

## Original Article

# Expression of autophagy related genes mTOR, Becline-1, LC3 and p62 in the peripheral blood mononuclear cells of systemic lupus erythematosus

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**Abstract:** To determine the expression of mTOR, Becline-1, LC3 and p62 in the peripheral blood mononuclear cells (PBMCs) of systemic lupus erythematosus (SLE) and assess their relationship with disease activity and immunologic features. The expression of mTOR, Becline-1, LC3 and p62 was detected by RT-PCR in 81 SLE subjects and 86 age- and sex-matched healthy controls. Data regarding demographics and clinical parameters were collected. Disease activity of SLE was evaluated according to the SLE Disease Activity Index (SLEDAI) score. Independent sample t-test was used to analyze the expression of mTOR, Becline-1, LC3, and p62 in the two groups. Pearson's or Spearman's correlation was performed to analyze their relationship with disease activity and immunologic features. The mean levels of Becline-1, LC3 and p62 mRNA were significantly higher in SLE patients than the controls ( $9.96 \times 10^{-4}$  vs  $7.38 \times 10^{-4}$  for Becline-1 with  $P < 0.001$ ;  $4.04 \times 10^{-5}$  vs  $2.62 \times 10^{-5}$  for LC3 with  $P < 0.001$ ;  $9.51 \times 10^{-4}$  vs  $7.59 \times 10^{-4}$  for p62 with  $P = 0.008$ ). However, the levels of mTOR mRNA in SLE patients were not significantly different from that in controls. Correlation analysis showed that Becline-1, LC3 and p62 mRNA levels correlated positively with SLEDAI, IgG and ds-DNA, negatively with C3. Our results suggested that autophagosomes formation were activated and their degradation were blocked in SLE. Moreover, the maintenance of autophagy balance can improve disease activity and immune disorders in SLE patients.

**Keywords:** Systemic lupus erythematosus, autophagy, mTOR, Becline-1, LC3, p62

## Introduction

Autophagy is an evolutionarily conserved lysosome-mediated catabolic process that maintains cellular homeostasis through the degradation of unwanted cytoplasmic constituents and recycling nutrients [1]. mTOR, Becline-1, LC3, and p62 are central autophagy related genes (Atgs) involved in the autophagy flux. Detailedly, mTOR regulates autophagy through the signaling pathways upstream of the autophagy machinery [2], Becline-1 and LC3 regulate autophagosomes formation [3, 4], and p62 is associated with the degradation of autophagosomes [5]. Autophagy participates in nearly all aspects of immunity, especially in the normal development and function of T and B lymphocyte populations [6-8]. When autophagy becomes inefficient, it may paradoxically lead to diverse autoimmune and chronic inflammatory diseases [9-13].

SLE is a prototype of chronic autoimmune inflammatory disease characterized by T and B cells dysfunction, and production of antinuclear antibodies, through mechanisms which are poorly understood [14]. Genome-wide association studies (GWAS) have linked polymorphisms in Atgs to SLE [15]. The role of autophagy as a key factor in lymphocyte biology has emerged over the last few years. However, few studies were available regarding the role of autophagy in SLE, what's more, the dynamic process of autophagy flux in SLE has never been published so far. Therefore, the present study is of great significance in SLE and may impact our clinical practice.

We analyzed the expression of Atgs mTOR, Becline-1, LC3, and p62 in the PBMCs of SLE patients and healthy controls at the first time. Furthermore, we also analyzed the correlations between the expression of Atgs and SLEDAI as

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**Table 1.** Baseline characteristics of SLE patients (N=81)

Characteristics	$\bar{x} \pm S/n$ (%)
Female sex	73 (90.12)
Age (year)	33.85±10.71
Disease duration (month)	50.93±48.48
Malar rash	12 (14.81)
Alopecia	38 (46.91)
Oral ulcer	9 (11.11)
Arthritis	11 (13.58)
Serositis	14 (17.28)
Central nervous system involvement	3 (3.70)
Vasculitis	3 (3.70)
Hematological system involvement	10 (12.35)
Renal involvement	38 (46.91)
SLEDAI	
≤4	31 (38.28)
5-9	25 (30.86)
>10	25 (30.86)
Immunologic features	
ANA positive	76 (93.82)
Anti-dsDNA positive (>100 IU/ml)	46 (56.79)
Low C3 levels (<0.9 g/L)	39 (48.14)
Low C4 levels (<0.1 g/L)	29 (35.80)
High IgG levels (>16 g/L)	28 (34.57)
Treatment, n (%)	
Glucocorticoids	78 (90.12)
Mycophenolate mofetil	39 (48.15)

