Decreased expression of *BECN1* mRNA is associated with lupus nephritis.

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Abstract

This study aimed to investigate the mRNA expression of the autophagy-related gene *BECN1* in Systemic Lupus Erythematosus (SLE) and to analyse the relationship of its expression with SLE clinical characteristics and disease activities. Quantitative polymerase chain reaction was performed to detect the mRNA expression levels of *BECN1* in 79 SLE patients and 45 healthy controls. *BECN1* was downregulated in the 79 patients with SLE (P<0.001), and those with downregulated *BECN1* mRNA exhibited a significantly increased incidence of nephritis (85.3% *vs.* 64.4%, P=0.038). *BECN1* mRNA downregulation, which may be related to deficiency in autophagosome formation, can promote the pathogenesis of SLE and nephritis.

Introduction

Systemic Lupus Erythematosus (SLE) is an autoimmune disease characterized by the generation of nuclear-targeting autoantibodies, consequently resulting in autologous systemic damages [1]. Although inflammation and vasculitis induced by the deposition of immune complexes are considered the pathological basis of SLE, its precise mechanism is still unknown [2,3]. Autophagy is a lysosome-mediated metabolic process that degrades and reuses cytoplasmic components specifically in old or damaged organelles, thus playing an important role in maintaining intracellular homeostasis [4-6]. In the immune system, autophagy can eliminate intracellular nucleic acids and other wastes; thus, it is an important process in innate immune responses. In addition, autophagy participates in the intrathymic-positive and -negative selection of lymphocytes; it eliminates intracellular antigenic materials, processes them into antigenic polypeptides, and commits them to immune cells, thus playing vital roles in acquired immunity [7,8]. Current studies have indicated that the abnormal expression of autophagy genes may be related to the occurrence of autoimmune diseases such as Crohn's disease, multiple sclerosis, or rheumatoid arthritis [9-11].

Autophagy begins with the formation of a complex that initiates phagophore membrane formation, involving *BECN1*, class III phosphoinositide 3-kinases, Vacuolar Protein Sorting 34 (VPS34), and other autophagy proteins. *BECN1* is a key protein in autophagy initiation and is considered the gatekeeper of autophagy. *BECN1* mRNA has been confirmed to be upregulated in murine experimental autoimmune neuritis, indicating that changes in *BECN1* expression are involved in the pathological process of autoimmune diseases [12,13].

It has been reported that the abnormal expressions of the autophagy genes *ATG5*, *ATG7*, *LC3*, *HSPA*, *UVRAG*, and *IRGM* are related to an imbalance of T-cell functions in patients with SLE [14], and this imbalance plays a key role in the pathogenesis of SLE. However, whether the expression of *BECN1*, the gatekeeper of autophagy, is altered in patients with SLE and related to the clinical manifestations of SLE remain unclear. Therefore, we determined *BECN1* expression in patients with SLE to investigate its expression pattern and relevance to the clinical manifestations of SLE.

Materials and Methods

Subjects

A total of 79 inpatients and outpatients were enrolled from Xinxiang People's Hospital, the Affiliated Hospital of North Sichuan Medical College, and West China Hospital of Sichuan University from June 2011 to December 2012. The patients included 13 males and 66 females, ranging from 15 to 58 years with a mean age of 33.2 years. Disease duration ranged from 0.5 to 173 months with an average of 29.7 months. All patients

met the classification criteria for SLE issued by the American Rheumatism Association (ARA) in 1997, and SLE Disease Activity Index (SLEDAI) were used to determine disease activity. In addition, 45 gender- and age-matched healthy volunteers were selected as the control group, including 5 males and 40 females, ranging from 16 to 60 years with a mean age of 30.4 years. This study followed the ethical standards, and was approved by the Ethics Committee of our department, and all study subjects signed the informed consent.

Reagents and equipment

The human lymphocyte separation medium Ficoll-Hypaque (Tianjin Haoyang Biological Manufacture Co., Ltd, Tianjinn China), the total RNA extraction reagent Trizol (Takara Co.), the ReverTra Ace qPCR RT Kit (BRK 100T), and the 7900 HT Fast Real-Time PCR System (ABI, USA) were used in this study.

