Association of TLR4 polymorphisms with Behçet’s disease in a Korean population

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Objectives. HLA-B51 is strongly associated with Behçet’s disease (BD) in any ethnic background. We recently reported that another gene, Toll-like receptor-4 (TLR4) is also implicated in BD in a Japanese population. To confirm these results, we investigated polymorphisms in the TLR4 gene in Korean patients with BD.

Methods. In this study, 119 patients with BD and 141 healthy controls were enrolled; every participant was a Korean. Nine single nucleotide polymorphisms previously detected in TLR4 gene in Korean patients with BD.

Results. The most frequent haplotype, TAGCGGTAA, was significantly increased in HLA-B*51-positive BD patients (49.5%), compared with healthy control participants [32.3%; \( P = 0.029 \); odds ratio (OR) = 2.01; 95% CI 1.25–3.23]. This haplotype was also significantly increased in BD patients with arthritis (48.2%; \( P = 0.003 \); OR = 1.96; 95% CI 1.26–3.26). There were no significant differences in the allele and genotype frequencies of patients and controls for each single nucleotide polymorphism.

Conclusions. The haplotype of TLR4 may increase the risk for developing BD and the complication of arthritis in the Korean population.

Key words: Behçet’s disease, Toll-like receptor 4, Korea, Polymorphism.

Introduction

Behçet’s disease (BD) is a refractory, multisystemic inflammatory disorder characterized by oral aphthous ulcers, ocular lesions, skin lesions and genital ulcers [1]. This disease is occasionally associated with inflammation throughout the body including the vascular system and joints [2]. In patients with BD, serum TNF and IFN-\( \gamma \) are significantly elevated [3, 4]. The strong association of HLA-B51 with BD was first described in 1973 and has been confirmed in patients from many ethnic groups [5, 6]. Epidemiologically, BD is scattered throughout the world but a higher prevalence has been found among Asian populations along the Silk Route, stretching into the countries of the Mediterranean region [6]. BD appears to be influenced by many susceptible genes, including endogenous and exogenous elements, because most HLA-B51-positive individuals do not suffer from BD throughout their lives, and \( \approx 50\% \) of the BD patients are negative for HLA-B51 [6, 7]. These observations suggest that there are other susceptible genes in addition to those that have been reported, which include TNF-\( \alpha \), IFN-\( \gamma \), IL-1a, -1\( \beta \), -2, -8, -10 and -12 and CD28 [5, 8, 9].

The Toll-like receptor (TLR)4 gene on chromosome 9q32–33 spans \( \approx 13\) kb and contains three exons encoding a protein consisting of 222-amino acid protein. The TLR proteins are a family of phylogenetically conserved receptors that recognize both self and non-self molecular patterns and play an important role in both the innate and adaptive immune systems [10–12]. Among TLR family members, TLR4 has been the most exhaustively investigated; it has been shown to be a principal receptor for lipopolysaccharide (LPS)-recognition. Single nucleotide polymorphisms (SNPs) in TLR4 have been reported to be associated with endotoxin hyporesponsiveness and Gram-negative infections, and they affect the risk for various inflammatory diseases, such as atherosclerosis, Crohn’s disease, ulcerative colitis, RA and prostate cancer [13–20].

Recently, we reported that TLR4 is significantly associated with BD in Japanese patients [21]. To confirm the reproducibility of this result, and to extend the investigation into the Korean population, we explored the association of TLR4 with BD in Korean patients using the same nine SNPs examined in the Japanese study. The information on linkage disequilibrium (LD) and haplotype structure defined by SNPs in a population is essential for the design of disease association studies. Genetic structure constructed by LD patterns and haplotype structure may be different among populations. Although differences in genetic structure between Korean and Japanese populations remain unclear, recent reports have presented strong genetic affinities [22, 23]. In the context of genetic similarity between Koreans and Japanese, we attempted to analyse disease susceptibility of the TLR4 gene for BD in Korean patients and differences in the haplotype structure in those SNPs examined.

