Concise Report

Polymorphisms in the endothelial nitric oxide synthase gene are associated with Behcét’s disease


Objective. Reduced plasma nitric oxide (NO) levels in Behcét’s disease (BD) patients have been implicated in the development of the endothelial abnormalities and thrombotic complications occurring in these patients. This study investigated the association of the endothelial NO Synthase (eNOS) gene polymorphisms with BD.

Methods. A case–control study was carried out using 193 unrelated Turkish BD patients and 106 healthy controls. All individuals were genotyped by PCR for two single-nucleotide polymorphisms (SNPs): −786 T→C in the promoter region and 894 G→T in exon 7 (Glu298Asp). A variable number of tandem repeats (VNTR) polymorphism in intron 4 was also investigated.

Results. The VNTR polymorphism was associated with BD, detected by an increased frequency of the b allele (odds ratio = 1.9, P = 0.0069) and h/b genotype (odds ratio = 2.2, P = 0.002) in patients. After the stratification of cases according to the family history, a significant difference between familial cases and controls in the −786 SNP was observed, with an increase in the frequency of the T allele (odds ratio = 2.5, P = 0.0016) and T/T genotype (odds ratio = 2.5, P = 0.0085), and the association of the VNTR polymorphism with BD became stronger. The −786*T and VNTR*b alleles were in linkage disequilibrium (D’ = 0.65, P < 0.0001), and the number of individuals homozygous for the −786*T/VNTR*b haplotype was significantly increased in the patients.

Conclusions. eNOS gene polymorphisms are associated with BD, which might contribute to the reduced NO activity observed in BD patients.

Key words: Behcét’s disease, Endothelial nitric oxide synthase gene (eNOS), Single-nucleotide polymorphism, Haplotype.

Behcét’s disease (BD) is an unclassified systemic vasculitis with a chronic course and unknown cause. Although, it was originally described as a triad of recurrent oral and genital ulcers and uveitis, it is now recognized as a multisystem disorder also affecting all types and sizes of blood vessels, the joints, the central nervous system, the lungs and the intestines [1].

The pathogenesis of BD is not known. However, it possibly involves complex interactions of genetic and environmental factors. The manifestations of BD are considered to be developed as a result of immunological dysfunction, which is suggested to be induced by microbial pathogens in genetically susceptible individuals and includes hyperreactivity of neutrophils, overexpression of several proinflammatory and Th1-type cytokines and several phenotypic and functional lymphocyte abnormalities [2, 3].

Histopathological studies have demonstrated that the predominant pathological finding at lesions is vasculitis, affecting both the vessel wall and perivascular tissues [1]. Several observations, including elevated plasma levels of von Willebrand factor and thrombomodulin and impaired flow-mediated dilation of brachial arteries, suggest endothelial dysfunction in BD. These immune-mediated endothelial changes have also been implicated in the pathogenesis of the increased thrombotic tendency observed in BD patients [3].

Vascular endothelium forms the single-cell lining of all blood vessels and plays a significant role in maintaining luminal integrity, which includes the production of nitric oxide (NO) [4]. NO is enzymatically synthesized from L-arginine with the catalytic help of nitric oxide synthase (NOS) [5]. Endothelial NOS (eNOS) is one of the three isoforms of NOS [6]; it is a soluble, constitutive enzyme that requires Ca2+/calmodulin for its activation. The gene encoding eNOS (NOS3) is located on chromosome 7 (7q35–36), and it is mainly expressed in the endothelium and at low levels in platelets [7]. eNOS is the most powerful endogenous vasodilator known. It also regulates cell adhesion to the endothelium and inhibits platelet aggregation and vascular smooth muscle cell proliferation [8, 9].

NO was reported to be reduced in patients with active BD [10], and this may have a critical role in the development of the endothelial abnormalities and thrombotic tendency observed in BD patients. Increased intravascular oxidative stress and consumption of NO may explain the reduced levels. However, polymorphisms in the eNOS gene, which might affect its transcription or function, may also contribute to the decreased NO activity in patients with BD.

