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Rare Variants of *ATG5* Are Likely to Be Associated With Chinese Patients With Systemic Lupus Erythematosus

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Abstract: Recently, common variants within or near *ATG5*, which is a key autophagy gene required for the formation of autophagosomes, have been identified as a candidate gene of systemic lupus erythematosus (SLE) by several genome-wide association studies. Moreover, elevated *ATG5* expression was observed in SLE as well as other autoimmune diseases. However, no significant associations between variants within *ATG5* and SLE were identified in several Chinese populations. The present study was conducted to further check the genetic role of *ATG5* by associating both common and rare variants of *ATG5* in Chinese patients with lupus nephritis (LN), a major phenotype with poor prognosis in SLE.

To detect the association of common variants of *ATG5* with LN, 7 tagging single nucleotide polymorphisms (SNPs) designed in immunochip and 4 SNPs reported to be associated with SLE were genotyped in 500 LN patients and 500 healthy controls. Furthermore, direct sequencing of exons and their flanking regions in 90 LN patients, 30 SLE patients, and 60 healthy controls were performed. Functional genomic annotation was performed by using public databases.

None of the 11 tagging SNPs was observed to be associated with LN. By sequencing, 13 variants were identified, including 5 common SNPs, 7 not previously described, and 1 reported as rare variants (<1%) in the Single Nucleotide Polymorphism Database or the 1000 Genome project. None of the 5 common SNPs showed significant association between patients and controls, whereas increased frequencies of rare or novel variants were observed in patients compared with healthy controls, with 6/90 in LN patients, 2/30 in SLE patients, and 1/163 in healthy controls. Although these rare variants were observed to be located in the flanking regions of exons instead of missense mutations, patients carrying them tended to have severe clinical phenotype, and in silicon analysis suggested their regulatory effects.

Increased frequencies of rare variants of *ATG5* were identified in patients with LN and SLE compared with healthy controls, highlighting a likely important role of rare *ATG5* variants in Chinese SLE patients.

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Abbreviations: CHB = Han Chinese in Beijing, ENCODE = the Encyclopedia of DNA Elements project, GLIF = GLI zinc finger, HEAT = heat shock protein, KLFS = Kruppel-like transcription factor, LN = lupus nephritis, MAF = minor allele frequency, miRNA = micro RNA, PCR = polymerase chain reaction, SLE = systemic lupus erythematosus, SNP = single nucleotide polymorphism, SSc = systemic sclerosis, 1000 Genome = 1000G.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex autoimmune disease with a strong genetic component.[1] To date, >50 loci showed robust association with SLE have been identified by hypothesis-free genome-wide association studies using common tagging single nucleotide polymorphisms (SNPs) with minor allele frequency (MAF) >5%,[2] significantly broadening our views about genetic pathogenesis of SLE. However, all these variants can explain no >15% of genetic risk in SLE. For the unexplained heritability, a further check for the rare variants may be helpful.

Autophagy is a phylogenetically ancient mechanism by which the cell can remove the long-lived proteins and damaged organelles through lysosomal degradation. Recently, genetic studies reported that polymorphisms in various autophagy-related genes, including *ATG5*, *ATG7*, *IRGM*, *DRAM1*, *CDKA1B*, *APOL1*, and *MTMR3*, were associated with SLE, suggesting the possible role of autophagy in SLE.[3–7] *ATG5* is an important gene in the initiation of autophagosome formation.[8] Several large-scale replication studies have repeatedly associated common variants within or near *ATG5* with susceptibility to SLE.[6,9] The functional studies targeting *ATG5* consistently demonstrated its pivotal role in autoimmune diseases. Elevated *ATG5* expression has been observed in a mouse model of autoimmune demyelination, as well as in blood and brain tissues from patients with multiple sclerosis.[10] Besides, the *ATG5* rs573775, T allele was reported to have effects on SLE susceptibility, cytokine production, and disease features.[3,11] Also in our previous study,[6] the risk alleles within *PRDM1-ATG5* region correlated with high *ATG5* instead of *PRDM1* messenger RNA expression in B cells, and higher *ATG5* expression was observed in B cells from SLE patients compared with normal controls. Thus, *ATG5* was suggested to be a strong candidate gene, which may play an important role in SLE.

