Research Article

Detecting Genetic Associations between ATG5 and Lupus Nephritis by trans-eQTL

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Objectives. Numerous loci were identified to perturb gene expression in trans. As elevated ATG5 expression was observed in systemic lupus erythematosus (SLE), the study was conducted to analyze the genome-wide genetic regulatory mechanisms associated with ATG5 expression in a Chinese population with lupus nephritis (LN).

Methods. The online expression quantitative trait loci database was searched for trans-expression single nucleotide polymorphisms (trans-eSNPs) of ATG5. Tagging trans-eSNPs were genotyped by a custom-made genotyping chip in 280 patients and 199 controls. For positive findings, clinical information and bioinformation analyses were performed. Results. Four trans-eSNPs were observed to be associated with susceptibility to LN (P < 0.05), including ANKRD50 rs17008504, AGA rs2271100, PAK7 rs6056923, and TET2 rs1391441, while seven other trans-eSNPs showed marginal significant associations (0.05 < P < 0.1). Correlations between the trans-eSNPs and ATG5 expression and different expression levels of ATG5 in SLE patients and controls were validated, and their regulatory effects were annotated. However, no significant associations were observed between different genotypes of trans-eSNPs and severity or outcome of the patients.

Conclusion. Using the new systemic genetics approach, we identified 10 loci associated with susceptibility to LN potentially, which may be complementary to future pathway based genetic studies.

1. Introduction

A strong body of evidence has suggested the complex genetic basis of systemic lupus erythematosus (SLE), and to date more than 50 loci have been identified, largely improving our insights into the pathogenesis of SLE [1, 2]. However, approximately 80%–90% of the associated variants were observed to be located in noncoding regions, which may have effects on gene expressions [3, 4]. As the gene expression level has been suggested to beheritable, the expression quantitative trait loci (eQTL) have been widely studied. Up to date, a number of eQTL were observed to influence gene expression through cis-acting regulatory effects (with the variants located within or near the target gene) [5], significantly broadening our understanding of genetic pathogenesis of diseases [6, 7]. However, trans-acting regulatory effects (with the variants distal to the target gene or on different chromosomes) have been seldom addressed.

Autophagy is a phyllogenetically ancient mechanism by which the cell can degrade and dispose of intracellular constituents or intracellular infectious agents in a regulated manner. Recently, genetic variants within or near ATG5—a gene product required for the formation of autophagosomes—have been identified to be associated with SLE by several genome-wide association studies (GWASs) [8, 9]. And elevated ATG5 expression level was observed in the splenic and renal macrophages of lupus mice and in peripheral blood mononuclear cells (PBMC) of SLE patients [10]. However, no significant associations between variants within ATG5 and SLE were observed in a Chinese population [9]. Also, in our
previous study [11], only variants in the PRDM1-ATG5 intergenic region not within ATG5 were detected to be associated with susceptibility to SLE, and they were detected to affect ATG5 expression level through a cis-eQTL effect. Animal studies suggested that ATG5-null mice were lethal within 24 hours of birth, indicating the important role of ATG5 for life. Thus, ATG5 was likely to be a strong susceptibility gene to SLE and its abnormal expression may be a key determinant in susceptibility. As a strong body of evidence supported that numerous loci perturb gene expression in trans [12–14], we thus hypothesized that genetic polymorphisms of autophagy genes may also function at the upstream of ATG5 by trans-eQTL effects to further modulate SLE susceptibility.

Lupus nephritis (LN), a major phenotype with poor prognosis of SLE, is possibly a kind of extreme phenotype. It was reported that autophagy inhibitions could decrease proteinuria levels, robustly reduced renal immune complex deposition, and remitted glomerulonephritis. Thus, by choosing trans-expression single nucleotide polymorphisms (trans-eSNPs) of ATG5 at the genome-wide level, the present genetic association study was conducted to search for the regulatory mechanisms associated with the gene expression of ATG5 in Chinese patients with LN.

