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Title: Association of Toll-like Receptor 9 Gene Polymorphism in Chinese Patients with Systemic Lupus Erythematosus in Taiwan

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Abstract: Objective: The purpose of this study was to determine whether Toll-like receptor 9 (TLR9) gene polymorphisms were markers of susceptibility to or severity of systemic lupus erythematosus (SLE) in Taiwanese patients.
Methods: The study included 211 healthy individuals and 167 Chinese patients with SLE. Polymorphisms of TLR9 (rs2066807 and rs187084 (-1486 T/C)) were typed from genomic DNA. The genotypes, allelic frequencies and carriage rates were compared between SLE patients and control subjects. The relationship between allelic frequencies and clinical manifestations of 167 SLE patients was evaluated.
Results: There was no statistically significant difference in TLR9 (rs2066807) gene polymorphism, allelic frequency, and carriage rate between the SLE and control groups. However, for the genotype of TLR9-1486 T/C (rs187084) polymorphism, there was a statistically significant difference between the SLE and the control groups ($p<0.001$, $\chi2 = 15.9$). Moreover, there was a significant association between the two groups in allelic frequency and carriage rate of the T allele ($p<0.001$, $\chi2 = 18.5$ and $p<0.01$, $\chi2 = 8.06$, respectively). We did not detect any association between the TLR9 genotype and the clinical or laboratory profiles in SLE patients.
Conclusion: The results suggest that the TLR9 -1486 T/C (rs187084), but not TLR9 (rs2066807), polymorphism is related to SLE in Taiwanese patients.

Association of Toll-like Receptor 9 Gene Polymorphism in Chinese Patients with Systemic Lupus Erythematosus in Taiwan

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Running title: Patients with SLE

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Conclusion: The results suggest that the TLR9 -1486 T/C (rs187084), but not TLR9 (rs2066807), polymorphism is related to SLE in Taiwanese patients.

Introduction

Systemic lupus erythematosus (SLE) is a prototypic immune complex-mediated disease with a broad spectrum of autoimmune phenomena and clinical symptoms. Many immunologic abnormalities have been found in SLE patients, including impaired T cell responses and dysregulation of B cell activation, leading to B cell hyperactivity and overproduction of auto-antibodies [1,2]. Auto-antibodies produced by differentiated B cells play an important role in the pathogenesis of SLE.

Toll-like receptor-9 (TLR-9) recognizes CpG motifs in microbial DNA[3]. TLR-9 signalling stimulates innate antimicrobial immunity and modulates adaptive immune responses including autoimmunity against chromatin, such as in SLE[4,5]. TLR-9 has recently been implicated in the activation of auto-reactive B cells in murine models of SLE [6-8]. Migita et al. showed that TLR-9 expression in B lymphocytes was increased in patients with SLE [9]. Genetic variations within *TLR* genes are known to be associated with a variety of inflammatory and infectious diseases[10]. Hur et al. presented their observation that four polymorphisms within a 4,334-bp segment of the TLR-9 gene demonstrated no significant association with susceptibility to SLE in a Korean population [11]. In addition, De Jager et al. also showed that there is no relationship between the TLR-9 genes and SLE or lupus nephritis in a UK population [12].

In this study, we evaluated the TLR9 (rs2066807 and rs187084 (-1486 T/C)) gene by comparing allelic and genotypic frequencies between 167 Chinese patients with SLE and 211 healthy individuals living in Central Taiwan. We also studied the association between TLR9 and SLE susceptibility and severity by focusing on single nucleotide polymorphisms (SNPs).

Patients and Methods

Patient selection

We enrolled 167 patients (149 women and 18 men) patients with definite SLE according to the 1982 revised American College of Rheumatology criteria [13], and 211 sex-, age-matched healthy individuals living in Central Taiwan. This study was approved by the Institutional Review Board (IRB) at China Medical University Hospital prior to patient enrollment. Informed consent was obtained from each patient and control subject involved.