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However, the SLE-associated variants of *ATG5* were observed in different populations, and moreover, no significant associations between variants within *ATG5* gene region and SLE were observed in several Chinese populations,[6,12] whereas, the rare variants of *ATG5* have been reported to be associated with several complex diseases, including prostate cancers, gastrointestinal cancers, and Parkinson disease.[13–15] So, it is of high interest to further check the genetic role of *ATG5* in SLE, especially rare variants of *ATG5* in the Chinese population. Lupus nephritis (LN), a major manifestation and fatal target-organ damage of SLE, is one of the strongest indicators of poor prognosis and possibly a kind of extreme phenotype. Thus, the present study was to further explore the genetic role of *ATG5* in a Chinese population by detecting the association of both common and rare variants of *ATG5* with LN.

MATERIALS AND METHODS

Patients and Controls

To replicate the association of common variants of *ATG5* with LN, 500 LN patients (31.9 ± 11.2 years, 423 women) and 500 healthy blood donors (40.0 ± 8.6 years, 140 women), who were of Han Chinese in Beijing (CHB) origin, were enrolled in the study. Furthermore, to detect the association of rare variants of *ATG5* with LN, exon sequencing was performed in 90 LN patients (26.4 ± 10.8 years, 57 women), 30 SLE patients (33.7 ± 10.0 years, 29 women), and 60 healthy blood donors (34.6 ± 10.3 years, 48 women).

The SLE patients met the revised SLE criteria of the American College of Rheumatology in this study.[16] All the LN patients were confirmed by renal biopsy using light microscopy, immunofluorescence, and electron microscopy, and the SLE patients without renal damage were defined as the none of proteinuria, abnormal urinary sediment, or renal biopsy evidence of nephropathy. The study was approved by the medical ethics committee of Peking University First Hospital, and all patients gave written informed consents.

SNP Selection and Genotyping

Seven SNPs designed in immunochip aiming for fine-mapping of immune-related genes in autoimmune diseases, including rs78200552, rs144506815, rs9386514, rs9373839, rs78937934, rs6906688, and rs510432, were genotyped by an Illumina Solexa HiSeq 2000 platform (Shenzhen Huada Gene Research Institute, ShenZhen, China). Of note, rs9373839 was reported to be one of the top associated variants with systemic sclerosis. Four SNPs, which were reported to be associated with SLE in whites,[3,9] including rs2245214, rs4945747, rs573775, and rs2757133, were also selected and genotyped by TaqMan allele discrimination assays (Applied Biosystems, Foster City, CA). Thus, a total of 11 common tagging SNPs were included in the present study. Among them, rs9386541, rs9373839, and rs4945747 were in the same block, whereas rs2757133 and rs573775 were in the same block. The genotyping data were verified by direct sequencing in selected samples with concordance of 100%.

Sequencing and Analysis

All 8 exons, including all coding exons and splice sites of *ATG5*, were sequenced using genomic DNA. Polymerase chain reaction (PCR) primers were designed using Primer3 based on the genomic sequence of human *ATG5* gene (GeneBank access number, NC_0000016.11). The PCR product sequencing was performed by Beijing Genomics Institute based on Sanger sequencing method. The sequencing results were analyzed by Chromas 15.0 (http://technelysium.com.au/?page id=13) and compared with wild type of *ATG5* gene reference sequences (http://blast.ncbi.nlm.nih.gov/). The PCR products were sequenced forward and backward, and repeated twice. Variants were considered to be common (previously reported with MAF $>1\%$), rare (previously reported with MAF $<1\%$), or novel (not previously reported in 1000 Genome (1000G) project or the Single Nucleotide Polymorphism Database). When a novel variant was identified, another pair of primers was designed to repeat the sequencing to make sure the novel variant was real.