2. Subjects and Methods

2.1. Subjects. A total of 280 LN patients from the Peking University First Hospital, who were of Han ethnicity living in north of China, were enrolled in the study. Their mean age was 33.2 ± 9.8 years and 247 of them were female. The controls were 198 geographically and ethnically matched healthy blood donors. Their mean age was 34.5 ± 10.3 years and 97 of them were female. One HapMap Han Chinese in Beijing (CHB) sample (NA18524) was included as a positive control for checking genotyping and no template control was taken as a negative control. All the patients met the revised SLE criteria of the American College of Rheumatology (ACR) [15] as a negative control. All the patients met the revised SLE for checking genotyping and no template control was taken in this study. In total, 1097 eSNPs were customized into a genotyping chip by an Illumina Solexa HiSeq 2000 platform (VC-201-0144). Beadpress Scanner and illumina Genomstudio were used for the analysis of chip data.

2.3. Statistical Analyses. For quality control analyses, trans-eSNPs were excluded if they had a call rate lower than 95% or a significant deviation from Hardy-Weinberg equilibrium in controls (P < 0.01). Similarly, we removed all the samples with a genotyping rate lower than 95% from further analysis. Principle component analysis (PCA) was used to detect population outliers in both cases and controls as previously described [16]. After quality control analyses for trans-eSNPs and samples, 68 trans-eSNPs in 279 LN patients and 199 controls were left for further genetic association analyses (Figure 1).

Allele frequencies were compared between cases and controls using chi-square tests and Fisher’s exact test was used when necessary. For the positive trans-eSNPs, we evaluated the associations between their genotypes and disease severity and outcome. Results of the measurement data were expressed as mean ± SD, and t-tests or one-way analysis of variance were used to analyze the difference. Statistical analyses were performed with SPSS16.0 software (SPSS Inc., Chicago, IL). A two-tailed P value of less than 0.05 was considered statistically significant.

2.4. Bioinformation Analyses. For noncoding variants, Regu- lomeDB and HaploRegv3 databases were searched for their regulatory effects. For exon variants, PolyPhen-2 (Polymorphism Phenotyping v2, http://genetics.bwh.harvard.edu/pph2/) was used to predict possible impact of an amino acid substitution on the structure and function of the protein.

To validate the trans-eQTL effects of the positive trans-eSNPs, the gene expression profiling of EBV-transformed lymphoblastoid cell lines of 270 unrelated HapMap individuals from the Gene Expression Variation project (GENEVAR project, http://www.sanger.ac.uk/humgen/genevar/) was used. Besides, the different expression levels of ATG5 were checked between SLE (E-GEOD-50772) and LN (E-GEOD-32592 and E-GEOD-32591) and healthy controls by using the ArrayExpress Archive database (http://www.ebi.ac.uk/arrayexpress/).

3. Results

3.1. Association Analyses of trans-eSNPs with LN. After quality control analyses, a total of 68 tagging trans-eSNPs were analyzed in 279 LN patients and 199 healthy controls. Four of them, including rs17008504 on ANKRD50 (OR = 0.645, 95% CI = 0.476 to 0.875, P = 0.005), rs2271100 on AGA (OR = 1.630, 95% CI = 1.105 to 2.405, P = 0.014), rs6056923 on PAK7 (OR = 0.546, 95% CI = 0.326 to 0.916, P = 0.022), and rs1391441 on TET2 (OR = 1.345, 95% CI = 1.039 to 1.743, P = 0.025), were observed to be associated with LN in the current study (Table 1). And, marginal significance was observed between 768 trans-eSNPs and susceptibility to LN (0.05 < P < 0.1), including rs712377 on SLC25A21 (OR = 1.283, 95% CI = 0.984 to 1.671, P = 0.065), rs391438 on TET2 (OR = 1.266, 95% CI = 0.978 to 1.638, P = 0.073), rs10878953 on CPSF6 (OR = 1.268, 95% CI = 0.977 to 1.647, P = 0.075), rs7529592 on AKNADI (VC-201-0144). Beadpress Scanner and illumina Genomstudio were used for the analysis of chip data.
(OR = 0.701, 95% CI = 0.463 to 1.062, P = 0.094), rs155098 on ITGA4 (OR = 1.264, 95% CI = 0.960 to 1.665, P = 0.095), rs7751485 on CDKAL1 (OR = 1.286, 95% CI = 0.975 to 1.698, P = 0.096), and rs7081173 on LINP (OR = 1.245, 95% CI = 0.962 to 1.611, P = 0.096). Thus, a total of 10 loci were suggested to play a potential role in the pathogenesis of LN through trans-eQTL effects.

