The *TLR7* 7926A>G polymorphism is associated with susceptibility to systemic lupus erythematosus

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Abstract. Systemic lupus erythematosus (SLE) is a systemic autoimmune disorder that predominantly affects women of childbearing age, with a female-to-male ratio of approximately 9:1. Previous findings indicated that male cases of SLE were associated with Klinefelter's syndrome (47, XXY), whereas females with Turner's syndrome (45, X0) did not contract SLE. Additionally, duplicated Toll-like receptor 7 (TLR7) was found to promote lupus-like disease. Consequently, the aim of this study was to evaluate whether the *TLR7* gene served as a genetic marker for the development of SLE. A case-control study was performed on one tag single nucleotide polymorphism TLR7 rs1634323 in a population with 507 SLE patients and 513 healthy controls. Genotyping was determined by the TaqMan genotyping assay using the ABI 7300 real-time reverse transcription polymerase chain reaction system. The results showed a significantly elevated risk of SLE with the rs1634323 AG genotype in females (P=0.040, OR=1.897, 95% CI 1.031-3.491), whereas a similar association was not replicated in males (P=0.303, OR=0.338, 95% CI 0.043-2.656). In a subgroup analysis by clinical manifestation of lupus nephritis, no significant differences were found. These findings indicate that the *TLR7* gene rs1634323 polymorphism may contribute to SLE susceptibility in females.

Introduction

Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disorder affecting multiple organ systems. It is characterized by B-cell hyperreactivity and the production of autoantibodies (1), and predominantly affects women of childbearing age, with a female-to-male ratio of approximately 9:1. Genetic factors are increasingly recognized as major contributors to SLE risk. Toll-like receptors (TLRs) are a family of pattern-recognition receptors that are important in the initiation of the cellular innate responses (2,3). TLRs are localized to either the cell surface or endosomes of certain cell types, most notably of antigen-presenting cells (APCs), such as dendritic (DCs) (4,5) and B cells (6). Currently, 13 TLRs (TLR1-TLR13) have been identified in mammalian species, including 10 in humans (7,8), all thought to have their own specific ligands and cell localization (9). TLRs, particularly TLR7 and TLR9, are critically involved in the activation of DCs and autoreactive B cells through the identification of endogenous nuclear autoantigens and the subsequent development of autoimmune responses against DNA- and RNA-related nuclear antigens. Stimulation of the TLR pathway enhances the transcription of several pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor-α (TNF-α) via the activated nuclear factor κB (NF-κB) by a downstream signaling pathway such as the myeloid differentiation factor 88 (MyD88) and interleukin-1R (IL-1R)-associated kinase (IRAK) (10-12).

Located at Xp22.3, a 4-Mb region of chromosome X, the *TLR7* gene and its functionally related gene *TLR8*, lying in close proximity to each other on chromosome X, encode proteins playing an important role in pathogen recognition and activation of innate immunity (13). Results of recent studies in animal models for SLE have shown that TLR7 was involved in the pathogenesis of murine lupus (14). TLR7 overexpression was associated with systemic autoimmunity in mice (15,16). By contrast, TLR7-deficient lupus-prone MRL/Mp$^{lpr/lpr}$ mice and TLR7-deficient mice backcrossed to the autoimmune MRL^{lpr} mouse strain showed an impaired production of antibodies to RNA-containing antigens, and developed less severe disease (17,18). In addition, the development of SLE was markedly suppressed in (NZBxNZW) F1 mice treated with a dual inhibitor of TLR7 (19) as well as in C57BL/6 (B6)-Fas^{lpr} mice bearing the Unc93b1 mutation, which impairs signaling via TLR7 (20). BXSB mice bearing the Yaa (Y chromosome-linked autoimmune acceleration) mutation,

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a translocation from the telomeric end of the X chromosome, containing the gene encoding TLR7, onto the Y chromosome (21,22), spontaneously developed an SLE-like disease that affects male animals much earlier than females. Moreover, Yaa-bearing mice demonstrated a 2-fold overexpression of TLR7 protein and mRNA (21,22).

