Nitric Oxide Synthase and Superoxide Dismutase Gene Polymorphisms in Behçet Disease

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Objective: To investigate the association of endothelial nitric oxide synthase (NOS), inducible NOS, manganese superoxide dismutase (SOD), and extracellular SOD gene polymorphisms with susceptibility to Behçet disease (BD) in Japan.

Methods: Seventy-eight consecutive Japanese patients with BD and 107 healthy control subjects were genotyped by polymerase chain reaction or polymerase chain reaction–restriction fragment length polymorphism methods for endothelial NOS polymorphisms in intron 4, exon 7, and promoter region; inducible NOS polymorphisms in exon 16 and promoter region; manganese SOD Ala16Val polymorphism; and extracellular SOD Arg213Gly polymorphism. HLA-B*51 alleles, which have been found to be associated with BD, were also determined.

Results: The frequencies of manganese SOD Val16 increased significantly in patients with BD. The manganese SOD-Val/Val genotype and HLA-B*5101 had a synergistic role in controlling susceptibility to BD. There was no significant difference in the frequencies of endothelial NOS, inducible NOS, and extracellular SOD gene polymorphisms between patients with BD and control subjects.

Conclusion: The manganese SOD Val16 allele is associated with the development of BD in Japan. Extracellular SOD, endothelial NOS, and inducible NOS gene polymorphisms do not constitute a risk factor for developing BD in Japan.

Clinical Relevance: The manganese SOD gene polymorphism seems to contribute to BD.

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Behçet disease (BD), a chronic inflammatory disease, is a multisystem disorder that affects eyes, mucous membranes, skin, joints, central nervous system, and blood vessels. The disease is prevalent in Middle Eastern and East Asian countries including Japan. The exact pathogenesis of BD remains unknown. Genetic, immunologic, and environmental factors have been suggested as contributing to the development of the disease. Although a strong genetic association identified in BD has been linked to the HLA-B*51 allele,1 non-HLA genes might also be related to the pathogenesis of BD.

Prominent features of BD are vasculitis and thrombosis resulting from endothelial dysfunction.2 Nitric oxide (NO) is responsible for endothelial vasorelaxation and inhibition of platelet adhesion. In addition, NO is produced in increased amounts under inflammatory conditions and might cause tissue injury by reacting with superoxide to yield peroxynitrite. Superoxide dismutase (SOD), a key antioxidant enzyme, scavenges superoxides and inhibits the formation of peroxynitrite, thereby suppressing the resulting injury and regulating the bioavailability of NO. In previous studies, serum NO levels were found to be significantly elevated or lowered in patients with BD compared with control subjects.3,4 An increase or a decrease in SOD activities has also been reported in patients with BD.3,5,6 Although these results conflict, they suggest that NO and SOD might have some role in the development of BD.

Nitric oxide is synthesized by a family of nitric oxide synthases (NOSs) in which 3 isoforms have been identified: 2 constitutive, that is, neuronal NOS and endothelial NOS (eNOS); and 1 inducible (iNOS). The eNOS gene is located on chromosome 7q35-36 and comprises 26 exons.10 When a variant of this gene causes deficient NOS,11 disease processes might ensue. Recent studies have reported that eNOS gene polymorphisms are associated with BD susceptibility in Italian, Korean, and Turkish populations.12-14 The iNOS gene is located on chromosome 17cen-q11.2 and comprises 27 exons.15 When a variant of this gene causes deficient NOS,11 disease processes might ensue. Recent studies have reported that eNOS gene polymorphisms are associated with BD susceptibility in Italian, Korean, and Turkish populations.
Superoxide dismutase constitutes a family of enzymes, including copper-zinc SOD, manganese SOD (MnSOD), and extracellular SOD (EC-SOD). Manganese SOD is present in mitochondria. The MnSOD gene is located on chromosome 6q25, consisting of 5 exons, and an alanine-to-valine substitution at codon 16 of human MnSOD might lead to misdirected intracellular trafficking followed by changes in MnSOD activity in the mitochondria. Extracellular SOD is found in the extracellular matrix of various tissues, including blood vessels, and is the major extracellular scavenger of the superoxide radical. The EC-SOD gene is located on chromosome 4p16.3-q21 and consists of 4 exons. Extracellular SOD has an amino acid substitution Arg213Gly in the heparin-binding domain. The glycine variant of the enzyme is responsible for high EC-SOD levels in serum. We investigated whether allelic variants of the eNOS, iNOS, MnSOD, and EC-SOD genes are involved in the etiology of Japanese BD. HLA-B*51 alleles, which have been found to be associated with BD in the Japanese, were also determined.

