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ASSOCIATION OF THE TOLL-LIKE RECEPTOR 9 GENE POLYMORPHISMS WITH BEHCET'S DISEASE IN A JAPANESE POPULATION

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Abstract : Bacterial infection (i.e., *Streptococcus sanguinis*) has been suggested to be related to pathogenesis and/or symptom of Behcet's disease (BD). Toll-like receptor 9 (TLR9) plays an important role in both the innate and adaptive immune systems by recognizing a component of bacterial DNA (i.e., CpG-DNA). Previous studies have demonstrated that single nucleotide polymorphisms (SNPs) in *TLR9* were associated with infectious and autoimmune/autoinflammatory diseases. In this study, we detected five SNPs with BD patients in a Japanese population. Allele frequency analysis of the three common SNPs (-1486 : T/C (promoter region), 1174 : A/G (intron 1), 2848 : G/A (exon 2 ; Pro545Pro)) showed no statistically significant difference between the BD patients and the healthy controls. However, genotyping analysis revealed that the homozygous genotypes -1486CC and 1174GG were significantly more frequent in the BD patients compared to the healthy controls (P=0.048 and P=0.027, respectively). The homozygous diplotype distribution C-G-A/C-G-A was significantly more frequent in the BD patients compared to the healthy controls (P=0.041). For reporter gene assay, the plasmid construct carrying diplotype distribution C-G/C-G of the -1486T/C and 1174A/G SNPs showed significantly higher luciferase activity compared to the plasmid construct carrying diplotype distribution T-A/T-A (P=0.019). These results suggested an association of the homozygous genotypes and homozygous diplotype configuration of the *TLR9* SNPs with susceptibility to BD in the Japanese population.

Key words : Toll-like receptor 9, Behcet's disease, Single nucleotide polymorphism

INTRODUCTION

Behcet's disease (BD) is a multisystem inflammatory disorder characterized with recurrent attacks of oral aphthous ulcers, genital ulcers, uveitis, and skin lesions. Although not included in the diagnostic criteria for BD[1)], there are some other features seen in patients with BD, such as thrombosis, gastrointestinal ulcerations and neurological involvement. BD is prevalent in a region that extends from the Mediterranean basin to Japan, overlapping with the ancient Silk-Road[2)].

The etiology of BD is unknown, however, it is generally considered as a multifactorial disease with important genetic and environmental components ; HLA-B51 positive, viral and/or bacterial infections, etc[3)]. Moreover, a relationship between bacterial infection (i.e., *Streptococcus sanguinis* ; previously called *Streptococcus sanguis*) and the pathogenesis of BD has been suggested as bacterial components and antibodies are frequently found in the oral flora and serum of patients with BD, respectively[4)].

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Toll-like receptors (TLRs) are a family of pattern recognition receptors that recognize distinct molecular patterns shared by a broad range of pathogens, including nucleic acids (i.e., CpG-DNA and single-stranded/double-stranded RNA)[5,6]. The arrays of 5´-Cytosine-Guanine-3´ in a DNA sequence, called CpG motifs, are commonly present in both bacterial and vertebrate DNAs. CpG motifs in bacterial DNA are unmethylated and induce a strong T helper 1 (Th1)-type immune response to antigens in mammals via TLR9 which is mostly expressed on plasma and surface membrane of B cells, dendritic cells and macrophages (plasma membrane >>> surface membrane)[5,7-9]. In contrast, most of CpG motifs in vertebrate DNA are methylated and immunologically inert[10].

To date, various studies have reported a significant association between *TLR9* gene polymorphisms and diseases such as bronchial asthma[11], Crohn's disease[12], cerebral malaria[13] and pulmonary aspergillosis[14]. In this study, we performed a systemic search for single nucleotide polymorphisms (SNPs) of the *TLR9* gene to assess whether the genetic components of TLR9 could be associated with the pathogenesis of BD.