Genotype data of 103 CHB controls were downloaded from 1000G project (http://www.ensembl.org/Homo_sapiens/Gene/Variation_Gene/Table?db=core;g=ENSG00000057663;r=6:106184476-106325820). Using these data and our sequencing data, association analysis and statistical analysis were performed.

Bioinformation Mechanism Prediction

Functional significance of the common or rare variants was annotated by the Encyclopedia of DNA Elements project data or related open databases. The variant regulatory effects, such as whether they were transcriptional factor binding sites, were searched by the Variant Effect Predictor (http://asia.ensembl.org/info/docs/tools/vep/index.html) and the Genomatix Database (http://www.genomatix.de/cgi-bin/tools/tools.pl). The RegRNA Database (http://regrna.mbc.nctu.edu.tw/html/tutorial.html) was used to identify the homologs of regulatory RNA motifs and elements. Moreover, to further predict whether genetic variants were micro RNA (miRNA) synthetic and target sites, the miRBase Database (http://www.mirbase.org/) and the miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/) were searched.

Statistical Analysis

The genotype frequencies of SNPs were tested for Hardy–Weinberg equilibrium separately in patients and controls. Associations between disease and SNPs were analyzed by χ^2 tests, and Fisher exact test was used when necessary. Results of the measurement data for clinical information were expressed as mean $\pm$ SD, and *t* tests were used to analyze the difference between patients carrying rare variants or not. The same procedure was applied to subgroups stratified according to sex and pathologic classes of LN patients. Statistical analysis was performed with SPSS16.0 software (SPSS Inc, Chicago, IL). A 2-tailed *P* value of <0.05 was considered statistically significant.

RESULTS

Association Analysis of the Tagging Common SNPs Within ATG5

A total of 11 tagging SNPs within *ATG5* were genotyped in 500 LN patients and 500 healthy controls. Deviation from Hardy–Weinberg equilibrium was not observed for any of the SNPs in the patients or controls ($P > 0.05$). At allele level, results of the case–control association analyses were shown in Table 1, and none of the SNPs were observed to be associated with LN significantly ($P > 0.05$). Sex ratio and pathologic classes of LN patients were 2 important confounding factors. In the current study, the female ratio of the patients was 84.6% (423/500), and percentages of class I, II, III, IV, V, and VI pathology of the 500 LN patients were 0%, 7.5%, 22.2%,

TABLE 1. Allelic association of the tagging SNPs in *ATG5* with LN

SNP (minor allele)	Position	MAF cases (500, %)	MAF controls (500, %)	*P*	OR (95% CI)
rs78200552(C)	106738660	7.7	8.6	0.459	0.885 (0.641–1.223)
rs144506815(T)	106742814	15.6	13.6	0.204	1.175 (0.916–1.508)
rs9386514(C)	106743595	5.5	4.3	0.214	1.295 (0.861–1.948)
rs9373839(C)	106762310	5.4	4.3	0.252	1.270 (0.843–1.914)
rs78937934(T)	106799684	8.2	9.1	0.474	0.892 (0.653–1.219)
rs6906688(A)	106835388	0.1	0.0	0.317	NA
rs510432(A)	106880723	40.4	42.4	0.363	0.921 (0.770–1.100)
rs4945747(T)	106748323	20.3	18.7	0.557	1.110 (0.783–1.575)
rs2245214(C)	106769434	49.3	45.8	0.333	1.153 (0.864–1.537)
rs2757133(A)	106829920	4.4	4.8	0.782	0.912 (0.475–1.749)
rs573775(T)	106871559	35.0	32.6	0.454	1.117 (0.836–1.491)

P values were calculated by χ^2 test using 2×2 contingency tables based on allele frequencies. None of the genotypes in the controls showed significant deviation from Hardy–Weinberg equilibrium. Chromosome positions were referred to GRCh36. CI = confidence interval; LN = lupus nephritis; MAF = minor allele frequency; OR = odds ratio.