METHODS

SUBJECTS

Seventy-eight patients with BD (58 men and 20 women aged 20-69 years [mean age, 42.7 years]) and 107 healthy control subjects (46 men and 61 women aged 28-84 years [mean age, 58.2 years]) previously enrolled in a multicenter case-control study of uveitis were included in this study. All patients fulfilled the International Study Group criteria for the diagnosis of BD and all had uveitis. All of the study subjects were Japanese. A peripheral blood sample was obtained from each subject after obtaining informed consent. The study protocol was carried out in accord with the principles of the second Helsinki Declaration.

DNA EXTRACTION

Genomic DNA was extracted from peripheral blood mononuclear cells by the agglutination partition method using a commercially available kit (SepaGene; Sanko Junyaku Co, Ltd, Tokyo, Japan).

GENOTYPING eNOS POLYMORPHISMS

The intron 4 variable number of tandem repeats polymorphism was detected by polymerase chain reaction (PCR) genotyping. Two primers, 5'-AGGCCTATGGTAGTGCCTT-3' and 5'-TCTCTATGTCGTGTCAC-3', based on the sequences flanking variable number of tandem repeats in intron 4 of the eNOS gene, were used to amplify the corresponding DNA fragment. The PCR products were separated by 3% agarose gel electrophoresis. The 420-base pair (bp) wild-type product contained five 27-bp repeats (allele b), and the 393-bp mutant type contained four 27-bp repeats (allele a).

The presence of Glu298Asp variants was determined by PCR-restriction fragment length polymorphism (RFLP) analysis. A set of primers, 5'-AAGGCAGAGACAGTGGATGGA-3' and 5'-CCAGTCACTCCCTTGGTGCTCA-3', was used to amplify the 248-bp fragment including the Glu298Asp mutation site. The amplified PCR products were digested with the restriction enzymes BanII (New England Biolabs, Beverly, Mass). The Asp298 variant had no cutting site for BanII and the wild-type −786T was not cleaved.

GENOTYPING MnSOD POLYMORPHISM

The presence of Ala16Val variants was determined by PCR-RFLP analysis using a set of primers, 5'-CATATGTAGGGAATACTGTATTTCA-3' and 5'-CTGAACTAGTCACTTGGAG-3'. The PCR products (220 bp) were digested with TaqI (New England Biolabs). Digestions resulted in 2 fragments of 176 bp and 44 bp (Ser608 variant) or in 3 fragments of 143, 44, and 33 bp (Leu608 variant).

The presence of −954G/C polymorphism was identified by PCR-RFLP analysis using a set of primers, 5'-CATATGTAGGGAATACTGTATTTCA-3' and 5'-CTGAACTAGTCACTTGGAG-3'. The PCR products (574 bp) were digested with BspWI (New England Biolabs). The −954C variant had no cutting site for BspI, whereas wild-type −954G was recognized, so the 574-bp PCR product was cleaved into 449- and 125-bp fragments.

GENOTYPING EC-SOD POLYMORPHISM

An Arg213Gly polymorphism in the signal peptide of the EC-SOD gene was evaluated by PCR-RFLP analysis using a set of primers, 5'-CGCTGTCTTTTCTCAGGCATGCGTGGC-3' and 5'-AAGCAGCTCGCTCAGGCATGCGTGGC-3'. The PCR products (122 bp) were digested with Fnu4HI (New England Biolabs). The Gly213 variant had no cutting site for Fnu4HI, whereas the wild-type Arg213 was recognized, so the 122-bp PCR product was cleaved into 82- and 30-bp fragments.

GENOTYPING HLA-B*51

HLA-B*51 alleles were determined by PCR using sequence-specific oligonucleotide probes, as described elsewhere.

STATISTICAL ANALYSIS

The χ² test was used to compare genotype and allele frequencies. When the expected cell number was fewer than 5, the Fisher exact test was used. Odds ratios and 95% confidence intervals were calculated when applicable.

RESULTS

The allele and genotype frequencies of the eNOS, iNOS, MnSOD, and EC-SOD gene polymorphisms in patients with BD and healthy control subjects are
given in Table 1. The genotype distributions of these genes in patients with BD and control subjects, respectively, were within the Hardy-Weinberg equilibrium. There was no sex- or age-dependent difference in genotype distribution among the control subjects (data not shown).

**POLYMORPHISMS OF THE eNOS GENE**

No significant difference was found in the distribution of allele and genotype frequencies of the variable number of random repeats polymorphism in intron 4, the Glu298Asp polymorphism, and the −786 T/C polymorphism.
morphism between patients with BD and control subjects.