MATERIALS AND METHODS

Subject

After giving informed consent, 39 BD patients were enrolled in this examination. They fulfilled the 1990 diagnostic criteria for BD[1]. The mean age of the patients was 44.4 years old (range 19-78 years old), and they were comprised of 17 men and 22 women. Of these BD patients, 14 (35.9%) were with complete-type and 25 (64.1%) were with incomplete-type. As a disease control group, we included 36 rheumatoid arthritis (RA) patients (9 men and 27 women, mean age 52.0 years old) who fulfilled the 1987 classification criteria for RA in our examination[15]. Because, similar to BD, there is an association between the pathogenesis of RA and Th1 cells, although a recent study showed a significant association of Th17 cells with development of the disease[16]. All patients were Japanese, and visited the collagen disease clinic of Fukushima Medical University Hospital, Fukushima, Japan between 2003 and 2007. The control subjects consisted of 43 healthy Japanese volunteers (22 men and 21 women, mean age 32.1 years old). Informed consent was obtained from the patients and healthy volunteers. This study was approved by the Ethics Committee of Fukushima Medical University.

DNA isolation

Genomic DNA was extracted from whole blood samples using PUREGENE® DNA purification kit (Gentra Systems, Minneapolis, MN, USA).

SNP discovery

All exons, introns, and approximately 1,500 bases of the 5´ flanking region and 1,500 bases of the 3´ flanking region of the *TLR9* gene were amplified from genomic DNA of eight BD patients (Fig. 1a). The genomic sequences were based on the GenBank reference sequence NM_017442 and AC097637.2 for *TLR9*. BD patients for the mutation study were selected at random. Nine *TLR9* gene segments were amplified by polymerase chain reaction (PCR) by using the primer pairs (Table 1A), *Taq* DNA polymerase (QIAGEN GmbH, Hilden, Germany), and genomic DNA from the BD patients as templates. Initial denaturation for PCR was carried out at 94°C for 3 min. Cycling conditions were : primer annealing at 55°C or 58°C for 60 s, polymerization at 72°C for 60 s and strand separation at 94°C for 30 s for thirty-five cycles. A final polymerization step at 72°C for 10 min was carried out to complete elongation processes. Amplified products were purified using the GFX™ PCR DNA or Gel Band Purification Kit (Amersham Biosciences UK Limited, Buckinghamshire, UK), and were subjected to direct sequencing with the GenomeLab™ DTCS-Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) on a CEQ2000 DNA Sequencer (Beckman Coulter).

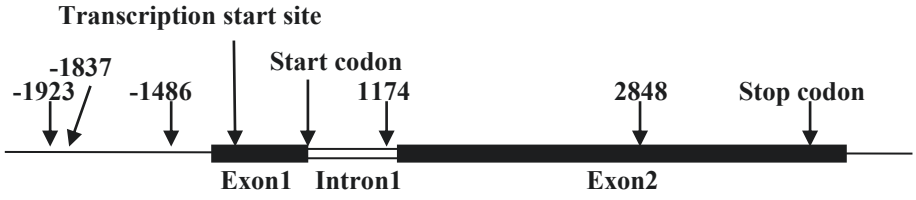

Fig. 1. Gene structure of *TLR9* and location of identified SNPs.

Table 1.

