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Association study of a single nucleotide polymorphism in the exon 2 region of toll-like receptor 9 (*TLR9*) gene with susceptibility to systemic lupus erythematosus among Chinese

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Abstract Toll-like receptor 9 (TLR9) plays an important role in the induction and regulation of the innate immune system or adaptive immune responses. Genetic variations within human *TLR9* have been reported to be associated with a range of immune-related diseases, such as asthma, systemic lupus erythematosus (SLE) and so on. Family-based association analysis was performed to further investigate whether a single nucleotide polymorphism (rs352140) in the exon 2 region of *TLR9* gene is associated with susceptibility to SLE in a Chinese population. A total of 77 patients with SLE from 74 nuclear families, aged from 12 to 63 years, were enrolled according to 1997 criteria of American College of Rheumatology (ACR), 211 family members of these patients were also included. Genotyping was performed by PCR-restriction fragment length polymorphism (PCR-RFLP) assay. Among 77 patients with SLE, the CC, CT and TT genetype frequencies of the SNP (rs352140) were 20.8, 61.0 and 18.2%, respectively. Single loci analysis suggested that the T allele at position of rs352140 was significantly associated with the susceptibility to SLE ($Z = 2.357$, $P = 0.018402$) in dominant model, but not in additive or recessive model. Genetype analysis showed that individuals with CT genetype had greater susceptibility to SLE than those without ($Z = 2.004$, $P = 0.045067$). Our study suggests that a single nucleotide polymorphism (rs352140) in the exon 2 region of *TLR9* gene may be a susceptibility factor for SLE in Chinese population.

Keywords Systemic lupus erythematosus · Polymorphism · Single nucleotide · TLR9 · exon 2

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Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease that the underlying cause is unknown. However, it is well known that both genetic and environmental factors have been implicated in this disease. Toll-like receptor 9 (TLR9) is a kind of pattern recognition receptor, which plays an important role in autoimmunity in patients with SLE [1]. The *TLR9* gene located on chromosome 3p21.3, which spans approximately 5 kb and has two exons, and the second of which is the major coding region [2]. Four single nucleotide polymorphisms (rs187084, rs5743836, rs352139 and rs352140) of which have been paid more attention since Lazarus' study, and studied mainly between Th2-driven atopic and Th1-dominated autoimmune diseases [3, 4]. The *TLR9* gene is one of the susceptibility regions for SLE [5], there are several studies on association between *TLR9* gene polymorphisms and SLE in different populations, however, the results are inconsistent. Hur et al. [6] showed that *TLR9* gene polymorphisms were not associated with SLE for a case-control study in Koreans, whereas a Japanese study [7] demonstrated that the presence of the G allele at position +1174

(rs352139) of TLR9 predisposed humans to an increased risk of SLE. Information concerning the association between *TLR9* gene polymorphisms and SLE in the Chinese population is also controversial. In a Hong Kong Chinese case-control study confirmed that four single nucleotide Polymorphisms (rs187084, rs5743836, rs352139 and rs352140) of the *TLR9* gene were not associated with SLE [8]. However, another case-control study through sequencing in China found that one SNP (rs352140) in the exon 2 region of *TLR9* gene is related to SLE [9]. In this study, we performed a family-based association study on the single nucleotide polymorphism (rs352140) to further ascertain whether it is a susceptible locus for SLE in the Chinese population.

Materials and methods

Subjects

A family-based association study was carried out for 288 subjects, including 77 patients (mean age 33.06 ± 11.55 years; range from 12 to 63 years) and 211 other family members from 74 nuclear families with SLE. All subjects were from Anhui Province in eastern China and were of Chinese Han ethnicity. All patients met the 1997 revised American College of Rheumatology criteria for SLE. They were enrolled from the Department of Rheumatology, Anhui Provincial Hospital and the Department of Rheumatology, First Affiliate Hospital, Anhui Medical University. The family criteria used for subject selection were as follows: (1) at least two parents and proband, (2) if one parent was missing, at least one normal sibling besides proband, and (3) if two parents were missing, at least two normal siblings besides proband. The detailed distributions of SLE families are presented in Table 1. All subjects in our study were given informed consent and blood samples, and the study protocol was approved by the Department of Epidemiology and Biostatistics at Anhui Medical University. The information about the SNP (rs352140) came from NCBI dbSNP, the SNP is synonymous mutation, and without change in the amino acid code.