To increase the detecting power, the genotype data of 136 CHB controls (except for the positive control NA18524) from the HapMap3 project, which were available for rs2271100, rs7529592, rs7081173, rs712377, rs1391438, and rs1391441, were downloaded. Using these data and our genotyping data, association analyses were performed. As can be seen in Table 1, almost all the loci showed more significant associations with combined data.

Besides, except for analyzing the association of the SNPs with LN susceptibility, we detected the association between trans-eSNPs genotypes and severity and outcome of LN patients in further, including their onset age, proteinuria, estimated glomerular filtration rate (eGFR), serum creatinine level, C3 level, systemic lupus nephritis disease activity index (SLEDAI) scores, percentage of crescent, different histological classes, response to treatment, and development of end stage renal disease (ESRD). Among them, response to treatment was measured by changes in proteinuria, and complete remission was defined as proteinuria <0.3 g per 24 hours while partial remission was defined as a decrease in proteinuria by at least 50% from the initial value and <3.5 g per 24 hours. Development of ESRD was defined as dialysis or death. However, no significant differences were observed between these clinical features and the different genotypes of the positive trans-eSNPs of ATG5 (see supplementary Table 1 in the Supplementary Material available online at http://dx.doi.org/10.1155/2015/153132), which may be due to the lower detecting power for subset analysis.

### Figure 1: The workflow of genetic association study of ATG5 by trans-eQTL

(a) eQTL: expression quantitative trait loci, eSNP: expression single nucleotide polymorphism, HWE: Hardy-Weinberg equilibrium, LN: lupus nephritis, MAF: minor allele frequency, PCA: principle component analysis, and SLE: systemic lupus erythematosus. (b) The online eQTL database: mRNA by SNP Browser v 1.0.1, http://www.sph.umich.edu/csg/liang/imputation/byGene.html. (c) HaploReg v3 database: http://www.broadinstitute.org/mammals/haploreg_v3.php. RegulomeDB database: http://regulome.stanford.edu/. (d) GENEVAR project: the Gene Expression Variation project, http://www.sanger.ac.uk/humgen/genevar/. (e) The ArrayExpress Archive database: http://www.ebi.ac.uk/arrayexpress/.

3.2. Functional Annotations by ENCODE Databases. RegulomeDB and HaploReg databases were used to annotate the regulatory effects of the positive trans-eSNPs. In RegulomeDB database, the trans-eSNPs with scores between 1 and 4 were listed (Supplementary Table 2); among them rs2271100, rs7081173, rs10788613, and rs1391441, were downloaded. Using these data and our genotyping data, association analyses were performed. As can be seen in Table 1, almost all the loci showed more significant associations with combined data.

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### Table 1: Allelic association analyses of the positive trans-eSNPs of ATG5 in LN.