SLE: Systemic lupus erythematosus; SLEDAI: Systemic lupus erythematosus disease activity index; ANA: Antinuclear antibodies; ESR: Erythrocyte sedimentation rate; CRP: C reactive protein; C3: Complement 3; C4: Complement 4; IgG: Immunoglobulin G; Reference range of C3: 0.9-1.8 g/L; Reference range of C4: 0.1-0.4 g/L; Reference range of IgG: 7-16 g/L; Reference range of dsDNA: 0-100 IU/ml.

well as immunologic features. Understanding autophagy and misregulation of this catabolic process has become an important goal in conceptualizing what goes wrong in SLE. We believe the present study will broaden our visions about SLE pathogenesis and provides a potentially new therapeutic avenue for SLE.

## Materials and methods

### Patients

This study was performed at the Department of Rheumatology, The First Affiliated Hospital of Zhengzhou University. All the participants signed informed consent forms according to

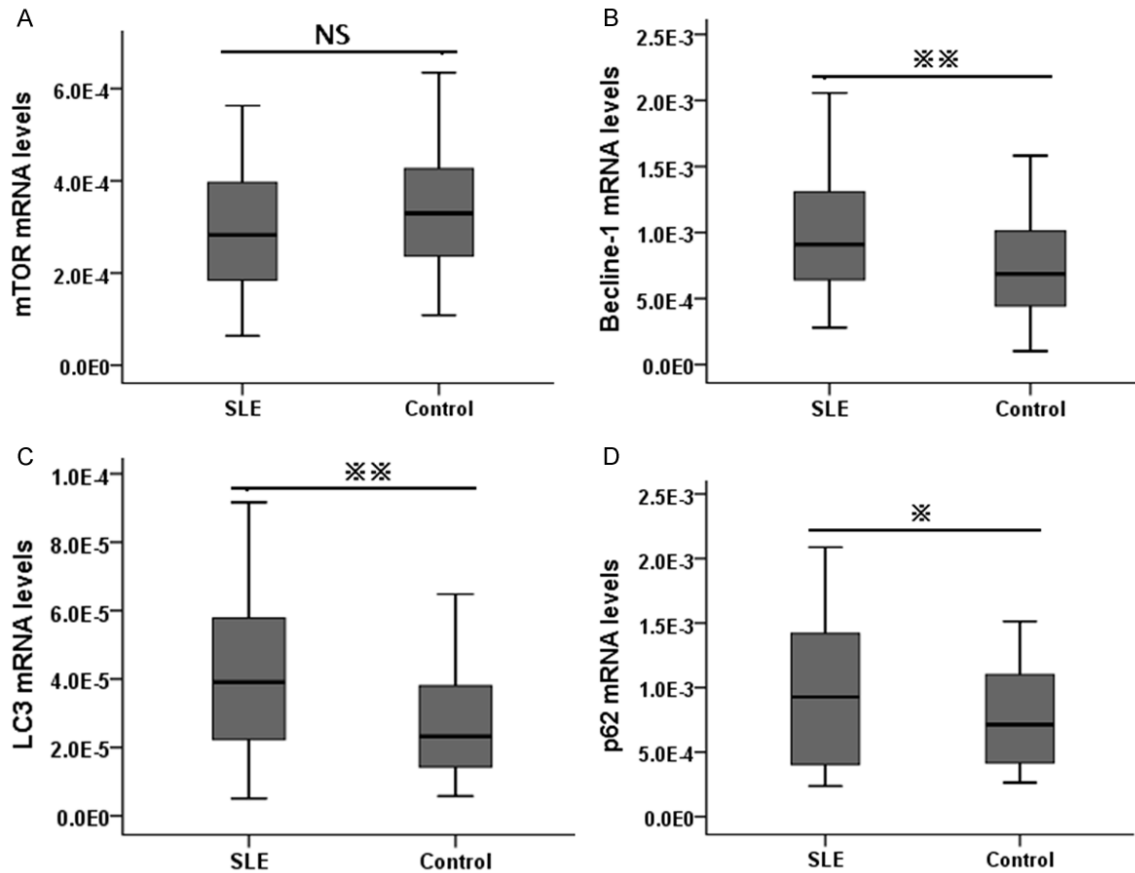
the Ethic Committee for the Conduct of Human Research protocol. Data regarding demographics and clinical parameters were collected.