Materials and methods

Participants

We recruited 119 Korean patients with BD from Seoul National University Hospital, Seoul, Korea. All the patients fulfilled the diagnostic criteria of the International Study Group for BD (ISGBD) [24], and the definition of the clinical manifestations followed the criteria by ISGBD [24]. Arthritis was defined as any episode of joint pain not related to direct trauma that was confirmed by a physician as the presence of either swelling or tenderness or pain on motion of the involved joints. The healthy blood donors were randomly recruited as healthy controls. Informed consents were obtained from all of the individuals. This study was approved by the ethical committee of Seoul University Hospital, Seoul, Korea.
were analysed using an ABI3130 sequencer (Applied Biosystems). DNA from the sequencing reactions. The sequencing reactions BigDye XTerminator Purification Kit was used to purify the USA) using either sense or anti-sense primers (Table 1). The Big Dye terminator v3.1 (Applied Biosystems, Foster City, CA, USA), the PCR products were sequenced with reactions (Table 1).

The standardized disequilibrium (D') of unphased genotypes using the expectation–maximization (EM) algorithms and of the Declaration of Helsinki.

Genotypic analysis

Differences in genotype frequency differences between case and control genotypes were assessed by using the χ²-test and Fisher’s exact test. The strength of LD between SNPs was quantified by the standardized disequilibrium (D') [25]. The maximum likelihood estimates of haplotype frequencies were estimated by pairs of unphased genotypes using the expectation-maximization (EM) algorithms and P-values were corrected by using the 1000 permutations test in Haploview software [26]. All P-values were derived from a two-sided test, and those that were <0.05 were considered to be statistically significant.

**TABLE 1.** PCR primers, product sizes and sequence primers for TLR4 polymorphisms

<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product size (bp)</th>
<th>Sequence primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs10759930</td>
<td>ATGCACGTAGCTAGCTTGGATG</td>
<td>ACCCTCTTTATCTCTTGGAC</td>
<td>295</td>
<td>Forward</td>
</tr>
<tr>
<td>rs1927914</td>
<td>GCTTATTAGGCTGTGAGCTTG</td>
<td>CTTGGATACCATCATGCTTG</td>
<td>251</td>
<td>Reverse</td>
</tr>
<tr>
<td>rs1927911</td>
<td>GTATGACGACAAAGATCTAGA</td>
<td>GGAGAAGTAGGCTAGAAGGC</td>
<td>400</td>
<td>Forward</td>
</tr>
<tr>
<td>rs12377632</td>
<td>AATCGATACCATACAGAGG</td>
<td>GCCCTAATTCAGAATCTCCT</td>
<td>473</td>
<td>Forward</td>
</tr>
<tr>
<td>rs2149356</td>
<td>AAGCTGTGATTAGCTTGGGA</td>
<td>TTTTGAGCTTGGAGGCTCTGT</td>
<td>419</td>
<td>Forward</td>
</tr>
<tr>
<td>rs11536889</td>
<td>CCTACTGCAGGTACCA</td>
<td>GCTTTTAGGACAGTGTCTGG</td>
<td>341</td>
<td>Reverse</td>
</tr>
</tbody>
</table>
| rs1554973 | GAGCTTCAAAGACAAAGGATA | TAGCGGTAA, was significantly increased in patients with BD complicated by arthritis when compared with healthy controls (OR = 1.96; 95% CI 1.26, 3.05; P = 0.030) or BD patients without arthritis (OR = 2.01; 95% CI 1.19, 3.40; P = 0.037) (Table 4).

**Discussion**

The TLR4 protein is expressed in a wide variety of human cells, and acts as a receptor in the activation of the innate/adaptive immune system in response to both endogenous and exogenous ligands. This transmembrane signalling receptor is the primary

**Results**

The patient group included 61 males (51.3%) and 58 females (48.7%). Oral aphthous ulcers were observed in all patients. Genital ulcers, skin lesions, ocular lesions, deep vein thrombosis and arthritis were observed in 77.3, 80.7, 37.8, 16.8 and 47.9% of the patients, respectively. We found 39.5% of the patients to be HLA-B*51 positive (Table 2).

