Investigation of *TLR5* and *TLR7* as candidate genes for susceptibility to systemic lupus erythematosus

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Abstract

Objective

The aim of this study was to evaluate the relevance of genetic variants of TLR5 *(rs5744168) and* TLR7 *(rs179008) gene in systemic lupus erythematosus (SLE) in a Spanish population.*

Material and methods

Our study population consisted of 752 SLE patients and 1107 healthy controls. All individual were of Spanish Caucasian origin. The TLR5 *and* TLR7 *polymorphisms were genotyped using a PCR system with pre-developed TaqMan allelic discrimination assay.*

Results

No statistically significant differences were observed when the allele and genotype distribution of TLR5 *rs5744168 and* TLR7 *rs179008 polymorphisms was compared between SLE patients and healthy controls. A significant increase frequency in the CC genotype of the* TLR5 *rs5744168 polymorphism among SLE patients without nephritis was found (93% vs. 87% in SLE patients with nephritis, p=0.03, OR=2.11 95%CI 0.93-3.51). However, this difference did not reach statistical significance in the allele frequencies (p=0.08).*

Conclusion

These results suggest that the tested variations of TLR5 *and* TLR7 *genes do not confer a relevant role in the susceptibility or severity to SLE in the Spanish population.*

Key words

Polymorphisms, systemic lupus erythematosus, susceptibility, toll-like receptors.

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Introduction

Systemic lupus erythematosus (SLE) is the prototypic systemic autoimmune disease and is characterized by B-cell hyperreactivity and the production of autoantibodies (1). Autoantibodies to DNA, RNA and associated proteins are common targets of the autoimmune response in SLE (2). The presence of these antinuclear antibodies has been detected in the serum of a majority of patients with SLE, and these antibodies result in the formation of immune complexes (ICs) that deposit in tissues and induce inflammation, thereby contributing to disease pathology. In fact, DNA and/or RNA can behave as autoantigens because they have the capacity to stimulate the innate immune system directly via Toll-like receptors (TLRs) or indirectly via Fcγ receptors (FcγR) and thereby promote the self-directed immune response, potentially leading to tolerance (3). The TLR family constitutes an important group of pattern-recognition receptors that play an essential role in the activation and regulation of innate and adaptive immunity through the recognition of specific molecular patterns of pathogens (4, 5). Currently, 11 TLR subtypes have been identified in humans, with each having specific ligands, cellular localization and expression profiles. Stimulation of the TLR pathway culminates in NFκB activation and transcription of immune response genes, such as cytokines and chemokines (5-7). Because of their central role in the regulation of inflammation and the immune response to pathogens, TLRs are excellent candidate genes in genetic susceptibility studies for autoimmune diseases, such as SLE.

TLR5 gene is known to recognize the bacterial flagellin and it located at 1q41 (8), a chromosome region liked with susceptibility to SLE in different populations (9, 10). A stop codon polymorphism in the ligand-binding domain of *TLR5* (*TLR5* rs5744168 also called Arg392Stop or C1174T) is unable to mediate flagellin signalling (11) and has been found associated with susceptibility to Legionnaires' disease (11), resistance to Crohn's disease (12) and SLE (13). These findings suggest that *TLR5* may be considered both a biological and a positional candidate gene for SLE.

TLR7 has recently been described as a potential functional relevance gene in SLE (14). TLR7 is involved in the recognition of singled-stranded viral RNA (15). Recent studies in congenic mice bearing the Y-linked autoimmune accelerator (*yaa*) lupus susceptibility locus, have showed that differences in expression of the *TLR7* gene as well as environmental factors that induce TLR7 responses may result in increased B cell sensivity to RNA-containing self-antigens (16, 17). In addition, TLR7 has the ability to induce the release of interferon-α (IFN-α), a cytokine that has been shown to have a relevant role in SLE (18).

Due to the central role of these *TLR* (*TLR5* and *TLR7*) genes within the innate immune system, the aim of this study was to determine the role of genetic variations in these genes with SLE in a Spanish population.

Material and methods

Patients

Peripheral blood samples were obtained after written informed consent from 752 SLE patients meeting the American College of Rheumatology criteria for SLE (19). These patients were recruited from nine Spanish hospitals: Hospital Virgen de las Nieves and Hospital Clínico (Granada), Hospital Virgen del Rocío (Seville), Hospital Carlos-Haya and Hospital Virgen de la Victoria (Málaga), Hospital Central (Oviedo), Hospital Parc Taullí (Sabadell), Hospital La Fe (Valencia) and Hospital Xeral-Calde (Lugo). Similarly, blood was taken from 1107 blood bank and bone marrow donors of the corresponding cities were included as healthy individuals. Both patient and control groups were of Spanish Caucasian origin and were matched for age by mean age and sex by frequency matching. Informed consent was provided by each individual included in the study. The samples were collected according to the Helsinki declaration. The study was approved by all local ethical committees from the corresponding centers. Demographic characteristics of the cases and controls in each population have been described previously (20).

