INTRODUCTION

Systemic lupus erythematosus (SLE) is a common chronic rheumatic disease. There are many immunological characters for this disease such as B and T cells dysfunction, immune complex deposition and pathogenic autoantibody production leading to various clinical manifestations and damage in many organs. The etiology of this disease is still undisclosed, but it is probably due to interaction among several factors such as genetic structure, hormones and environment which includes pathogens, chemicals, and radiation. These factors may provoke the immune dysfunction leading to the pathogenesis of SLE disease.

Autophagy is a highly conserved self-digested process that is mediated by lysosome and keeps homeostasis and integrity of cells through the degradation of unwanted cytoplasmic constituents, malfunctioning organelles, misfolded proteins and recycling nutrients. Genome-wide association studies (GWAS) have shown more than 60 candidate genes conferring susceptibility to SLE and also have related single nucleotide polymorphisms (SNPs) in autophagy-related gene 5 (ATG-5) to SLE. Beclin-1, microtubule-associated protein-light chain 3 (LC-3) and interleukin (IL)-10 transcripts were significantly higher in SLE patients compared to healthy controls. The previous transcripts were positively correlated with SLE Disease Activity Index (SLEDAI). Beclin-1 and LC-3 transcripts were negatively correlated to complement component 3 (C3) levels. Only LC-3 transcripts were negatively correlated to complement component 4 (C4). The rs573775 SNP of ATG-5 with the variant allele was significantly associated with disease susceptibility, conferring a higher risk of SLE development. This variant allele was more prevalent in patients below 30 years, patients with anemia and in patients with anti-double-stranded DNA (dsDNA), confirming the essential role of ATG-5 polymorphism in the susceptibility of Egyptian patients to SLE.
chain 3 (LC-3) are vital autophagy-related genes (Atgs) involved in the autophagy flux by regulating autophagosomes formation.\textsuperscript{5,9,10} Autophagy shares in nearly all steps of immunity, especially in the usual development and function of B and T lymphocytes.\textsuperscript{11,12}

Disturbances in autophagy may surprisingly lead to various autoimmune and chronic inflammatory diseases.\textsuperscript{6,13,14}

In this study, we analyzed the transcript levels of Atgs Beclin-1, LC-3, ATG-5 and interleukin-10 (IL-10) gene in the peripheral blood mononuclear cells (PBMCs) of SLE patients and healthy controls. The correlations between Atgs, IL-10 gene expression and SLEDAI as well as C3 and C4 levels and the distribution of ATG-5 rs573775 SNP in a sample of Egyptian SLE patients and controls were analyzed to prove their probable involvement to disease susceptibility.

2 | PATIENTS AND METHODS

2.1 | Study population

The present study is a case-controlled study included 70 patients with SLE admitted to the Rheumatology Unit of Internal Medicine Department in Assiut University Hospital from July 2018 to July 2019. From each patient an informed consent was obtained to be enrolled in this study. The study protocol was accepted by the local Ethics Committee of Faculty of Medicine, Assiut University (IRB NO.17200219). According to the American College of Rheumatology, patients achieved at least 4 criteria of SLE (ACR 1997).\textsuperscript{15} These criteria included malar rash, discoid rash, oral or nasal rash, photosensitivity, arthritis, serositis, renal, neurological, hematological involvements and immunological abnormalities. Patients were excluded from the study if they had any of the following criteria: (a) age <18 years or >60 years; (b) coexistence of other autoimmune diseases; (c) lactation or pregnancy. Disease activity was evaluated according to the SLE Disease Activity Score (SLEDAI) as the follow-

2.2 | Total RNA isolation and RT-PCR analysis

PBMCs were prepared using the standard Ficoll-Histopaque density-gradient centrifugation method (Sigma-Aldrich). Total RNA was extracted using Trizol RNA extraction reagent (Invitrogen). RNA concentration and purity were measured by Nano Drop™ 8000 Spectrophotometer (Applied Biosystems). RNA (1 μg) was used to synthesize the complementary DNA (cDNA) using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s protocol. Reverse-transcription polymerase chain reactions (RT-PCR) were carried out in a total volume of 20 μL. Reaction mixtures included 10 μL 2 × SYBR-green I mix (Applied Biosystems), 1 μL forward primer, 1 μL reverse primer, and 1 μL cDNA. The transcript level was normalized to housekeeping gene (β-actin) and the relative gene expression was determined according to the $2^{- ΔΔC T}$ method.\textsuperscript{17} The primer sequences used were as follows: Beclin-1, forward 5′-TGAGGGATGGAAGGGTCTAAG-3′ and reverse 5′-GCCCTGCGCTGTGGTAAGTAATC-3′; LC-3, forward 5′-CATGAGCGAGTTGTTCAAGATG-3′ and reverse 5′-TCGTCCTTTCTCCTGCTGTAG-3′; β-actin, forward 5′-TAGTTGCGTTACACCTTTCTTGTG-3′ and reverse 5′-TCACCTTCACCCCTGTCACAG-3′; ATG-5, forward 5′-AGGCAACCTGACCAAGAACA-3′ and reverse 5′-GAGGAAAGCAAGGTGATGC-3′; IL-10, forward 5′-TTCTTG-3′ and reverse 5′-GCCTGGGCTGCTGGTAAGTAATC-3′; and reverse 5′-ATTCTTACACCTGTCCACGGCTT-3′.\textsuperscript{19}