53.9%, 15.9%, and 0.4%, respectively. To investigate the possible association of the common SNPs with different sex and pathologic classes of LN patients, we further stratified the patients into subgroups. However, no association was observed between the LN subsets and any of the 11 SNPs investigated (*P* values ranged from 0.07 to 0.83).

Association Analysis of the Sequencing Variants of ATG5

All 8 exons, including essential splice sites were sequenced by Sanger technology and 13 variants were identified. Among them, 5 were common SNPs previously reported in CHB population, including rs510432, rs138203657, rs1624701, rs41292420, and rs77859116, whereas 8 were rare or novel (see the Sequencing and Analysis section for definitions). For the 5 common SNPs, they were found in patients and controls with similar frequencies ($P > 0.05$) (supplementary Table 1, http://links.lww.com/MD/A286).

Among the 8 rare or novel variants, none was reported in 1000G database or rare variant database except 106773567C>T (only reported in a Puerto Rico population with MAF 0.5%), indicating they were novel variants (supplementary Fig. 1, http://links.lww.com/MD/A286). Five heterozygous variants, including 106773751C>T, 106773700G>T, 106773530T>G, 106773567C>T, and 106696229T>C, were identified in 1 LN patient each, and 2 variants, including 106740994G>A and 106740756->TTAT, were observed in 1 SLE patient each. Although 106696268G>T was identified in 1 LN patient and 1 healthy control, the ratio of rare variants in LN patients, SLE patients, and reference controls (the 60 healthy controls in the present study and 103 CHB controls in 1000G database) were 6/90 (6.7%), 2/30(6.7%) and 1/163 (0.6%) with calculated odds ratio 11.6 between LN or SLE patients and controls ($P < 0.05$) (Table 2). Moreover, among all of the patients, the LN patients carrying the rare variants tended to have severe clinical manifestations (Table 3). Thus, further analysis of the clinical information in the subset LN patients with rare variants or not were taken (Table 4). As for the relatively higher male ratio of the sequencing LN patients, we first compared the clinical information between male and female LN patients. In this study, patients carrying the rare variants tended to be predominantly male (male ratio 83.3% vs 33.3%, $P < 0.05$). However, it seemed to be interesting that the patients carrying the rare variants tended to be earlier onset, having heavier proteinuria, and with worse renal function, higher Systemic Lupus Erythematosus Disease Activity Index Scores, and lower levels of C3 and C4. The same procedure was applied to subgroups stratified according to the sex of LN patients without rare variants, and similar results were observed, highlighting a likely important role of rare variants. However, due to the relatively small sample size, only age onset, quantity of urine protein, and

TABLE 2. Association analysis of the rare or novel sequencing variants of *ATG5*

Variant location	Type	Referring base	All bases	Number in patients (120)	Number in controls (163)	*P* value
106773751	Heterozygous	C	C/T	1	0	—
106773700	Heterozygous	G	G/T	1	0	—
106773530	Heterozygous	T	T/G	1	0	—
106696268	Heterozygous	G	G/T	1	1	—
106773567	Heterozygous	C	C/T	1	0	—
106696229	Heterozygous	T	T/C	1	0	—
106740994	Heterozygous	G	G/A	1	0	—
106740756	Insert	—	–/TTAT	1	0	—
In total				8	1	0.004

P values were calculated by Fisher exact test frequencies of variants in patients and controls. Chromosome positions were referred to GRCh37.

