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Original Article

Associations of genetic polymorphisms of TLR5, TLR9 and transduction molecules in MyD88 signaling pathway with systemic lupus erythematosus in Zhuang and Han ethnics of Guangxi province, China

Si-Jian Wen[1*], Fei-Yun Wu[1*], Ling Fang[2*], Hui Liu[1], Wen-Jun Zheng[1], You-Kun Lin[1]

[1]*Department of Dermatology, First Affiliated Hospital of Guangxi Medical University, Nanning 530021, China;* [2]*Department of Cell Biology and Genetics, Guangxi Medical University, Nanning 530021, China.* [*]*Equal contributors.*

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Abstract: Objectives: To discuss the associations of SNPs of TLR5, TLR9 and transduction molecules in MyD88 signaling pathway with systemic lupus erythematosus (SLE) risk in Zhuang and Han ethnics and to compare the difference between the two ethnics. Methods: PCR and direct sequencing method were used to detect gene polymorphisms of TLR5, TLR9 and transduction molecules in MyD88 signaling pathway in 77 patients with SLE and 72 healthy controls, in order to explore their relationships with SLE incidence and compare the differences in genotypes and allele frequencies between groups. Results: TLR5 rs5744168 gene polymorphism was unrelated with SLE susceptibility of Guangxi Zhuang and Han. Among the Han population, there was a statistically significant difference in TLR9 rs352140 genotype frequency between SLE group and control group (P = 0.043). In the Han and Zhuang populations, there were no significant differences in MyD88 rs7744 genotype and allele frequencies (all P > 0.05) between SLE group and control group; but there was a statistically significant difference in allele frequencies between the case group and the control group (P = 0.033). For TRAF6 rs5030472, GA + AA genotype frequency of Zhuang SLE group was significantly higher than that of control group and the difference was statistically significant (P = 0.006); an allele frequency was also significantly higher. IRF5 rs2004640 GG/TT genotype and the corresponding G allele frequencies of Zhuang SLE group were significantly higher than that of control group, with statistically significant differences (P = 0.008 and P = 0.000, respectively). Conclusion: TLR5 rs5744168 gene polymorphism may have no correlation with SLE susceptibility in Guangxi Zhuang and Han populations; TLR9 rs352140 gene polymorphism may be associated with SLE susceptibility in Guangxi Han population, while TRAF6 rs5030472 and IRF5 rs2004640 gene polymorphisms may relate to SLE susceptibility in Guangxi Zhuang population.

Keywords: Zhuang nationality, Han nationality, SLE, TLR5, TLR9

Introduction

Systemic Lupus Erythematosus (SLE) is a typical systemic autoimmune disease, with features of autoantibody production and immune complex formation. The incidence is high and it is common in women of childbearing age. It has many clinical manifestations and can affect any organs and systems. Due to the complex clinical manifestations, severe illness and high incidence, SLE gave rise to a huge loss; at present, its etiology and pathogenesis are still unclear. Most scholars believe that it may be related to genetic factors, infections, drugs, physical factors, hormones, other environmental and psychological factors, and pregnancy [1]. There are a lot of evidences to prove that genetic factors and infection factors play a major role in the pathogenesis of SLE, and influence the development and activity index of the disease.

In recent years, the study on the relationship of HLA (Human Leukocyte Antigen) with SLE further confirmed the genetic susceptibility of SLE. With the development of SLE genetics research, a total of 50 loci had been found to be related with SLE, including TLR5 and TLR9 [2]. TLRs are transmembrane receptors, which combined with corresponding Pathogen Associated Mo-

Table 1. General demographic characteristics of participants

Group		Number of Male	Number of Female	age
SLE group	Zhuang ethnics	1	32	35.7 ± 8.6
	Han ethnics	4	40	36.4 ± 8.3
Control group	Zhuang ethnics	8	24	36.3 ± 9.1
	Han ethnics	8	32	37.1 ± 9.3

lecular Pattern (PAMP) by the identification of associated pathogens to start signal transduction pathways and induce the expressions of some immune response molecules (including inflammatory cytokines). Studies have shown that TLR5, TLR9 and their gene polymorphisms may play a role in the pathogenesis of SLE, and there may be ethnic differences [3-5].