Specimen collection and peripheral blood mononuclear cell (PBMC) separation

From each study subject, 5 ml of peripheral blood was collected early in the morning. PBMC was separated from the collected blood, using Ethylenediaminetetraacetic acid (EDTA)-K_2 anticoagulant and Ficoll-Hypaque following the density gradient centrifugation method according to protocol. Monocytes were then removed according to the wall adherence method [14]. The cells obtained were then counted, and peripheral blood lymphocytes were collected for total RNA extraction.

Total RNA extraction

According to protocol, total RNA was extracted using Trizol reagent, dissolved in Diethyl Pyrocarbonate (DEPC)-treated deionized water, and stored at-80°C. UV spectrophotometry was performed to analyse its purity and concentration.

cDNA synthesis

cDNA was synthesized according to the instructions of the ReverTra Ace qPCR RT Kit. The oligo (dT) 18 Primer was selected, and 9 μL of RNA solution was reverse transcribed into cDNA and stored at-20°C.

Reverse transcription-polymerase chain reaction (RT-PCR)

The primers of the target gene (Gene ID: 8678) and the internal reference GAPDH (Gene ID: 2597) for RT-PCR were synthesized by BIONEER Co., (Pudong, Shanghai, China).

The total volume (20 μl) consisted of 8 μl of ddH$_2$O, 9 μl of SYBR mixture, 0.5 μl of forward primer (10 μmol/L), 0.5 μl of reverse primer (10 μmol/L), and 2 μl of cDNA. PCR cycling conditions were 95°C for 30 s, 95°C for 5 s, 52°C for 5 s, and 72°C for 30 s, with a total of 40 cycles. The results of RT-PCR are expressed in cycle number (Ct), the point in the reaction at which the fluorescence signal in each tube reached the preset

threshold; the greater the C_t value, the lower the amount of the starting template, and vice versa. The ΔC_t value was obtained by subtracting the C_t value of the housekeeping gene GAPDH from the gene C_t value; the greater the ΔC_t value, the lower the gene expression level. RQ=$2^{-(\Delta\Delta Ct)}$ was used to determine the mRNA expression level; the higher the RQ value, the higher the mRNA expression level [3]. The reaction was performed using the ABI 7900HT Fast Real-Time PCR System, and each run included a template-free control. All experiments with the specimens and controls were repeated to obtain an average value.

Statistical analysis

Measurement data are expressed as $\bar{x} \pm s$. The comparison of measurement data between two groups was performed by t-test, comparison of count data was performed by Pearson's Chi-square (χ^2) test, and comparison of measurement data among multiple groups was performed by one-way ANOVA. All data were processed using the SPSS 11.5 software package with P<0.05 considered as statistically significant.

Results

RT-PCR

The expression level of *BECN1* mRNA (RQ) in patients with SLE was lower than that in healthy controls (P <0.001) (Figure 1).

Correlations between BECN1 mRNA expression level and SLE disease activity

The results revealed a significant difference in the expression levels of *BECN1* mRNA between patients with SLE and healthy controls. To determine whether this difference may affect SLE, we further analysed the correlations between *BECN1* mRNA expression level and clinical manifestations of patients. The mean expression level of *BECN1* mRNA in the control group (-2 SD, specifically RQ=1.207) was set as the normal lower limit, and RQ<1.27 was defined as *BECN1* mRNA downregulation. The results showed that SLE patients with increased *BECN1* mRNA downregulation had a higher disease activity score than healthy controls (8.06 ± 2.83 *vs.* 6.01 ± 2.56, P=0.004), and further analysis revealed that the expression level of *BECN1* mRNA was negatively correlated with SLEDAI (r=-0.267, P=0.001).

Correlations between BECN1 mRNA expression level and clinical features of SLE

To investigate whether the expression level of *BECN1* mRNA was related to certain clinical features of SLE, we further analysed and compared the clinical features and laboratory results of patients with SLE who had different *BECN1* mRNA expression levels. Patients with SLE with downregulated *BECN1* mRNA exhibited a significantly increased incidence of nephritis (85.3% *vs.* 64.4%, P=0.038) (Table 1).
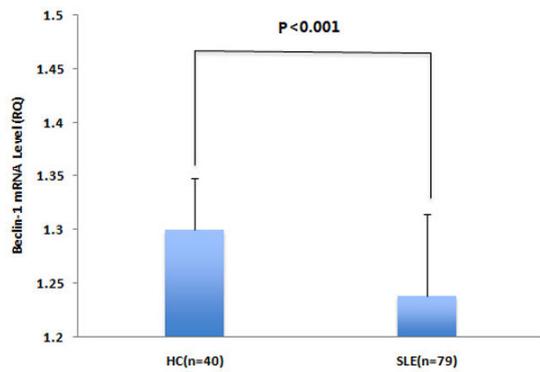
Figure 1. *BECN1 mRNA expression levels in PBMC in two groups ($\bar{x} \pm s$).*

