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Original Article

Association of miR-181 cluster polymorphisms with systemic lupus erythematosus risk

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Abstract: Systemic lupus erythematosus (SLE) is an autoimmune disease manifested by self-reactive antibodies due to failure of selection in both B and T lymphocytes. miR-181, expressed in hematopoietic cell lineage, has been proven to be an important modulator of B- and T-cell differentiation, maturation, and function. This study aimed to investigate association of three polymorphisms (rs16927589 T>C, rs77418916 A>T, and rs8108402 C>T) of the miR-181 cluster with risk of SLE in a Chinese population. In this study, 202 patients with SLE and 299 control subjects were included. miR-181 polymorphisms were discriminated by Snapshot SNP genotyping assay and DNA sequencing methods. It was found that T allele, TT, and TC genotypes, and the TT/CT vs. CC and CC/CT vs. TT models of rs8108402 C>T polymorphism were associated with increased risk of SLE (T vs. C: adjusted OR=1.51, 95% CI: 1.13~2.00, *P*=0.005; TT vs. CC: adjusted OR=2.65, 95% CI: 1.18~5.98, *P*=0.019; TC vs. CC: adjusted OR=1.50, 95% CI, 1.03~2.19, *P*=0.033; TT/CT vs. CC: adjusted OR=1.61, 95% CI: 1.12~2.31, *P*=0.010; CC/CT vs. TT: adjusted OR=2.23, 95% CI: 1.01~4.93, *P*=0.048). Haplotype analysis showed that TAT haplotype was associated with increased risk of SLE (OR=1.48, 95% CI: 1.09~2.00, *P*=0.011), while TAC haplotype was associated with decreased risk of SLE (OR=0.68, 95% CI: 0.53~0.89, *P*=0.004). However, significant association between the other two polymorphisms and SLE risk was not observed. In conclusion, for the first time, it was found that rs8108402 C>T polymorphism is associated with increased risk of SLE, in a Chinese population.

Keywords: miR-181, gene, polymorphisms, systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is a severe systemic autoimmune disease characterized by self-reactive antibodies due to failure of selection in both B and T lymphocytes. It ultimately results in immune intolerance and is accompanied by an increasing rate of apoptosis and deficiency in the clearance of apoptotic cells [1, 2]. SLE seriously threatens human health, usually accompanied by many complications that make it more difficult to cure. At present, several non-genetic risk factors such as infection, hormones, cytokines dysregulation, drugs, and environmental factors have been identified as contributing to the pathogenesis of SLE [3-5]. However, the exact pathogenic mechanism of SLE has not been fully elucidated. Existing reports have demonstrated that genetic factors may play important roles in development of SLE [6-8].

MicroRNAs (miRNAs) are small non-coding single-stranded RNAs that regulate posttranscriptional gene expression and play important roles in various biological functions including inflammation, atherosclerosis, and autoimmune response [9, 10]. The miR-181 family constitutes four members (miR-181a~d). As one of the members of this family, miR-181a has been closely related to the pathogenesis of SLE. miR-181a can be expressed by many human organs, such as the brain and lungs. However, miR-181a is expressed highest in the thymus [11],

Table 1. Primer sequences used for detecting rs16927589, rs77418916, and rs8108402 polymorphisms

Reference SNP ID	PCR primers
rs16927589	F: 5'-TAATTAGGGGCAAGGGGAGGAT-3'
	R: 5'-TATGTGGGGATATGTGGGCACT-3'
	EF: 5'-TTTTTTTTTTTTTTTTTTCAAAGTGAAATGTGATAAGAACTGTC-3'
rs77418916	F: 5'-TGCACAGTCTATCCCACAGTTCATT-3'
	R: 5'-CTGGCCTGATAGCCCTTCTTCA-3'
	EF: 5'-TTTTTTTTTTTTTTTTTTTTTTTTTTCAGTTCATTAGTTCTCTGCTGCACAC-3'
rs8108402	F: 5'-ACTCTGTGCGATCACTGGAGGA-3'
	R: 5'-GGAATCACAACAGCGTGAGTGG-3'
	EF: 5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTATGCTGTGTGACCGCGCACG-3'