A. Primer sequences used for screening of the whole *TLR9* gene

Fragment	Position	Forward	Reverse	bp
TLR9-1	-2109 to -1293	5´-CCAAGGGACTCTGGGAAAG-3´	5´-CATGTCACCCTCTCAACAGGG-3´	817
TLR9-2	-1380 to -376	5´-ATAGACCAGGCAAAGGAGCTC-3´	5´-TAGCACCAGTAGCGGGTACA-3´	1005
TLR9-3	-460 to 434	5´-TGTGGTGCAGGAGCCAAG-3´	5´-GCAGTTTGGGAAGCCGAAGT-3´	894
TLR9-4	351 to 1061	5´-AGAGCCCAGGGTTTCTCCATG-3´	5´-GCTTGTGGGGAACCTGAAC-3´	711
TLR9-5	976 to 1899	5´-CTGGATTCTAGGTCTCAGTCC-3´	5´-AGGACAACAGCAGATACTCC-3´	924
TLR9-6	1812 to 2520	5´-AACCTCACCCACCTGTCAC-3´	5´-TGGCTGTCAGCTCCGAA-3´	709
TLR9-7	2407 to 3350	5´-TGCAGATGAACTTCATCAACC-3´	5´-GCTGTTGCAGCTGACATC-3´	944
TLR9-8	3267 to 4057	5´-CAGGAAACCAGCTGAAGG-3´	5´-CGCAAGAGACCACTGAC-3´	791
TLR9-9	3864 to 4827	5´-GCAGACTGGGTGTACAACGA-3´	5´-GAACAGTCCCCATAGCTGCTG-3´	1309

B. Primer sequences used for SNP genotyping

SNP position†	Forward	Reverse	bp
-1486 (rs187084)	5´-CAGCCTTCACTCAGAAATACCC-3´	5´-GGCCAACAAGGCCCTATG-3´	532
1174 (rs352139)	5´-CTGGTTCTGAAGCCTAATTC-3´	5´-AGCATCAGGATGTTGGTATGG-3´	692
2848 (rs352140)	5´-TGCAGATGAACTTCATCAACC-3´	5´-GCTGTTGCAGCTGACATC-3´	944

C. Primer sequences used for making plasmids of reporter assay‡

Fragment	Forward	Reverse	bp
I	5´-GCTCTAGACAGCCTTCACTCAGAAATACCC-3´	5´-CCCAAGCTTGCCAGGGTGTAGCTTGA-3´	1074
II	5´-GCTCTAGACATGGGAGCAGAGACATAATG-3´	5´-CCCAAGCTTCTGGAGCTCACAGGGTAGGAA-3´	2624
III	5´-GCTCTAGACAGCCTTCACTCAGAAATACCC-3´	5´-CCCAAGCTTCTGGAGCTCACAGGGTAGGAA-3´	2954

†Positions were calculated taking the A of the *TLR9* ATG start codon as position 1 based on Genbank Accession No. NM_017442.

‡"TCTAGA" and "AAGCTT" indicate the restriction sites of XbaI and HindIII, respectively.

Genotyping of TLR9 SNPs

Three common SNPs (-1486T/C (promoter region ; rs187084), 1174A/G (intron 1 ; rs352139), 2848G/A (exon 2 : *P545P* ; rs352140) ; frequency higher than 10%) were analyzed for genotyping. The -1486T/C SNP was genotyped by reaction fragment length polymorphism (RFLP). Briefly, genomic DNA fragments containing -1486T/C SNP were amplified by PCR. The PCR conditions were the same as described in the SNP discovery section. The PCR products were then digested with the restriction enzyme BfrI (TOYOBO, Osaka, Japan), run on a 3% agarose gel and subsequently stained with ethidium bromide to visualize the bands. Digested bands were denoted as -1486T and undigested bands were denoted as -1486C. Genotyping of the other two SNPs (1174A/G, 2848G/A) were performed by direct sequencing method. Primers used for PCR or sequencing are shown in Table 1B. Then, we inferred three-SNP haplotypes and estimated the distribution of diplotype configuration (diplotype distribution) for the three-SNP haplotypes by using the EM algorithm.