Table 1. *Correlations of different ATG6/Beclin-1 mRNA expression levels with clinical manifestations and laboratory results of SLE.*

ATG6/Beclin-1	RQ<1.207 (n=34)	RQ ≥ 1.207 (n=45)	P-value
Age (years)	32.4 ± 16.1	33.8 ± 14.3	0.371
Female, n (%)	29 (85.3)	37 (82.2)	0.715
SLE disease duration (months)*	32.7 ± 31.3	27.4 ± 22.6	0.143
Rash, n (%)	21 (61.8)	23 (51.1)	0.345
Light sensitivity, n (%)	10 (29.4)	14 (31.1)	0.871
Oral ulcers, n (%)	13 (38.2)	17 (37.8)	0.967
Arthritis, n (%)	20 (58.8)	23 (51.1)	0.496
Serositis, n (%)	7 (20.6)	11 (24.4)	0.686
Kidney damage, n (%)	29 (85.3)	29 (64.4)	0.038
Blood system damage, n (%)	14 (41.2)	24 (53.3)	0.284
CNS involvement, n (%)	3 (8.80)	1 (2.20)	0.42
ANA, n (%)	34 (100.0)	44 (97.8)	1
Anti-dsDNA antibody-positive, n (%)	22 (64.7)	24 (53.3)	0.31
Anti-Sm antibody-positive, n (%)	12 (35.3)	18 (40.0)	0.67
ANA-positive, n (%)	21 (61.8)	23 (51.1)	0.345

Note: ANA: Anti-Nuclear Antibodies; SLE: Systemic Lupus Erythematosus; Sm=Smith; CNS: Central Nervous System.

Discussion

Abnormal immune features of SLE are associated with abnormal activation of T and B lymphocytes, producing a variety of nuclear-targeting autoantibodies and resulting in systemic damages [13,15]. Given the regulatory roles of autophagy in innate and acquired immune systems, eliminating dead cells and intracellular DNA and RNA, and maintaining the generation of T and B lymphocytes, autophagy is currently considered as a key pathogenic contributor to SLE-associated abnormalities [8,12,16-20]. Genomic studies have shown that

loci with Single Nucleotide Polymorphisms (SNPs) rs548234, rs6937876, and rs656843 on the autophagy gene locus *ATG5* can upregulate *ATG5*, thus demonstrating a relationship with the occurrence and development of SLE. Further studies have shown that the upregulation of *ATG5* is related to the activation of autophagy induced by complement-inactivated serum in patients with SLE [21,22]. Another study reported that the abnormal expressions of the autophagy genes *ATG7*, *LC3*, *HSP4*, *UVRAG*, and *IRGM* are related to an imbalance of T-cell functions in patients with SLE [12]. These results suggest that the changes in autophagy gene expressions may play important roles in the pathogenesis or progression of SLE. The formation of autophagosomes is mediated by a group of evolutionarily conserved Autophagy-Related Genes (ATGs) that encode ATG proteins [23], and nearly 36 types of ATG proteins have been found in mammals, among which *BECN1* (equivalent to *ATG6* in yeast) is a key protein in autophagy initiation; thus, it is considered the gatekeeper of autophagy. As the first known gene directly related to autophagy activation in mammals, *BECN1* is a target for inhibiting autophagy activity. After cells receive autophagy-inducing signals, a small membrane-like structure that is similar to a liposome will form in the cytoplasm. This structure is known as a phagophore (equivalent to an autophagic precursor), promoting the activation of downstream autophagic signals and resulting in the interaction of *BECN1* with *ATG14*, *VPSl5*, and *VPS34* to form the *PI3KC3* complex. In addition, it recruits FYVE- or PX-containing proteins in the cytoplasm for autophagic membrane formation and to promote other ATG proteins to bind to the phospholipids on autophagic precursors; thus, these autophagic precursors can continue to extend, wrap around cytoplasmic components that need to be reduced (including organelles and other biological macromolecules), and form autophagosomes [24]. Many studies have demonstrated that *BECN1* plays crucial roles in inhibiting the occurrence and development of tumors, preventing aging, regulating the immune system, and protecting the nervous system [25-28]. *BECN1* biallelic deficiency was shown to result in early placental death in mice, and *BECN1* monoallelic deficiency may lead to sporadic tumors and paraplasm of germinal center B cells and easily result in degenerative changes in the nervous system. *BECN1* was found to be downregulated in Alzheimer's disease [29-31]. To investigate the roles of *BECN1* expression in the pathogenesis and progression of SLE, we determined and compared *BECN1* mRNA expression in the mononuclear cells of the peripheral blood of patients with SLE and of healthy controls. The results demonstrated that the mRNA expression of *BECN1* in patients with SLE was significantly reduced (P<001). Further analysis of *BECN1* mRNA expression and SLE clinical features revealed that *BECN1* mRNA expression level in patients with SLE was significantly negatively correlated with SLEDAI (r=-0.267, P=0.001). Considering that the expression of *BECN1* can regulate autophagy, a high expression of *BECN1* is consistent with an increased number of autophagosomes. In a previous study, *BECN1*-deficient rats were found to have significantly reduced number of autophagic vacuoles [24]. Similarly, this study revealed the downregulation of *BECN1* mRNA in patients with