**POLYMORPHISMS OF THE iNOS GENE**

No significant difference was observed in the distribution of allele and genotype frequencies of the Ser608Leu polymorphism and the −954 G/C polymorphism between patients with BD and control subjects.

**Ala16Val POLYMORPHISM OF THE MnSOD GENE**

A significant difference in the allele distribution of the MnSOD gene was found between patients with BD and control subjects; the frequency of the Val allele in the patients was significantly higher than in the control subjects (0.936 vs 0.864, respectively; odds ratio, 2.07; 95% confidence interval, 1.00-4.28; \( P = .047 \); Table 1). In addition, a high frequency of Val/Val genotype in the patients compared with the control subjects was statistically significant (87.2% vs 73.8%, respectively; \( P = .03 \)), while the frequency of the Ala/Val genotype decreased in the patients with BD (Table 1).

**Arg213Gly POLYMORPHISM OF THE EC-SOD GENE**

No significant difference was found in the distribution of allele and genotype frequencies of the Arg213Gly polymorphism between patients with BD and control subjects.

**MOLECULAR TYPING OF THE HLA-B*51 FAMILY OF ALLELES**

Only subtypes HLA-B*5101 and HLA-B*5102 were detected in both patients with BD and control subjects. The frequencies of HLA-B*5101 in the patients with BD were significantly elevated compared with those in the control subjects (0.276 vs 0.065, respectively; odds ratio, 5.43; 95% confidence interval, 2.85-10.37; \( P < .001 \); Table 2).

**MnSOD POLYMORPHISM AND HLA-B*51 POLYMORPHISM IN ASSOCIATION WITH BD**

Because both the MnSOD-Val/Val genotype and HLA-B*5101 showed statistically significant associations with BD, we investigated the relation between the MnSOD polymorphism and the HLA polymorphism for the risk of BD using a 2-locus analysis as described by Svejgaard and Ryder.\(^28\) The basic data for the test and various 2×2 comparisons are given in Table 3. There was no significant association between the MnSOD-Val/Val genotype and HLA-B*5101 in patients with BD and control subjects. The MnSOD-Val/Val genotype increased the risk in the HLA-B*5101–negative individuals compared with the HLA-B*5101–positive individuals, although it was not statistically significant. Similarly, HLA-B*5101 increased the risk in the MnSOD-Val/Val–negative individuals compared with the MnSOD-Val/Val–positive individuals. The HLA-B*5101 association was stronger than and independent of the MnSOD-Val/Val genotype, as demonstrated by a significant association in both MnSOD-Val/Val–positive and MnSOD-Val/Val–negative individuals. The presence of both disease-associated genetic markers showed a higher risk (odds ratio, 16.47; \( P < .001 \)) than that in the presence of only 1 marker.

**COMMENT**

We investigated the association of polymorphisms in the eNOS gene, iNOS gene, MnSOD gene, and EC-SOD gene with genetic susceptibility to BD in Japanese subjects. In the control subjects, the allele and genotype frequencies of each gene were compatible with those previously reported in Japanese control populations.\(^26,28-32\) We identified a significant disease association with the MnSOD Ala16Val polymorphism. The Val/Val genotype of the MnSOD gene was a novel genetic marker of risk factor for BD in Japan. No significant associations were identified with eNOS, iNOS, and EC-SOD gene polymorphisms.

Our results differ from those previously reported. In Taiwan, there was no significant difference in the allele and genotype frequencies of the MnSOD Ala16Val polymorphism between patients with BD and control subjects.\(^33\) In Italian and Korean populations, a significant association of Glu298Asp polymorphisms of the eNOS gene with BD was observed, but no difference in intron 4 polymorphism between patients and control subjects was detected.\(^12,13\) In Turkey, a strong association of the eNOS variable number of random repeats intronic polymorphism with BD was reported, and no significant difference was observed in the distribution of the allele and genotype frequencies of the Glu298Asp polymorphism between patients with BD and control subjects.\(^19\) Racial or ethnic differences might be one reason for the incon-

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**Table 2. HLA-B*51 Allele Frequencies**

<table>
<thead>
<tr>
<th>HLA Alleles</th>
<th>With Behçet Disease (n = 78)</th>
<th>Healthy Control Subjects (n = 107)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>No. AF (%)</td>
<td>No. AF (%)</td>
</tr>
<tr>
<td>B*5101</td>
<td>43 27.6</td>
<td>14 6.5</td>
</tr>
<tr>
<td>B*5102</td>
<td>2 1.3</td>
<td>0 0.0</td>
</tr>
<tr>
<td>B<em>5103 - B</em>5121</td>
<td>0 0.0</td>
<td>.001 5.43 (2.85-10.37)</td>
</tr>
</tbody>
</table>