This study investigated the association of known eNOS gene polymorphisms with BD in a group of Turkish patients and healthy controls.
Materials and methods

Study population

A case-control study was carried out using 193 unrelated Turkish BD patients (112 male and 81 female), all fulfilling the International Study Group (ISG) criteria [11]. The ethical committee of Istanbul Faculty of Medicine approved the study, and all individuals provided informed consent prior to blood collection in compliance with the principles of the Declaration of Helsinki. The study group included 173 consecutive cases and also 19 unrelated patients from multicase BD families, who were recruited for genome-wide linkage analysis. A total of 51 patients described another patient with BD among their relatives. A group recruited for genome-wide linkage analysis. A total of 51 patients also 19 unrelated patients from multicase BD families, who were previously [13]. The intronic 27 bp insertion/deletion VNTR was genotyped using PCR amplification and ethidium bromide-stained agarose gel electrophoresis [14].

Genotyping

The study group was genotyped for 2 single-nucleotide polymorphisms (SNPs), −786 T→C in the promoter region and 894 G→T in exon 7 (Glu298Asp), and also a variable number of tandem repeats (VNTR) polymorphism in intron 4. For the promoter region polymorphism, a 282 bp DNA was amplified using the forward primer of 5'-ATG ACT CAA GTG GGG; and the reverse primer of 5'-AGC CCC TCA GAT GAC ACA GA-3'. The amplified product was digested with the MspI restriction enzyme (New England Biolab, Hitchin, UK), which resulted in 194 and 88 bp bands for the −786T allele, and 149, 88 and 45 bp bands for the −786C allele. Digested PCR products were genotyped by ethidium bromide-stained agarose gel electrophoresis. The missense 894 G→T polymorphism (Glu298Asp) was genotyped by PCR amplification and BanII restriction enzyme (New England Biolab) digestion as described previously [13]. The intronic 27 bp insertion/deletion VNTR was genotyped using PCR amplification and ethidium bromide-stained agarose gel electrophoresis [14].

Statistical analysis

A power calculation was conducted to determine the number of samples for both cases and controls required to perform this study in order to achieve 80% power to detect an odds ratio (OR) of 2.5 at 5% significance level, assuming both autosomal dominant and recessive models of inheritance. This was achieved by using the EPI Info software package version 6. The number of samples required depends on the allele frequencies for each marker; therefore, the number was different for each marker.

The distribution of the control genotypes was checked for Hardy–Weinberg equilibrium. The overall distribution of alleles and genotypes for each polymorphism was compared between cases and controls using χ² analysis. ORs and 95% confidence intervals (CIs) were calculated to assess the risk associated with particular alleles and genotypes.

The estimation of haplotype frequencies and pairwise linkage disequilibrium calculations were carried out using the EH program, which also provides log likelihood, χ² and the degrees of freedom under hypotheses of with (H1) and without (H0) allelic association [15]. To test for heterogeneity in haplotype frequencies between cases and controls, the likelihood ratio test was used [16].

Single nucleotide polymorphism spectrum decomposition (SNPspD) was used to calculate the Meff value to correct for multiple testing [17].

Results

The allele and genotype frequencies of the three investigated eNOS gene polymorphisms in BD cases and healthy controls are given in Table 1. No significant difference was found in the distribution

