 25(OH)D3 levels <10 ng/ml and 10-30 ng/ml were defined as severe vitamin D deficiency and vitamin D insufficiency, respectively.

Determination of T-cell subsets

Two milliliters of ethylenediaminetetraacetic acid (EDTA) anticoagulated venous peripheral blood were collected from the patients for the detection of T-cell subsets. Cells were labeled with PC5 anti-human CD3 antibody, FITC anti-human CD4 antibody, PE anti-human CD8 antibody (Beckman Coulter) for 30 minutes at room temperature. T-cell subsets were determined by Coulter Epics XL Flow Cytometer (Beckman Coulter) and elucidated based on differentiation of T cells. Autophagy might be activated in HIV-infected CD4+ T cells [13] and in vitro-aged human CD8+ T cells [14]. Clarke AJ, et al showed that autophagy was activated in CD4+ T cells in SLE and required for plasmablast development [15]. Autophagic vacuoles were more frequent in T cells from lupus patients compared with healthy controls [16]. Hence, we speculated that vitamin D may affect T-cell subsets via regulating autophagy. In order to confirm the speculation, we first investigate whether vitamin D levels affect the expression of Atgs and the counts of T-cell subsets in active SLE. Also, we assess the possible association between Atgs and T-cell subsets. This study aimed at describing the association between vitamin D levels, Atgs and T-cell subsets and may thus lead to design new therapeutic options for SLE.

Materials and methods

Subjects

This study was performed at the Department of Rheumatology, The First Affiliated Hospital of Zhengzhou University after the approval of the study protocol by the ethics committee (2015-53). All participants signed informed consent forms.

Table 1. Baseline characteristics of SLE patients
SLE and autophagy

CD3+ for T cells, CD3+ and CD4+ for CD4+ T cells, CD3+ and CD8+ for CD8+ T cells.

Total RNA isolation and RT-PCR analysis

Five milliliters of EDTA anticoagulated venous peripheral blood were collected for the detection of mTOR, LC3, and p62 mRNA levels in PBMCs. The PBMCs were isolated using the standard Ficoll-Hypaque density-gradient centrifugation method. Total RNA was extracted using a Trizol RNA extraction kit (Invitrogen, USA). RNA concentration and purity were measured by TU-1901 ultraviolet spectrophotometer (Beijing’s Purkinje General Instrument Co., Ltd., China). A total of 0.8 µg RNA was used to synthesize cDNA using the AMV First Strand cDNA Synthesis Kit (BBI Life Science Co., Ltd., China) according to the manufacturer’s instructions. Quantitative PCR was performed with 2 µl of cDNA, 0.4 µl of each primer (10 umol/µl) and 10 µl of SYBR Green qPCR Master Mix (BBI Life Science Co., Ltd., China) and analyzed with the LightCycler480 Software (Roche, Switzerland). The cycle threshold values were used to calculate the normalized expression of mTOR, LC3, and p62 against β-actin using the Q-Gene software [19]. The values of genes were normalized to the β-actin levels. The sequences of the primer pairs are listed below:

- β-actin, 5'-TAGTTGCGTTACACCCTTTCTTG-3' /5'-TCACCTTCACCCTCCAGTT-3';
- mTOR, 5'-TCACCTTCACCCTCCAGTT-3';
- Beclin-1, 5'-TGAGGGGATGGAAGGTTCTAAG-3'/5'-GCCTGGGCTGTGGTAAGTAATC;
- LC3, 5'-CATGAGCGAGTTGGTCAAGAT-3'/5'-TCGTTCTTTCTCTCCAGT-3';
- p62, 5'-GGGGACTGGTTGCTTTT-3'/5'-CAGCCCATCGCAGATCACATT-3'.

Statistical analysis

Statistical analyses were performed using the statistical software SPSS 21.0. The normality of continuous variables was established by means of one sample K-S test. Data were presented as the mean ± SD. Univariate comparisons between nominal variables were performed by the Chi-square test. Comparisons of continuous variables between two groups were done using the independent sample t-test in the cases of normal variables and the Mann-Whitney U-test in the cases of non-normal variables. For correlations between two continuous

Figure 1. The mRNA levels of Atgs in PBMCs in active SLE patients with different degree of vitamin D deficiency; A: The levels of mTOR mRNA were higher in severe vitamin D deficiency group compared to vitamin D insufficiency group, P=0.036; B: The levels of LC3 mRNA were lower in severe vitamin D deficiency group compared to vitamin D insufficiency group, P<0.001; C: The levels of p62 mRNA has no significant difference between severe vitamin D deficiency and vitamin D insufficiency group, P=0.667.
variables, Pearson's or Spearman's correlation was used for normal or non-normal variables, respectively. \( p < 0.05 \) was considered as statistically significant.