Recent studies [6-8] prompted that TLR9-MyD88-TRAF6-IRF5 signaling pathway had a certain relationship with the development of SLE, and genetic factors were the main pathogenic factors for SLE; therefore, the study of the relationship between the gene polymorphism of the signaling pathway and the genetic susceptibility of SLE is a hot research currently.

Tao et al [6] found that the TLR9 gene polymorphisms in Japanese had some relevance with SLE susceptibility. But it had not been validated in Koreans and Britons [7, 8]. Sandling et al [9] found that there was an obvious relevance between TRAF6 gene polymorphism and Swedish patients with SLE; TRAF6 gene polymorphism was the risk factor of Swedes SLEs, which had no association with US SLEs [9]. Study [10] also found that in Mexican population, SLE susceptibility was related with IRF5 gene polymorphism. However, the relationships between above gene polymorphisms and Guangxi Zhuang and Han populations are unclear.

This study was conducted to discuss the correlations of SNPs of TLR5, TLR9 and transduction molecules in MyD88 signaling pathway with SLE pathogenesis in Zhuang and Han ethnics.

Subjects and methods

Subjects

77 SLE patients, hospitalized in the Department of Dermatology of the First Affiliated Hospital of Guangxi Medical University from March 2010 to November 2011, were selected, aged 9 to 72 years old. Inclusion criteria were in line with diagnostic criteria for classification of SLE revised by ACR (American college of Rheumatology) in 1997 [11]; all patients did not receive treatments of corticosteroids, immunosuppressants and immunomodulators before admission. Such patients with type I diabetes, rheumatoid arthritis, ankylosing spondylitis, dermatomyositis, scleroderma and other autoimmune diseases confirmed by history, physical and laboratory examinations; and the patients complicated with pneumonia, hypertension, heart disease, cancer and other infectious or major chronic diseases; and pregnant or lactating women were excluded. The normal control group (n = 72) enrolled the healthy individuals in the same period in the First Affiliated Hospital of Guangxi Medical University; by medical history, physical and laboratory examinations, type I diabetes, rheumatoid arthritis, systemic lupus erythematosus, leather polymyositis, scleroderma and other autoimmune diseases, as well as pneumonia, hypertension, heart disease, cancer and other infectious and major chronic diseases were excluded. The ages were between 12-68 years old. The general characteristics of the subjects were shown in **Table 1**. All subjects were ethical; the study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Guangxi Medical University.

Methods

DNA isolation: 2 ml venous blood of each subject was collected and anticoagulated with EDTA; Bioteke whole blood genome DNA isolation kit (spin column) was used to extract leukocyte genomic DNA, which was dissolved in elution buffer EB, -80°C for preservation.

Primer design: Referring to DNA sequences of TLR5, TLR9, MyD88, TRAF6 and IRF5 genes in GenBank, primers were designed using primer5. 0 (sequences were shown in **Table 2**), which were synthesized by Shanghai Sangon biotechnology company.

PCR amplification

The total volume of the PCR reaction system was 50 μl, including the template DNA 6 μl, 2 ×

Table 2. The primer sequences and annealing temperature of each SNPs

Gene	Primers (5'-3')	Primers (3'-5')	Products (bp)	Annealing temperature (°C)
TLR5	GGACTTGACAACCTCCAAGTTC	AAGGGGTTTGATCTCCACTACA	409	54
TLR9	AAAGAAGGCCAGGTAATTGTCA	TGCTAGACCTGTCCCACAATAA	431	58
MyD88	CCCACCAACTTTGTACCTTGAT	TAAATTGCTCTGGGAAGGAGAG	447	58
TRAF6	GAGCAGAAGCAAAGGCATACTT	ATCGCCATAATTCAAGCTGTCT	282	57
IRF5	CGGGATGAAGACTGGAGTAG	ACGCTTTAACCTCAACCTCTTT	376	57

Power Taq PCR Master Mix 25 μl, upstream and downstream primers respectively 2 μl, with sterile double distilled water to make up to 50 μl. PCR cycle parameters: pre-denaturation at 95°C for 3 minutes, denaturation at 95°C for 30 s, annealing and renaturation at 54°C for 30 s, extension at 72°C for 45 s, totally 40 cycles, finally extension at 72°C for 10 minutes. Above reactions were performed on the German Bi-ometru T personal PCR instrument.