For this study, 81 patients with SLE were enrolled from October of 2015 to May of 2016. All participants met at least four of the American College of Rheumatology (ACR) [16] revised criteria for SLE. Patients were excluded in the study if they met the following criteria: (1) age <18 years old or >60 years old; (2) pregnancy or lactation; (3) treatment with immunosuppressive agents other than mycophenolate mofetil; (4) coexistence of other autoimmune diseases such as rheumatoid arthritis, systemic sclerosis, or chronic diseases affecting autophagy such as systemic infection, cancers, diabetes mellitus, neurodegeneration, and heart diseases, etc. Disease activity was assessed in accordance with the SLE Disease Activity Score (SLEDAI 2000 (SLEDAI-2K)) [17]. C3, C4, IgG were detected by immuno turbidimetry, dsDNA was detected by enzyme linked immuno sorbent assay (ELISA).

In addition, 86 healthy volunteers matched for age, sex were recruited as the control group. Five milliliters of ethylenediaminetetraacetic acid (EDTA) anticoagulated venous peripheral blood were collected from the patients and healthy volunteers for the detection of mTOR, Becline-1, LC3, and p62 mRNA levels in PBMCs.

### Total RNA isolation and RT-PCR analysis

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 was 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 µmol/µl) and 10 µl of SybrGreen 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, Becline-1, LC3, and p62 against β-actin using the Q-Gene software [18]. The sequences of the



**Figure 1.** The mRNA levels of mTOR, Beclin-1, LC3, p62 in PBMCs of SLE patients. A. The mRNA levels of mTOR in PBMCs of SLE patients ( $P=0.080$ ); B. The mRNA levels of Beclin-1 in PBMCs of SLE patients ( $P<0.001$ ); C. The mRNA levels of LC3 in PBMCs of SLE patients ( $P<0.001$ ); D. The mRNA levels of p62 in PBMCs of SLE patients ( $P=0.080$ ). \*\* refers to  $P<0.001$ ; \* refers to  $P<0.05$ .

primer pairs are listed below:  $\beta$ -actin, 5'-TAG-TTGC GTTACACCCTTTCTTG-3'/5'-TCACCTTCA-CCGTTCCAGTT-3'; mTOR, 5'-TCACATTACCCCT-TCACCA-3'/5'-TCAGCGAGTTCTTGCTATTCC-3'; Beclin-1, 5'-TGAGGGATGGAAGGGTCTAAG-3'/5'-GCCTGGGCTGTGGTAAGTAATC; LC3, 5'-CATGAGCGAGTTGGTCAAGAT-3'/5'-TCGTCTTTCTCCTGCTCGTAG-3'; p62, 5'-GGGACTTGGTTGCCTT-3'/5'-CAGCCATCGCAGATCACATT-3'.

#### Statistical analysis

All 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. Variables were summarized using the mean and standard deviation (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 case of