**Abbreviations:** AF, allele frequency; CI, confidence interval; OR, odds ratio.
The Ala/Val variation in the MnSOD leader signal affects the processing efficiency of the enzyme. The Ala type of MnSOD might have an α-helical structure that is a common conformation of mitochondrial leader signals, while the Val type might change its conformation from α-helix to β-sheet with a substitution at position 16.18 The α-helical structure is important for the effective transport of precursor proteins into mitochondria. The Val type is less efficiently transported into mitochondria than the Ala type of the enzyme. The processing study of these 2 leader signals has suggested that the basal level of the MnSOD activity might be higher for Ala/Ala, followed by Ala/Val and then Val/Val.31 The Val variant of the MnSOD might be present at a lower concentration in mitochondria, and homozygous Val/Val should have lower resistance to oxidative stress than in patients with other MnSOD variants. Therefore, the observed positive association of BD with the MnSOD-Val/Val genotype could be explained, at least in part, by the Val16 of the MnSOD leader signal itself being related to the etiology of BD via its less efficient mitochondrial transport. The Ala allele of the MnSOD gene was more widespread than the Val allele in healthy white populations.31 In contrast, the frequency of the Ala variant is significantly lower in Asian populations than in most white populations and the Val/Val genotype is most common in Asian populations. This might be why BD is found with greater frequency in Asian populations than in white populations.

We also performed HLA-B*51 subtyping, and a significantly strong association of HLA-B*5101 with BD was found, which is in accord with previously reported findings.1 The relation between the MnSOD-Val/Val genotype and HLA-B*5101 for risk of BD was investigated. There was no linkage disequilibrium between the MnSOD-Val/Val genotype and HLA-B*5101. However, no statistically significant association of the MnSOD-Val/Val genotype with BD was found for either HLA-B*5101–positive or HLA-B*5101–negative individuals, possibly owing to the few individuals in the positive and negative categories. Although we could not conclude that MnSOD-Val/Val contributed to susceptibility independently of HLA-B*5101, the MnSOD-Val/Val genotype had a synergistic role with HLA-B*5101 in controlling susceptibility to the pathogenesis of BD, because individuals having both markers showed higher risks than those having only 1 marker.

In summary, we confirmed the association of BD with HLA-B*5101 in Japanese patients. We also demonstrated that the Val allele of the MnSOD gene, especially in the homozygous state, is associated with BD in Japan.

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Table 3. Detecting the Strongest Disease Association With MnSOD-Val/Val or HLA-B*5101 According to the Method of Svejgaard and Ryder28

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Odds Ratio</th>
<th>P Value</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>A vs non-A</td>
<td>2.4</td>
<td>.03</td>
<td>MnSOD-Val/Val associated</td>
</tr>
<tr>
<td>B vs non-B</td>
<td>8.16</td>
<td>&lt;.001</td>
<td>HLA-B*5101 associated</td>
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<tr>
<td>+ + vs + +</td>
<td>1.27</td>
<td>.56</td>
<td>MnSOD-Val/Val not associated in HLA-B*5101−positive individuals</td>
</tr>
<tr>
<td>+ − vs − −</td>
<td>2.33</td>
<td>.08</td>
<td>MnSOD-Val/Val not associated in HLA-B*5101−negative individuals</td>
</tr>
<tr>
<td>+ + vs + −</td>
<td>7.07</td>
<td>&lt;.001</td>
<td>HLA-B*5101 associated in MnSOD-Val/Val−positive individuals</td>
</tr>
<tr>
<td>+ − vs + +</td>
<td>13.0</td>
<td>.008</td>
<td>HLA-B*5101 associated in MnSOD-Val/Val−negative individuals</td>
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<tr>
<td>+ + vs + +</td>
<td>0.18</td>
<td>.04</td>
<td>MnSOD-Val/Val and HLA-B*5101 associations differ</td>
</tr>
<tr>
<td>+ + vs − −</td>
<td>16.47</td>
<td>&lt;.001</td>
<td>MnSOD-Val/Val and HLA-B*5101 associated vs their absence</td>
</tr>
<tr>
<td>Association between A and B in patients with Behçet disease</td>
<td>1.27</td>
<td>.49</td>
<td>MnSOD-Val/Val and HLA-B*5101 not associated</td>
</tr>
<tr>
<td>Association between A and B in control subjects</td>
<td>2.32</td>
<td>.23</td>
<td>MnSOD-Val/Val and HLA-B*5101 not associated</td>
</tr>
</tbody>
</table>

Abbreviations: +, positive; −, negative.

*Data are given as number (percentage) unless otherwise indicated.
REFERENCES