suggesting that miR-181a may play an important role in the function and maturation of T lymphocytes. Moreover, miR-181a can dynamically regulate thymocytes differentiation and is expressed highest in early stages of T-cell differentiation, especially in double positive and double negative thymocytes, indicating that miR-181a may be involved in T-cell selection [12]. Additionally, miR-181a can modulate TCR-mediated T-cell activation and augment both the strength and sensitivity of TCR signaling to strong and weak agonists, suggesting that miR-181a may play crucial role in the development, function, and maintenance of T-cell tolerance [13]. SLE is characterized by the presence of auto-antibodies such as antinuclear antibodies (ANA), anti-DNA antibodies, and anti-Smith antibodies (Anti-Sm). It is also characterized by the defection of B- and T-cell tolerance and maturation, resulting in the increase of these auto-antibodies [14]. Taken together, these evidences indicate that the miR-181 family may play a crucial role in the pathogenesis of SLE.

rs16927589 T>C and rs77418916 A>T polymorphisms are located on the intron of mir-181b and mir-181a genes, respectively. The rs8108402 C>T polymorphism is located on the promoter of the miR181c/d gene. Previous studies have reported that polymorphisms on intron and promoter of human genes are associated with susceptibility of different kinds of human diseases [15-17]. Recently, Jing et al. [18] reported that rs1891385 polymorphism, located on the first intron of IL-33, was associated with increased risk of SLE. Wei et al. [19] found that rs4705342 polymorphism of the mir-143/145 was associated with decreased risk of ischemic stroke. To the best of our knowledge, association between these three polymorphisms and risk of SLE has not been reported. Thus, in this present study, association between rs16927589 T>C, rs77418916 A>T, and rs8108402 C>T polymorphisms and risk of SLE, in a Chinese population, was investigated.

Materials and methods

Study population

This case-control study was approved by the Ethics Committee of Affiliated Hospital of Youjiang Medical University for Nationalities. Informed consent was obtained from all participants. The case group included 202 SLE patients consecutively recruited from the departments of Dermatology, Nephrology, and Rheumatology of the Affiliated Hospital of Youjiang Medical University for Nationalities, from January 2013 to September 2016. There were 180 women and 22 men with a mean age of 41.25±14.63 years. All SLE patients recruited into the study met the 1997 revised American College of Rheumatology (ACR) SLE criteria. Moreover, 299 age and gender matched controls, who came to the same hospital for routine medical checkups during the same period, were recruited. There were 261 women and 38 men with a mean age of 40.86±9.87 years. According to thorough clinical and laboratory evaluations, none of them were found to have any medical conditions. Clinical information such as age, gender, and lupus nephritis were obtained from Medical Record Review. All participants were unrelated individuals from the same geographic region.

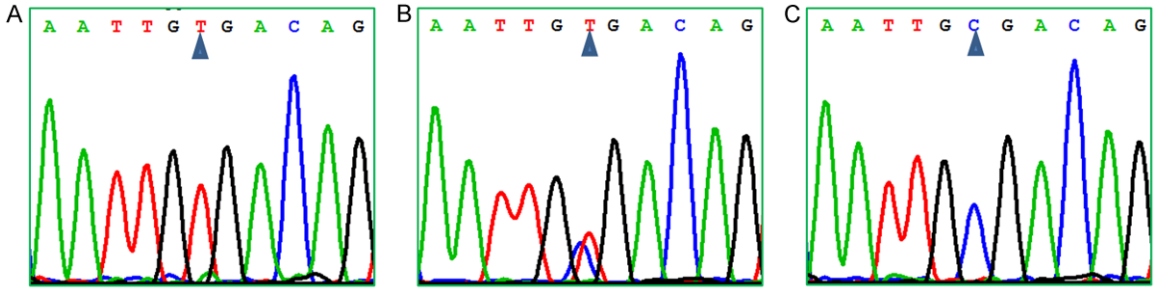

Figure 1. Sequencing diagram of genotype for miR-181 gene rs16927589 polymorphism. Note: The arrow of figure (A-C) shows TT, TC, and CC genotypes, respectively.