Sequencing of PCR products: PCR products were sequenced by Guangzhou Branch of Shanghai Invitrogen Biotechnology Co., Ltd. sequenced. After results returned, Chromas software was used to open the DNA sequence to read and note.

Statistical analysis

The genotype and allele frequencies in each group were calculated; genotype and allele frequencies of polymorphic loci were compared by chi-square test using statistical software SPSS13.0; correlation analysis between gene polymorphism and SLE clinical features and laboratory examinations was performed using the chi-square test; $P < 0.05$ was considered statistically significant.

Results

Sequencing of PCR products

Direct sequencing of the PCR product was shown in **Figure 1**.

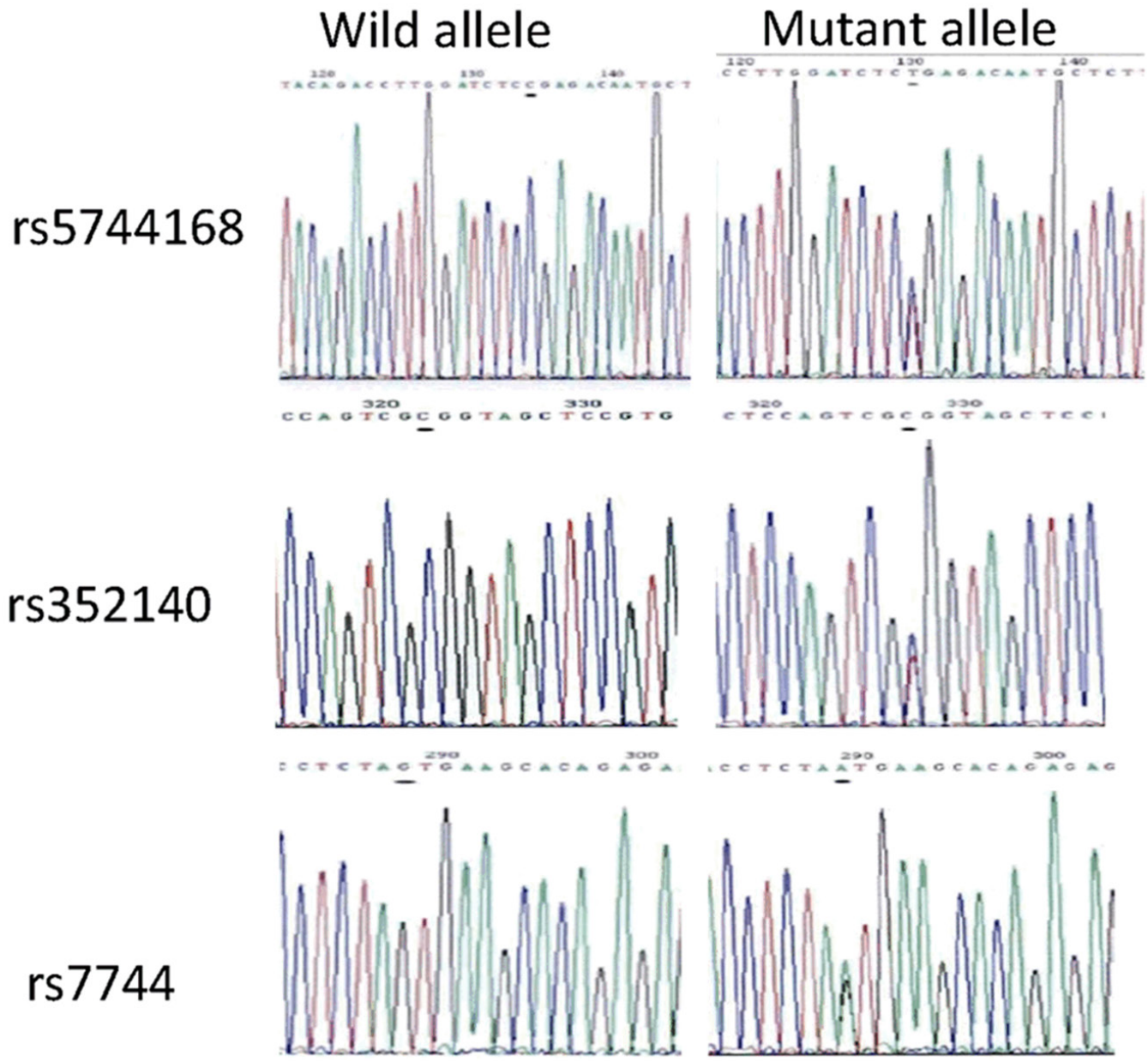
Distribution of genotypes and alleles in the two groups