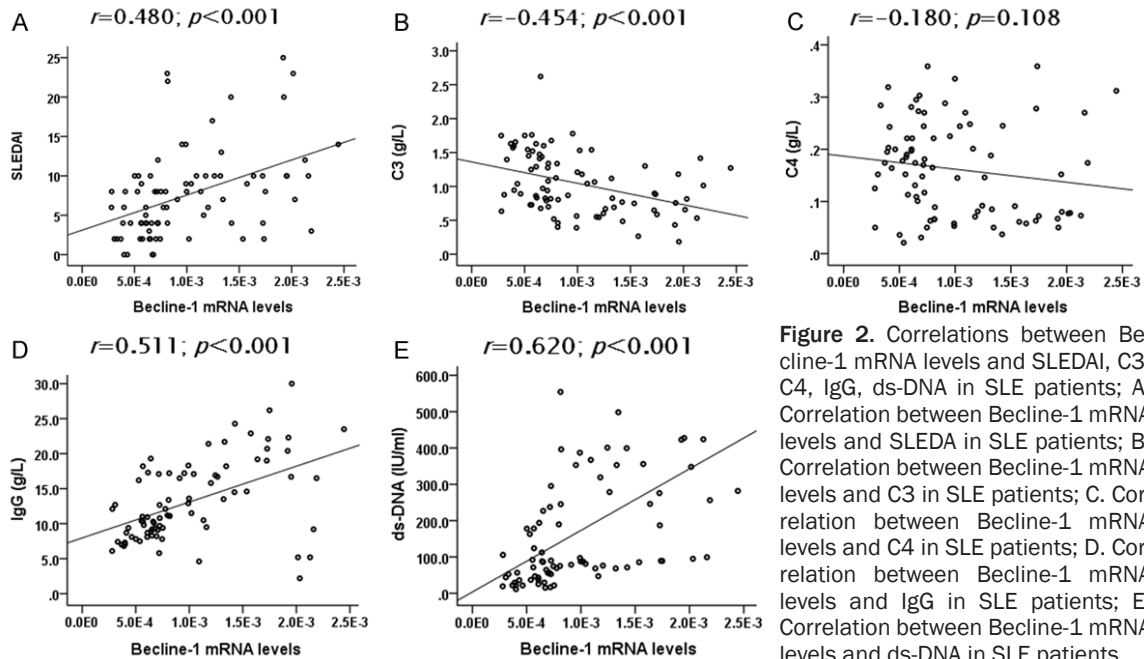
normal variables and the Mann-Whitney U-test in the case of non-normal variables. For correlations between two continuous variables, Pearson's or Spearman's correlation was used for normal or non-normal variables, respectively.  $P<0.05$  was considered as statistically significant.

#### Result

##### Baseline characteristics of study subjects

The baseline characteristics of the 81 SLE patients included in this study are summarized in **Table 1**. Among these patients, 73 (90.12%) were female, with the mean age at the time of the study being  $33.85\pm 10.71$  years and the mean disease duration of  $50.93\pm 48.48$  months. The SLEDAI score was below 4 in 31 patients (38.28%), 5-9 in 25 patients (30.86%), and above 10 in the remaining 25 patients (30.86%). Some 73 (90.12%) patients were tak-

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**Figure 2.** Correlations between Beclin-1 mRNA levels and SLEDAI, C3, C4, IgG, ds-DNA in SLE patients; A. Correlation between Beclin-1 mRNA levels and SLEDAI in SLE patients; B. Correlation between Beclin-1 mRNA levels and C3 in SLE patients; C. Correlation between Beclin-1 mRNA levels and C4 in SLE patients; D. Correlation between Beclin-1 mRNA levels and IgG in SLE patients; E. Correlation between Beclin-1 mRNA levels and ds-DNA in SLE patients.

ing prednisone, 39 (48.15%) were taking Mycophenolate mofetil.

For comparison, 86 healthy subjects were recruited to this study. Among them, 77 (89.53%) were female with a mean age of  $34.47 \pm 9.72$  ( $P=0.303$ ) compared with SLE patients.

### *The expression of mTOR, Beclin-1, LC3, and p62 in the PBMCs of SLE patients*

The levels of mTOR, Beclin-1, LC3, p62 mRNA in PBMCs of SLE patients are shown in **Figure 1**. The mean level of mTOR mRNA were  $2.97 \times 10^{-4}$ ,  $3.31 \times 10^{-4}$  in SLE patients and controls respectively, the difference between the two groups was not significant ( $P=0.080$ ). The mean level of Beclin-1 mRNA was significantly higher ( $P<0.001$ ) in SLE patients ( $9.96 \times 10^{-4}$ ) in comparison with that in controls ( $7.38 \times 10^{-4}$ ). The mean level of LC3 mRNA was  $4.04 \times 10^{-5}$  in SLE patients, which was significantly different from  $2.62 \times 10^{-5}$  in controls ( $P<0.001$ ). In addition, the mean level of p62 mRNA in SLE patients ( $9.51 \times 10^{-4}$ ) was also higher ( $P=0.008$ ) than the controls ( $7.59 \times 10^{-4}$ ).

Difference in Beclin-1 ( $P=0.355$ ), LC3 ( $P=0.299$ ), p62 ( $P=0.185$ ) mRNA levels between patients treated with mycophenolate mofetil ( $n=39$ ) compared to patients who didn't receive ( $n=42$ ) were not significant. And there

were no significant correlations between Beclin-1 ( $r=0.056$ ;  $P=0.355$ ), LC3 ( $r=0.070$ ;  $P=0.536$ ), p62 ( $r=-0.021$ ;  $P=0.852$ ) mRNA levels and glucocorticoid dosage.

