Association Study of Toll-like Receptor 5 (*TLR5*) and Toll-like Receptor 9 (*TLR9*) Polymorphisms in Systemic Lupus Erythematosus

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ABSTRACT. ***Objective.*** Toll-like receptors (TLR) play an important role in both adaptive and innate immunity. Variations in TLR genes have been shown to be associated with various infectious and inflammatory diseases. We investigated the association of *TLR5* (Arg392Stop, rs5744168) and *TLR9* (–1237T→C, rs5743836) single nucleotide polymorphisms (SNP) with systemic lupus erythematosus (SLE) in Caucasian American subjects.

Methods. We performed a case-control association study and genotyped 409 Caucasian women with SLE and 509 Caucasian healthy female controls using TaqMan® allelic discrimination (rs5744168) or polymerase chain reaction-restriction fragment length polymorphism analysis (rs5743836).

Results. None of the 2 *TLR* SNP showed a statistically significant association with SLE risk in our cohort.

Conclusion. Our results do not indicate a major influence of these putative functional *TLR* SNP on the susceptibility to (or protection from) SLE. (First Release May 15 2007; J Rheumatol 2007;34:1708–11)

Key Indexing Terms:
TLR5 *TLR9* TOLL-LIKE RECEPTOR LUPUS SYSTEMIC LUPUS ERYTHEMATOSUS

Systemic lupus erythematosus (SLE) is a prototypic multisystem autoimmune disease that affects predominantly premenopausal women. The disease is characterized by systemic chronic inflammation associated with the production of autoantibodies against multiple antigens, including nucleic acids and nucleoproteins. SLE has a complex genetic basis and is caused by a complex interaction of unknown environmental factors and multiple genetic susceptibility loci on different chromosomes[1].

Members of the Toll-like receptor (TLR) family are type I transmembrane proteins that play a key role in the activation and regulation of both innate and adaptive immune responses. At least 10 different *TLR* have been cloned from the human genome to date. The role of TLR proteins in various human diseases has been investigated by the analysis of sequence variants in genes that participate in TLR signaling. These studies have shown that variation in TLR functions may affect several diseases, including sepsis, immunodeficiencies, atherosclerosis, asthma, and inflammatory disorders[2]. Because recognition of microbial components by TLR triggers activation of not only innate immunity but also adaptive immunity, *TLR* are also excellent candidate genes to be examined in relation to autoimmune diseases[3]. Several autoimmune disorders have been shown to be associated with infection and dysregulation of innate immunity, suggesting that autoimmunity can be induced by crosstalk between adaptive and innate immune pathways[3]. Two major theories proposed to account for the correlation between infection and the onset and/or exacerbation of autoimmunity are molecular mimicry and bystander activation[4]. In addition to mediating immunity against pathogens, growing evidence indicates that TLR stimulation may also potentially contribute to autoimmune responses[5].

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A recent report by Rahman and Eisenberg[5] has provided an in-depth review of the role of TLR in SLE pathogenesis. TLR-mediated activation of dendritic cells and B cells has multiple consequences that can affect other aspects of the immune system contributing to SLE pathology. Activation of TLR in dendritic cells leads to production of type I interferons that are critical to the link between innate and adaptive immunity[3,6]. Pathogen-associated molecular patterns can costimulate autoreactive B cells through TLR interaction and participate in the breakage of B cell tolerance[7]. Genetic variation affecting TLR signaling is therefore expected to affect the threshold of dendritic cell and/or B cell activation, which in turn may influence susceptibility to SLE.

TLR5 (chromosome 1q41-q42, GenBank accession number NM_003268) is known to recognize the bacterial flagellin. A stop codon polymorphism in the ligand-binding domain of human *TLR5* (c.1174C→T, p.Arg392Stop, refSNP ID: rs5744168) was shown to result in decreased flagellin signaling[8]. *TLR5*/Arg392Stop variant was found to be associated with Legionnaires' disease[8] and Crohn's disease[9]. Given the critical role of TLR5 protein in inflammatory signaling pathways and the linkage studies that have mapped a major SLE susceptibility locus to human chromosome 1q41 where *TLR5* resides[10], *TLR5* has been considered both a biological and a positional candidate for SLE. The only SLE study to date with *TLR5* stop codon polymorphism has suggested a protective effect on SLE[11].