Polymerase chain reaction

The genomic DNA was prepared from peripheral blood by a genomic DNA isolation reagent kit (Genomaker, Taiwan). Polymerase chain reaction (PCR) was used to identify the genotypes of the TLR9 (rs2066807 and rs187084) polymorphisms. Polymerase chain reaction of the polymorphisms was performed in a total volume of 50 μL, containing genomic DNA (2–6pmol of each primer), 1X Taq polymerase buffer (1.5mM $MgCl_2$) and 0.25 units of AmpliTaq DNA polymerase (Perkin Elmer; Foster City,CA,USA). For the TLR9 (rs2066807) gene promoter region, the primers used were upstream 5'- TGGGCTGTTTCTCCATACAA-3' and downstream 5'-AAACCAGCTGAGGGATTCAGT-3'. For the TLR9 -1486 T/C (rs187084), the primers used were upstream 5'-CCCAGCAGCAACAATTCAT-3' and downstream 5'-TTATTCCCCTGCTGGAATGT-3'. Polymerase chain reaction amplification was performed in a programmable PCR thermal cycler (GeneAmp PCR System 2400, Perkin Elmer). The PCR cycling conditions for TLR9 (rs2066807) and TLR9 (rs187084) gene polymorphisms examination were as follows: 35 cycles at 95^0 C for 30 seconds, 56^0 C for 45 seconds, and 72^0 C for 30 seconds. They were then left to stand at 72^0 C, then held at 4^0 C.

The polymorphisms of the respective genes of interest were discerned by digestion with Tth111I and Hpy188III, respectively. The PCR products were mixed separately using the aforementioned enzymes and reaction buffer according to the manufacturer's instructions. Both reactions were incubated for three hours at 37^0 C. After this, 10µl of each product was loaded into a 3% agarose gel containing ethidium bromide and subjected to electrophoresis. The resultant genotypes were classified into the categories of non-excisable homozygote allele (CC for TLR9 (rs2066807) and TT for TLR9 (rs187084) genes, respectively), excisable homozygote allele (GG for TLR9 (rs2066807) and CC for TLR9 (rs187084) genes, respectively), and heterozygote allele (CG for TLR9 (rs2066807) and CT for TLR9 (rs187084) genes, respectively).

Statistical analysis

The carriage rate of an allele is the number of individuals carrying at least one copy of the allele relative to the total number of individuals. Allelic frequency was expressed as a percentage of the total number of alleles. The genotype distributions, allelic frequencies, and carriage rates for the TLR9 (rs2066807) and TLR9 (rs187084) gene polymorphisms for SLE patients and the controls were compared using the chi-square test. Results were considered statistically significant when the probability of findings occurring by chance was less than 5% ($p < 0.05$). The odds ratios (OR) were calculated from genotypic frequency and allelic frequency with a 95% confidence interval (95% CI) for the TLR9 (rs2066807) and TLR9 (rs187084) gene polymorphisms.

Results

The frequencies of the genotypes of TLR9 (rs2066807) in the SLE and the control groups are shown in Table 1. In the SLE group, 155 patients (94.5 %) had the GG genotype, 9 patients (5.5 %) had the C/G genotype, and no patient (0 %) had the CC genotype. In the

control group, 200 people (96.2 %) had the GG genotype, 8 (3.8 %) had the C/G genotype, and no patient (0 %) had the CC genotype. There were no significant differences in the distribution of the TLR9 (rs2066807) C/G polymorphism between the SLE and the control groups (χ^2= 0.57, p=0.45). We also analyzed the relationship between the two groups in allelic frequencies and carriage rate of TLR9 (rs2066807) (Table 1). There was no significant association in frequency (p=0.46) and carriage rate (p=0.47) between SLE patients and normal subjects.

Table 2 showed the frequencies of the genotypes of TLR9 (rs187084) in the SLE and the control groups. In the SLE groups, 123 patients (73.6 %) had the TT genotype, 39 patients (23.4 %) had the C/T genotype, and 5 patients (3.0 %) had the CC genotype. In the control group, 119 people (56.4 %) had the TT genotype, 67 (31.8 %) had the C/T genotype, and 25 patients (11.8 %) had the CC genotype. For the genotype of TLR9 (rs187084) polymorphism, there were statistically significant differences between the SLE and the control groups (chi-squared test, p<0.001, χ^2 = 15.9). We also analyzed the relationship between the two groups in allelic frequencies and carriage rate of TLR9 (rs187084) (Table 2). There was a significant association in the frequency of the T allele between SLE patient and normal subjects (χ^2= 18.5, p<0.001), giving an odds ratio of 2.23 (95% confidence interval 1.45-3.42). Similarly, there was a significant association in the carriage rate of the T allele between SLE patient and normal subjects (χ^2= 8.06, p<0.01), giving an odds ratio of 1.82 (95% confidence interval 1.14-2.91).