Genotyping of TLR5 and TLR7 polymorphisms

DNA was obtained from peripheral blood, using QIAamp DNA Blood Maxi Kit (Qiagen, Hilden, Germany). *TLR5* and *TLR7* genotyping were performed using a TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, CA). The PCR reaction was carried out in a total reaction volume of 4 μl, containg 50 ng genomic DNA as template, 2 μl of Taqman genotyping master mix, 0.1 μl of 20x assay mix and ddH_2O up to 4 μl of final volume. The amplification protocol used was: initial denaturation at 95ºC for 10 min followed by 40 cycles of denaturation at 92ºC for 15 s, and annealing / extension at 60ºC for 1 min. After PCR, the genotype of each sample was automatically attributed by measuring the allele-specific fluorescence in the ABI Prism 7900 Sequence Detection System, using the SDS 2.2.2 software for allele discrimination (Applied Biosystems).

Statistic analysis

Allele and genotype frequencies were obtained by direct counting and for the statistical analysis to compare allelic and genotypic distributions we used the χ^2 test. We assessed the quality of the genotype data by testing for Hardy-Weinberg equilibrium in the case and control samples, using Fisher's exact test (p>0.05). Odds ratio (OR) with 95% confidence intervals (95%CI) were calculated according to Woolf's method. The software used was StatCalc program (Epi Info 2002; Centers of Disease Control and Prevention, Atlanta, GA, USA). *P*-values below 0.05 were regarded as statistically significant. The power of each study was computed as the probability of detecting an association between *TLR5* and *TLR7* polymorphisms and SLE at the 0.05 level of significance, assuming an OR of 1.5 (small effect size). Power analysis was estimated using the Quanto v 0.5 software (Department of Preventive Medicine University of Southern California, CA, USA).

Results

All the genotype frequencies in cases and healthy controls were not significantly different from those predicted by Hardy-Weinberg equilibrium.

Table I shows the distribution of genotypes and alleles of the *TLR5* rs5744168 polymorphism studied in SLE patients and controls. No statistically significant differences were observed when the allele and genotype distribution was compared between SLE patients and healthy controls. The allele frequencies found in our controls population are in good agreement with allele frequencies observed in other Caucasian populations (21). We next considered whether the *TLR5* stop codon showed a preferential association with particular clinical manifestations of SLE (Table II). A significant increase frequency in the CC genotype among SLE patients without nephritis was found (93% *vs*. 87% in SLE patients with nephritis, p=0.03, OR=0.47 95%CI 0.28-1.07). However, this difference did not reach statistical significance in the allele frequencies (p=0.08).

No *TLR7* polymorphisms have been described to date that influence the course of human diseases. Nevertheless, a recent study detected a variants with a frequency over 5% (rs179008), which results in an aminoacid change from glutamine to leucine at codon 11 (Q11L) (22). We analyzed the *TLR7* Q11L polymorphism in our cohort of SLE patients. No statistically significant differences were observed between allele frequencies of SLE patients and healthy controls (Table III). In addition, we found

Table I. Genetic and allelic distribution of *TLR5* rs5744168 polymorphism in SLE patients and healthy controls.

TLR5 rs5744168	SLE patients n=752 (%)	Healthy controls n=1107 (%)	*p*-value	OR (95% CI)
Genotypes				
CC	673 (89.5)	1009 (91.1)	0.2	0.83 (0.60-1.14)
CT	74 (9.8)	96 (8.7)	0.4	1.15 (0.83-1.60)
TT	5 (0.7)	2 (0.2)	0.1	3.70 (0.64-27.54)
Alleles	2n=1504	2n=2214		
C	1420 (94.4)	2114 (95.5)	0.1	0.80 (0.59-1.09)
T	84 (5.6)	100 (4.5)	0.1	1.25 (0.92-1.70)

Table II. Distribution of *TLR5* rs5744168 genotypes (%) by clinical features of the SLE patients.

SLE feature		CC	CT	TT	C	T
Nephritis	+	0.87*	0.13	0	0.93**	0.07
	–	0.93	0.6	0.08	0.97	0.03
Malar rash	+	0.93	0.07	0	96.4	3.6
	–	0.88	0.10	0.02	93.3	6.7
Discoide	+	0.87	0.1	0.03	0.92	0.08
	–	0.91	0.08	0.01	0.095	0.05
Oral ulcer	+	0.88	0.12	0	0.94	0.06
	–	0.93	0.06	0.01	0.96	0.04
Photosensivity	+	0.92	0.08	0.01	0.96	0.04
	–	0.89	0.10	0.01	0.94	0.06
Arthritis	+	0.92	0.09	0.01	0.95	0.05
	–	0.093	0.06	0.01	0.96	0.4
ANA	+	0.91	0.08	0.01	0.95	0.5
	–	1.00	0	0	1.00	0
Anti-dsDNA Ab	+	0.90	0.09	0.01	0.95	0.5
	–	0.96	0.03	0.01	0.97	0.03

*p=0.03, OR=0.47 95%CI 0.28-1.07.
**p=0.08.