| Chr | Position | Locus | SNP (minor allele) | MAF Case/control (%) | Allele OR by minor allele (95% CI) | Allele P values | Current population | Combined with HapMap CHB population |
|-----|----------|-------|-------------------|----------------------|-----------------------------------|----------------|-------------------|-------------------|
| 4   | 124718662 | 5’ of ANKRD50 | rs7008504(A) | 19.2/26.9 | 0.645 (0.476, 0.875) | 0.005 | — |
| 4   | 177438525 | AGA | rs2271100(G) | 16.1/10.6 | 1.630 (1.105, 2.405) | 0.014 | 0.012 |
| 20  | 9840271  | 5’ of PAK7 | rs6056923(G) | 4.9/8.5 | 0.546 (0.326, 0.916) | 0.022 | — |
| 4   | 105207603 | TET2 | rs1391441(G) | 49.8/42.3 | 1.345 (1.039, 1.743) | 0.025 | 0.002 |
| 14  | 36867767 | SLC25A21 | rs712377(C) | 42.3/36.4 | 1.283 (0.984, 1.671) | 0.065 | 0.146 |
| 4   | 105230686 | TET2 | rs1391438(G) | 51.6/45.7 | 1.266 (0.978, 1.638) | 0.073 | 0.009 |
| 8   | 69280407 | 3’ of CPSF6 | rs10878953(G) | 45.0/39.2 | 1.268 (0.977, 1.647) | 0.075 | — |
| 1   | 108823932 | AKNAD1 | rs7529592(G) | 9.0/12.3 | 0.701 (0.463, 1.062) | 0.094 | 0.080 |
| 2   | 181482227 | ITGA4 | rs155098(G) | 35.3/30.2 | 1.264 (0.960, 1.665) | 0.095 | — |
| 6   | 21127081 | CDKAL1 | rs7751485(G) | 34.8/29.3 | 1.286 (0.975, 1.698) | 0.096 | — |
| 10  | 88787819 | 3’ of LIPN | rs7081173(A) | 50.2/44.7 | 1.245 (0.962, 1.611) | 0.096 | 0.116 |

(a) CHB: Han Chinese in Beijing. 95% CI: 95% confidence interval, chr: chromosome, LN: lupus nephritis, OR: odds ratio, SNP: single nucleotide polymorphism.

(b) P values were calculated by chi-square test using 2 × 2 contingency tables based on allele frequencies.

(c) None of the genotypes in the controls or patients showed significant deviation from Hardy-Weinberg equilibrium.

(d) Chromosome positions were referred to GRCh38.

#### 3.3. Validation of trans-eQTL Effects of the Positive trans-eSNPs and the Differential Expression Level of ATG5 in SLE.

The 11 positive trans-eSNPs were significantly associated with the expression level of ATG5 in our selective database of lymphoblastoid cell lines from 400 children (correlation coefficient ranged from 0.666 to 0.999, and \( P \) ranged from 0.00015 to \( 6.75 \times 10^{-6} \)). Their trans-eQTL effects were validated in 270 unrelated HapMap individuals. As shown in Table 2, except for rs155098 and rs7751485, the significant associations between the positive trans-eSNPs and ATG5 expression level were consistently verified.

Furthermore, we ascertained whether ATG5 was expressed differently in SLE patients and healthy controls. The expression level of ATG5 was significantly higher in SLE PBMC and LN tubulointerstitial samples than those of controls (1794.00 ± 240.22 versus 1541.73 ± 201.64, \( P = 6.27 \times 10^{-5} \); 6.75 ± 0.19 versus 6.46 ± 0.20, \( P = 1.82 \times 10^{-5} \) ) while in glomeruli samples of LN only marginal significant association was observed (7.86 ± 0.22 versus 7.73 ± 0.15, \( P = 0.053 \)).

#### 4. Discussion

Hypothesis-free GWASs have significantly broadened our views about genetic pathogenesis of SLE [1]. However, the majority of the associated variants were noncoding variants and with modest effects (OR = 1.1–1.5), which can only account for a small proportion of heritability of SLE. Gene expression level was suggested to be heritable, and the detection of the related eSNPs was considered to be an efficient way to reconstruct gene networks [17]. As a disease-predisposing gene, higher ATG5 expression level [10,11] and variants with cis-eQTL effects within or near ATG5 [11] were observed in SLE. Thus, we suspect that any variants that perturb the expression of ATG5 in trans would also be related to the susceptibility to SLE. Thus, the present study was conducted to analyze the genome-wide genetic regulatory mechanisms associated with the gene expression of ATG5 in Chinese LN patients. By searching the online eQTL database, 78 tagging trans-eSNPs of ATG5 were genotyped for the association study. The results showed that 4 trans-eSNPs, including rs7008504, rs2271100, rs6056923, and rs1391441, showed significant associations with susceptibility to LN (\( P < 0.05 \)), while 7 trans-eSNPs, including rs712377, rs1391438, rs10878953, rs7529592, rs135098, rs7751485, and rs7081173, showed marginal significant associations (0.05 < \( P < 0.1 \)). However, no significant associations between the SNPs and severity or outcome of patients with LN were observed in the current study. In silico analysis suggested their regulatory effects. Besides, compared with healthy controls, higher expression level of ATG5 was observed in PBMC, tubulointerstitial, and glomeruli samples of SLE patients, and
the correlation between the positive trans-eSNPs and ATG5 expression level was validated in 270 unrelated HapMap individuals, suggesting the potential role of these loci in the pathogenesis of LN through perturbing the expression of ATG5.