As for the human population, an increased level of TLR7 mRNA was observed in SLE patients using real-time reverse transcription-polymerase chain reaction (RT-PCR) (23). Results of a recent study revealed that an increase in the TLR7 copy number (CN) was a risk factor for childhood-onset SLE in a Mexican population and provided new evidence for the X-linked gene dose in SLE susceptibility (24). Furthermore, several studies reported cases of male SLE patients associated with Klinefelter's syndrome (47, XXY) (25-27), whereas female patients with Turner's syndrome (45, X0) did not contract SLE (28,29). Based on these observations, the possible association of *TLR7* polymorphism with SLE and particular clinical manifestations of SLE were examined.

Materials and methods

Patients and controls. A total of 507 SLE patients (468 female and 39 male), meeting at least four of the American College of Rheumatology (ACR) 1997 revised criteria for the classification of SLE, were enrolled in the present study (30). These patients were recruited from Anhui Provincial Hospital and the First Affiliated Hospital of Anhui Medical University, China. Renal involvement of SLE was defined according to the ACR criteria, i.e., any one of the following: i) persistent proteinuria ≥0.5 g/day; ii) the presence of active cellular casts; or iii) biopsy evidence of lupus nephritis (LN). Demographic, personal and clinical data were collected from hospital records or by questionnaire and reviewed by experienced physicians. Population-based controls (315 female and 198 male) were randomly selected from healthy blood donors, without any rheumatologic conditions or any allergic status. Patient and control groups were from a Chinese population from the same area. The mean age was 37.38±12.208 years for the cases (female: 37.60±12.306 years, range 15-79; male: 34.72±10.758 years, range 17-59) and 35.01±16.291 years for the controls (female: 38.42±15.931 years, range 19-78; male: 29.60±15.402 years, range 11-75). This study was reviewed and approved by the local ethical committees from the corresponding centers and written informed consent was obtained from all participants prior to initiation of the study.

Selection of SNPs investigated. We performed a systemic search for *TLR7* single nucleotide polymorphisms (SNPs) with a minor allele frequency (MAF) >0.05 in the Han Chinese population of Beijing, China, as listed in the international HapMap project databank and the NCBI SNP databank. Haploview version 4.1 software was used to tag SNP selection. One tag SNP rs1634323 (7926A>G) in the intronic region of the *TLR7* gene, which overlaps four SNPs (rs1638596, rs1634319, rs1620233, rs5741880), was selected based on our inclusion criteria.

Genomic DNA extraction and SNP genotyping. Genomic DNA was extracted from EDTA-anticoagulated peripheral blood leukocytes from the buffy coat of each blood sample by centrifugation of 5 ml of whole blood using Flexi Gene DNA kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genotyping was performed by the TaqMan SNP assay using Assay-on-Demand probes and primers (Applied Biosystems, Foster City, CA, USA; catalogue nos. C-7625702-10 for rs1634323). RT-PCR analysis was carried out in 96-well plates in the ABI Prism 7300 real-time PCR system. Reaction conditions were: initial denaturation at 95˚C for 10 min followed by 50 cycles of denaturation at 95˚C for 15 sec, and annealing/extension at 60˚C for 1 min.

To confirm the genotyping results from the TaqMan SNP assay, we randomly selected 60 individuals for direct sequencing using an automated sequencer (ABI model 3739XL genetic analysis; Perkin-Elmer Applied Biosystems). The concordance rate was 100%.

Statistical analysis. Data were analyzed using SPSS 10.01 software (SPSS Inc., 2000). Differences of genotypic and allelic frequency distributions in SLE patients and healthy controls were analyzed by the Chi-square test or Fisher's exact test. Odds ratio (OR) with 95% confidence interval (CI) was calculated using unconditional logistic regression. Power calculations were performed using the G*Power program (31). All tests were two-tailed, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

The *TLR7* 7926A>G genotype and allele distributions in SLE patients and control subjects are shown in Tables I and II. When the 7926AA genotype was used as the reference group, the 7926AG genotype was associated with an elevated risk (P=0.040, OR=1.897, 95% CI 1.031-3.491) in female individuals, whereas a similar association was not replicated in male subjects (P=0.303, OR=0.338, 95% CI 0.043-2.656). In addition, we did not observe any statistical evidence for other genotype and allele comparisons.

To determine whether the *TLR7* 7926A>G has a preferential association with particular clinical manifestations of lupus nephritis (LN) or SLE, we subdivided the patients into a LN group and a SLE without nephritis group. Given that there was a limited number of male cases, we only performed statistical analysis in female subjects. The genotype frequencies of *TLR7* 7926A>G were 90.5 (AA), 8.8 (AG) and 0.7% (GG) in female cases with LN and 91.0 (AA), 8.7 (AG) and 0.3% (GG) in female patients without LN. No statistical differences were found for genotypic and allelic analyses. Detailed information is listed in Table III.