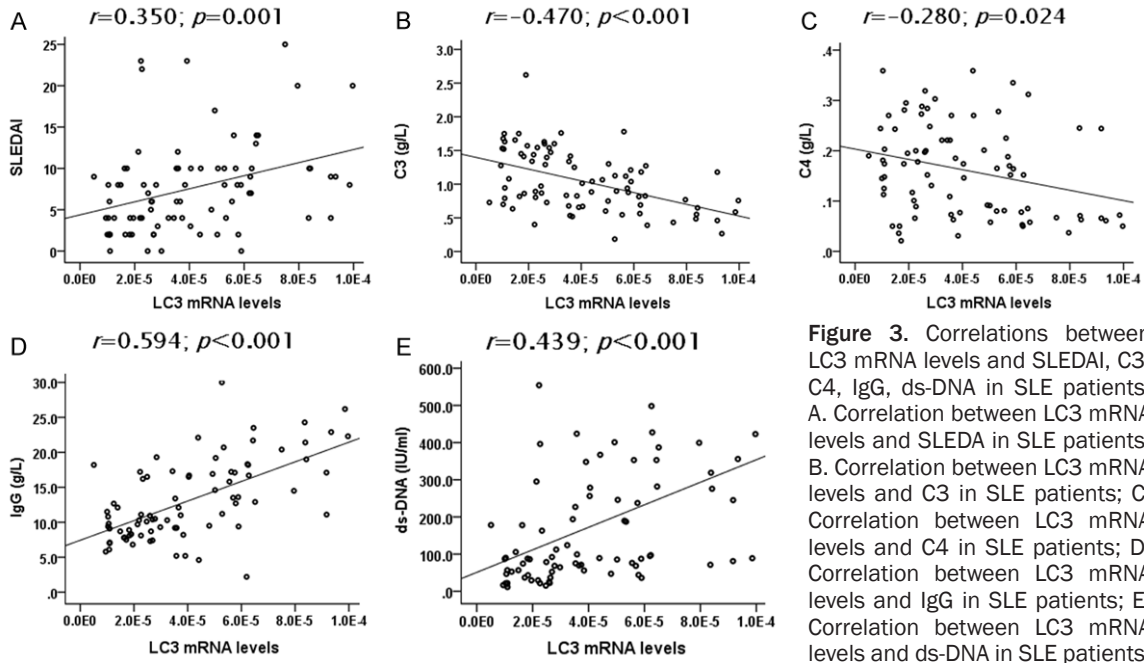
### *Correlations between the expression of Beclin-1, LC3, p62 in PBMCs and SLEDAI as well as immunologic features*

There were positive correlations between Beclin-1 mRNA levels and SLEDAI ( $r=0.480$ ;  $P<0.001$ ), IgG ( $r=0.511$ ;  $P<0.001$ ), as well as anti-dsDNA ( $r=0.620$ ;  $P<0.001$ ), negative correlation between Beclin-1 mRNA levels and C3 ( $r=-0.454$ ;  $P<0.001$ ). No significant correlation was found between Beclin-1 mRNA levels and C4 ( $r=-0.180$ ;  $P=0.108$ ) (**Figure 2**).