Construction of luciferase reporter plasmids

We designed plasmid constructs for use in luciferase reporter gene assays. The plasmid constructs contained genomic DNA corresponding to the *TLR9* promoter, exon 1, intron 1 and part of exon 2. The genomic DNA were amplified by PCR using gene-specific primers containing either a XbaI (forward primer) or a HindIII (reverse primer) restriction site at the 5´ end of the primers (Table 1C). These fragments were then cloned into the XbaI-HindIII site of the pGL3-Basic vector (Promega, Madison, WI, USA) (Fig. 1b). Fragment I corresponds to the 5´-flanking region of the TLR9 gene (from -1626 to -553) carrying either SNP -1486T or -1486C. Fragment II corresponds to the 5´-flanking region, exon 1, intron1, and a part of exon 2 (from -1296 to 1328) carrying either SNP

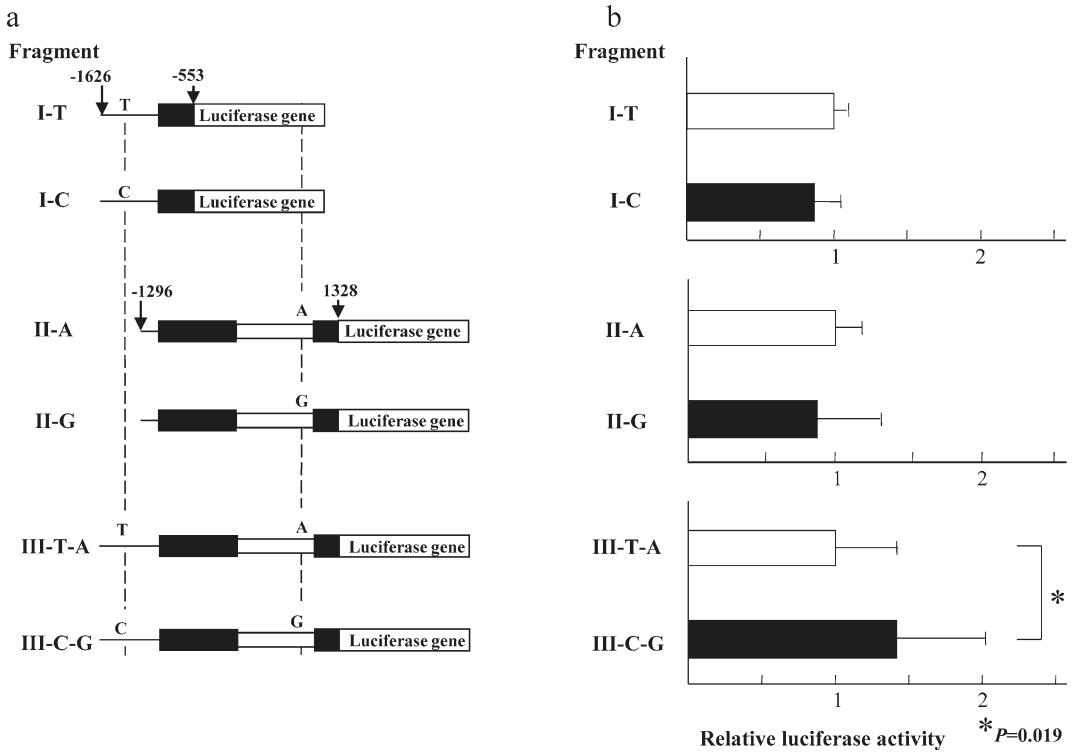

Fig. 2. a) Luciferase reporter gene plasmid constructs carrying *TLR9* SNP(s) -1486T, -1486C, 1174A, 1174G, -1486T/1174A (haplotype T-A) or -1486C/1174G (haplotype C-G). *TLR9* segments were inserted upstream from the firefly luciferase gene.
b) Transcriptional activity of the *TLR9* segments-luciferase fusion genes. Each expression vector (20 μg) was co-transfected with phRL-TK (0.4 μg) into Raji cells by electroporation. Firefly luciferase activity was assayed 48 h after transfection and normalized to *Renilla* luciferase activity. Results represent relative luciferase activity when compared to the plasmid constructs carrying -1486T, 1174A or -1486T/1174A (white bars=1 ; data shown represent the mean±SD for three independent experiments in each comparison).