TLR9 (chromosome 3p21.3, GenBank accession number NM_017442) is required for the recognition of CpG-DNA motifs (short sequences of unmethylated cytosine-guanine-rich DNA that are predominantly present in bacterial DNA)[12]. The immunostimulatory effects of CpG-DNA can initiate and/or modulate autoimmunity by inducing the inflammatory cells and production of cytokines[13]. A *TLR9* polymorphism (T→C at –1237 of the ATG, refSNP ID: rs5743836) was shown to be associated with susceptibility to asthma[14] and Crohn's disease[15]. TLR9 protein signaling has been suggested to be involved in lupus activity that is triggered by unmethylated microbial DNA[12]. Mammalian DNA can also stimulate *TLR9* when present in immune complexes, which are commonly observed in the circulation of patients with SLE[2,6]. SLE DNA immune complexes have been reported to induce proliferation of self-reactive B cells and production of cytokines by dendritic cells in a *TLR9*-dependent manner[16]. Reports that evaluated association of *TLR9* SNP with SLE risk have provided controversial results[17-20].

We performed a case-control genetic association study to replicate a reported association[11,17] of 2 TLR SNP (*TLR5*/Arg392Stop and *TLR9*/–1237T→C) with the susceptibility to (or protection from) SLE.

MATERIALS AND METHODS

Subjects. Peripheral blood leukocyte DNA samples from 409 Caucasian women with SLE (341 from Pittsburgh, PA, 68 from Chicago, IL) and 509 Caucasian female controls (452 from Pittsburgh, 57 from Chicago) were studied.

The SLE cases were recruited for a multicenter study designed to determine the prevalence of cardiovascular disease and associated risk factors in women with SLE. Cases were 18 years of age or older, and met the 1982 and 1997 revised American College of Rheumatology classification criteria for SLE[21]. All SLE subjects were participants in either the Pittsburgh Lupus Registry or the Chicago SOLVABLE study (Study of Lupus Vascular and Bone Longterm Endpoints). Demographic and clinical details of the patient population have been described[22-24]. Controls with no apparent history of SLE were matched geographically and recruited either from the Central Blood Bank of Pittsburgh or from the SOLVABLE study in the Chicago site.

Blood samples were obtained at the baseline visit. All subjects provided written informed consent for the study of SLE genetics, in accord with protocols that were approved by the University of Pittsburgh and the Northwestern University institutional review boards.

Genotyping. *TLR5* SNP (Arg392Stop) genotyping was performed by TaqMan® allelic discrimination using a pre-made TaqMan® SNP Genotyping Assay (C_25608804_10; Applied Biosystems, Foster City, CA, USA). For the *TLR9* SNP (–1237T→C), the genotyping was performed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis using the restriction enzyme *BstN* I. Primer sequences for PCR-RFLP analysis were as follows: forward 5'-GCT GGA TGG CCC TGT TGA-3'and reverse 5'-GCC TCA GGG CCT TGG GAT-3'. *BstN* I digestion resulted in a single fragment of 123 bp for TT genotype, 3 fragments of 123, 87 and 36 bp for TC genotype, and 2 fragments of 87 and 36 bp for CC genotype.

Statistical methods. Allele frequencies were calculated by the allele counting method. Observed genotype frequencies were compared to Hardy-Weinberg equilibrium and the significance of deviations was tested by the chi-squared goodness-of-fit test. Fisher's exact test and standard Z-test of 2 binomial proportions were used to compare the genotype and allele frequencies, respectively, between cases and controls. All computations, logistic regression, and analysis of variance (ANOVA) were performed using R statistical software package (version 2.3.1; http://www.r-project.org). Power for association studies of genes was calculated using the Quanto program (http://hydra.usc.edu/GxE).

RESULTS

Distribution of TLR5 and TLR9 SNP. Distribution of genotypes and allele frequencies for *TLR5*/Arg392Stop and *TLR9*/–1237T→C SNP are summarized in Table 1. Allele and genotype frequencies of both SNP were comparable between SLE cases and controls in our combined Pittsburgh–Chicago sample (for *TLR5* SNP $p = 0.514$ and $p = 0.462$, for *TLR9* SNP $p = 0.517$ and $p = 0.815$, respectively).

Interaction between TLR5 and TLR9 SNP. In order to determine whether *TLR5* (Arg392Stop) and *TLR9* (–1237T→C) SNP can act synergistically on disease susceptibility, we examined the simultaneous effects of these 2 SNP on SLE risk. We fit a logistic regression model with the following independent variables: age, recruitment site, *TLR5*, *TLR9*, and *TLR5* × *TLR9* interaction. No statistically significant independent effects on SLE were detected ($p = 0.954$ for *TLR5* and $p = 0.694$ for *TLR9*); however, a marginally significant interaction was observed ($p = 0.040$ for *TLR5* × *TLR9*).

DISCUSSION

Allele frequencies of the *TLR5* and *TLR9* SNP that we observed in our combined control population were similar to

Table 1. Frequency of TLR SNP in SLE patients and controls. Only the individuals who were successfully genotyped for one or both SNP were included in the table.