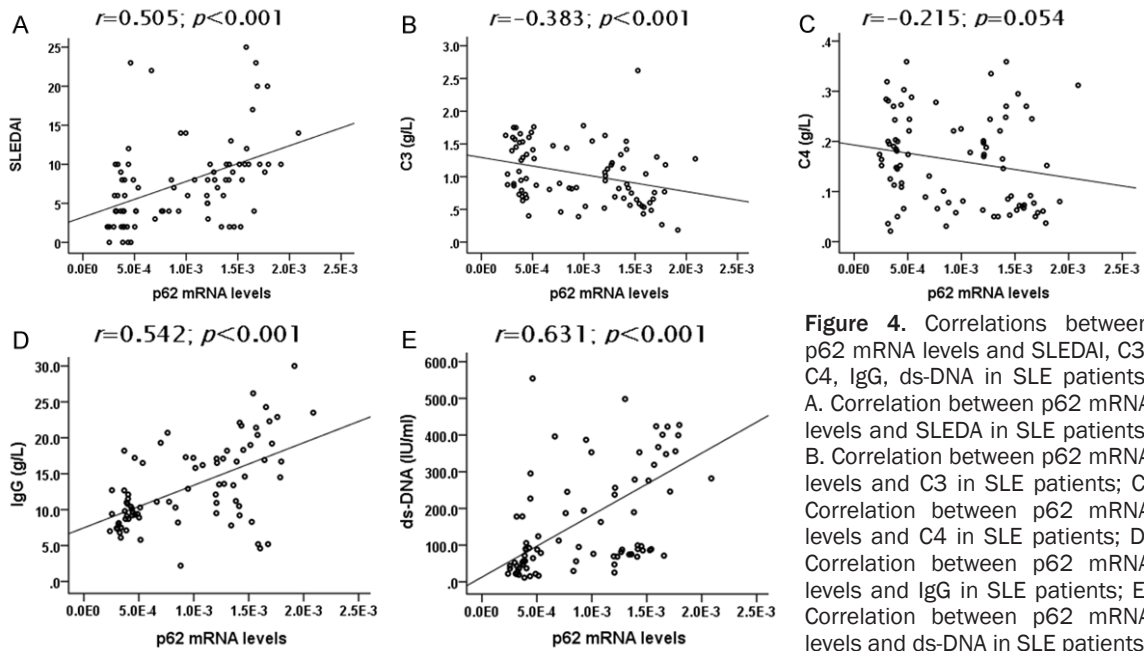
LC3 mRNA levels showed positive correlations with SLEDAI ( $r=0.350$ ;  $P=0.001$ ), IgG ( $r=0.594$ ;  $P<0.001$ ) and anti-dsDNA ( $r=0.439$ ;  $P<0.001$ ), negative correlations with C3 ( $r=-0.470$ ;  $P<0.001$ ) and C4 ( $r=-0.251$ ;  $P=0.024$ ) (**Figure 3**).

p62 mRNA levels correlated positively with SLEDAI ( $r=0.505$ ;  $P<0.001$ ), IgG ( $r=0.542$ ;  $P<0.001$ ) and anti-dsDNA ( $r=0.631$ ;  $P<0.001$ ), negatively with C3 ( $r=-0.383$ ;  $P<0.001$ ). The correlation between p62 mRNA levels and C4 ( $r=-0.215$ ;  $P=0.054$ ) was not significant (**Figure 4**).

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**Figure 3.** Correlations between LC3 mRNA levels and SLEDAI, C3, C4, IgG, ds-DNA in SLE patients; A. Correlation between LC3 mRNA levels and SLEDAI in SLE patients; B. Correlation between LC3 mRNA levels and C3 in SLE patients; C. Correlation between LC3 mRNA levels and C4 in SLE patients; D. Correlation between LC3 mRNA levels and IgG in SLE patients; E. Correlation between LC3 mRNA levels and ds-DNA in SLE patients.



**Figure 4.** Correlations between p62 mRNA levels and SLEDAI, C3, C4, IgG, ds-DNA in SLE patients; A. Correlation between p62 mRNA levels and SLEDAI in SLE patients; B. Correlation between p62 mRNA levels and C3 in SLE patients; C. Correlation between p62 mRNA levels and C4 in SLE patients; D. Correlation between p62 mRNA levels and IgG in SLE patients; E. Correlation between p62 mRNA levels and ds-DNA in SLE patients.

## Discussion

The complete autophagic flux can be divided into several stages, including initiation, elongation and closure, maturation of autophagosomes, fusion with lysosomes as well as breakdown and release of macromolecules back to the cytosol. This work showed for the first time a clear deregulation of autophagic flux in the PBMCs of SLE.

Measurement the expression of Becline-1 and LC3 are widely used to reliably quantify autophagosomes formation. No more than one report [19] showed that Becline-1 was significantly upregulated in the PBMCs of SLE patients. Several studies have indicated that the expression of LC3 was increased in SLE patients or lupus-prone mice. Alexander J [20] and colleagues demonstrated that LC3 were increased in T and B cells in human SLE. A

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French report [21] showed that LC3-II was significantly increased in T cells from lupus-prone mice. In our present study, we found that Becline-1 and LC3 were both high expressed in the PBMCs of SLE, and the results were in accordance with theirs to some extent. Based on these studies, it was possible to conclude that autophagosomes formations were activated in SLE. Apart from this, we found that the expression of Becline-1 and LC3 correlated positively with SLEDAI as well, which may suggest that activated autophagosomes formation was not only involved in the occurrence but also the progression of lupus.