The chi-square test showed that there were no statistically significant differences in TLR5 rs5744168 genotype frequency between SLE group and the control group of both Zhuang and Han populations ($P > 0.05$), also without statistically significant difference in the corresponding C and T allele frequencies ($P > 0.05$), shown in **Table 3**. There were no statistically significant differences in genotype and allele frequencies of TLR5 rs5744168 between the Zhuang SLE group and Han SLE group, Zhuang control group and Han control group ($P > 0.05$), also without statistically significant differences between the SLE group and control group of Zhuang, the SLE group and control group of Han ($P > 0.05$), shown in **Table 3**.

There were no statistically significant differences in TLR9 rs352140 genotype and allele frequencies between SLE group and the control group of both Zhuang and Han populations ($P > 0.05$, **Table 4**).

There were no statistically significant differences in TLR9rs352140 genotype and allele frequencies between SLE group and the control group of Zhuang population (all $P > 0.05$), but there were statistically significant differences in TLR9rs352140 genotype frequency between SLE group and the control group of Han population ($P = 0.043$), without statistically significant differences in TLR9rs352140 allele frequency ($P > 0.05$). There were no statistically significant differences in genotype and allele frequencies of TLR9rs352140 between the Zhuang SLE group and Han SLE group, Zhuang control group and Han control group (all $P > 0.05$), shown in **Table 4**.

There were no statistically significant differences in MyD88 rs7744 genotype frequency between SLE group and the control group of both Zhuang and Han populations ($P = 0.085$), while with statistically significant differences in MyD88 rs7744 allele frequency ($P = 0.033$); A allele frequencies of Zhuang and Han SLE groups were significantly higher than that of the Zhuang and Han control groups, while the corresponding G allele frequencies were significantly lower, shown in **Table 7**. There were no statistically significant differences in My-