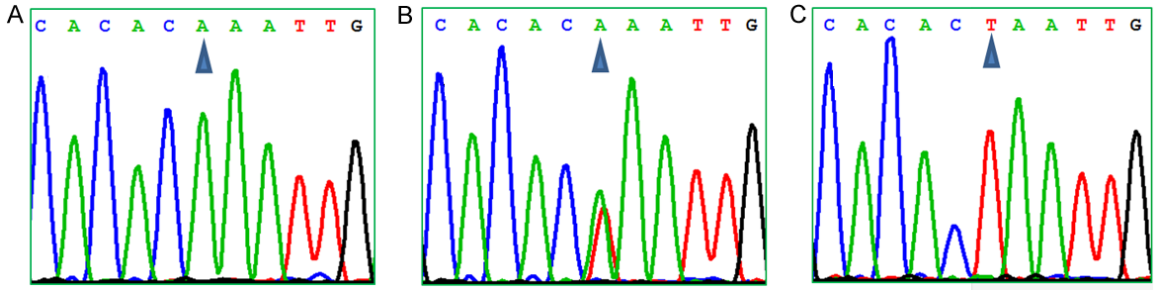

Figure 2. Sequencing diagram of genotype for miR-181 gene rs77418916 polymorphism. Note: The arrow of figure (A-C) shows AA, AT, and TT genotypes, respectively.

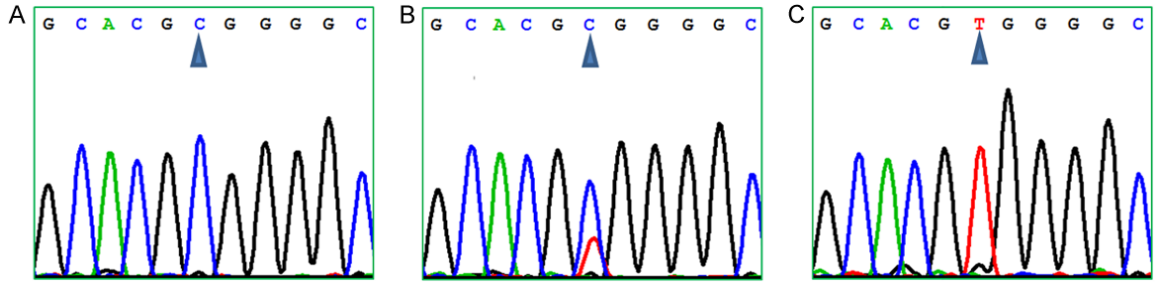

Figure 3. Sequencing diagram of genotype for miR-181 gene rs8108402 polymorphism. Note: The arrow of figure (A-C) shows CC, CT, and TT genotypes, respectively.

DNA isolation and genotyping

Genomic DNA was extracted from peripheral blood by using a whole-blood genome DNA extraction reagent kit (DP318, QIANGEN, China), according to manufacturer instructions. PCR primers were designed based on the GenBank reference sequence of the miR-181 cluster. Their sequences are listed in **Table 1**. Genotyping methods have been described in detail, previously [20]. Briefly, genotypes of rs16927589 T>C, rs77418916 A>T, and rs8108402 C>T polymorphisms were detected by Snapshot SNP genotyping assay. Genotyping results were read by using GeneMapper4.1 (Applied Biosystems). Results were read by two independent research assistants, blind to the cases and controls. DNA sequencing method was used to confirm results, which were 100% concordant (**Figures 1-3**).

Lymphocyte subsets analysis by flow cytometry

Peripheral blood for lymphocyte subsets analysis was collected in EDTA tubes. Fifty μL peripheral blood was incubated with 10 μL fluorescent dye-conjugated monoclonal antibodies [Becton Dickinson (BD), Franklin Lakes, NJ, USA] in the dark at room temperature for 15 minutes. Afterward, erythrocytes were lysed

Table 2. Demographic and clinical characteristics of the study participants

Variable	Cases	Controls	*P* value
Age (M ± SD)	41.25±14.63	40.86±9.87	0.441
Genders (Female/Male)	180/22	261/38	0.539
24 h urinary protein, +ve/-ve	91/26	-	-
Lupus nephritis (Yes/No)	96/106	-	-
Anti-ANA, +ve/-ve	152/17	-	-
Anti-dsDNA, +ve/-ve	63/106	-	-
ANCA, +ve/-ve	35/103	-	-
Anti-Sm, +ve/-ve	67/103	-	-
Anti-RIB, +ve/-ve	62/107	-	-

M ± SD, mean ± standard deviation; ANA, antinuclear antibodies; Anti-Sm, anti-Smith antibodies; +ve, positive; -ve, negative; ANCA, antineutrophil cytoplasmic antibodies; Anti-RIB, Anti ribosomal P protein antibody.