SLE, indicating that patients with SLE have defects in autophagic function. Another study examined and compared *BECN1* mRNA expression in the PBMCs of three patients with SLE and three healthy controls and found that *BECN1* mRNA was significantly upregulated in the former [32]. Although the observed *BECN1* mRNA upregulation in that study is in contrast to our observation of *BECN1* mRNA downregulation in patients with SLE, the previous study may not accurately represent the expression of *BECN1* mRNA in patients with SLE because of the limited number of cases. T and B lymphocytes are associated with increased autophagy in SLE, which is also believed to extend the survival period of autoreactive T and B cells to participate in the pathogenesis of SLE [11,19,33]. However, a previous study did not find an increase in autophagy in the T lymphocytes of patients with SLE; conversely, T lymphocytes in SLE were found to resist the stimulation induced by autophagy [34]. In addition, autophagy genes were reported to be downregulated in the macrophages of patients with SLE [34]. Notably, glucocorticoids, the preferred drugs for SLE treatment, may also upregulate autophagy, thus playing a therapeutic role [35,36]. Rapamycin is notable as a specific autophagy activator; in a murine model of lupus (NFBXNZWF1), it was observed that rapamycin can effectively attenuate the reduction of autoantibodies and the deposition of immune complexes and urinary proteins in vasculitis, thereby significantly prolonging the survival period [37]. Clinically, rapamycin has been found to not only suppress SLE disease activity but also significantly reduce the levels of corticosteroids in patients [38]. These results suggest that autophagy deficiency may be related to the pathogenesis or disease activity of SLE, and the results of *BECN1* downregulation observed in our study may be associated with existing autophagy deficiency in SLE. Lupus Nephritis (LN) is the most common involvement of a major organ in SLE, and we found that patients with SLE with downregulated *BECN1* mRNA had a higher incidence of LN than healthy controls (85.3% *vs.* 64.4%, P=0.038). The occurrence of LN is thought to be related to anti-dsDNA antibody and Anti-Nuclear Antibody (ANA), and certain evidence has indicated that the autoantibody and autophagy deficiencies in patients with SLE are related to the non-effective removal of apoptotic cells [39]. Therefore, the finding indicating that patients with SLE with downregulated *BECN1* mRNA are more susceptible to LN may be attributed to these antibodies. Our results that demonstrated the higher anti-dsDNA antibody levels and ANA-positive rates in patients with SLE with downregulated *BECN1* mRNA also support this association.

Overall, our findings are preliminary, and the precise mechanisms leading to the downregulation of *BECN1* expression in patients with SLE are not yet well understood. ATG proteins are transcriptionally regulated by several factors such as genes positively or negatively regulating autophagy. An overexpression of genes negatively regulating autophagy has been demonstrated in patients with SLE [34], which might explain the downregulated *BECN1* mRNA in our patients. Future studies investigating the role of *BECN1* expression in

autophagic flux and identifying its downregulation mechanisms may improve our understanding of the biological significance of this gene and its key roles in SLE pathogenesis.

Conflict of Interest

All authors have no conflict of interest regarding this paper.

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