**Result**

**Baseline characteristics**

Among the 50 active SLE patients, 25(OH)D3 levels <10 ng/ml was found in 22 patients (44%), 10-30 ng/ml in 28 patients (56%) and none >30 ng/ml. Our previous study demonstrated that a serum concentration of vitamin D of less than 10 ng/ml increased the risk of higher disease activity. Thus, serum 25(OH)D3 levels <10 ng/ml and 10-30 ng/ml were defined as severe vitamin D deficiency and vitamin D insufficiency, respectively. The baseline characteristics of the 50 active SLE patients are summarized in **Table 1**.

The mRNA levels of mTOR, LC3, p62 in PBMCs of active SLE patients with different degree of vitamin D deficiency are shown in **Figure 1**. The mean level of mTOR mRNA was 3.98×10^{-4} in severe vitamin D deficiency group compared to 3.08×10^{-4} in vitamin D insufficiency group (\( P=0.036 \)). The mean level of LC3 mRNA was 3.97×10^{-5} in severe vitamin D deficiency group, which was significantly different from 7.89×10^{-5} in vitamin D insufficiency group (\( P<0.001 \)). However, the mean level of p62 mRNA has no significant difference between severe vitamin
The counts of CD3+, CD4+, CD8+ T cells and the CD4/CD8 ratio of active SLE patients with different degree of vitamin D deficiency

The counts of CD3+, CD4+, CD8+ T cells and the CD4/CD8 ratio of active SLE patients with different degree of vitamin D deficiency are shown in Figure 2. The mean counts of CD3+ T cells were 812/µl, 790/µl in severe vitamin D deficiency and vitamin D insufficiency group, respectively, the difference was not significant (P=0.667). Whereas, the mean counts of CD4+ T cells were significantly higher (P=0.001) in severe vitamin D deficiency group (393/µl) in comparison with that in vitamin D insufficiency group (296/µl) and the CD4/CD8 ratio was also significantly higher (P<0.001) in severe vitamin D deficiency group when compared with that in vitamin D insufficiency group. In contrast, significantly lower of CD8+ T cells counts were observed in severe vitamin D deficiency group (381/µl VS 459/µl; P=0.012).

Correlation between the levels of mTOR, LC3 mRNA in PBMCs and the counts of CD3+, CD4+, CD8+ T cells in active SLE patients

Correlation analysis was carried out to identify the possible correlations between the levels of mTOR, LC3 mRNA in PBMCs and the counts of CD3+, CD4+, CD8+ T cells in active SLE patients. There were no significant correlations between the levels of mTOR mRNA and the counts of CD3+ T cells (r=0.116, P=0.423), as well as CD8+ T cells (r=-0.136, P=0.348), while a significant positive correlation was observed between the levels of mTOR mRNA and the counts of CD4+ T cells (r=0.326, P=0.021) (Figure 3).

No significant correlation was observed between the levels of LC3 mRNA in PBMCs and the counts of CD3+ T cells, (r=0.007, P=0.946). A negative correlation was observed between the levels of LC3 mRNA in PBMCs and the counts of CD4+ T cells (r=-0.302, P=0.033), and a positive correlation was observed between the levels of LC3 mRNA in PBMCs and the counts of CD8+ T cells (r=0.299, P=0.035), respectively (Figure 4).

Correlation between serum 25(OH)D3 levels and the counts of CD3+, CD4+, CD8+ T cells in active SLE patients

There was no significant correlation between serum 25(OH)D3 levels and the counts of CD3+
SLE and autophagy

Discussion

Low levels of vitamin D are prevalent among SLE patients and have been shown to negatively correlate with disease activity [20-22], yet the underlying mechanisms remain largely unknown. Therefore, it is meaningful to research vitamin D. Previous data showed that autophagy induced by vitamin D inhibited both Mycobacterium tuberculosis and human immunodeficiency virus type 1 [23], which suggests that autophagy might play a general role in multiple health-promoting effects of vitamin D. The role of autophagy in the pathogenesis of SLE has been received considerable attention since the association between the PRDM1-ATG5 gene region and SLE was found by GWAS [24]. However, no consensus has been reached regarding vitamin D and its immunomodulatory role via autophagy and there were few studies of the correlation between autophagy and serum 25(OH)D3 levels in active SLE. In this study, we found that vitamin D levels affected the expression of Atgs and T-cell subsets in active SLE and Atgs were correlated with the counts of T-cell subsets.