Table 3. Lymphocyte subsets analysis of SLE patients and healthy controls

Variable	Cases	Controls	*P* value
$CD3^+$			1.321×10^{-12}
955-2860	16 (25.8)	39 (97.5)	-
<955	46 (74.2)	1 (2.5)	-
$CD4^+$			2.400×10^{-11}
550-1440	11 (17.7)	34 (85.0)	-
<550	51 (82.3)	6 (15.0)	-
$CD8^+$			5.483×10^{-9}
320-1250	25 (40.3)	39 (97.5)	-
<320	37 (59.7)	1 (2.5)	-
NK			2.972×10^{-12}
150-1100	12 (19.4)	36 (90.0)	-
<955	50 (80.6)	4 (10.0)	-
Th/Ts			1.445×10^{-4}
0.7-2.8	40 (645)	39 (97.5)	-
<0.7	21 (35.5)	1 (2.5)	-

with 450 μL of Lysing Solution (BD) and incubated in the dark at room temperature for 15 minutes. Finally, acquisition was analyzed using a 6-color FACS Canto (BD) for 15,000 events per sample. Data were processed using FACS Diva software (BD).

Statistical analysis

Statistical analyses were calculated using SPSS 23.0 statistical software package (SPSS Inc., Chicago, IL, USA). Gender, $CD3^+$, $CD4^+$, $CD8^+$, NK, and Th/Ts were categorical variables. Comparison of data was measured by Chi-square test. Continuous variables, such as age, are displayed as mean ± SD. If these data were normally distributed, Student's t-test was used, otherwise, rank-sum test was used. Hardy-Weinberg equilibrium (HWE) was tested by Chi-square test. Logistic regression was used to assess association between the three polymorphisms and risk of SLE. Odds ratio (OR) and 95% confidence interval (CI) of the three polymorphisms were adjusted based on age and gender. Haplotype analysis was performed by online SHEsis software [21]. Statistical significance was set at $P<0.05$.

Results

In this case-control study, 202 patients with SLE and 299 healthy individuals were included. Demographic and clinical characteristics of study participants are listed in **Table 2**. There were no significant differences between the two groups based on age and gender. In patients with SLE, 24-hour urinary protein levels were significantly increased. Antibodies such as anti-ANA, anti-dsDNA, anti-Sm, and rib-P were positive in many patients with SLE.

Lymphocyte subsets analysis was performed and the results are presented in **Table 3**. It was found that levels of $CD3^+$, $CD4^+$, $CD8^+$ positive cells, and NK end Th/Ts cells were significantly decreased in patients with SLE, compared with healthy controls.

Genotype and allele frequencies of the three polymorphisms are shown in **Table 4**. Genotype distributions of the three polymorphisms, in both cases and controls, were in HWE, with *P* values of 0.200 and 0.245 for rs16927589 T>C, 0.200 and 0.275 for rs77418916 A>T, and 0.161 and 0.316 for rs8108402 C>T, respectively (**Table 4**). The allele distribution of rs8108402 C>T polymorphism was significantly different between SLE patients and control subjects and T allele was found to associate with an increased risk of SLE compared with C allele (T vs. C: adjusted OR=1.51, 95% CI: 1.13~2.00, P=0.005). Moreover, significantly increased risk was also observed in genotypes and model analysis (TT vs. CC: adjusted OR=2.65, 95% CI: 1.18~5.98, *P*=0.019; TC vs. CC: adjusted OR=1.50, 95% CI, 1.03~2.19, *P*=0.033; TT/CT vs. CC: adjusted OR=1.61,

Table 4. Genotype distributions of miR-181 polymorphisms between control patients and SLE group

