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Association of *TLR9* gene polymorphisms with lupus nephritis in a Chinese Han population

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ABSTRACT

Objective. *Although increasing data have supported the possible role of* TLR-9 *in the pathogenesis of lupus nephritis (LN), the effect of* TLR-9 *on the lupus phenotype remains controversial. The aim of this study was to associate common variants in the* TLR9 *gene with susceptibility to lupus nephritis in the Chinese population to further ascertain whether it is a susceptible locus for SLE, especially its likely role in LN from a comparatively large population.*

Methods. *Two previously reported SLE associated single nucleotide polymorphism (rs352139, rs352140) were investigated in a case-control study comprised of 315 patients with biopsy proven lupus nephritis and 338 matched healthy controls. Single nucleotide polymorphisms (SNPs) were typed by TaqMan allele discrimination assays.*

Results. *Both rs352139 (p=0.040, OR: 0.713, 95%CI: 0.516-0.985) and rs352140 (p=0.048, OR: 0.723, 95%CI: 0.525-0.997) were associated with LN in dominant model. A trend for an association between genotypes and the disease activity indexes was observed. However, no significance was achieved.*

Conclusions. *The present study suggested that* TLR9 *gene have a role in establishing an autoimmune background and pathogenesis in human LN.*

Introduction

For many years, investigations into the pathogenesis of systemic lupus erythematosus (SLE) have focused on abnormalities in adaptive immunity and in particular, on the emergence and persistence of autoreactive T and B cells. Recent experimental and clinical studies have placed new emphasis on the role of the innate immune system in SLE. The Toll-like receptors (TLRs) constitute an ancestral family of innate immune activation molecules that function to discriminate "self" from microbial "non-self". Systemic lupus erythematosus appears to be one of the conditions in which self-nucleic acid formats can activate innate viral nucleic acid recognition receptors such as *TLR-7* or *TLR-9* (1-3).

Although increasing data supported the possible role of *TLR-9* in the pathogenesis of the disease, the effect of *TLR-9* on the lupus phenotype remains controversial. For example, it was suggested that both immune cells and nonimmune renal cells express a limited set of functional Toll-like receptors that trigger local cytokine and chemokine release in lupus nephritis upon Toll-like receptor activation (4-7). However, lupus nephritis can occur with the help of *TLR-9* dependently or independently (8-12). In addition, some genetic association studies taken to evaluate the role of *TLR-9* in human also get controversial conclusions (13-18). In this study, we performed a case-control study through TaqMan Genotyping on two previously SLE associated single nucleotide polymorphism of *TLR-9* gene (rs352139, rs352140) to further ascertain whether it is a susceptible locus for SLE in the Chinese population, especially its likely role in LN from a comparatively large population.

Methords

Patients and controls

Three hundred and fifteen (315) LN patients were recruited from Peking University First Hospital, all of Han ethnicity living in north of China. Their mean age was 32.4±11.1 years and 84.8% were females (female to male ratio of 5.6). All SLE patients met ACR classification according to the revised SLE criteria of the American College of Rheumatology (ACR) (19). Lupus nephritis was confirmed by renal biopsy and classified according to International Society of Nephrology/Renal Pathology Society classification of lupus nephritis (ISN/RPS) 2003 revised classification system (15-16). Activity index (AI) and chronicity index (CI) were evaluated and SLE activity index was measured by SLE-DAI scoring.

Three hundred and thirty-eight (338) healthy controls were geographically and ethnically matched. Their mean age was 31.7±9.1 years and 55.8% were females (female to male ratio of 1.3).

The study was approved by the medical ethics committee of Peking University. All patients gave informed consent.

SNP selection and genotyping

Two SNPs of *TLR9* gene (rs352139, rs352140) which were previously reported to be associated with SLE were selected for case control study. rs352139 (+1174) was located within intron 1, and rs352140 (+1635) was located within exon 2, which were in one block based on the HapMap data of Han Chinese in Beijing (CHB) population. The variants were genotyped by TaqMan allele discrimination assays (catalogue nos. C_2301953_10 for rs352139, C_2301954_20 for rs352140, Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. In short, 20 nanogram genomic DNA was introduced into a reaction mixture consisting of TaqMan Genotyping Master Mix, forward and reverse primers, and two TaqMan Probes labeled by FAM and VIC dye respectively. The thermal cycling condition was as follows: a pre-run at 95°C for 10 min; 40 cycles with a 15 s denaturation step at 95°C, followed by a 60°C annealing step for 1 minute. All assays were conducted on an ABI Prism 7500 Instrument. The SDS software V1.3.1 plotted the results of the allelic discrimination on a scatter plot. Random samples were repeated to check the concordance of the assay and negative controls were included to ensure accuracy of genotyping.