Wild allele
Mutant allele
rs5744168
rs352140
rs7744

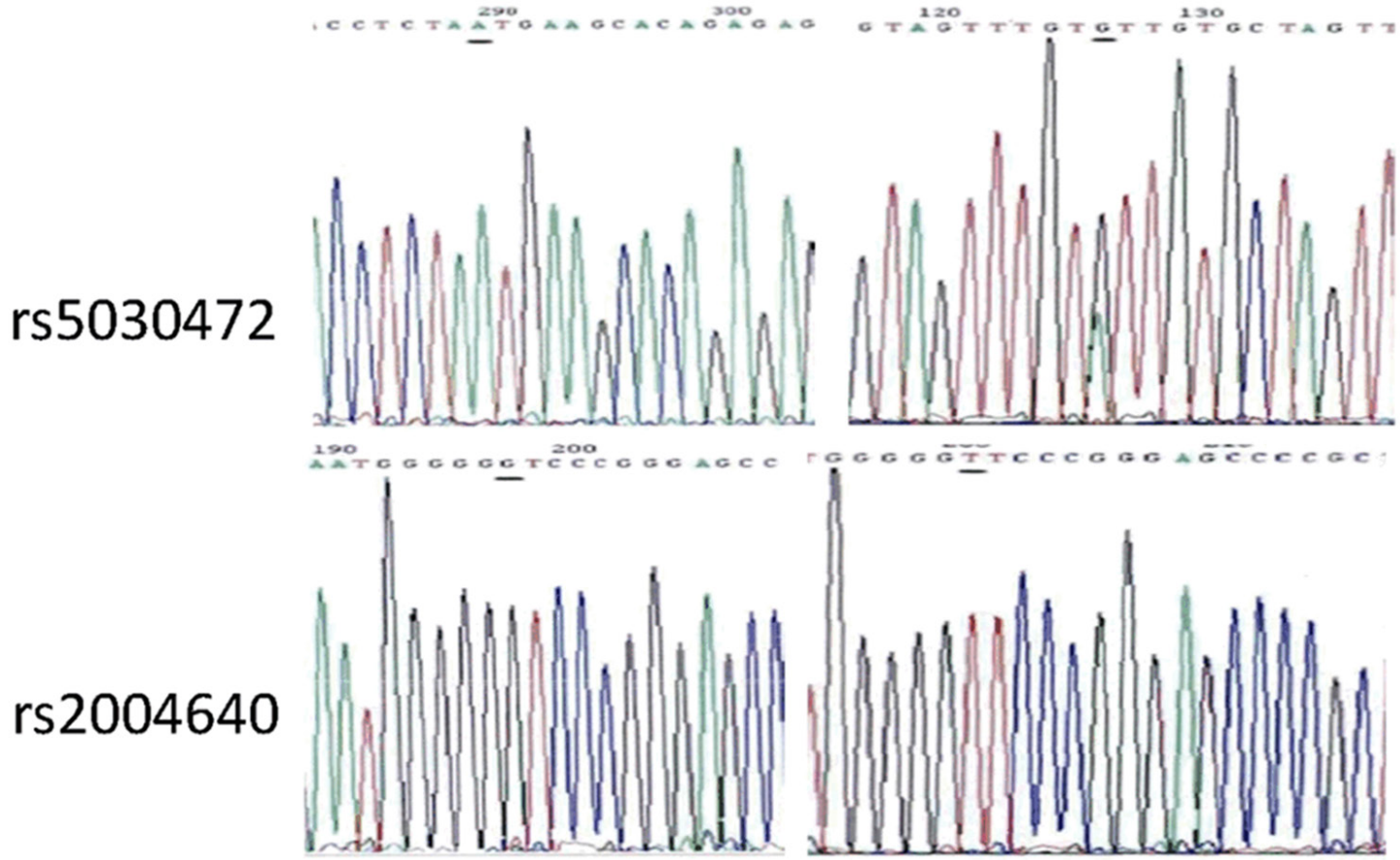


Figure 1. The sequencing results of each SNPs.

Table 3. Genotype and allele frequency distribution in rs5744168 between the SLE group and control group

TLR5 rs5744168	Total			Zhuang			Han		
	SLE, n (%)	Control, n (%)	*P* value	SLE, n (%)	Control, n (%)	*P* value	SLE, n (%)	Control, n (%)	*P* value
CC	74 (96.1)	68 (94.4)	0.632	31 (93.9)	32 (100.0)	0.157	43 (97.7)	36 (90.0)	0.135
CT	3 (3.9)	4 (5.6)		2 (6.1)	0 (0)		1 (2.3)	4 (10.0)	
TT	0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)	
C	151 (98.1)	140 (97.2)	0.637	64 (97.0)	64 (100.0)	0.160	87 (98.9)	76 (95.0)	0.141
T	3 (1.9)	4 (2.8)		2 (3.0)	0 (0)		1 (1.1)	4 (5.0)	

Table 4. Genotype and allele frequency distribution in rs352140 between the SLE group and control group

TLR9 rs352140	Total			Zhuang			Han		
	SLE, n (%)	Control, n (%)	*P* value	SLE, n (%)	Control, n (%)	*P* value	SLE, n (%)	Control, n (%)	*P* value
CC	34 (44.2)	28 (38.9)	0.160	16 (48.5)	12 (37.5)	0.648	18 (40.9)	16 (40.0)	0.496
CT	33 (42.9)	40 (55.6)		13 (39.4)	16 (50.0)		20 (45.5)	24 (60.0)	
TT	10 (13.0)	4 (5.6)		4 (12.1)	4 (12.5)		6 (13.6)	0 (0)	
C	101 (65.6)	96 (66.7)	0.844	45 (68.2)	40 (62.5)	0.043	56 (63.6)	56 (70.0)	0.382
T	53 (34.4)	48 (33.3)		21 (31.8)	24 (37.5)		32 (36.4)	24 (30.0)	