1174A or 1174G. Fragment III corresponds to full length of Fragments I+II (from -1626 to 1328) carrying either SNPs -1486T/1174A (haplotype T-A) or -1486C/1174G (haplotype C-G). The source of this genomic DNA was from BD patients carrying haplotype T-A and/or C-G. Expand High Fidelity PCR System (Roche Diagnostic GmbH, Penzberg, Germany) was used for PCR amplification. All plasmid constructs were sequenced to ensure that the fragments had a correct nucleotide sequence.

Cell culture and luciferase assay

Transfect host cells (Raji cells (ATCC CCL 86)) for luciferase assays were maintained at 37°C in a humidified 5% CO_2 incubator and in RPMI 1640 medium (Sigma-Aldrich) with 10% FCS (Sigma-Aldrich). The cells (7.0×10^6 cells in 0.7 ml) were co-transfected with 20 μg of the test construct and 0.4 μg of phRL-TK (Promega) by electroporation with a Gene Pulser II (Bio-Rad, Hercules, CA, USA) at 260 V and 975 μF. After transfection, the cells were incubated in 20 ml of RPMI 1640 with 10% FCS for 48 hours. The cells were then washed and lysed in 250 μl of 1×Passive Lysis Buffer (Promega), and luciferase activity in 20 μl of cell lysates was measured using the Dual Luciferase Reporter Assay System, according to manufacturer's protocols. Firefly luciferase activity of individual cell lysates was normalized against *Renilla* luciferase activity.

Statistical analysis

Statistical analyses were performed using StatView version 5 for windows (SAS Institute Inc., Cary, NC) and SNPAlyze version 4.1 (Dynacom Co. Ltd., Yokohama, Japan). To compare cases and controls, unpaired two-tailed Student's *t* test (Fig. 1c) was used for continuous items and the Fisher's exact probability test (Table 3, 5) for categorical items. A value of $P<0.05$ was considered significant. We examined Lewontin's D´ (D') and the linkage disequilibrium coefficient r^2 between each pair of SNPs using Expectation-Maximization (EM) algorithm in SNPAlyze software.

Table 2. Allele frequencies of *TLR9* SNPs in healthy controls, BD and RA patients

Position[†]	Allele	Healthy controls *N = 43*	BD patients *N = 39*	RA patients *N = 36*	dbSNP[‡]	JSNP[§]
-1923	C/A	0.965 / 0.035	0.974 / 0.026	n.d. [¶]	0.963 / 0.037	n.d. [¶]
-1837	C/T	0.942 / 0.058	0.923 / 0.077	n.d. [¶]	0.991 / 0.009	n.d. [¶]
-1486*	T/C	0.477 / 0.523	0.434 / 0.564	0.431 / 0.569	0.603 / 0.397	n.d. [¶]
1174*	A/G	0.488 / 0.512	0.434 / 0.564	0.431 / 0.569	0.464 / 0.536	0.517 / 0.483
2848*	G/A	0.523 / 0.477	0.434 / 0.564	0.431 / 0.569	0.542 / 0.458	0.517 / 0.483

[†]Positions were calculated taking the A of the *TLR9* ATG start codon as position 1 based on Genbank Accession No. NM_017442.
[‡]dbSNP : http://www.ncbl.nlm.nih.gov/SNP.
[§]JSNP : a database of common gene variations in the Japanese population[23,24].
http://snp.ims.u-tokyo.ac.jp/index.html.
[¶]n.d. indicates not determined.
*SNPs were in Hardy-Weinberg equilibrium (by permutation test, all $p > 0.1$).