The magnitude of LD between any two of the nine SNPs showed an extremely high value among all SNPs, with pair-wise LD valued at D' > 0.68 in controls and > 0.74 in cases (Fig. 1). There was no significant difference between the allele or genotype frequencies of the cases and controls. In our clinically stratified analysis, we investigated the presence of some of the clinical features, such as oral aphthous ulcers, genital ulcers, skin lesions, ocular lesions, deep vein thrombosis and arthritis. None of these clinical findings was significantly associated with the nine SNPs (data not shown).

Table 3 shows haplotype frequencies of the patients with BD and healthy controls. Only possible haplotypes were estimated to have a frequency > 0.05 in both case and control groups using an EM algorithm. The patients with BD were divided into two groups, those with or those without HLA-B*51 and arthritis. The healthy controls were also divided into with or without HLA-B*51.

Table 4 summarizes the analysis of the haplotypes, comparing BD patients with controls. The most frequent haplotype, TAGCGGTAA, was significantly increased in HLA-B*51-positive patients compared with healthy controls [odds ratio (OR) = 2.01; 95% CI 1.25, 3.23; P = 0.029]. This haplotype was also increased in patients with BD complicated by arthritis when compared with healthy controls (OR = 1.96; 95% CI 1.26, 3.05; P = 0.030) or BD patients without arthritis (OR = 2.01; 95% CI 1.19, 3.40; P = 0.037) (Table 4).

**TABLE 2.** Clinical features of the Korean patients with BD

<table>
<thead>
<tr>
<th>Feature</th>
<th>Cases, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>61</td>
</tr>
<tr>
<td>Female</td>
<td>58</td>
</tr>
<tr>
<td>Oral aphthous ulcers</td>
<td>119 (100)</td>
</tr>
<tr>
<td>Genital ulcers</td>
<td>92 (77.3)</td>
</tr>
<tr>
<td>Skin lesions</td>
<td>96 (80.7)</td>
</tr>
<tr>
<td>Ocular lesions</td>
<td>45 (37.8)</td>
</tr>
<tr>
<td>Deep vein thrombosis</td>
<td>20 (16.8)</td>
</tr>
<tr>
<td>Arthritis</td>
<td>57 (47.9)</td>
</tr>
<tr>
<td>HLA-B*51</td>
<td>47 (39.5)</td>
</tr>
</tbody>
</table>

![Fig. 1. D' score for the nine SNPs studied across the TLR4 haplotype. Upper triangle, patient population; lower triangle, control population. A black cell means D' > 0.8.](image-url)
Furthermore, HSPs stimulated significantly higher in BD patients than in controls [31–33]. The development of BD through cross-reactive immunopathological responses [27–30]. Notably, the expression of HSPs is up-regulated at lesions of BD and the serum level of HSPs is significantly higher in BD patients than in controls [31–33]. Furthermore, HSPs stimulated \( yt \)-cell responses from BD patients but not in controls in \( \textit{vitro} \) [31, 34]. TLR4 reportedly recognizes and interacts with HSP and LPS, regarded as antigens in BD. Recent studies have reported TLR4 polymorphisms and the risk of various diseases including inflammatory diseases [16, 18, 35]. It has been reported that two non-synonymous TLR4 SNP sites, Asp299Gly (rs4986790) and Thr399Ile (rs4986791), are associated with an elevation of serum cytokines in the Caucasian population [18]. However, no other studies have detected these non-synonymous mutations in Asian populations, including Koreans [36]. Thus, we investigated nine intronic SNPs, the same SNPs described in our earlier work focused on a Japanese population [21].