Table 1 Detailed distribution of SLE families

Category	1 sib	2 sibs	3 sibs	4 sibs	5 sibs
2 parents	22	10	10	3	2
1 parent	0	7	10	3	1
0 parent	0	0	6	0	0
Total	22	17	26	6	3

DNA isolation

Forearm venous blood samples from each study subject were collected in 10 ml Vacuum tube containing EDTA and citrate, the plasma were excluded from the cell pellet by pipetting after being centrifuged at 2,000 revolutions for 10 min. Genomic DNA was extracted from the cell pellet in whole blood (QIAamp blood kit; Qiagen) and stored at −80°C before test.

Genotyping of *TLR9* gene polymorphism

The polymorphisms of the SNP (rs352140) were genetyped by PCR restriction fragment length polymorphism (PCR-RFLP) using the following primers: Forward 5′CTTGGC TGTGGATGTTGTTG3′; Reverse 5′TCAATGGCTCCCA GTTCC3′. The 237 bp PCR amplicon was digested into three fragments of 101, 136, 237 bp (Fig. 1) by the AccII restriction enzyme [total reaction volume 15 μl, including PCR product 10 μl, 10 × buffer 1.5 μl, restriction enzyme 0.5 μl (10 units/μl), H_2O 3.0 μl]. Each reaction mixture contained 20 ng of genome DNA, 0.2 μM of each primer, 0.3 mm dNTPs, and 2.5 U Taq (heat-resistant) DNA polymerase (Takara; Otsu, shiga, Japan), T-gradient thermocycler (Biometra, gottingen, Germany) was used. The amplification procedure consisted of intial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 45 s, annealing at 56.8°C for 45 s, and extension at 72°C for 45 s, followed by a final extension at 72°C for 7 min. All families DNA samples, as well as negative controls (without DNA) were included in each round of amplification to check for contamination.

Statistical analysis

An extension of the transmission/disequilibrium test, the unified approach, family-based association test (FBAT) [10], was carried out using dominant, recessive and additive genetic models to avoid the bias of population

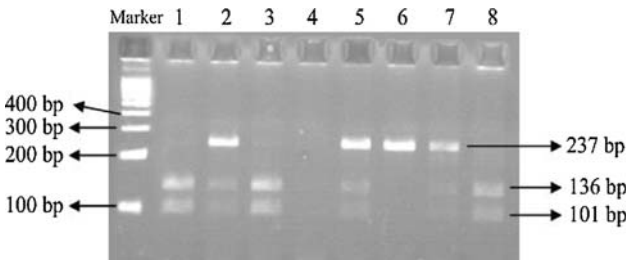

Fig. 1 Genetyping of the *TLR9* gene polymorphism (rs352140) in the exon 2 region by AccII RFLP, DNA size marker (100 bp ladder). PCR fragments containing CC are digested into two fragments (101, 136 bp), Whereas PCR fragments containing CT are digested into three fragments (101, 136, 237 bp), and TT are undigested (237 bp)

admixture arising from a population-based association study. The informative families which determined the power of FBAT were dependent on the genetic models. Single-marker FBAT analysis was used to estimate the single loci frequencies. Each test counted how often a specific locus was present in informative families with SLE. The positive Z value of each locus indicated a specific single locus was more transmitted from parents to patient than excepted under the null hypothesis of no linkage and no association. The Hardy–Weinberg equilibrium was also performed for families with ambiguous results. A threshold P value of less than 0.05 was considered as suggestive evidence of association.

Results

A total of 77 patients with SLE, as well as 211 family members from 74 nuclear families for whom the complete genotype was available were analyzed. Table 1 shows the detailed distribution of the enrolled SLE families.

The genetype frequencies of the SNP (rs352140) in patients with SLE were as follows: CC, CT, TT were 20.8, 61.0, and 18.2% respectively. The C and T allele frequencies were 51.3 and 48.7%, and the genetype distributions met Hardy–Weinberg's expectation ($\chi^2 = 1.689$, $P = 0.430$) (Table 2).