RESULTS

SNPs discovery and their allele frequencies

We sequenced exons, introns, and approximately 1,500 bases of the regions 5´ and 3´ of TLR9 gene in eight BD patients. We identified a total of five SNPs in the regions sequenced ; three common SNPs (-1486T/C (promoter region ; rs187084), 1174A/G (intron 1 ; rs352139), 2848G/A (exon 2 : *P545P* ; rs352140), that were characterized as being at least 10% polymorphic in Japanese populations, and two rare SNPs -1923C/A and -1837C/T that were located at promoter region (Table 2 and Fig. 1a). Previous studies in European population showed that -1237C allele in *TLR9* gene (rs5743836) was associated with bronchial asthma[11], pulmonary aspergillosis[14] and atopic eczema[17]. However, no SNP at -1237 was found in the eight BD patients in our study. This result was consistent with reports indicating that the -1237C allele was rare (<2%) in Japanese people[18,19]. As shown in Table 2, allele frequencies of the three common SNPs were in Hardy-Weinberg equilibrium (by permutation test, all $p > 0.1$). The other two rare polymorphisms (-1923C/A and -1837C/T) were not in Hardy-Weinberg equilibrium (Table 2). The allele frequencies of the three common SNPs were identical in both BD and RA patients. In contrast, they varied in healthy controls. However, there was no statistically significant difference of allele frequencies between all five identified SNPs among BD, RA and healthy controls.

TLR9 genotypes in BD patients, RA patients and healthy controls

We then focused on three common SNPs (-1486T/C, 1174A/G, and 2848G/A ; frequency higher than 10%), and analyzed their genotype distributions among BD, RA and healthy controls. For -1486 and 1174 SNPs, the homozygous genotypes -1486CC and 1174GG were significantly more frequent in the BD patients compared to the healthy controls (Table 3). The homozygous genotype 2848AA was also more frequent in the BD patients compared to the healthy controls, however it did not reach statistical significance. Overall, the BD patients had frequent homozygous genotype distributions in the three common SNPs (-1486CC, 1174GG, 2848AA), while the healthy controls had frequent heterozygous genotype distributions (-1486TC, 1174AG, 2848GA). Regarding the RA patients, the genotype distributions in the three common SNPs were similar to the BD patients (i.e. frequent heterozygous genotype distributions). However, there were no statistically significant differences between the RA patients and the healthy controls (Table 3).

There is a strong linkage disequilibrium among the three common SNPs

We observed identical allele frequencies for the three common SNPs in both BD and RA patients (Table 2). Thus, it is suggested that there is a strong linkage disequilibrium (LD) between the three common SNPs in the BD patients, the RA patients and the healthy controls (Table 4). As shown in Table 5, a total of 4 of the 8 possible three-SNP haplotypes were estimated in healthy con-

Table 3. Genotype distributions of *TLR9* SNPs

Polymorphisms	Genotype	Healthy controls $N=43$	BD patients $N=39$	RA patients $N=36$
-1486T/C	TT	7 (16.3%)	10 (25.6%)	8 (22.2%)
	TC	27 (62.8%)	14 (35.9%)	15 (41.7%)
	CC	9 (20.9%)	15 (38.5%)	13 (36.1%)
	P value		$P=0.048$	$P=0.164$
1174A/G	AA	7 (16.3%)	10 (25.6%)	8 (22.2%)
	AG	28 (65.1%)	14 (35.9%)	15 (41.7%)
	GG	8 (18.6%)	15 (38.5%)	13 (36.1%)
	P value		$P=0.027$	$P=0.101$
2848G/A	GG	10 (23.4%)	10 (25.6%)	8 (22.2%)
	GA	25 (58.1%)	14 (35.9%)	15 (41.7%)
	AA	8 (18.6%)	15 (38.5%)	13 (36.1%)
	P value		$P=0.086$	$P=0.203$

P value by Fisher's Exact test ; Healthy controls vs. BD or RA patients.