Statistical analysis

The genotype frequencies of SNPs were tested for Hardy-Weinberg equilibrium separately in cases and controls. Disease associations were analysed by chi-square tests. Linkage disequilibrium (LD) test and haplotype association were analysed by Haploview V 3.32 and Haplo.Stats V 1.4.3 respectively. Differences of the means between two groups were tested with the Student's *t*-test. Statistical analyses were performed with SPSS12.0 software (SPSS Inc., Chicago, IL). A two-tailed *p*-value of less than 0.05 was considered statistically significant. Although multiple comparisons should affect the interpretation of the statistical significance, because of the lack of a universally accepted method of correcting the *p*-values for multiple SNPs in linkage disequilibrium, we decided to present unadjusted *p*-values.

Table I. *TLR9* polymorphisms in LN patients and healthy controls in Chinese.

	SNP	Patients (n=315)	Controls (n=338)	P	OR(95%CI)
rs352139	Genotype frequency				
	G/G	48 (15.2%)	54 (16.0%)	0.111	
	A/G	145 (46.0%)	179 (53.0%)		
	A/A	122 (38.7%)	105 (31.1%)		
	Allele frequency				
	G (vs. A)	241 (38.3%)	287 (42.5%)	0.122	
	Recessive model				
	G/G (vs. A/G + A/A)	48 (15.2%)	54 (16.0%)	0.795	
	Dominant model				
	G/G + A/G (vs. A/A)	193 (61.3%)	233 (68.9%)	0.040	0.713 (0.516-0.985)
rs352140	Genotype frequency				
	T/T	49 (15.6%)	53 (15.7%)	0.116	
	T/C	141 (44.8%)	176 (52.1%)		
	C/C	125 (39.7%)	109 (32.2%)		
	Allele frequency				
	T (vs. C)	239 (37.9%)	282 (41.7%)	0.163	
	Recessive model				
	T/T (vs. T/C + C/C)	49 (15.6%)	53 (15.7%)	0.965	
	Dominant model				
	T/T + T/C (vs. C/C)	190 (60.3%)	229 (67.8%)	0.048	0.723 (0.525-0.997)

P-values were calculated by chi-square analysis using 2×3 (comparison of genotype frequencies) or 2×2 (other comparisons) contingency tables. Significant *p*-values were in bold and OR values were presented only for significant associations.

Results

TLR9 *polymorphisms in LN patients and healthy controls in Chinese*

The SNPs rs352139 and rs352140 in the *TLR9* gene were genotyped in 315 Chinese LN patients and 338 healthy controls. Deviation from Hardy-Weinberg equilibrium was not observed for any of the SNPs in the control or LN samples (p=0.160–0.715).The allele, genotype frequencies of the identified markers of the *TLR9* gene were calculated and presented in Table I for the healthy controls and the LN subjects. At allele-type level, although allele A of rs352139 and allele C of rs352140 seemed to be more frequent in LN patients than in healthy controls, there was no statistical significance. At genotype level, both rs352139 (p=0.040, OR: 0.713, 95%CI: 0.516-0.985) and rs352140 (p=0.048, OR: 0.723, 95%CI: 0.525-0.997) were associated with LN in dominant model.

Linkage disequilibrium in healthy Chinese individuals and haplotype with LN patients

The LD analysis revealed that r^2 values between rs352139 and rs352140 in healthy controls and LN patients were 0.97 and 0.94 respectively.

By the haplo.cc (implemented in the haplo.stats program) analysis, we have calculated the different significance of association between the susceptibility to LN and the haplotypes. The frequency of haplotype of GT was higher in LN cases than that in controls (41.72% *vs*. 37.46%), although it did not have statistical significance (p=0.11).

Association between genotypes and disease activity/severity indexes

When genotypes were correlated with disease activity, including activity index, chronicity index, presence of anti-dsDNA autoantibody, serum complement 4 level, proteinuria, and active proliferative glomerulonephritis, a trend for an association between genotypes and the clinical indexes was observed, however, no significance was achieved (Table II).

Discussion

Nephritis is a major cause of morbidity in patients with lupus. A variety of immune mechanisms, both humoral and cellular, have been implicated in both the initiation and amplification of the

Table II. Disease activity in different TLR9 genotype groups.