Polymorphisms	Controls n=299 (%)	Cases n=202 (%)	Logistic Regression		Logistic Regression	
			OR (95% CI)	*P* value	OR (95% CI)†	*P* value†
rs16927589						
TT	220 (73.6)	140 (69.3)	1.00 (Ref)		1.00 (Ref)	
CT	76 (25.4)	59 (29.2)	1.22 (0.82~1.82)	0.331	1.23 (0.82~1.84)	0.317
CC	3 (1.0)	3 (1.5)	1.57 (0.31~7.90)	0.583	1.51 (0.30~7.60)	0.618
Dominant model						
CC/CT vs. TT			1.23 (0.83~1.83)	0.297	1.24 (0.83~1.84)	0.288
Recessive model						
TT/CT vs. CC			1.49 (0.30~7.44)	0.629	0.1.43 (0.28~7.17)	0.665
T	516 (86.3)	339 (83.9)	1.00 (Ref)			
C	82 (13.7)	65 (16.1)	1.21 (0.85~1.72)	0.297	1.21 (0.85~1.72)	0.295
HWE	0.200	0.245				
rs77418916						
AA	220 (73.6)	141 (69.8)	1.00 (Ref)		1.00 (Ref)	
AT	76 (25.4)	58 (28.7)	1.19 (0.80~1.78)	0.395	1.20 (0.80~1.79)	0.381
TT	3 (1.0)	3 (1.5)	1.56 (0.31~7.84)	0.589	1.50 (0.30~7.55)	0.624
Dominant model						
TT/AT vs. AA			1.21 (0.81~1.79)	0.356	1.21 (0.81~1.79)	0.347
Recessive model						
AA/AT vs. TT			1.49 (0.30~7.44)	0.629	1.43 (0.28~7.17)	0.665
A	516 (86.3)	340 (84.2)	1.00 (Ref)			
T	82 (13.7)	64 (15.8)	1.19 (0.83~1.69)	0.349	1.19 (0.83~1.69)	0.347
HWE	0.200	0.275				
rs8108402						
CC	175 (58.5)	94 (46.5)	1.00 (Ref)		1.00 (Ref)	
CT	113 (37.8)	92 (45.5)	1.52 (1.05~2.20)	0.029	1.50 (1.03~2.19)	0.033
TT	11 (3.7)	16 (7.9)	2.71 (1.21~6.07)	0.016	2.65 (1.18~5.98)	0.019
Dominant model						
TT/CT vs.CC			1.62 (1.13~2.32)	0.008	1.61 (1.12~2.31)	0.010
Recessive model						
CC/CT vs. TT			2.25 (1.02~4.96)	0.044	2.23 (1.01~4.93)	0.048
C	463 (77.4)	280 (69.3)	1.00 (Ref)			
T	135 (22.6)	124 (30.7)	1.52 (1.14~2.02)	0.004	1.51 (1.13~2.00)	0.005
HWE	0.161	0.316				

OR, odds ratio; CI, confidence interval; Ref, reference category; †, Adjusted by age, gender.

95% CI: 1.12~2.31, *P*=0.010; CC/CT vs. TT: adjusted OR=2.23, 95% CI: 1.01~4.93, *P*= 0.048). However, significant association between the other two polymorphisms (rs16927589 T>C and rs77418916 A>T) and risk of SLE was not observed.

Haplotype analysis and linkage disequilibrium (LD) analysis of the three polymorphisms, between cases and controls, were performed and results are shown in **Table 5** and **Figure 4**, respectively. It was found that rs16927589 T>C was in strong LD with rs77418916 A>T polymorphisms (D'=1.00, r=0.99). As shown in **Table 5**, it is evident that TAT haplotype was associated with increased risk of SLE (OR=1.48, 95% CI: 1.09~2.00, P=0.011). TAC haplotype, however, was associated with decreased risk of SLE (OR=0.68, 95% CI: 0.53~0.89, P=0.004).

Discussion

To the best of our knowledge, this was the first study to evaluate association between

Table 5. Haplotype analysis of the 3 polymorphisms and risk of SLE

Haplotypes	Controls (%)	Cases (%)	OR (95% CI)	*P* value
T T C	21 (3.6)	19 (4.8)	1.36 (0.73~2.55)	0.334
T A T	114 (19.0)	104 (25.6)	1.48 (1.09~2.00)	0.011
T A C	402 (67.3)	235 (58.3)	0.68 (0.53~0.89)	0.004
C T C	61 (10.1)	45 (11.0)	1.10 (0.73~1.66)	0.636
C T T	5 (1.2)	4 (0.7)	-	-
C A C	0 (0.0)	0 (0.0)	-	-
C A T	0 (0.0)	1 (0.2)	-	-