TABLE 3. Clinical characters of patients carrying the rare variants

Patient no.	1	2	3	4	5	6	7	8
Rare variant	106773751 C>T	106773700 G>T	106773530 T>G	106696268 G>T	106773567 C>T	106696229 T>C	106740994 G>A	106740756 ->TTAT
Sex	Male	Male	Male	Male	Female	Male	Female	Female
Age onset, years	28*	20	15	15	21	19	45	18
SLEDAI	16	18	27	22	14	26	—	—
Hematuria	Yes	Yes	Yes	Yes	Yes	Yes	—	—
Quantity of urine protein, g/L	11.20	4.72	2.89	7.00	4.55	4.00	—	—
C3, g/L	0.23	0.53	0.12	0.17	0.36	0.53	—	—
Pathological types	IV-G (A/C)	IV-G (A)	III-(A)	IV-G (A)	III-(A)	IV-G (A)	—	—

Patient 1, 2, 3, 4, 5, and 6 referred to LN patients, and patient 7 and 8 referred to SLE patients without renal damage. LN = lupus nephritis; SLEDAI = Systemic Lupus Erythematosus Disease Activity Index.
* Although the patient was diagnosed at 28 years first, he had a 14-year history of leg edema.

C3 level of the LN patients carrying rare variants showed significant or marginally significant difference from the controls, suggesting the necessity to validate the association of these rare variants and LN in a larger cohort.

Bioinformatics Analysis of the Sequencing Rare Variants

Among the 8 rare or novel variants, 4 variants (106696229 T>C, 106696268G>T, 106740756->TTAT, and 106740994 G>A) located in the intron region, 2 (106773530T>G and 106773567C>T) in the 5′UTC region and 2 (106773700G>T and 106773751C>T) in the promoter region. By searching Variant Effect Predictor, although the variants were not missense mutations, they have several types of features, including transcript, regulatory feature, and motif feature. Moreover, by searching Genomatix, a variant in promoter region, 106773700G>T, was identified to be the binding site of several transcription factors, including Kruppel-like transcription factors (KLFSs), GLI zinc finger (GLIF), and the family member of heat shock proteins (HEATs). In addition, by searching RegRNA Databases, the rare variants may play a role in SLE through regulation of RNA transcription, splicing, and miRNA binding. Among the involved miRNAs, the association of miRNA-155 with *ATG3* was also identified in miRTarBase (supplementary Table 2, http://links.lww.com/MD/A286).

DISCUSSION

Recent studies showed that SNPs, rs2245214 and rs573775, in *ATG5* were associated with SLE susceptibility in white populations instead of Asian populations.[3,6,9,12] As

TABLE 4. Pooled analysis of associations with the rare variants of *ATG5* in the subsets of sequencing LN patients

	Sex		Rare variants			
				Patients without rare variants		
Group	Female patients (n = 57)	Male patients (n = 33)	Patients with rare variants (n = 6)	Female (n = 56)	Male (n = 28)	In total (n = 84)
Age onset, years	24.14 ± 9.77	**30.39 ± 14.04 (0.028)**	20.33 ± 5.13	24.20 ± 9.85	**32.21 ± 14.35 (0.002)**	26.87 ± 12.07
Quantity of urine protein, g/L	4.17 ± 3.39	**1.36 ± 2.74 (1.30×10^{-4})**	5.30 ± 3.97	4.16 ± 3.42	**0.60 ± 1.46 (0.033)**	3.01 ± 3.37
eGFR, mL/min/1.73m^2	35.11 ± 52.75	33.55 ± 46.56	56.57 ± 64.02	33.23 ± 51.27	32.38 ± 45.94	32.95 ± 49.32
Serum creatinine, μmol/L	137.53 ± 151.98	182.00 ± 244.77	204.50 ± 345.30	138.79 ± 153.06	172.73 ± 220.93	149.83 ± 177.32
C3, g/L	0.43 ± 0.25	0.35 ± 0.29	0.23 ± 0.19	**0.44 ± 0.25 (0.06)**	0.38 ± 0.30	0.42 ± 0.27
C4, g/L	0.06 ± 0.08	0.05 ± 0.08	0.04 ± 0.04	0.07 ± 0.08	0.05 ± 0.09	0.06 ± 0.08
Percentage of crescent, %	33.85 ± 30.39	**17.69 ± 29.31 (0.017)**	20.84 ± 32.51	34.47 ± 30.32	16.34 ± 28.79	28.50 ± 30.86
SLEDAI	17.63 ± 6.36	17.32 ± 7.08	20.75 ± 6.70	17.70 ± 6.41	16.42 ± 6.94	17.33 ± 6.54