mTOR is a master regulator of cellular metabolism and plays a crucial role in regulating autophagy. Growing evidence indicates that there was a tight, inverse coupling of autophagy induction and mTOR activation. Pattingre S and his colleagues [22] demonstrated that when energy sources were deficiency, mTOR activity was low, autophagy was upregulated to recycle nutrients. When energy and nutrients were readily available, mTOR was active and autophagy was downregulated. Meijer AJ [23] also pointed out that activated mTOR inhibited autophagosome formation. Surprisingly, in the present study, we didn't demonstrate a significant inverse relationship between autophagosomes formation and mTOR activation. The reason for this phenomenon remains unclear, but several possibilities should be considered. Firstly, autophagy is regulated by multiple signaling pathways, including mTOR signaling pathway, Ras/cAMP-dependent protein kinase pathway [24] and the LKB1-AMPK signal pathway, etc [25]. In SLE, other signaling pathways rather than mTOR may play more important role in regulating autophagy. More mechanism researches are needed in the future to clarify which signaling pathway is the primary regulator of autophagy in SLE. Secondly, mTOR includes two distinct signaling complexes, mTORC1 and mTORC2. Activation of mTORC1 leads to inhibition of autophagy, while mTORC2 is not a direct autophagy regulator, and the molecular mechanism of mTORC2 regulation by upstream effectors is largely unknown. Furthermore, the activity of mTOR is determined by plenty of regulators, such as nutrients, growth factors, energy, and stress, etc [2].

The accumulation of autophagic vacuoles is due to disequilibrium between autophagosome

formation and capacity of lysosomal degradation. p62 is a central marker of autophagic degradation. The loss of Atgs or factors required for the fusion of autophagosomes with lysosomes all results in a prominent increase of p62-positive aggregates [5]. Our present study showed that the expression of p62 in SLE was higher than controls, which may suggest that there was a blockage of downstream actions of autophagy flux. Moreover, p62 plays a role in the induction of autophagy. A recent report found TLR7-induced autophagy depended on p62 expression [26]. In addition, p62 can relay the signal from amino acids to the mTORC1 pathway to prevent excessive autophagy [5], which may also explain why the expression of mTOR was not decreased in SLE. In consideration of the high expression of Becline-1, LC3 and p62, we speculate that autophagic vacuoles were accumulated in SLE.

Autophagy integrates with the degradation of waste proteins, if autophagy flux is inefficiency, invalid proteins will accumulate. Some evidence have emerged to support that impaired clearance of apoptosis proteins may increase the amount of nuclear antigens presented to T lymphocytes. And that, this process was accompanied by autoimmune responses that can lead to the development of lupus [27]. In this study, we demonstrated that the expression of Becline-1, LC3 and p62 correlated positively with SLEDAI, and also correlated with certain immunologic features in SLE. Based on these observations, it was possible for us to postulate that autophagy might play an important role in the pathogenesis of SLE.

Different immunosuppressive agents may have different effect on the autophagy process, and yet there was no clear evidence have proved that mycophenolate mofetil could affect the expression of Atgs Becline-1, LC3 and p62. We eliminated those patients who treated with immunosuppressive agents other than mycophenolate mofetil in our work. In order to exclude the influence of mycophenolate mofetil on Becline-1, LC3 and p62 mRNA levels, we used a subanalysis to explore differences between patients who treated with mycophenolate mofetil and those who didn't, which showed no difference in Becline-1, LC3, p62 mRNA levels between patients treated with mycophenolate mofetil and patients who didn't receive. Correlation analysis showed that there were

no significant correlations between Beclin-1, LC3, p62 mRNA levels and glucocorticoid dosage. However, we may not be able to avoid the influence of mycophenolate mofetil and glucocorticoids completely, thus, further experimental and clinical studies are needed.

The present study will broaden our visions of autophagy flux and may facilitate the identification of precise therapeutic targets of SLE. Most compounds have been described as potential regulators of the autophagy process now, however, most of them are poorly selective. Through in-depth understanding of autophagy process and definitizing problematical stages, we could deal with selective tools to manipulate autophagy.

mTOR includes two distinct signaling complexes, mTORC1 and mTORC2. LC3 also involves two forms, LC3-I and LC3-II. Unfortunately, we didn't detect the levels of different hypotype of mTOR and LC3. However, even with these limitations, the findings of this study revealed the vital position of autophagy in SLE to a certain degree. This represents an important area for future research.

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## Disclosure of conflict of interest

None.

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