Table 5. Genotype and allele frequency distribution in rs7744 between the SLE group and control group

MyD88 rs7744	Total			Zhuang			Han		
	SLE, n (%)	Control, n (%)	*P* value	SLE, n (%)	Control, n (%)	*P* value	SLE, n (%)	Control, n (%)	*P* value
AA	31 (40.3)	20 (27.8)	0.085	16 (48.5)	12 (37.5)	0.381	15 (33.3)	8 (20.0)	0.196
AG	38 (49.4)	36 (50.0)		13 (39.4)	12(37.5)		26 (57.8)	24 (60.0)	
GG	8 (10.4)	16 (22.2)		4 (12.1)	8 (25.0)		4 (8.9)	8 (20.0)	
A	100 (64.9)	76 (52.8)	0.033	45 (68.2)	40 (56.3)	0.106	56 (62.2)	40 (50.0)	0.109
G	54 (35.1)	68 (47.2)		21 (31.8)	24 (43.7)		34 (37.8)	40 (50.0)	

D88rs7744 AA/AG/GG genotype and A/G allele frequencies between SLE group and control group in Zhuang and Han populations (all P > 0.05), shown in **Table 6**. There were also no statistically significant differences between Zhuang SLE and Han SLE groups, Zhuang control and Han control groups (all P > 0.05), shown in **Table 5**.

Due to the less of GA and AA genotypes in the SLE group and control group, the GA and AA were combined to compare with the GG genotype. The chi-square test showed that for TRAF6 rs5030472, GA + AA genotype frequency of Zhuang SLE group was significantly higher than that of Zhuang control group and Han SLE group, with statistically significant differences (P = 0.006; P = 0.024); and the corresponding A allele frequency was also significantly higher (P = 0.004; P = 0.014). But between the Han SLE group and control group, the Han and Zhuang control groups, there were no statistically significant differences in the genotype and allele frequencies (all P > 0.05), shown in **Table 6**.

IRF5 rs2004640 GG/TT genotype and the corresponding G allele frequencies of Zhuang SLE group were significantly higher than that of the control group, with statistically significant differences (respectively P = 0.008 and P = 0.000). But the difference between the Han

Table 6. Genotype and allele frequency distribution in rs5030472 between the SLE group and control group

TRAF6 rs5030472	Total			Zhuang			Han		
	SLE, n (%)	Control, n (%)	*P* value	SLE, n (%)	Control, n (%)	*P* value	SLE, n (%)	Control, n (%)	*P* value
GG	68 (88.3)	68 (94.4)	0.185	26 (78.8)	32 (100.0)	0.006	42 (95.5)	36 (90.0)	0.332
GA+AA	9 (11.7)	4 (5.6)		7 (1.2)	0 (0)		2 (4.5)	4 (10.0)	
G	144 (93.5)	140 (97.2)	0.130	58 (68.2)	64 (100)	0.004	86 (97.7)	76 (95.0)	0.341
A	10 (6.5)	4(2.8)		8 (31.8)	0 (0)		2 (2.3)	4 (5.0)	

Table 7. Genotype and allele frequency distribution in rs2004640 between the SLE group and control group

IRF5 rs2004640	Total			Zhuang			Han		
	SLE, n (%)	Control, n (%)	*P* value	SLE, n (%)	Control, n (%)	*P* value	SLE, n (%)	Control, n (%)	*P* value
GG	44 (57.1)	28 (38.9)	0.026	19 (57.6)	8 (25.0)	0.008	25 (56.8)	20 (50.0)	0.531
GT+TT	33 (42.9)	44 (61.1)		14 (42.4)	24 (75.0)		19 (43.2)	20 (50.0)	
G	88 (57.1)	56 (38.9)	0.002	38 (57.6)	16 (25.0)	0.000	50 (56.8)	40 (50.0)	0.376
T	66 (42.9)	88 (61.1)		28 (42.4)	48 (75.0)		38 (43.2)	40 (50.0)	

SLE group and Han control group was not statistically significant (P = 0.531), and the difference in corresponding G and T allele frequency was also not statistically significant (P = 0.376). There were no statistically significant differences in IRF5rs2004640 genotype and allele frequencies between the SLE groups of Zhuang and Han (all P > 0.05), but with a statistically significant difference between the control groups of the two ethnics (all P < 0.05), shown in **Table 7**.