2.3 | SNP selection and genotyping

Genomic DNA was obtained from peripheral blood by using GeneJET Genomic DNA purification Kit (Thermo-Fisher Scientific). SNP in the ATG-5 gene (rs573775) was analyzed by TaqMan SNP genotyping technology for allelic discrimination using a minor groove binder-specific allelic probe (C_910347_20) and 7500 real-time PCR system (Applied Biosystems). Reaction was performed as follows: an initial cycle for 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

2.4 | Statistical analysis

Statistical analyses were achieved using GraphPad Prism version 7.0 software (GraphPad Software Inc). Data are presented as the mean ± SEM P value was assessed using unpaired two-tailed Student’s t test and one-way analysis of variance test. Chi-square test was used to analyze the correlation between Beclin-1, LC-3, ATG-5 and IL-10 gene expression levels and patients’ immunological features (C3 and C4 levels) and SLEDAI.

3 | RESULTS

3.1 | Demographic, laboratory, and clinical characteristic of the patients

The characteristics of the 70 SLE patients involved in this study are summarized in Table 1. Among these patients, 63 (90%) were females, the mean age was 28.9 ± 0.93 years, and the mean disease
TABLE 1  Clinical characteristics of systemic lupus erythematosus patients

<table>
<thead>
<tr>
<th>Parameters</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female n (%)</td>
<td>63 (90%)</td>
</tr>
<tr>
<td>Age at diagnosis, mean ± SEM</td>
<td>28.9 ± 0.93 y</td>
</tr>
<tr>
<td>Disease duration, mean ± SEM</td>
<td>2.24 ± 0.46 y</td>
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</table>

Clinical manifestations
- Malar rash: 21 (30%)
- Alopecia: 36 (51.4%)
- Discoid rash: 10 (14.29%)
- Photosensitivity: 11 (15.7%)
- Bleeding tendency: 9 (12.9%)
- Oral ulcers: 20 (28.6%)
- Serositis: 11 (15.7%)
- Vasculitis: 5 (7.1%)
- Arthritis: 29 (41.4%)
- Renal involvement: 45 (64.3%)
- Neurological involvement: 8 (11.4%)

SLEDAI
- No activity (SLEDAI = 0): 2 (2.9%)
- Mild activity (SLEDAI = 1-5): 5 (7.1%)
- Moderate activity (SLEDAI = 6-12): 21 (30%)
- High activity (SLEDAI = 13-20): 32 (45.7%)
- Very high activity (SLEDAI > 20): 10 (14.3%)

Immunologic features
- ANA positive: 65 (92.9%)
- Anti-dsDNA positive: 55 (78.57%)
- Low C3 levels, <0.8 g/L: 57 (81.4%)
- Low C4 levels, <0.12 g/L: 60 (85.7%)

Treatment
- Steroids: 63 (90%)
- Immunosuppressive drugs: 61 (87.14%)

Abbreviations: ANA, antinuclear antibodies; dsDNA, double-stranded DNA; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.

duration was 2.24 ± 0.46 years. The SLEDAI score was 0 in 2 patients (2.9%), 1-5 in 5 patients (7.1%), 6-12 in 21 patients (30%), 13-20 in 32 patients (45.7%), and above 20 in the remaining 10 patients (14.3%).

3.2  | Expression of Beclin-1, LC-3, ATG-5 and IL-10 in the PBMCs of SLE patients

The relative mRNA expression level of Beclin-1 in the PBMCs of SLE patients was significantly higher than controls (P = .024) Also, the transcript expression of LC-3 and IL-10 were significantly higher in SLE patients than controls (P < .0001), while the expression level of ATG-5 was comparable in SLE patients and controls (P = .27) (Figure 1).