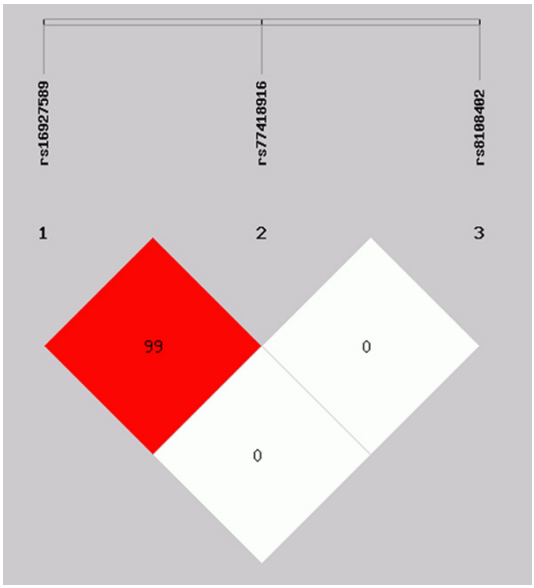

Figure 4. Linkage disequilibrium analyses of the three polymorphisms. rs16927589 and rs77418916 polymorphisms were in strong linkage disequilibrium (R2=0.99). The right polymorphisms were in relative no linkage disequilibrium.

rs16927589 T>C, rs77418916 A>T, and rs8108402 C>T polymorphisms and risk of SLE. It was demonstrated that T allele, TT, and TC genotypes and TT/CT vs. CC and CC/CT vs. TT models of the rs8108402 C>T polymorphism were associated with increased risk of SLE. Moreover, we found that TAT haplotype was associated with increased risk of SLE but TAC haplotype was associated with decreased risk of SLE.

SLE is a chronic and systemic autoimmune disease characterized by polyclonal B-cell activation, elevated pathogenic autoantibodies production, impaired immune complex clearance, and inflammatory responses, causing severe organ damage [22-25]. At present, the etiology and pathogenesis of SLE have not been fully elucidated. miR-181 cluster is a newly discovered member of the microRNA family with various biological functions [26]. The human miR-181 family constitutes four members (miR-181a~d). They are encoded by three different transcripts located on three different chromosomes. Previous studies have demonstrated that the miR-181 family may play important roles in differentiation, maturation, and function of T- and B-cells [11-13, 27]. Moreover, several studies have implicated critical roles for miR-181 family members in cardiovascular inflammation and immune cell homeostasis [2, 26, 28]. Additionally, Carlsen AL et al. [26], Neilson JR et al. [12], and Shen N et al. [29] have confirmed that miR-181 plays a crucial role in occurrence and development of systemic lupus erythematosus by promoting CD4 and CD8 double-positive T-cell development. However, not all subjects with altered miR-181 expression will finally develop SLE, suggesting that genetic variants may be related to risk of SLE.

rs8108402 C>T polymorphism is located on the promoter of the miR-181c/d gene. Previously, a large number of studies have reported that single nucleotide polymorphisms in the promoter region of human miRNA genes are associated with susceptibility to different kinds of human diseases [10, 30, 31]. Gao et al. [32] confirmed that rs4938723 polymorphisms in the promoter region of pri-miR-34b/c is a protective factor for development of colorectal cancer. Wei et al. [19] has found that rs4705342 in the promoter of miR-143/145 is a protective factor for ischemic stroke, probably by reducing levels of miR-145. To the best of our knowledge, genetic association studies between miR-181 polymorphisms and risk of SLE have not been carried out. In this present case-control study, association between three polymorphisms of miR-181 cluster and risk of SLE, in a Chinese population, was investigated. For the first time, it was found that rs8108402 T allele, TT, and TC genotypes and TT/CT vs. CC and CC/CT vs. TT models of the rs8108402 C>T polymorphism were associated with significantly increased risk of SLE. Data from this study suggests that rs8108402 C>T polymorphism may serve as a risk factor for predicting SLE in the Chinese population.

Although the results are promising, there were several limitations to this study. First, this study was based on hospitalized controls, possibly leading to selection bias. Thus, population-based replication studies will be important in confirming these findings. Second, genotype-phenotype analysis could not be performed due to a lack of clinical data of the patients.

In summary, this study provides evidence that rs8108402 C>T polymorphism is associated with an increased risk of SLE, in the Chinese population. These findings, if validated by larger cohort studies with different ethnic groups, will help us to better understand the precise effects of polymorphisms on the etiology of SLE.

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Disclosure of conflict of interest

None.

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