<table>
<thead>
<tr>
<th>ENOS −786</th>
<th>T</th>
<th>C</th>
<th>Genotypes % (n)</th>
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<tbody>
<tr>
<td>Patients (n = 191)</td>
<td>71 (272)</td>
<td>29 (110)</td>
<td>51 (98)</td>
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<td>Patients with family history (n = 51)</td>
<td>81 (83)</td>
<td>19 (19)</td>
<td>65 (33)</td>
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<td>Controls (n = 102)</td>
<td>64 (130)</td>
<td>36 (74)</td>
<td>42 (43)</td>
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<thead>
<tr>
<th>ENOS 27 bp VNTR</th>
<th>b</th>
<th>a</th>
<th>Genotypes % (n)</th>
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<tbody>
<tr>
<td>Patients (n = 191)</td>
<td>88 (335)</td>
<td>12 (47)</td>
<td>77 (148)</td>
</tr>
<tr>
<td>Patients with family history (n = 50)</td>
<td>92 (96)</td>
<td>8 (8)</td>
<td>86 (43)</td>
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<tr>
<td>Controls (n = 104)</td>
<td>79 (165)</td>
<td>21 (43)</td>
<td>61 (63)</td>
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<thead>
<tr>
<th>ENOS +894</th>
<th>G</th>
<th>T</th>
<th>Genotypes % (n)</th>
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<tr>
<td>Patients (n = 189)</td>
<td>76 (287)</td>
<td>24 (91)</td>
<td>59 (112)</td>
</tr>
<tr>
<td>Patients with family history (n = 51)</td>
<td>70 (71)</td>
<td>30 (31)</td>
<td>47 (24)</td>
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<tr>
<td>Controls (n = 105)</td>
<td>73 (155)</td>
<td>27 (57)</td>
<td>51 (54)</td>
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1. Overall distribution of genotype frequencies in cases vs controls, P = 0.18; and in familial cases vs controls, P = 0.008, P_corr = 0.03.
2. T allele frequency in cases vs control, OR = 1.4, 95% CI 0.9–2.1, P = 0.06, P_corr = 0.17.
3. T allele frequency in familial cases vs controls, OR = 2.5, 95% CI 1.4–4.7, P = 0.0016, P_corr = 0.006.
4. TT genotype in cases vs controls, OR = 1.5, 95% CI 0.9–2.4, P = 0.14, P_corr = 0.4.
5. TT genotype in familial cases vs controls, OR = 2.5, 95% CI 1.2–5.4, P = 0.0085, P_corr = 0.03.
6. Overall distribution of genotype frequencies in cases vs controls, P = 0.006, P_corr = 0.017; and in familial cases vs controls, P = 0.005, P_corr = 0.019.
7. Allele frequency in cases vs controls, OR = 1.9, 95% CI 1.2–2.9, P = 0.0009, P_corr = 0.02.
8. Allele frequency in familial cases vs controls, OR = 3.1, 95% CI 1.4–6.8, P = 0.0035, P_corr = 0.014.
9. bb genotype in familial cases vs controls, OR = 2.2, 95% CI 1.3–3.8, P = 0.0002, P_corr = 0.006.
10. pp genotype in familial cases vs controls, OR = 4.0, 95% CI 1.7–9.5, P = 0.0014, P_corr = 0.005.
11. Overall distribution of genotype frequencies in cases vs controls, P = 0.26; and in familial cases vs controls, P = 0.81.
12. T allele frequency in cases vs controls, OR = 1.2, 95% CI 0.8–1.7, P = 0.41.
13. T allele frequency in familial cases vs controls, OR = 0.9, 95% CI 0.5–1.4, P = 0.55.
of allele and genotype frequencies of the −786 promoter region polymorphism between cases and controls. However, in a subgroup analysis of patients with a positive family history, a significant difference in the overall distribution of −786 genotypes was observed between familial cases and controls (χ²=9.6, P = 0.008). This was reflected by increases in the frequency of the T allele (OR = 2.5, 95% CI 1.4–4.7, P = 0.0016) and the T/T genotype (OR = 2.5, 95% CI 1.2–5.4, P = 0.0085).

No significant difference was observed in the distribution of the allele and genotype frequencies of the +894 polymorphism (Glu298Asp) between cases and controls, even when the family history was taken into consideration (Table 1).

The overall distribution of the intronic 27 bp insertion/deletion VNTR polymorphism genotypes was found to be significantly different between BD cases and controls (χ²=10.1, P = 0.006). This was reflected in increases in the frequency of the b allele (OR = 1.9, 95% CI 1.2–2.9, P = 0.0069) and the b/b genotype (OR = 2.2, 95% CI 1.3–3.8, P = 0.002). Furthermore, in a subgroup analysis of patients with a positive family history, more significant results were obtained (χ²=10.7, P = 0.005), which reflected increases in the frequency of the b allele (OR = 3.1, 95% CI 1.4–6.8, P = 0.0035) and the b/b genotype (OR = 4.0, 95% CI 1.7–9.5, P = 0.0014) in familial cases compared with healthy controls (Table 1).

The haplotype analysis revealed that the promoter region −786*T and intron 4 VNTR*b alleles were in linkage disequilibrium (D = 0.65, P < 0.0001) (Table 2). A difference in linkage disequilibrium was observed using the log likelihood test between cases and controls, which did not reach significance (P = 0.065). To test the role of the −786*T/VNTR*b haplotype as a marker for increased risk of developing BD, the frequencies of the T/T genotype at position −786 of the promoter region and the b/b genotype at the intron 4 VNTR markers were examined. The number of individuals homozygous for the −786*T/VNTR*b haplotype was significantly different between BD cases and controls. Among 189 patients, 90 were homozygous for the −786*T/VNTR*b haplotype, while 29 out of 100 controls carried two copies of this haplotype (χ² test, P = 0.0022, OR = 2.2, 95% CI 1.3–3.7). This association became stronger when patients with a positive family history were compared with healthy controls: 31 of the 50 patients were homozygous for the −786*T/VNTR*b haplotype (P < 0.0001, OR = 4.6, 95% CI 2.2–9.5).