3.3  | Correlations between the expression of Beclin-1, LC-3, ATG-5, IL-10 in PBMCs and SLEDAI, C3 and C4 levels

There was a strong positive correlation between Beclin-1 mRNA level and SLEDAI (r = .7315; P < .0001) and moderate negative correlation with C3 level (r = -.3309; P = .0051). No significant correlation was obtained with C4 level (r = -.2074; P = .0849) (Figure 2). Similarly, there was a strong positive correlation between LC-3 mRNA level and SLEDAI (r = .8474; P < .0001) and moderate negative correlation between LC-3 mRNA level and C3 level (r = -.5714; P < .0001) and in addition C4 level (r = -.4688; P < .0001) (Figure 3), while there were no significant correlations between ATG-5 mRNA level and SLEDAI (r = .0724; P = .5514), C3 level (r = -.07831; P = .5293) and C4 level (r = -.0063; P = .9584). There was a strong positive correlation between IL-10 mRNA levels and SLEDAI (r = .836; P < .0001). No significant correlations were observed with C3 level (r = -.0131; P = .836), and in addition C4 level (r = -.1324; P = .2747) (Figure 4).

3.4  | Association of ATG-5 rs573775 SNP with SLE among Egyptian patients

The distribution of ATG-5 genotypes and alleles in healthy individuals and in SLE Egyptian patients are recorded in Table 2. A significant association was observed between SLE susceptibility and the variant alleles. There was a higher risk of disease development in variant allele carriers than wild type allele carriers (odds ratio [OR] = 3.14, P = .0366 at the genotypic level and OR = 3.4, P = .0109 at allelic level).

3.5  | Effect of ATG-5 SNP rs573775 on the expression of Beclin-1, LC-3, IL-10 and ATG-5 genes in PBMCs of SLE patients

No significant differences were observed in Beclin-1, LC-3, ATG-5, and IL-10 gene expressions between various genotypes for rs573775 SNP.

3.6  | Genotype and phenotype analysis

Genotypic and phenotypic correlation analyses between the variant alleles of rs573775 SNP and clinical characteristics of patients were carried out. The variant allele significantly existed in patients who were diagnosed at age below 30 years 3.013 times more than patients who were diagnosed at age equal to or above 30 years (OR = 3.013, P = .0463). The variant allele significantly existed in anemic patients 3.5 times more than non-anemic patients (OR = 3.536, P = .0282) and also significantly existed in patients with anti-dsDNA 4.4 times more than patients without anti-dsDNA (OR = 4.471, P = .0170). There were no statistically significant correlations observed between the variant allele and other clinical characteristics of SLE.
patients (renal involvement, neurological involvement, ANA, malar rash, alopecia, discoid rash, photosensitivity, bleeding tendency, oral ulcers, serositis, vasculitis and arthritis).

4 | DISCUSSION

Autophagy plays an important role in many immunological processes and defects in this process are associated with several autoimmune and neurodegenerative disorders.20

This process involves several stages, including formation of phagophores, formation of autophagosomes, fusion of autophagosomes with lysosomes and autolysosomes formation which is required for degradation of defective proteins. Beclin-1, LC-3 and ATG-5 genes regulate autophagosomes formation.21,22

Estimation of Beclin-1 and LC-3 gene expression are broadly used to reliably measure auto-phagosomes formation. Two studies5,23 showed that Beclin-1 was significantly upregulated in the PBMCs of SLE patients. Only one study24 showed that Beclin-1 expression was significantly downregulated in the PBMCs of SLE patients. A

The expression level of Beclin-1 (A), microtubule-associated protein-light chain 3 (LC-3) (B), autophagy-related gene 5 (ATG-5) (C), and interleukin (IL)-10 (D) transcripts in peripheral blood mononuclear cells (PBMCs) of patients with SLE and compared with the controls.
British study demonstrated that LC-3 expression was elevated in B and T lymphocytes in human SLE. Another study showed that LC-3 expression was significantly upregulated in the PBMCs of SLE patients. A French report reported that LC3-II expression was significantly increased in T cells from lupus-prone mice. In our current study, we reported that Beclin-1 and LC-3 genes were both highly expressed in the PBMCs of SLE patients compared to controls, and these results agreed with previous studies to some extent. According to these studies, it was possible to deduce that the formation of autophagosomes was activated in SLE. We also found that the Beclin-1 and LC-3 gene expressions were correlated positively with SLEDAI, which may suggest that activated formation of autophagosomes was not only involved in the incidence but also involved in the disease progression. So upregulation of Beclin-1 and LC-3 gene expressions may contribute in disease incidence and increasing severity of the disease. In the current study, we recorded that IL-10 gene was highly expressed in PBMCs of SLE patients. This result agreed with several studies. B-cell survival, proliferation, differentiation and antibody production were elevated by IL-10. Also the apoptosis of auto-reactive B-cell was decreased by IL-10 through increasing Bcl-2.

