Title: Lack of association of Toll-like receptor 9 gene polymorphism with Behçet's disease in Japanese patients

Short title:*TLR9* gene polymorphism in Japanese patients with Behçet's disease

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Abstract: Toll-like receptors (TLR) play an important role in the induction of defense mechanisms of the innate and adaptive immune responses to microbial pathogens.   Genetic polymorphisms within the *TLR9* gene have been reported to be associated with a variety of inflammatory and infectious diseases. Behçet's disease (BD) is a chronic inflammatory disease, and the etiology of BD has yet to be fully elucidated. We investigated the potential association of the *TLR9* gene with susceptibility to BD by analyzing the frequency of nine single nucleotide polymorphisms (SNPs) in a population of 200 Japanese BD patients and 102 randomized controls. Our results demonstrated that SNPs in the *TLR9* gene were not significantly associated with susceptibility to BD.

Behçet's disease (BD) is a chronic systemic inflammatory disorder characterized by four major symptoms: recurrent uveortinitis, oral aphthosis, genital ulcers, and skin lesions. BD is occasionally associated with inflammation in tissues and organs throughout the body, including the vascular system, gastrointestinal tract, central nervous system, lungs, kidneys, joints and epididymis (1). The disease generally arises in young adults, although childhood-onset has also been reported (2), and usually runs a course of unpredictable exacerbations and remissions that gradually abate with time. BD patients have been diagnosed world-wide, though BD is found most commonly in Mongoloids and rarely in Caucasians, with a particularly high prevalence in countries along the ancient Silk Route from Japan to the Middle East and the Mediterranean basin (3).

While the immunopathogenic mechanisms of BD remain uncertain, various genetic and environmental factors, immune mechanisms, and infectious agents are implicated in being involved in the onset of BD. Our group, as well as others, has presented evidence for an association between BD and human leukocyte antigen (HLA) class I antigen, HLA-B51 (3). In addition, we suggested that the HLA-B*51 allele was a potential candidate to indicate susceptibility of developing BD (4). In addition, the streptococcal antigens (5), bacterial superantigens (6), and mycobacterial antigens (7) and Type 1 herpes virus (8) have been considered to function as contributory factors towards the development of BD.

Toll-like receptors (TLRs), a family of evolutionarily conserved pathogen recognition receptors, play a pivotal role in innate recognition of foreign material. TLR activation leads to induction of both the innate and adaptive immune responses directed against invading pathogens (9-11). Notably, genetic variations in genes involved in innate immunity are associated with a range of inflammatory disorders (12).

TLR9 is expressed in a wide variety of human cells but mainly in plasmacytoid dendritic cells (pDC) and B cells. TLR9 recognizes unmethylated CpG motifs common in bacterial and viral DNA but rarely found in mammalian DNA (13). Activation of TLR9 in pDC induces Th1 cytokines such as interferon-α or interleukin-12 (IL-12) and Th1-biased immune response (14). The *TLR9* gene,

located on chromosome 3p21.3, spans approximately 5kb and contains two exons with the second being the major coding exon (15). Various studies have reported an association between *TLR9* polymorphisms and diseases such as asthma (16), Crohn disease (17), and systemic lupus erythematosus (SLE) (18), but no genetic study on the relationship between *TLR 9* polymorphisms and BD has yet been reported. To evaluate the potential candidacy of *TLR9* as a BD susceptibility gene, we investigated the association of *TLR9* single nucleotide polymorphisms (SNPs) and BD using case control analysis.

The total subject group consisted of 200 Japanese patients diagnosed with BD and 102 healthy Japanese controls. The BD patients were diagnosed according to the standard criteria proposed by the Japan Behçet's Disease Research Committee at the Uveitis Clinic of Yokohama City University or Hokkaido University, and classified as complete-type BD or incomplete-type BD, according to these criteria (19). All the control subjects were healthy volunteers, and similar in ethnic origin to the patients; control subjects were unrelated to each other and to the BD patients. The research methods were in compliance with the Declaration of Helsinki guidelines. Details of the study were explained to all patients and controls, and consent to genetic screening was obtained. Peripheral blood lymphocytes were collected, and genomic DNA was extracted from peripheral blood cells using the QIAamp DNA Blood Maxi Kit (QIAGEN).

Nine SNPs (rs187084, rs5743836, rs5743841, rs352139, rs5743842, rs5743843, rs352140, rs5743845, and rs5743846; named SNP1 - SNP9) within the TLR9 gene were genotyped by the TaqMan 5' exonuclease assay using primers supplied by ABI. Probe fluorescence signal was detected by TaqMan Assay for Real-Time PCR (7500 Real Time PCR System, Applied Biosystems) following the manufacturer's instructions.

Allelic frequencies of all detected SNPs were tested for Hardy-Weinberg equilibrium (HWE). Differences of genotype frequency between case and control were assessed by χ^2 test and Fisher's exact test. The maximum likelihood estimates of haplotype frequencies were estimated by pairs of unphased genotypes using the expectation-maximization (EM) algorithms in the R package 'haplo.stats' (20). Statistical analyses were performed using the Statview

software (ver.5.0, SAS Institute Inc., USA). The correction of P values (Pc) was calculated by the Bonferroni᾿s correction where the coefficient was the total number of the contingency tables tested. P value < 0.05 and Pc value < 0.1 were considered as statistically significant. The strength of linkage disequilibrium (LD) between SNPs was measured with LD coefficient (Lewontin᾿s D') (21), obtained from the R package 'genetics' in the R Project for Statistical Computing (http://www.r-project.org/).