The Meff value calculated by SNPSpD was 2.8547, which was used to multiply the P value instead of Bonferroni correction. The corrected P values are presented in Table 1.

Discussion
This study demonstrates a strong association of the eNOS gene intron 4 27 bp insertion/deletion VNTR polymorphism with BD. The association of the eNOS gene intron 4 polymorphism and −786 promoter region polymorphism with BD was stronger in patients with a positive family history.

Salvarani et al. investigated the association of two eNOS polymorphisms, +894 (Glu298Asp) in exon 7 and the 27 bp VNTR in intron 4, in 73 BD cases and 135 controls of Italian origin [18]. They observed a significant association of Glu298Asp polymorphism with BD (P corr = 0.0009), but could not detect any difference for the intronic marker between the patients and controls. Similarly, Kim et al. reported a significant increase in the Asp298 allele in Korean BD patients in an analysis of 65 patients and 80 healthy controls, and no difference for the intron 4 polymorphism between the patients and controls[19]. As discussed by the authors, the conservative substitution in exon 7 may not have a direct functional effect, but it may be in linkage disequilibrium with a regulatory polymorphism on the same haplotype [19]. It is very difficult to pinpoint the real regulatory polymorphisms among the DNA variations within a selected haplotype block. The increased transmission of the −786*T/VNTR*b in our patients suggests that the regulatory polymorphism may be located on this haplotype. However, the characteristics of this haplotype may show differences between different populations, and it may explain the discrepancy in the associated eNOS polymorphisms between Italian, Korean and Turkish BD patients. On the other hand, lack of association of the intron 4 polymorphism in Italian and Korean patients may also be due to the small sample size and the high frequency of the associated allele in the controls (frequency of b allele = 0.78 and 0.88, respectively). Power calculations revealed that the number of samples used in this study has 80% power to detect a gene conferring a genotyping relative risk of 2.5 at the 5% significance level for −786 eNOS and 894 exonic markers and is only a few samples short of the number needed to have this power for 27 bp intronic marker (31 cases and 18 controls) under the dominant model. Under the recessive model, the samples used in this study had adequate power to detect association.

The functional significance of eNOS polymorphisms has been investigated in a number of studies carried out in different populations, which have revealed inconsistent findings. Nakayama et al. observed reduced endothelial NO synthesis with a T → C change at position −786 of the eNOS gene in a functional study conducted in a Japanese population [20]. However, Sim et al. found that the intron 4 VNTR polymorphism could have contributed over 25% of the phenotypic variation in the healthy Caucasian families in which the eNOS gene was observed, where the minor allele, a, causes an increase in the NO level [7]. Apart from different associations between haplotypes and regulatory polymorphisms, various environmental factors, which affect the level of oxidative stress, may also modify the phenotypic expression potentials of
the DNA variants in the eNOS gene, making functional studies of the eNOS gene more difficult to conduct.

The $P$ values reported in this study were corrected for multiple testing using the SNPSpD method, which takes into account the strong linkage disequilibrium between the polymorphic sites and the fact that they are not independent observations, hence eliminating the overcorrection that might result from adopting Bonferroni correction. The Meff value was 2.8547, which was multiplied by the $P$ value. To correct for stratification, the value 3.8547 was used (Meff value + 1 stratification). For all the results, all $P_{corr}$ values remained significant ($P_{corr} = 0.019$ for the intron 4 VNTR, and $P_{corr} = 0.03$ for the −786 promoter region polymorphism).

In conclusion, our findings confirm that eNOS gene polymorphisms are associated with BD. These polymorphisms may contribute to the reduced NO activity and inflammatory endothelial changes observed in patients with BD. The increased frequency of the promoter region −786*T and intron 4 VNTR*b haplotype in BD patients suggests that the regulatory polymorphism may be located on this particular haplotype. However, the present work should be regarded as a hypothesis-testing study with its limitations, and further studies are needed to pinpoint the regulatory polymorphism or haplotype and its effects on the development of certain manifestations of BD.

<table>
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<th>Key messages</th>
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<td>- The association of eNOS gene polymorphisms with BD was confirmed.</td>
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<td>- eNOS polymorphisms might contribute to the reduced NO activity and inflammatory endothelial changes seen in BD patients.</td>
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<td>- Further studies are needed to pinpoint the regulatory polymorphism in the eNOS gene.</td>
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The authors have declared no conflicts of interest.

References