Discussion

Systemic lupus erythematosus is an autoimmune disease, which has a certain relationship with hereditary. Zhang et al. [12] reported that the frequency of genotype and allele of TLR9 rsl87084C/T were associated with the SLE of Han population. Our results were in line with Zhang et al.'s study. We found that there was a difference between patients with SLE and the healthy controls of Han in TLR9rs352140 gene polymorphism. However, there was no difference between Zhuang and Han in SLE patients and the health of both group, respectively, indicating that genotype TLR9rs352140TT may be a risk genotype of Guangxi Han with SLE. The result of the study did not show that there was obvious correlation between TLR9rs352140 gene polymorphism and SLE group with Guangxi Zhuang and Han. Also, our research found that the polymorphism of MyD88 rs7744 may have no relevance with SLE susceptibility of Guangxi Zhuang and Han. In addition, we also found that the frequencies of MyD88 rs7744AA/AG/GG genotype showed no statistical significant difference between SLE Zhuang and the Han control group. But there are significant differences in the frequency of A, G allele between the two groups. As for TRAF6 rs5030472 polymorphism, we found it correlated with the SLE susceptibility of Guangxi Zhuang, while it did not correlate with that of SLE group in Guangxi Han. The result was similar with the literature, and it further supported TRAF6 rs5030472 polymorphism correlated with SLE and the difference existed in different ethnics. Reddy et al [10] found that all the three SNP loci of IRF5 gene had genetic correlations in Mexican SLE patients (including the SLE children in the family); the frequency of hazard haplotype in the Mexican healthy individuals was significantly higher than that of European healthy individuals, and it was even higher than that of the Mexican Indian healthy individuals. We found that IRF5 rs2004640GG/TT genotype and the corresponding G allele frequencies of Zhuang SLE group were significantly higher than those of the control group; there were statistically significant differences in IRF5rs2004640 genotype and allele frequencies between SLE group

and control group in Zhuang and Han populations; there were also statistically significant differences in IRF5 rs2004640GG and TT genotype and allele frequencies between Zhuang and Han control groups, suggesting that the correlations between IRF5 rs2004640 polymorphisms and SLE susceptibility may also exist ethnic differences. The correlation analysis of IRF5 rs2004640 polymorphisms and clinical and laboratory parameters in SLE groups showed that there were statistically significant differences between the IRF5 rs2004640 GG/TT genotype and G/T allele frequencies.

Through these studies, we found that some transduction molecule in gene polymorphism of TLR9 and its MyD88 pathway and its signaling pathway correlated with the SLE susceptibility of Guangxi Zhuang and Han to a certain degree. Some of them may still exist ethnic differences. We also found that gene polymorphism of few levels of transduction molecules in signaling pathway correlated with clinical manifestations and laboratory activity of Guangxi Zhuang and Han with SLE, which provided a clue for further study that whether the IRF5 rs2004640 polymorphism was involved in regulating the disease activity of SLE and indicated that blocking the pathway at any level were likely to become targets for active immunization, so as to provide a reference for active immunized intervention therapy of SLE.

However, it should be noted that in the development and prognosis of SLE, both genetic and environmental factors played an important role. Although genetic factor was the main factor, the presence of genetic factors of SLE was complexity. Individual genome may contain multiple susceptibility genes, or multiple susceptibility loci in a susceptibility gene. Therefore, we should increase the sample size and susceptibility gene polymorphisms in the following studies in order to further clarify the relationship between SLE and susceptibility gene and further improve, perfect the diagnosis effect of SLE and refine efficacy of the determination system, and then provide more precise reference in the future for individual-specific immunotherapy research.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. You-Kun Lin, Department of Dermatology, First Affiliated Hospital of Guangxi Medical University. NO. 6 Shuangyong Road, Nanning 530021, Guangxi Zhuang Autonomous Region, China. Tel: +86-0771-5356514; Fax: +86-0771-5356514; E-mail: liyoukn@126.com

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