Nine SNPs in TLR9 were genotyped, four of which (SNP1, SNP4, SNP5, SNP7) were monopolymorphic, while six (SNP3. SNP5, SNP6, SNP7, SNP8, and SNP9) are in the coding exon and SNP4 is in the intron. Allelic frequencies of 9 SNPs in cases and controls are listed in Table 1. No statistically significant association (Fisher᾿s exact test, P<0.05) was observed for any of the SNPs (Table 1). The subjects used in this study were justified by the Hardy-Weinberg᾿s exact test and no genetic bias was observed for each SNP.

We also evaluated the LD indexes for the specific LD block using 5 SNPs in *TLR9* in both control and patient subjects (Table 2). Pairwise LD mapping confirmed that all five of these alleles have a comparatively strong LD index of >0.8 for D' and >0.4 for r^2. The haplotype frequencies in BD patients were similar to those observed in controls, with no detected significant difference (Table 3).

Although the etiology of BD is still uncertain, herpes simplex virus immunopathology, autoimmunity to oral mucosa or cross-reactive microbial antigens, and streptococcal infection seem to be potential candidates in inducing BD. TLR9 plays a pivotal role in the induction of first-line defense mechanisms of the innate immune system and triggers effective adaptive immune responses to different bacterial and viral pathogens. Furthermore, it has been speculated that polymorphisms in the TLR9 gene might influence the functional capability of TLR9 to elicit effective defense mechanism against microbial pathogens, rendering a high susceptibility to microbial infections (16, 22-24). Despite the predicted candidacy of TLR9 gene as a susceptibility marker for BD, our study clearly demonstrates that no relationship was found.

In conclusion, our study in a group of Japanese demonstrated that TLR9 gene polymorphisms were not significantly associated with the susceptibility to BD.

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Table 1 Association of 9 SNPs of *TLR9* gene with Behçeť disease

Name	SNP rs	Function	AA change	Allele	Case (%) n=200	Control (%) n=102	χ^2	P
SNP1	rs187084	5'Untranslated		A	141(70.5)	75(73.5)	0.304	NS
				G	151(75.5)	73(71.6)	0.545	
SNP2	rs5743836	5'Untranslated		T	200(100)	102 (100)		NS
				C	0(0)	3(2.9)		
SNP3	rs5743841	Exon1	synonymous	C	200(100)	102(100)		NS
				T	0(0)	0(0)		
SNP4	rs352139	Intron1		T	141(70.5)	76(74.5)	0.537	NS
				C	150(75.0)	72(70.6)	0.675	
SNP5	rs5743842	Exon2	Arg to Cys	C	200(100)	102(100)		NS
				T	0(0)	0(0)		
SNP6	rs5743843	Exon2	Gln to His	C	200(100)	102(100)		NS
				A	0(0)	0(0)		
SNP7	rs352140	Exon2	synonymous	T	150(75)	71(69.6)	1.001	NS
				C	109(54.5)	55(53.9)	0.009	
SNP8	rs5743845	Exon2	Arg to Gln	C	200(100)	102(100)		NS
				T	1(0.5)	3(2.9)		
SNP9	rs5743846	Exon2	Ala to Thr	C	200(100)	102(100)		NS
				T	0	0		

SNPrs: public reference SNP number from the dbSNP database; numbers in parentheses indicate the percentage

AA change: amino acid change

NS: not significant by χ^2 test 2x2 contingency table (df=1)

Table 2 Pairwise linkage disequilibrium coefficients (D' and r^2) between SNPs on *TLR9* gene

D`

		SNP	1 rs187084	2 rs5743836	4 rs352139	7 rs352140	8 rs5743845
	control	1		0.990	0.976	1.000	0.326
	patient	rs187084		-	0.990	0.990	0.965
	control	2	0.015		0.987	0.986	0.994
	patient	rs5743836	-		-	-	-
r^2	control	4	0.606	0.010		1.000	0.932
	patient	rs352139	0.970	-		1.000	0.965
	control	7	0.574	0.008	0.364		0.999
	patient	rs352140	0.970	-	1.000		0.965
	control	8	0.019	0.085	0.100	0.100	
	patient	rs5743845	0.003	-	0.003	0.003	

The degree of LD is shown as the LD index of Lewontin correlation (D') in the upper right triangle and Pearson correlation (r^2) in the lower left triangle.

Table 3 Estimated haplotype frequencies of the *TLR9* gene between controls and patients with Behçet's disease

SNP rs	1 187084	2 5743836	3 5743841	4 352139	5 5743842	6 5743843	7 352140	8 5743845	9 5743846	Frequency Control	BD
Hp1	A	T	C	T	C	C	C	C	C	0.5098	0.4700
Hp2	G	T	C	C	C	C	T	C	C	0.4657	0.5200
Hp3	G	C	C	C	C	C	C	T	C	0.0147	0.0000
Hp4	G	T	C	T	C	C	C	C	C	0.0098	0.0050
Hp5	A	T	C	C	C	C	T	C	C	0.0000	0.0025
Hp6	A	T	C	C	C	C	C	T	C	0.0000	0.0025

BD: Behçet's disease