The analysis of clinical information was applied to subgroups stratified according to sex and rare variants of the sequencing LN patients. Five of the 6 LN patients with rare variants were men. Only the *P* values for significant or marginal significant associations were presented in brackets. LN = lupus nephritis; SLEDAI = Systemic Lupus Nephritis Disease Activity Index.

ATG5 plays an important role in immune responses, a deep exploration of genetic variants of *ATG5* in SLE is of importance to get a better understanding of its genetic role in SLE. Identifying rare variants by targeted gene sequencing is an efficient approach to identify gene mutations, including low frequency and novel variants for complex diseases.[17] In the present study, we tested 11 tagging SNPs within *ATG5* in LN patients. Furthermore, exon sequencing of *ATG5* was performed in LN patients, SLE patients, and healthy controls. By sequencing, 5 common SNPs and 8 rare variants were detected. The results showed that no significant association was found with the 11 tagging and the 5 sequencing common SNPs in *ATG5*, whereas the elevated frequencies of rare variants of *ATG5* in LN patients and SLE patients were detected. Moreover, LN patients carrying the rare variants tended to have more severe clinical manifestations.

As many previous genetic studies about *ATG5* reported that the rare variants of *ATG5* were associated with complex diseases, such as prostate cancer, stomach cancer, colon cancer, and Parkinson disease,[13–15] our results suggested the potential role of rare variants of *ATG5* in LN and SLE patients in the present population. Although the rare variants did not change the amino acids, bioinformation mechanism prediction indicated their regulatory effects on transcription. One rare variant in the promoter region was identified to be the binding site of several transcriptional factors, including KLFS, GLIF, and HEAT. Moreover, by searching the RegRNA Database, the rare variants may be involved in the pathogenesis of LN by regulating RNA transcription, RNA splicing, and miRNA binding. Among the binding miRNAs, miRNA143 and miRNA155 have been reported to be associated with the regulation of cell proliferation, apoptosis, and autophagy.[18,19] Mutation of *ATG5* in kidney epithelium resulted in tubulointerstitial disease, loss of organ function, and death,[20] and all *ATG5*-null mice died within 24 hours of birth,[8] suggesting the vital role of *ATG5* for life. Our data may be consistent with the data from *ATG5* knockout mice that noncoding variants rather than missense mutations may be involved in SLE susceptibility without lethal effect at birth. As we also observed that patients with *ATG5* mutations tended to be younger in age onset, future studies targeting SLE patients with family history or patients died in early age would be of importance.

However, in this study, except for age onset, quantity of urine protein, and C3 level, no significant difference of the clinical features, including serum creatinine, and pathological classes, existed in LN patients carrying rare variants and controls, which may be mainly due to the limitation of our relatively small sample size. In the future, the validation of the association of these rare variants and LN in a larger cohort is necessary. Besides, as both SLE and LN were still a complex phenotype with obscure pathogenesis, extreme phenotype was still difficult to be clearly defined. Thus, identifying the rare variants in extreme-phenotype LN patients may be helpful. Furthermore, as only the exons and flanking regions of *ATG5* were sequenced in our study, the noncoding variants emerging as important contributors to genetic disease but missed by exon capture, are still needed to be widely investigated.

Taken together, this study indicates that interrogation of rare variants will enable identification of potential genetic variants that can refine current pathogenesis models for the disease. The study provided further genetic data for *ATG5* variants in Chinese SLE patients, highlighting a likely important role of rare variants.

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