Table III. Allelic distribution of *TLR7* rs179008 polymorphism in SLE patients and healthy controls.

TLR7 rs179008	SLE patients n=752 (%)	Healthy controls n=1107 (%)	*p*-value	OR (95% CI)
Alleles				
C	1104 (81.2)	1439 (80.6)	0.7	1.04 (0.87-1.23)
T	256 (18.8)	346 (19.4)	0.7	0.97 (0.80-1.54)

no association of this polymorphism and genotype frequencies in female patients with SLE (data not shown). We also estimated the allele frequencies in male with SLE and no deviation in the distribution compared with allele frequencies in male controls was observed. In addition, available clinical features of patients with SLE were analyses for possible association with the different alleles or genotypes of *TLR7* polymorphism. However, when we stratified SLE patients according to the presence of renal involvement, no statistically significant differences were observed in the distribution of this polymorphism between SLE patients with or without lupus nephritis (data not shown). Similarly, no significant differences were observed between this genetic variant and the following variables, age at onset, articular involvement, cutaneous lesions, photosensivity, hematological alterations, neurological disorders and serositis (data not shown).

Discussion

Due to the central role of TLRs within the innate immune system, genetic variation in this gene family may alter susceptibility to some diseases. Genetic variations in the *TLR* genes have been associated with many inflammatory and/or autoimmune diseases (13, 23-26). Accumulating evidence indicates a role of TLRs in the recognition of endogenous ligands which might be involved in these disorders (3, 27).

In the present study we analysed a large cohort of SLE patients and healthy controls to assess the role of *TLR7* gene in the susceptibility to SLE, and tried to replicate the previously association reported by Hawn *et al*. between the *TLR5* rs5744168 polymorphism and SLE (13). Our data revealed no evidence of association of these variants with SLE in a Caucasian cohort from Spain. These findings contradict a recent family based report, which found that *TLR5* 1174C allele confers protection from developing SLE (13). However, an in agreement with our data a study in two Caucasians populations from North-America could not replicate the initial association (21). Several reasons could be underlying this discrepancy. The first result could emerge as a consequence of a type I error (false positive), as a result of examining the statistical power of the study using allelic OR results, and it concluded that it is underpowered to detect an association (<40%). On the contrary, the existence of a type II error (false negative) in our and Demirci *et al*. studies is unlikely because of the high statistical power of these studies (97% and 73%, respectively) to detect an effect similar to that observed in the first study.

Although *TLR7* genetic variants are largely unexplored, Pisitkun *et al*. have reported that a genomic segmental duplication, which included the murine *Tlr7* gene, and the translocation of this segment to the *Yaa* locus were associated with autoreactive B cell responses to RNA-related antigens. (16). However, the genomic increase in *Tlr7* in a murine model of lupus cannot be translated directly to humans with SLE, since no significant concordance between the relative number of gene copies and the SLE phenotype was found (28). Although these variants in the *TLR7* seem not play a relevant role in human SLE, several evidences show a role of this gene in the development of the disease. Since *TLR7* is located on chromosome X and there is an increase prevalence of SLE in women (29), it is suggested an X-linked genetic component in SLE. In addition, IFN-α, a cytokine critically involved in the pathogenesis of SLE (18, 30), markedly increases B cell expression of TLR7 by plasmocytoid dendritic cells (31).

In the present study we investigated for the first time a *TLR7* polymorphism (Q11L) to test SLE susceptibility, and we found no evidence of association. The possibility that this lack of association could have arisen due to type II error seems unlikely, since we estimated that our cohort has enough power (>99%) to detect the effect of the polymorphism, taking into consideration an OR of 1.5 at a 5% significance level. Furthermore, allele frequencies in our control population are similar to those reported in the SNP database (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=179008) in other Caucasian-European populations. However, we cannot exclude the possibility that other polymorphisms of the *TLR7* gene that are not in linkage disequilibrium with the alleles tested may contributed to the development of SLE. In addition, this SNP is located in a region of known copy number variation, which should be taken with caution in genotyping studies since the alleles may differ according to the number of copies carried.

TLR5 and TLR7 signalling involves the adaptor protein myeloid differentiation factor 88 (MyD88), since mice rendered MyD88-deficient are unresponsive to ligands for these TLRs (32) and that activation leads to the production of proinflammatory cytokines such as TNF-α, IL6, IL1β and IL12. In addition, TLR stimulation generally leads to the production of IL12 and IL23 and thereby favours a Th1-type response (33). However, in previous studies, we failed to find an association between genetic variants in these cytokines and other TLRs with SLE in our population (34-37).

In conclusion, although the clinical relevance of *TLR5* rs5744168 and *TLR7* rs179008 polymorphisms indicates the possible physiological effect of other polymorphisms in chronic inflammatory diseases, these variants seems not play a relevant role in SLE in our population. However, this finding cannot rule out a possible role of TLR5 and TLR7 in SLE pathogenesis therapeutic targets (38).

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