Assuming a type I error rate of 5% and OR of 1.5, we estimate that our study has a statistical power of 89.1% to detect a true-positive association.

Discussion

SLE is a prototypic autoimmune disease that affects nine times as many females as males. However, the reason for SLE primarily affecting females has not been clearly elucidated. Various studies have reported cases of male SLE patients associated with Klinefelter's syndrome (47, XXY) (25-27), while

Table I. Genotype and allele distributions of the *TLR7* rs1634323 (7926A>G) polymorphism in female SLE patients and healthy controls.

Polymorphism (rs1634323)	SLE N (%)	Control N (%)	P-value	OR (95% CI)
Genotype frequency				
AA	425 (90.8)	295 (93.7)		Reference
AG	41 (8.8)	15 (4.8)	0.040	1.897 (1.031-3.491)
GG	2 (0.4)	5 (1.6)	0.127	0.278 (0.054-1.441)
AG+GG	43 (9.2)	19 (6.4)	0.154	1.494 (0.861-2.592)
Allele frequency				
A	891 (95.2)	605 (96.0)		Reference
G	45 (4.8)	25 (4.0)	0.431	0.818 (0.496-1.349)

OR, odds ratio; CI, confidence interval; SLE, systemic lupus erythematosus.

Table II. Genotype and allele distributions of the *TLR7* rs1634323 (7926A>G) polymorphism in male SLE patients and healthy controls.

Polymorphism (rs1634323)	SLE N (%)	Control N (%)	P-value	OR (95% CI)
Genotype frequency				
AA	38 (97.4)	183 (92.4)		Reference
AG	1 (2.6)	15 (7.6)	0.303	0.338 (0.043-2.656)[a]
GG	-	-	-	-
Allele frequency				
A	77 (98.7)	382 (96.0)		Reference
G	1 (1.3)	15 (4.0)	0.489	3.024 (0.394-23.230)[b]

[a]With adjustment for age. [b]Fisher's exact test. OR, odds ratio; CI, confidence interval; SLE, systemic lupus erythematosus.

other studies found that female patients with Turner's syndrome (45, X0) did not contract SLE (28,29). Scofield *et al* (32) found that male individuals with Klinefelter's syndrome had approximately a 14-fold higher risk of developing SLE than 46 XY males. Smith-Bouvier *et al* (33) showed that the XX sex chromosome complement, as compared with XY, conferred greater susceptibility to autoimmune diseases, including SLE. A single 46, XX male child has also been reported with severe SLE from an early age (34). Similar to the Turner's syndrome patients, however, such patients may ultimately prove to be highly informative if a critical portion of the X chromosome can be identified as associated with disease based on deletion and triplication (34). Thus, X-linked genes may be risk factors for SLE.

Strong evidence supported the contribution of the *TLR7* gene, mapped at the X sex chromosome, to the development of autoimmunity in a SLE model. Nickerson *et al* (17) reported that lupus-prone mice deficient in TLR7 failed to generate Abs to RNA-containing antigens (Ags), such as Smith (Sm) Ag, and showed ameliorated disease. In contrast, Deane *et al* (35) found the upregulated production of RNA-related autoantibodies and spontaneous autoimmunity in transgenic mice beyond a 2-fold increased expression of TLR7. These results underscored the importance of tightly regulating the expression of TLR7 to prevent the spontaneous triggering of harmful autoreactive and inflammatory responses. Furthermore, an analysis of C57BL/6 mice congenic for the Nba2 (NZB autoimmunity 2) locus (B6.Nba2) bearing the Yaa mutation revealed that introduction of the TLR7 null mutation on the X chromosome significantly reduced serum levels of IgG autoantibodies against DNA and ribonucleoproteins, as well as the incidence of LN (21).

SNPs are the most common form of genetic variants in the human genome, some of which have potential functional effect on the susceptibility to human diseases including SLE. It is valuable to build a comprehensive catalogue of SNPs in candidate genes in the human population to better understand the roles of common genetic variants in diseases such as autoimmune diseases. Several studies have been performed to evaluate the association of *TLR7* gene polymorphisms with SLE. In the first case-control study of 752 SLE patients and 1107 healthy controls, Sánchez *et al* (36) found no evidence of

Table III. Genotype and allele distributions of the *TLR7* rs1634323 (7926A>G) polymorphism in female patients with LN and female SLE patients without LN.