Since ATG5 was reported to be expressed in B cells, the eQTL database of lymphoblastoid cell lines from 400 children was chosen for searching trans-eSNPs of ATG5, for its relatively larger sample size and that environmental factors tend to have less effects on children. However, though the correlation between the trans-eSNPs and ATG5 expression was validated in EBV-transformed lymphoblastoid B cell lines from the 270 unrelated HapMap individuals (JPT, CHB, CEU, and YRI), the association significance seemed to be weaker. This may be due to the smaller sample size, and also it was presumed that trans-eQTL effects are often more indirect and therefore weaker [14], indicating the necessity to replicate the associations in a larger cohort in the future.

The present study, 4 loci, including ANKRD50, AGA, PAK7, and TET2, were identified to be associated with susceptibility to LN significantly. ANKRD50 (ankyrin repeat domain 50) was reported to have an essential role in the SNX27-retromer-mediated endosome-to-plasma-membrane recycling [18], but its exact mechanism was still needed to be studied further. AGA (aspartylglucosaminidase) is a lysosomal hydrolase that participates in the degradation of glycoproteins. Like other lysosomal enzymes, the deficiency of AGA leads to lysosomal storage disorder and AGA mutation was suggested to be weakly associated with chronic arthritis [19]. Thus, whether the dysfunction of AGA is associated with susceptibility to SLE by affecting autophagy—a degradation process conducted by lysosome—should be studied. PAK7 (p21 protein-activated kinase 7) is a member of Ser/Thr protein kinases, which has effects on cytoskeletal dynamics, cell proliferation, and survival [20]. For both ATG5 and microtubule associated light chain 3 (LC3) plays important roles in the extension of autophagosome membrane, PAK7 may regulate the expression of ATG5 through microtubule system.

TET2 (tet methylcytosine dioxygenase) is a methylcytosine dioxygenase that catalyzes the conversion of methylcytosine to 5-hydroxymethylcytosine. It was involved in chromatin modifications and other cellular processes through the interaction with O-linked β-N-acetylgalactosamine transferase and it was also reported to be associated with several myeloproliferative disorders [21, 22]. Future studies are needed to uncover the mechanism of epigenetic and signaling networks wired with TET2 in SLE. Besides, another 6 loci with marginal associations, including SLC25A21, CPSF6, AKNAD1, ITGA4, CDKAL1, and LINP, were identified. SLC25A21 (solute carrier family 25 member 21) is a homolog of the S. cerevisiae ODC proteins. It is reported that HIV-1 Vif downregulates the expression of SLC25A21 in Vif-expression T cells [23]. And, virus infection was closely associated with SLE, indicating the potential role of SLC25A21 in SLE. CPSF6 (cleavage and polyadenylation specific factor 6) is one subunit of a cleavage factor required for 3′ RNA cleavage and polyadenylation processing. The interaction between CPSF6 and RNA is involved in the assembly of the 3′ end processing complex and facilitates the recruitment of other processing factors. AKNAD1 (AKNA domain containing 1) contains a domain found in an AT-hook-containing transcription factor and its alternative splicing can result in multiple transcript variants. ITGA4 (alpha 4 subunit of VLA-4 receptor) belongs to the integrin alpha chain family proteins, which can mediate adhesion of cells [24] and participate in B cell apoptosis. Besides, gene expression of integrins and their ligands was found to be upregulated in rheumatoid arthritis [25]. CDKAL1 (CDK5 regulatory subunit associated protein 1-like 1) is a member of the methylthiotransferase family and GWASs have linked intronic SNPs of CDKAL1 with susceptibility to type 2 diabetes [26]. As autophagy is also strongly suggested to be associated with the pathogenesis of metabolic diseases including diabetes, the interaction between CDKAL1 and ATG5 could exist. LINP (lipase, family member N) is imperfect.