Table 4. Linkage disequilibrium analysis among *TLR9* SNPs in healthy controls, BD and RA patients

Polymorphisms		Healthy controls *N=43*		BD patients *N=39*		RA patients *N=36*	
		D'	r^2	D'	r^2	D'	r^2
-1484T/C	1174A/G	1	0.872	1	1	1	1
-1486T/C	2848G/A	1	0.834	1	1	1	1
1174A/G	2848G/A	1	0.956	1	1	1	1

Lewontin's D´ (D') and the linkage disequilibrium coefficient r^2 between each pair of SNPs were examined by EM algorithm in SNPAlyze software.

trols. Interestingly, only two three-SNP haplotypes, T-A-G and C-G-A, were estimated in the BD and RA patients, indicating that these SNPs were in strong LD in those patient groups (Table 4). The usage of haplotype C-G-A was more frequent in both BD (56.4%) and RA (56.9%) patients compared to the healthy controls (47.7%), however the difference did not reach statistical significance between the groups (Table 5) (BD vs. healthy controls ; $P=0.238$, RA vs. healthy controls ; $P=0.267$).

A total of five possible diplotype configurations were estimated in the healthy controls (Table 5). In contrast, only three possible diplotype configurations were estimated in the BD and RA patients because of the strong LD among the three common SNPs. Notably, the homozygous diplotype C-G-A/C-G-A was significantly more frequent in patients with BD than in the healthy controls ($P=0.041$). The diplotype distribution in the RA patients was similar to the BD patients, however there was no statistically significant difference between the RA patients and healthy controls ($P=0.135$) (Table 5).

We also evaluated the distribution of *TLR9* gene polymorphisms in the subgroups of BD patients with or without HLA-B51, a BD-associated HLA-B allele, however there was no statistically significant difference between the two groups (data not shown).

Luciferase assay

We performed a luciferase assay to determine the effects of *TLR9* SNPs (-1486T/C and 1174A/G) on transcriptional activity. Figure 2 a shows the six luciferase reporter gene plasmid constructs carrying SNP(s) -1486T, -1486C, 1174A, 1174G, -1486T/1174A (haplotype T-A) or -1486C/1174G (haplotype C-G). As shown in Figure 2 b, there was no difference in luciferase activity between the plasmid constructs carrying a single polymorphism (i.e., Fragment I ; -1486 T vs. -1486 C, Fragment II ; 1174 A vs. 1174 G). Interestingly, the Fragment III-C-G construct carrying SNPs -1486C and 1174G showed higher luciferase activity compared to the Fragment III-T-A construct carrying SNPs -1486T and 1174A ($P=0.019$). Of note, the

Table 5. Haplotype/diplotype distributions of *TLR9* SNPs

Haplotype/diplotype†	Healthy controls	BD patients	RA patients
Haplotype	2*N*=86	2*N*=78	2*N*=72
T-A-G	41 (47.7%)	34 (43.6%)	31 (43.1%)
C-G-A	41 (47.7%)	44 (56.4%)	41 (56.9%)
C-A-G	3 (3.5%)	0	0
C-G-G	1 (1.1%)	0	0
		P=0.238	*P*=0.267
Diplotype	*N*=43	*N*=39	*N*=36
T-A-G/T-A-G	7 (16.3%)	10 (25.6%)	8 (22.2%)
T-A-G/C-G-A	24 (54.4%)	14 (35.9%)	15 (41.7%)
C-G-A/C-G-A	8 (18.6%)	15 (38.5%)	13 (36.1%)
T-A-G/C-A-G	3 (6.1%)	0	0
C-G-A/C-G-G	4 (6.1%)	0	0
		P=0.041	*P*=0.135

P value by Fisher's Exact test ; Healthy controls vs. BD or RA patients.
†The allele order is -1486T/C, 1174A/G, and 2848G/A SNPs of *TLR9*.

Fragment III-C-G construct is a part of the common diplotype configuration C-G-A/C-G-A in the BD patients (Table 5).