Population Group	*TLR5* (Arg392Stop, rs5744168) Patients	*TLR5* (Arg392Stop, rs5744168) Controls	*TLR9* (–1237T→C, rs5743836) Patients	*TLR9* (–1237T→C, rs5743836) Controls
Pittsburgh	n = 323	n = 427	n = 339	n = 451
Genotypes	n (%)	n (%)	n (%)	n (%)
CC	294 (91.02)	383 (89.70)	9 (2.65)	12 (2.66)
CT	27 (8.36)	43 (10.07)	94 (27.73)	125 (27.72)
TT	2 (0.62)	1 (0.23)	236 (69.62)	314 (69.62)
	p = 0.513		p = 1.000	
Alleles				
C	0.952	0.947	0.165	0.165
T	0.048	0.053	0.835	0.835
	p = 0.679		p = 1.000	
Chicago	n = 67	n = 55	n = 59	n = 53
Genotypes	n (%)	n (%)	n (%)	n (%)
CC	60 (89.55)	46 (83.64)	1 (1.69)	3 (5.66)
CT	6 (8.96)	8 (14.55)	12 (20.34)	16 (30.19)
TT	1 (1.49)	1 (1.82)	46 (77.97)	34 (64.15)
	p = 0.699		p = 0.246	
Alleles				
C	0.940	0.909	0.119	0.208
T	0.060	0.091	0.881	0.792
	p = 0.362		p = 0.072	
Combined	n = 390	n = 482	n = 398	n = 504
Genotypes	n (%)	n (%)	n (%)	n (%)
CC	354 (90.77)	429 (89.00)	10 (2.51)	15 (2.98)
CT	33 (8.46)	51 (10.58)	106 (26.63)	141 (27.98)
TT	3 (0.77)	2 (0.41)	282 (70.85)	348 (69.05)
	p = 0.462		p = 0.815	
Alleles				
C	0.950	0.943	0.158	0.170
T	0.050	0.057	0.842	0.830
	p = 0.514		p = 0.517	

those previously published[14,25] or reported in public databases.

Of 13 missense SNP of *TLR5* evaluated by Merx, *et al*[25], the Arg392Stop was among 3 *TLR5* SNP that showed a significant influence on receptor function in a cell culture system. A recent study by Hawn, *et al*[11] used a transmission disequilibrium testing (TDT) analysis in a Caucasian SLE cohort of subject/parent trios (199 affected patients, 75 unaffected siblings, and 326 parents) and reported that the *TLR5*/Arg392Stop variant was associated with protection from developing SLE. However, we found no evidence to support a significant association between the *TLR5*/Arg392Stop variant and SLE using a case-control study design. This discrepancy may be related to the methodology (TDT vs case-control) and/or population sampling differences between the 2 studies. It is possible that the effect of *TLR5* is more pronounced in familial SLE than in sporadic SLE. Alternatively, the effect size of *TLR5*/Arg392Stop variant may be small and can only be detected in very large case-control samples. Given the ~5% minor allele frequency of this SNP, the power of our sample size to detect an odds ratio (OR) of 1.8 was 80%. Several candidate genes showing initial positive associations have generated negative findings in replication studies due to issues with insufficient power or sample heterogeneity. More studies with large cohorts are necessary to characterize the role of this *TLR5* SNP in the etiology of SLE.

TLR9/–1237T→C SNP is located at the 5' upstream of the gene where it could influence transcription regulation. An initial study conducted family-based TDT in 224 Caucasian SLE patients and their parents, and reported that *TLR9*/–1237T→C variant was associated with protection from SLE and lupus nephritis[17]. However, 2 independent case-control studies failed to find a significant association between *TLR9* polymorphisms and susceptibility to SLE in Koreans (350 SLE patients and 330 controls)[18] and Chinese (467 SLE patients and 799 controls)[19]. A more recent study also reported no association with SLE susceptibility and *TLR9* SNP using a

TDT approach in 362 mostly European SLE-subject/parent trios[20]. Consistent with these reports, our case-control association study in an independent Caucasian SLE cohort revealed no association between the *TLR9*/–1237T→C SNP and SLE. Given the ~15% minor allele frequency of this SNP, our study had 80% power to detect an OR of 1.5.

To our knowledge, this is the first report of analysis of *TLR5*/Arg392Stop and *TLR9*/–1237T→C SNP in a Caucasian SLE cohort using a case-control study design. Our results do not indicate a major influence of the *TLR5*/Arg392Stop and *TLR9*/–1237T→C SNP on susceptibility to (or protection from) SLE. Although the development of SLE does not seem to be strongly affected by the independent effects of these 2 SNP, they might still have combined effects on disease progression (as also suggested by our results showing a marginally significant genetic interaction between the 2 loci) due to the central role of TLR in immune system function. However, given the multiple comparisons that we performed, this marginal significance ($p = 0.04$) should be considered provisional, until replicated by large independent case-control studies.

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