The clinical manifestations and laboratory findings of the SLE patients, given as ACR criteria, are shown in Tables 3 and 4. The association of TLR9 (rs2066807) and TLR9 (rs187084) with particular clinical features of SLE was examined in the 158 and 160 patients,

respectively. We observed increased frequencies of mucosal ulcer among patients with the G allele when compared with patients with the C allele in Table 3. However, the differences did not reach statistical significance ($\chi^2 = 3.05$, $p=0.08$). In Table 4, we found increased frequencies of photosensitivity among patients with the T allele when compared with patients with the C allele. The differences also did not reach statistical significance ($\chi^2 = 5.17$, $p=0.07$). Similarly, we did not find any relationship among TLR9 (rs2066807), TLR9 (rs187084), and severity of SLE (i.e., no association with renal or neurologic involvement) in the Chinese patients in this study.

Discussion

Members of the TRL 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 TRL have been cloned from the human genome to date. TLR-9 is an interesting candidate for an SLE susceptibility gene given its costimulatory role in activating murine autoreactive B cells and in activating human dendritic cells. Migita et al. showed that TLR-9, not TLR-2 or TLR-4, expression in B lymphocytes was increased in patients with SLE [9]. The importance of the TLR9-dependent pathways will depend on the levels of TLR9 expression. The differential expression of TRL9 may correlate with the responsiveness to CpG DNA, and the altered TRL9 expression could potentially affect the B cell immune response to chromatic or immunocomplex (IC) in

patients with SLE[14,15]. This upregulated TLR9 expression may activate B lymphocytes through the interaction between TLR9 and its ligands and may be related to the pathogeneses of lupus.

TLR9 has been mapped to chromosome 3p21.3. It spans approximately 5 kb and has two exons, the second of which is the major coding region[16]. TLR9– 1237C/T SNP is located at the 5' upstream of the gene where it could influence transcription regulation. A few studies about the relationship of such polymorphisms with SLE have been reported. However, the results are conflict. An initial study conducted family-based TDT in 224 Caucasian SLE patients and their parents, and reported that TLR9– 1237C/T variant was associated with protection from SLE and lupus nephritis[17]. However, Hur et al. [11] reported no association with SLE in four polymorphisms of the TLR9 gene (-1486 T/C, -1237 C/T, +1174 A/G, and +2848 G/A) in 680 Korean people (350 SLE patients and 330 controls). The genotype frequencies of the four SNPs in the Koreans studied were similar to those of the Chinese population from Hong Kong. Their study included 799 Hong Kong Chinese healthy blood donors and 467 SLE patients and showed no relationship of TLR9 (-1486 T/C (rs187084), -1237 C/T (rs5743836), IVSI-44A/G (rs352139), and +1635 A/G (rs352140) gene

polymorphisms with SLE[18]. Although no significant association was found, the TT genotype of TLR9-1486 tended to be over-represented in patients with serositis (61.1%) compared with those without serositis (44.1%) (p= 0.056)

Furthermore, De Jager et al. [12] genotyped 362 SLE-affected subjected/parent trios for 10 SNP2 covering a 68742-bp genomic segment that contains the TLR-9 and ~60 kb of flanking sequence and demonstrated that there was no association between 362 SLE and 10 TLR9 (including -1486 T/C and -1237 C/T) in the UK population. They also found no evidence of association of susceptibility to lupus nephritis with any of the TLR-9 SNPs or haplotypes. Similarly, Dimirci et al.[19] performed a case-control association study and genotyped 409 Caucasian women with SLE and 509 Caucasian healthy female control and revealed no significant association of TLR9 (-1237 C/T, rs5743836) with SLE in Caucasian-American subjects. In Japan, DNA samples were obtained from 220 Japanese patients with SLE and 203 controls. The genotype of TRL9 +1174 is closely associated with an increased risk of SLE in Japanese people[20] (p=0.029), but patients with SLE tend to have C allele at position -1486 (p=0.11).

In contrast, our study examined the TLR9 (rs2066807 and rs187084) gene by comparing allelic and genotypic frequencies between 167 Chinese patients with SLE and 211 healthy individuals living in Central Taiwan. In surveying this genomic segment, we found that the genotype of TLR9 -1486 T/C (rs187084) ($p<0.001$, $\chi^2 = 15.9$), but not TLR9 (rs2066807) polymorphism, has a statistically significant difference between the SLE and the control groups. However, we did not find any relationship between TLR9 (rs2066807 and

rs187084) gene and severity of SLE (i.e., no association with renal or neurologic involvement) in Chinese patients. Previous studies have not demonstrated any genetic variations of TLR9 -1486 T/C associated with an increased risk of SLE in UK, Korean, Japanese and Hong Kong Chinese populations. This difference in the distribution of TLR9 observed at position +1486 between these studies may be due to ethnic factors.