In the present study, we examined TLR4 because it was implicated in that previous study as a candidate gene in Japanese patients with BD [21]. It is widely accepted that \( \text{HLA-B}^{*}51 \) affects the development of BD, and the frequency of \( \text{HLA-B}^{*}51 \) is much higher in male patients, patients with ocular lesions and complete-type BD patients [6]. The association of the nine SNPs with BD could not be explained by any single SNP, but the data in this study show that the frequency of the TAGCGGTAA haplotype is significantly increased in \( \text{HLA-B}^{*}51 \)-positive BD. TLR4 is located on the long arm of chromosome 9, whereas \( \text{HLA} \) is on the short arm of chromosome 6; thus, to elucidate the genetic influence of \( \text{HLA-B}^{*}51 \) on TLR4, we divided \( \text{HLA-B}^{*}51 \)-positive participants from controls. Among healthy controls, there were no significant differences between healthy participants with and without \( \text{HLA-B}^{*}51 \). In addition, there were no significant differences between \( \text{HLA-B}^{*}51 \)-positive patients and controls. These results suggest that the most frequently occurring haplotype, TAGCGGTAA, may be influenced by \( \text{HLA-B}^{*}51 \) and dependently associated with \( \text{HLA-B}^{*}51 \)-positive BD patients.

The TLR4 gene has been well investigated in the context of RA [18, 35, 37], and arthritis classified as one of the minor symptoms of BD. In fact, 40% of the BD patients have the complication of arthritis, and it is the most common minor symptom [38]. In the present study, we successfully examined 57 BD patients (47.9%) with arthritis. When we examined the haplotype frequencies for BD patients with and without arthritis, the most frequently occurring haplotype, TAGCGGTAA, which is associated with a higher risk of BD, was significantly increased in patients with arthritis. When we examined the haplotype frequencies for BD patients with and without arthritis, the most frequently occurring haplotype, TAGCGGTAA, is associated with a higher risk of BD, was significantly increased in patients with arthritis.
It has been reported that intronic SNPs play important roles in disease development [41]. Other studies analysing complex traits have indicated that haplotypes within the gene interact and have a large effect on the observed phenotype, which could be explained by SNPs [42, 43]. Our present results support the hypothesis that the TLR4 haplotype, TAGCGGTAA, increases the risk for BD, as well as the complication of arthritis. Although there have been many reports of a negative association of non-synonymous SNPs [Asp299Gly (rs4986790) and Thr399Ile (rs4986791)] with BD, a positive association has also been demonstrated in an analysis of intronic SNPs similar to that undertaken in the present study [35, 44, 45]. Our results are consistent with the previous results showing that genetic contributions to inflammatory diseases can be successfully detected by an analysis of intronic SNPs among Asian populations. A recent report has suggested that the TLR4 polymorphisms may only be maintained by evolutionary pressure from infectious disease [46]. It is intriguing to consider whether or not the function and the polymorphisms of TLR4 can be changed alternatively against exogenous elements. Analysis of TLR4 may be helpful in elucidating the aetiology of BD along the historic Silk Road.

Previously, we reported a significant association at a single SNP (rs70377117), especially in incomplete BD patients, but did not show a significant haplotype link among Japanese patients [21]. However, we successfully found a significant difference of haplotype frequencies among Korean BD patients in the current work. Thus, there is a small variation between the results of these two studies, although our findings are, in general, in close agreement with those for the Japanese population [21]. There are three possible sources of bias that could distort these comparisons between results. First, the Japanese investigation involved 200 patients and 102 controls, and there were 119 patients and 141 controls in the current work. Having an insufficient number of samples may have resulted in the small variation between the two studies. A larger sample size for each population would be needed in follow-up studies. Secondly, in the present work, we used the ISGD criteria; however, the other study selected patients based on the Japanese Committee’s Criteria. This use of different criteria may have led to some minor differences in comparisons of the haplotype frequencies. Finally, the nature of the populations was different between the two reports: ocular features were seen in only 37.8% of the participants in this work, but 89.5% in the Japanese paper. Also, the arthritis rate was 47.9% in the present study, but only 35.5% in the other investigation [21]. All samples in the current work were collected at the rheumatology clinic, but patients were recruited in the ophthalmology clinic in the Japanese population study. Different patient characteristics may also have been a source of bias.

In conclusion, we have identified TLR4 as a susceptible gene for HLA-B*51-positive BD and the complication of arthritis in Koreans using a case-control study of SNPs. The present findings are consistent with the interpretation that the immune response against various exogenous and/or endogenous TLR4 ligands plays an important role in the development of BD.

**Rheumatology key message**

- TLR4 is a susceptible gene for HLA-B*51-positive BD and the complication of arthritis in Koreans.

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**References**