DISCUSSION

In this study, we detected significant association of *TLR9* SNPs with susceptibility to BD in a Japanese population. Although the allele frequencies of the three common SNPs showed no statistically significant differences between the BD patients and healthy controls, the BD patients had significantly more frequent homozygous genotypes -1486CC and 1174GG, compared to the healthy controls. Furthermore, diplotype analysis showed that the BD patients had significantly more frequent homozygous diplotype C-G-A/C-G-A, the part of which consisted of the homozygous genotypes -1486CC and 1174GG, compared to the healthy controls.

The pathogenesis of BD is largely unknown, however, Th1-dominant immune response has been suggested to be the major inflammatory process in BD. IFN-γ is a key cytokine of Th1 immune response and increased levels of serum Th1-type cytokines including IFN-γ and increased numbers of Th1 cells have been reported in BD patients[20]. Recently, Sam-Agudu *et al.* reported significant associations of *TLR9* gene polymorphisms with serum IFN-γ levels in children with cerebral malaria[13]. In their report, patients carrying the -1237C or 1174G alleles had higher levels of IFN-γ than those without these alleles. In our study, no polymorphism was detected at -1237 of *TLR9* in the BD patients, likely due to ethnic diversity of gene polymorphisms, because the -1237C allele is a rare SNP in Japanese populations[18,19]. They also showed that patients carrying the homozygous genotype 1174GG had significantly increased serum IFN-γ levels compared to patients carrying 1174AA. Of interest, the homozygous genotype 1174GG is one of the genotypes that showed significant association with BD in our study. Kim *et al.* reported positive correlations between TLR expression levels and cytokine expression levels in patients with dermatomyositis and polymyositis[21]. In this report, significant positive correlations between TLR9 expression and inflammatory cytokines including IFN-γ were shown. These previous reports and our results suggest that *TLR9* gene polymorphisms, which show significant association with BD, affect *TLR9* gene expression levels.

To define whether the *TLR9* gene polymorphisms affect its expression levels, we performed a luciferase assay. It has not been reported any cis-element sequences at the *TLR9* SNPs positions. In our study, we observed that the plasmid constructs carrying the allele 1174G alone showed no difference in luciferase activity compared to other plasmid constructs. However, the plasmid constructs carrying the alleles -1486C and 1174G (haplotype C-G) showed significantly increased luciferase activity compared to the plasmid constructs carrying the alleles -1486T and 1174A (haplotype T-A). These

results suggested that the synergistic effect between -1486G and 1174C. Of note, the usage of diplotype distribution C-G/C-G was more frequent in the BD patients compared to the healthy controls. These results suggested the association of the *TLR9* gene polymorphisms with its expression levels.

In our study, allele and haplotype frequencies of *TLR9* SNPs showed no statistically significant differences between the BD patients and healthy controls, while genotype and diplotype distribution of *TLR9* SNPs showed significant differences between the BD patients and healthy controls. When we study an association between gene polymorphism and disease, associations of genotype or diplotype distributions are more important than those of allele or haplotype distributions, because genotypes are linked to an individual's traits and phenotypes. On the other hand, allele and haplotype frequencies express dissemination patterns of SNPs in a certain population[22], rather than an individual's traits/phenotypes. Previously, Ito *et al.* analyzed and discussed allele frequencies and haplotypes, and reported that there was no association between TLR9 polymorphisms and BD[19]. However, they did not use their analysis result of pairwise LD coefficients in analysis of haplotype frequencies.

We additionally analyzed *TLR9* SNPs in patients with RA as a disease control group. The genotype and the diplotype distributions of the three common SNPs of *TLR9* in the RA patients were similar to the BD patients, however there were no statistical differences between the RA patients and healthy controls.

In conclusion, our data showed significant association of the homozygous genotypes and homozygous diplotype configuration of the *TLR9* SNPs with susceptibility to BD in the Japanese population. The luciferase assay suggested that those *TLR9* SNPs might affect *TLR9* gene expression levels. Additional studies are required to define the mechanisms of inflammatory processes via TLR9 in BD.

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