To our knowledge, this is the first report of analysis of TLR9 rs2066807 and TLR9– 1486T/C SNP in a Chinese SLE cohort using a case-control study design. Our results indicate a major influence of the TLR9– 1486T/C SNP on susceptibility to SLE. This might suggest that the genetic variations of TLR9 -1486 T/C (rs187084) is specific for Taiwanese SLE population.

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Disclosures - none

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Table 1. Comparison of TLR9 (rs2066807) genotype distributions, allelic frequencies, and carriage rates observed in SLE patients and healthy control subjects

TLR9 (rs2066807)	SLE patients n=164 (%)	Controls n=208 (%)	*P*	OR (95% CI)
Genotype				
GG	155 (94.5)	200 (96.2)	0.45	
C/G	9 (5.5)	8 (3.8)		
CC	0	0		
Allelic frequencies				

G	319 (97.3)	408 (98.1)	0.46	0.69 (0.27~1.82)
C	9 (2.7)	8 (1.9)		1.44 (0.56~3.67)
Carriage rate				
C	164 (100)	208 (100)	0.47	0.70 (0.27~1.81)
G	9 (5.5)	8 (3.8)		1.43 (0.55~3.68)

All *p* values represent chi-square test results.

Table 2. Comparison of TLR9 (rs187084) genotype distributions, allelic frequencies, and carriage rates observed in SLE patients and healthy control subjects

TLR9 (rs187084)	SLE patients n=167 (%)	Controls n=211 (%)	*P*	OR (95% CI)
Genotype				
TT	123 (73.6)	119 (56.4)	<0.001	
C/T	39 (23.4)	67 (31.8)		
CC	5 (3.0)	25 (11.8)		
Allelic frequencies				

T	285 (85.3)	305 (72.3)	<0.001	2.23 (1.45~3.42)
C	49 (14.7)	117 (27.7)		0.45 (0.29~0.69)
Carriage rate				
T	162 (97.0)	186 (88.2)	<0.01	1.82 (1.14~2.91)
C	44 (26.3)	92 (43.6)		0.55 (0.34~0.88)

All *p* values represent chi-square test results.

Table 3. Relationship between the TLR9 (rs2066807) genotype and clinical signs and findings in patients with SLE

	GG n=150 (%)	C/G n=8 (%)	Total n=158 (%)	***P***
Malar rash	72 (48.0)	2 (25.0)	74 (46.8)	0.20
Discoid lupus	22 (14.7)	0 (0)	22 (13.9)	0.24
Photosensitivity	60 (40.0)	2 (25.0)	62 (39.2)	0.39
Mucosal ulcer	42 (28.0)	0 (0)	42 (26.2)	0.08
Arthritis	74 (49.3)	3 (37.5)	77 (48.7)	0.51
Serositis	30 (20.0)	0 (0)	30 (18.9)	0.16
Glomerulonephritis	56 (37.3)	4 (50.0)	60 (37.9)	0.47
Neuropsychiatric	18 (12.0)	0 (0)	18 (11.4)	0.29
Hematological	76 (50.7)	2 (25.0)	78 (49.4)	0.16
Immunology	121 (80.7)	6 (75.0)	127 (80.4)	0.69
ANA	144 (96.0)	7 (87.5)	151 (95.6)	0.25

Table 4. Relationship between the TLR9 (rs187084) genotype and clinical signs and findings in patients with SLE

	CC n=5 (%)	C/T n=38 (%)	TT n=117 (%)	Total n=160 (%)	***P***
Malar rash	1 (20.0)	16 (42.1)	60(51.3)	77 (48.1)	0.27
Discoid lupus	0 (0)	3 (7.8)	21 (17.9)	24 (15.0)	0.20
Photosensitivity	1 (20.0)	10 (26.3)	53 (45.3)	64 (40.0)	0.07
Mucosal ulcer	0 (0)	8 (21.1)	36 (30.8)	44 (27.5)	0.19
Arthritis	4 (80.0)	26 (68.4)	70 (59.8)	100 (62.5)	0.45
Serositis	0 (0)	12 (31.6)	29 (24.8)	41 (25.6)	0.29
Glomerulonephritis	2 (40.0)	18 (47.4)	44 (37.6)	64 (40.0)	0.56
Neuropsychiatric	0 (0)	1 (2.6)	11 (9.4)	12 (7.50)	0.31
Hematological	4 (80.0)	20 (52.6)	58 (49.8)	82 (51.3)	0.40
Immunology	5 (100)	30(78.9)	98 (83.8)	133 (82.1)	0.47
ANA	5 (100)	35 (92.1)	108 (92.3)	148 (92.5)	0.81