Polymorphism (rs1634323)	LN N (%)	SLE without nephritis N (%)	P-value	OR (95% CI)[a]
Genotype frequency				
AA	133 (90.5)	292 (91.0)		Reference
AG	13 (8.8)	28 (8.7)	0.957	1.019 (0.512-2.030)[a]
GG	1 (0.7)	1 (0.3)	-	-
AG/GG	14 (9.5)	29 (9.0)	0.865	0.943 (0.483-1.844)[a]
Allele frequency				
A	279 (94.9)	612 (95.3)		Reference
G	15 (5.1)	30 (4.7)	0.776	0.912 (0.483-1.722)

[a]With adjustment for age. OR, odds ratio; CI, confidence interval; LN, lupus nephritis; SLE, systemic lupus erythematosus.

an association between the *TLR7* rs179008 polymorphism and SLE in a Spanish population. Nevertheless, a recent report on the same loci showed that the T allele significantly elevated the risk of SLE in a southern Brazilian population (37). Moreover, an association of rs3853839 with SLE was found by one group in an Eastern Asian population (38) using the Beadstation Infinium II platform (Illumina), and a similar association was also observed by another group (39) in a Japanese population using the TaqMan genotyping assay on an ABI 7300 real-time PCR system. In addition, Kawasaki *et al* (39) observed a correlation of two SNPs in the intron 2 (rs179019 and rs179010) with SLE, which exhibited no apparent correlation in Chinese and Korean subjects. There are several reasons for this discrepancy, including sample size, genetic background, cohort demographics (such as educational background and occupation), study design and genotyping methods.

In this study, we investigated a cohort of SLE patients and healthy controls to assess the role of the *TLR7* gene rs1634323 polymorphism in SLE susceptibility for the first time in a Chinese population, and the results revealed evidence of an association with SLE in female subjects. These findings provided evidence supporting our previous hypothesis that susceptibility to SLE may be associated with the gene dose effect of the X chromosome (40).

Previous studies (36-39) have evaluated the associations between the *TLR7* gene polymorphism and clinical subsets of SLE. For example, Sánchez *et al* (36) observed no statistically significant differences in the distribution of rs179008 between SLE patients with or without LN, age of onset, articular involvement, cutaneous lesions, photosensitivity, hematological alterations, neurological disorders and serositis. By contrast, results from a study by Shen *et al* (38) revealed a weak association of the rs3853839 G allele with the presence of anti-RNA binding protein, but not the age of disease onset, presence of mucocutaneous manifestations, LN or dsDNA. Recently, a study conducted in Japan detected a slightly higher risk of renal disorder compared to SLE patients, although a statistically significant association was not observed in the case-only analysis (SLE patients with renal disorder versus those without renal disorder) (39). To determine whether *TLR7* rs1634323 was associated with a clinical LN phenotype, we performed a subgroup analysis. Our study revealed no significant association of this genetic variant and LN in female patients (Table III).

It is suggested that unique predispositions may exist for male LN. Findings of several studies have demonstrated that male SLE patients present a typical manifestation, tend to have a significantly worse prognosis and have more serious kidney involvement (41-43). More specifically, a recent study has addressed the gender-based differences in Brazilian lupus patients and demonstrated that male patients had more severe LN, as indicated by higher creatinine levels and by comparison of the histopathological activity index values when compared to females diagnosed with this renal abnormality (44). Moreover, by promoting the expression of nephritogenic gp70 autoantigen (45-47), TLR7 is involved in the acute phase expression of serum envelope glycoprotein gp70 in murine lupus. Genetic studies have revealed a marked correlation of serum levels of gp70 IC, detected close to the onset of renal disease in the circulation and found within diseased glomeruli of lupus mice (48,49), with the development of severe lupus nephritis (50-53). TLR7 may also enhance the production of endogenous retroviruses (53), thereby providing another source for nephritogenic IC formation. Therefore, further studies based on male patients with a large sample size should be conducted.

In conclusion, our study suggested that the *TLR7* gene rs1634323 polymorphism may contribute to the etiology of female SLE in the Chinese population.

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