**Figure 3** The correlation between microtubule-associated protein-light chain 3 (LC-3) and Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and complement levels. The correlation between LC-3 messenger RNA (mRNA) level and SLEDAI, C3 and C4 levels. A, Correlation between LC-3 mRNA level and SLEDAI. B, Correlation between LC-3 mRNA levels and C3 level. C, Correlation between LC-3 mRNA level and C4 level.

**Figure 4** The correlation between interleukin (IL)-10 and Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and complement levels. The correlation between IL-10 messenger RNA (mRNA) level and SLEDAI, C3 and C4. A, Correlation between IL-10 mRNA level and SLEDAI in SLE patients. B, Correlation between IL-10 mRNA level and C3 level in SLE patients; (C) Correlation between IL-10 mRNA level and C4 level in SLE patients.
expression, which leads to increasing of autoantibody production in SLE patients. The transcript level of IL-10 gene was found correlated positively with SLEDAI, which may recommend the role of IL-10 in the disease through intensifying and saving an inflammation cycle.

In the current study, we found that ATG-5 gene expression unchanged in the PBMCs of SLE from healthy controls. However, these results disagreed with the study of Li, Yue23 who found the significant upregulation of ATG-5 expression in both macrophages of lupus mice and PBMCs of SLE patients. Another report showed elevated ATG-5 expression in Chinese patients with lupus nephritis (LN).31 This difference may be attributed to the heterogeneity of the disease, different patient populations and different sample sizes.

ATG-5 gene was one of many genes involved in the autophagy process which was identified as a risk factor for SLE susceptibility by some GWAS.7,32 Few studies reported many SNPs in ATG-5 gene in various inhabitants and verified association with SLE, for example, in Chinese,33 European34 and Italian studies.35 A significant association was found between the variant alleles of rs573775 and predisposition to SLE. In Italian35 and European34 studies, they reported a significant association between the variant alleles and predisposition to SLE. However, in 2 Chinese studies33,36 and another Spanish study8 observed that rs573775 variant allele was not associated with SLE.

The results which we obtained agreed with Italian35 and European34 studies and disagreed with Chinese33,36 and Spanish8 studies. This difference in results may be due to the heterogeneity of the disease and different races.

The current study showed the effect of ATG-5 SNP rs573775 on the transcript levels of Beclin-1, LC-3 and IL-10 genes in PBMCs of SLE patients. No significant difference was observed in Beclin-1, LC-3 and IL-10 gene expression between various genotypes for rs573775. López, Alonso-Pérez8 reported that the rs573775 mutant allele and the high IL-10 producing genotype carriers were at higher risk for SLE. In the current study, there was not a significant difference perceived in ATG-5 gene expression between different genotypes for rs573775 SNP (P = .33, .12). These results were in accordance with that was reported by Ciccacci, Perricone.35 In the current study, genotype and phenotype correlations between clinical characteristics of SLE patients and ATG-5 rs573775 variants were done. The variant allele significantly existed in SLE patients who diagnosed at age below 30 years 3.013 times more than patients who acquired the disease at age equal or above 30 years. Also it was found that variant allele significantly existed in anemic patients 3.5 times more than non-anemic patients and also significantly existed in patients with anti-dsDNA 4.4 times more than patients without anti-dsDNA. There was no significant difference between different genotypes in SLE patient with different SLEDAI and other clinical characteristics of SLE patients. Our results disagreed with Alonso-Perez, Suarez-Gestal34 who reported there was an association between the older age at onset (>30 years) and the variant allele of ATG-5.

5 | CONCLUSION

Beclin-1, LC-3 and IL-10 transcripts are upregulated in SLE patients. The previous transcripts were positively correlated with SLEDAI. The rs573775 SNP of ATG-5 with the variant allele was significantly associated with disease susceptibility, conferring a higher risk of SLE development in the Egyptian population. This highlights the preservation of autophagy balance as a tool for improving the disease activity in SLE patients. The genetic variations in autophagy genes could contribute to autoimmune diseases susceptibility and require further research.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Mohamed A. El-Feky perceived and planned the experiments with Ayat M. Kamel. Ayat M. Kamel performed the experiments and wrote the manuscript with support from all authors. Ghada H. Ahmed contributed to sample collection. Mohamed S. Badary, Wegdan A. Mohamed, Mohamed. A. El-Feky contributed to the interpretation of the results.

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REFERENCES