In single loci analysis of the SNP (rs352140), the association between *TLR9* polymorphism and SLE by family-based association test (single-marker FBAT analysis) suggested that the T allele at position of rs352140 was significantly associated with the susceptibility to SLE ($Z = 2.357$, $P = 0.018402$) in dominant model, but not in additive or recessive model (Table 3). Genetype analysis showed that individuals with CT genetype had greater susceptibility to SLE than those without ($Z = 2.004$, $P = 0.045067$) (Table 4).

Table 2 The genotype distribution of rs352140 polymorphism and Hardy–Weinberg's expectation test in SLE

SNP	Genetype	Observed	Expected	χ^2	P
rs352140	CC	16	20	1.689	0.430
	CT	47	39		
	TT	14	18		

Discussion

There has been much dispute over the relationship between the *TLR9* gene polymorphisms and SLE. Therefore, we carried out this study to further ascertain whether a single nucleotide polymorphism (rs352140) of *TLR9* gene is associated with susceptibility to SLE in a Chinese population. In the present study, we found that T allele at position of rs352140 was associated with the susceptibility to SLE in dominant model, but not in additive or recessive model. The susceptible genetype was CT, which was contradicted to the single loci analysis to some extent, and that might caused by the lower frequency of the TT genetype (Table 4) and the limited number of the SLE families. Our results showed that dominant inheritance might play an important role in the susceptibility to SLE in the Chinese population at the position of rs352140, and T allele at the position of rs352140 predisposed humans to

Table 3 Association between the *TLR9* polymorphism and SLE by family-based association test (single marker FBAT analysis) in additive, dominant and recessive models

Allele	Additive model			Dominant model			Recessive model		
	No. of families[a]	Z[b]	P	No. of families	Z	P	No. of families	Z	P
C	51	−1.598	0.110055	25	0.210	0.834035	34	−2.357	0.018402
T	51	1.598	0.110055	34	2.357	0.018402	25	−0.210	0.834035

[a] Informative families: families may have two heterozygote parents or multiple offspring, if the number of informative families is less than 10, the test will not be performed

[b] The negative Z value indicates that the frequency of transmitted allele is negative association with susceptibility to SLE

Table 4 Association between *TLR9* genetype and SLE by family-based association test

Genetype	Frequency	Families	S	$E(S)$	Var(S)	Z	P
CC	0.299	34	8.000	14.583	7.799	−2.357	0.018402
CT*	0.542	51	33.000	25.917	12.493	2.004	0.045067
TT	0.158	25	10.000	10.500	5.694	−0.210	0.834035

* $P < 0.05$

an increased risk of SLE. Therefore, we consider that *TLR9* gene may play a certain role in improving the susceptibility to SLE. TLR9 has been presumed important in the induction of first-line defense of the innate immune system and regulation of adaptive immune response to its ligand, unmethylated CpG DNA, which exists in microbial DNA or endogenous DNA of patients with SLE [11], and the potential of unmethylated CpG-DNA for activating B cells and plasmocytoid dendritic cells in SLE patients suggest that TLR9 may be involved in the pathogenesis of lupus [12–14]. Based on TLR9 function in SLE, genetic variation could affect the threshold for B cell or dendritic cell activation leading to a different individual's susceptibility to SLE. However, the results are inconsistent among studies of association between *TLR9* gene polymorphisms and SLE [6–9, 15, 16]. The allele or genetype frequencies of the same locus among various studies were also different, such discrepancies might be caused by different genetic background of each population, for example, the −1237T/C (rs5743836) of *TLR9* gene is non-polymorphic in a Chinese or a Korea study population [6, 8], while it is common in US ethnic groups [4]. The method used in study may be another factor for the different results [17]. Our results suggested that *TLR9* gene may be a susceptible gene of SLE in the Chinese population. However, significant linkage disequilibria were also observed between the alleles of *TLR9* gene family, and the status of linkage disequilibrium has been considered to be different in various populations [4, 17]. Thus our results may either arise from the linkage disequilibrium with another primarily associated gene.

There are several limitations in our study. The number of the studied families is limited, and the SDT (considering patients and other normal siblings) test cannot be carried out. The association between the clinical symptoms and the genetypes didn't be analyzed either. The advantage of our study is that the family-based association study has been used.

Conclusion

The present study has suggested that T allele at the position of rs352140 is a risk factor of SLE by family-based association test, further studies are needed to elucidate the role of TLR9 or *TLR9* gene in the pathogenesis of SLE.

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