### Table 2: Correlation between genotypes of the positive trans-eSNP with ATG5 expression in public databases.

| Gene          | Positive trans-eSNPs (n = 400) | HapMap LCL (n = 270) |
|---------------|-------------------------------|----------------------|
| ANKRD50       | rs7008504                     | rs1027497           |
|               | 9.10 × 10⁻⁵                   | 0.92                 |
| AGA           | rs2271100                     | rs2271100           |
|               | 1.20 × 10⁻⁴                   | 1                    |
| PAK7          | rs6056923                     | rs6056922           |
|               | 1.10 × 10⁻⁴                   | 1                    |
| TET2          | rs1391441                     | rs1391441           |
|               | 5.90 × 10⁻⁶                   | 1                    |
| TET2          | rs1391438                     | rs7655890           |
|               | 7.10 × 10⁻⁶                   | 0.99                 |
| SLC25A21      | rs712377                      | rs712377            |
|               | 6.70 × 10⁻⁵                   | 1                    |
| CPSF6         | rs10878953                    | rs7308481           |
|               | 6.40 × 10⁻⁵                   | 0.99                 |
| AKNAD1        | rs7529592                     | rs7529592           |
|               | 6.00 × 10⁻⁵                   | 1                    |
| ITGA4         | rs155098                      | rs155099            |
|               | 1.20 × 10⁻⁴                   | 1                    |
| CDKAL1        | rs7751485                     | rs10946430          |
|               | 1.00 × 10⁻⁶                   | 0.97                 |
| LINP          | rs7081173                     | rs7081173           |
|               | 1.50 × 10⁻⁴                   | 1                    |

(a) LCL: lymphoblastoid cell lines, trans-eSNP: expression single nucleotide polymorphism.
(b) Children LCL refers to lymphoblastoid cell lines from 400 children from families recruited through a proband with asthma.
(c) HapMap LCL refers to Epstein-Barr virus-transformed lymphoblastoid cell lines from 270 HapMap CEU, CHB, JPT, and YRI individuals (http://www.sanger.ac.uk/humgen/genevar/).
(d) Only the SNP on each locus with strongest associated significance with ATG5 expression level was shown.
a lipase that is highly expressed in granular keratinocytes in the epidermis and plays a role in the differentiation of keratinocytes. Mutations in this gene were reported to be associated with lamellar ichthyosis type 4 [27]. In the present study, a missense variant rs10788611 within LIPN showed potential association with LN, which has caused the amino acid change from Threonine (T) to Asparagine (N) at position 244. However, its effects on protein function were seldom studied. Overall, the data above provided some clues for understanding the potential role of ATG5 in the genetic pathogenesis of SLE.

However, there were still some limits in the present study. Due to the low allelic frequency of some trans-eSNPs and the relatively small sample size, the associations between trans-eSNPs and susceptibility to LN seemed to be weak, which could be enhanced by adding the referred CHB controls in HapMap. Besides, the smaller sample size of genotype subsets could be difficult to provide enough power for analyzing the association between trans-eSNPs genotypes and clinical features. Thus, to replicate the associations in a larger population is still needed in the future.

In summary, the present study established a relationship between trans-eSNPs of ATG5 and LN in a northern Han population from China. By this new systemic genetics approach, 10 loci have been identified to be associated with LN potentially, widely broadening our understanding of the genetics role of ATG5 in LN. Although these variants showed moderate associations, our approach allows for analysis of association data from a new perspective, and the results may be complementary to future pathway-based genetic studies in SLE.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors’ Contribution

Yue-miao Zhang and Fa-juan Cheng contributed equally to this work.

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