Parameters*	Genotype (rs352139) G/G + A/G vs. A/A	*p*-values	Genotype (rs352140) T/T + T/C vs. C/C	*p*-values
AI	7.56 ± 4.89 vs. 8.10 ± 5.34	0.534	7.59 ± 4.91 vs. 8.05 ± 5.31	0.598
CI	2.80 ± 2.19 vs. 2.95 ± 2.09	0.687	2.79 ± 2.20 vs. 2.97 ± 2.08	0.623
SLE-DAI	18.07± 6.53 vs. 18.41 ± 7.84	0.748	18.16± 6.35 vs. 18.27 ± 7.99	0.923
Presence of anti ds-DNA	57.9% vs. 64.6%	0.409	57.3% vs. 60.4%	0.702
Serum C4, g/l	0.117± 0.079 vs. 0.126 ± 0.086	0.512	0.118± 0.079 vs. 0.125 ± 0.086	0.628
Proteinuria (g/24h)	4.16± 4.14 vs. 4.95 ± 4.10	0.198	4.22± 4.17 vs. 4.84 ± 4.09	0.309
Presence of active proliferative glomerulonephritis (class IV + III)	62.2% vs. 66.8%	0.241	61.3% vs. 65.7%	0.274

All parameters were detected at the time of renal biopsy. Data were expressed as mean ± standard deviation or proportion. *AI: activity index; Ci: chronicity index; SLE-DAI: Systemic Lupus Erythematosus Disease Activity Index; Renal pathology classification according to International Society of Nephrology/ Renal Pathology Society classification of lupus nephritis (ISN/RPS) in 2003.

inflammatory response within kidney; innate immunity pathways may further amplify inflammatory reactions and renal resident cells activated after injury may also participate in subsequent destructive restorative processes (1). Nucleosomes (representing DNA-histone complexes) either free or in complexes with antibodies have been found both in the serum and in renal lesions of patients with lupus raising the possibility of *in-situ* stimulation of infiltrating peripheral blood cells and resident renal cells in patients with nephritis (12, 20).

To date, a great amount of evidence supports the independent role of TLR9 in the pathogenesis of LN; however, there has been much dispute over the relationship between TLR9 and LN, as well as gene polymorphisms association study. Therefore, we firstly checked the possible association between *TLR9* gene polymorphisms and LN from a large Chinese population, the biggest LN cohort with homogeneous ethnicity compared to former study. We found that *TLR9* gene was associated with susceptibility to LN in dominant model, which was similar to data for rs352140 from family-based association study (13). However, the significance was weak, which may indicate that it just exerts moderate effects in combination with the environments (21, 22). Previously, it was reported that rs352139 associated with SLE in Japanese population (p=0.029) (14) and rs352140 was associated with SLE in dominant model from a family based Chinese population (p=0.018) (13). It was obvious that the association was marginal, as in the present study. So although there is a lack of association between rs352139 and SLE in Hong Kong Chinese, Korean and Caucasian populations (15-17), such negative results cannot rule out the role of TLR9 in the pathogenesis of SLE, especially since the data from Caucasians suggested a marginally genetic interaction between *TLR5* and *TLR9*. Moreover, the fact that different cohorts contained different proportions of LN and different haplotype structure within the *TLR9* gene between different populations should also taken into consideration. Instead of SLE, targeting LN maybe have more indicative significance for two reasons: the ligand for *TLR9* is CpG DNA (lupus nephritis, a clinical phenotype associated with the presence of anti–double-stranded DNA) and *TLR9* is expressed in renal nonimmune cells (5-7, 23). We further examined whether the association could be reinforced, and we found a correlation between genotypes and clinical indexes. However, we only observed a trend for an association without statistical significance. The relative risk group of patients had higher levels of anti-dsDNA autoantibody, C4, more severe pathology manifestation and worse renal function. Thus, our results suggested that *TLR9* gene be a susceptible gene of LN in the Chinese population. However, significant linkage disequilibria were also observed between the alleles of the *TLR9* gene, thus our results may either arise from the linkage disequilibrium with another associated polymorphism or another gene. No previous study simultaneously investigated rs352139 and rs352140, so previously reported single SNP association, rs352139 or rs352140, may simply be due to their linkage disequilibrium. At this point, a meta-analysis should be carried out. However, because of the very limited LN patients involved in former studies and the different genetic backgrounds of each population, it may require a more extensive replication study.

In summary, these data suggested that *TLR9* genetic variations have a role in establishing an autoimmune background and pathogenesis in human LN. Although the precise mechanisms are unclear, those results suggest that TLR9 is involved in LN and provide an interesting clue for elucidating the mechanism of pathogenesis in LN.

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