The role of vitamin D in autophagy is inconsistent in different diseases. In the Parkinson’s disease model with rotenone-induced neurotoxicity, 1,25(OH)2D3 decreased mTOR levels and increased levels of LC3 and beclin-1 to attenuate the neurotoxicity [25]. In addition, treatment of a C6 glioblastoma cell line with temozolomide (TMZ) and vitamin D resulted in significantly increasing number of LC3 puncta compared with the cells treated with TMZ alone [26]. Consistent with these previous observations, higher expression of mTOR mRNA and lower expression of LC3 mRNA in severe vitamin D deficiency group were observed in the present study, which suggested that autophagy was decreased in severe vitamin D deficiency patients with active SLE. However, a report showed that 1,25(OH)2D3 decreased the number of autophagosomes and the protein expression of LC3-II and p62 in experimental autoimmune myocarditis mice to improve cardiac function and diminish cell infiltration [27]. This is probably because vitamin D may regulate autophagy at different levels. It can induce autophagy to reduce cell apoptosis and atten-

Figure 4. Correlation between the levels of LC3 mRNA in PBMCs and the counts of CD3+, CD4+, CD8+ T cells in active SLE patients; A: There was no significant correlation between the levels of LC3 mRNA in PBMCs and the counts of CD3+ T cells, r=-0.007, P=0.946; B: The levels of LC3 mRNA in PBMCs correlated negatively with the counts of CD4+ T cells, r=-0.302, P=0.033; C: The levels of LC3 mRNA in PBMCs correlated positively with the counts of CD8+ T cells, r=0.299, P=0.035.

T cells (r=-0.069, P=0.632). Serum 25(OH)D3 levels correlated negatively with the counts of CD4+ T cells (r=-0.423, P=0.002) and correlated positively with the counts of CD8+ T cells (r=0.318, P=0.024) (Figure 5).
SLE and autophagy

Increasing evidences showed that vitamin D was crucial for lymphocytes development and differentiation. In the present study, higher CD4+ T cells counts and CD4/CD8 ratio and lower CD8+ T cells counts were observed in severe vitamin D deficiency group. What’s more, serum 25(OH)D3 levels correlated negatively with CD4+ T cells counts and correlated positively with CD8+ T cells counts. Study by Bang U and colleagues [28] reported a positive correlation between 1,25(OH)2D3 and activated CD4+ T lymphocytes in HIV infected males. In animals with experimental autoimmune encephalomyelitis [29], apoptotic CD4+ T cells were evident and the CD4+ T cells numbers reduced by 63% with calcitriol treatment, which indicated that vitamin D may decreased CD4+ T cells by increasing its sensitivity to apoptotic signals. In addition, inhibition of vitamin D against differentiation of Th17 cells was found in animal studies [30] and in CD4+ T cells culture of SLE patients [31]. Also, vitamin D has been found to have a direct immunomodulatory effect on CD8+ T cells of patients with early multiple sclerosis and healthy control subjects [32]. Smolders J, et al [33] showed an association between 25(OH)D3 levels and Treg function in multiple sclerosis patients and skewing of the Th1/Th2 balance towards Th2, which suggest the important role of vitamin D in T cell regulation. However, the association between 25(OH)D3 levels and the individual percentages of CD4+ T helper cells was not observed.

Autophagy has been reported to play a role in CD4+ T cells death induced by HIV [13]. Additionally, autophagy was also essential for effector CD8+ T cells survival and memory formation in response to influenza infection [34]. In mice, ATG5 or ATG7 deficiency was shown to reduce CD4+ and CD8+ T cells numbers in both thymus and periphery [35, 36]. In our study, the counts of CD4+ T cells correlated positively with mTOR mRNA and negatively with LC3 mRNA. On the contrary, a significant positive correlation was observed between LC3 mRNA and the counts of CD8+ T cells. However, no significant correlations were observed between the levels of mTOR mRNA and LC3 mRNA and CD3+ T cells. Our data indicated that au-
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tophagy may influence the differentiation of CD4+ and CD8+ T cells but not T-cells counts.

Unfortunately, we could not avoid the influence of glucocorticoids and immunosuppressants completely, since all the participants were active SLE. Thus, further experimental and clinical studies are needed. In our follow-up studies, we will ameliorate our study design, probably by expanding the sample size and in vitro method.

In conclusion, severe vitamin D deficiency affects the expression of autophagy related genes in PBMCs and T-cell subsets in active systemic lupus erythematosus, and further mechanism will be researched in the follow-up studies.

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Disclosure of conflict of interest

None.

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