Endothelial nitric oxide synthase gene Glu298Asp polymorphism is associated with Behçet's disease

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ABSTRACT

Objective. *The 894 G→T (Glu298Asp) polymorphism in exon 7 of the endothelial nitric oxide synthase (eNOS) gene was previously reported to be associated with Behçet's Disease (BD) susceptibility in Italian origin and Korean patients, but not in a group of unrelated Turkish patients. We analyzed whether this polymorphism is associated with BD, in another group of Turkish patients.*

Methods. *We studied 132 consecutive Turkish BD patients being followed up by Ege University Rheumatology Department and 91 healthy controls. All individuals were genotyped by PCR-RFLP for 894 G→T in exon 7 (Glu298Asp).*

Results. *The frequency of the T allele in BD group (101/264) was significantly higher than in healthy controls (OR 1.88, %95 CI 1.27-2.49, p < 0.001). The frequency of the homozygote (TT) Glu298Asp polymorphism in BD (27/132) was also significantly higher than in healthy controls (5/91) (OR 3.72, %95 CI 3.44-4.0, p < 0.001). However, no association was found between the Glu298Asp polymorphism and clinical parameters in BD.*

Conclusions. *In this study, we found that Glu298Asp polymorphism of the eNOS gene was associated with BD in Turkish patients.*

Introduction

Behçet's disease (BD), which was first described as the triple symptom complex of recurrent oral and genital ulcers and iritis, is a unique systemic vasculitis, which affects almost all types and sizes of blood vessels (1). Although the exact pathogenesis of BD is not known, it is believed to occur in the context of a susceptible genetic background under the influence of various environmental factors, especially microbial pathogens (1, 2). The immunological abnormalities in BD may be summarized as hyperreactivity of neutrophils, overexpression of several proinflammatory and Th1-type cytokines and several phenotypic and functional lymphocyte abnormalities (2).

Thrombotic tendency is also a well known feature in BD (1, 2). Although hypercoagulable/prothrombotic state may also be important in developing thrombosis, endothelial cell dysfunction (ECD) is accepted as the main immune-mediated factor causing thrombosis in BD (1-5). ECD is a physiological phenomenon which basically reflects impaired flow-mediated dilatation of brachial arteries, mostly due to reduced nitric oxide (NO) levels (6). NO is the most powerful endogenous vasodilator known, and is enzymatically synthesized by normal endothelium, from L-arginine with the catalytic help of endothelial nitric oxide synthase (eNOS) (7, 8). In the presence of endothelial injury, as in the case of systemic vasculitis, NO production will be reduced, causing impaired flow-mediated dilatation (6-8). So, reduced plasma NO levels reported in active BD (9), may contribute to the endothelial abnormalities and thrombotic tendency observed in BD.

Although increased intravascular oxidative stress and consumption of NO may contribute to the reduced NO levels in BD, polymorphisms in the eNOS gene may also play a role (10). From this standpoint, well-known eNOS gene polymorphisms in BD had already been investigated in previous studies (10-12). Among these, Glu298Asp polymorphism in exon 7 of the eNOS gene was found to be significantly associated with BD patients from Italy (11) and Korea (12). However similar association could not be found in a group of Turkish BD patients (10). In this study, we aimed to investigate the association of Glu298Asp polymor-

phism in exon 7 of the eNOS gene once again, in another group of Turkish BD patients, as well as to find out whether this polymorphism was correlated with clinical parameters.

Materials and methods

Study population

We studied 132 consecutive Turkish BD patients (M/F:73/59; mean age 38.3 ± 8.7 years), being followed up Ege University Rheumatology department and all fulfilling the International Study Group (ISG) criteria (13). Ninety one healthy controls with no history or signs of BD or recurrent oral ulceration were also included in this study. Both the Behçet's patients and healthy controls were Turkish, residing in Turkey for at least one generation. All the Behçet's patients were unrelated. The ethical committee of Ege University Faculty of Medicine approved the study, and all individuals provided informed consent prior to blood collection.

In Behçet's patients, the presence of at least one of the following clinical findings including eye involvement, deep vein thrombosis, large vessel involvement and central nervous system involvement was accepted as major organ involvement.

Genotyping

The study group was genotyped for the missense single-nucleotide polymorphism (SNP) of 894 G→T in exon 7 (Glu298Asp) by using the PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) technique (11, 14). Each amplification was performed using 250 ng of genomic DNA in a total volume of 50 BL containing 1.5 U AmpliTaq DNA polymerase (Perkin-Emler), 20 pmol of each primer and 200 nmol of each dNTP on a Perkin-Elmer 9600 Thermal Cycler (AmpliCycle, Perkin-Elmer, Roche Molecular Systems, NJ, USA) under the following conditions: The initial denaturation was made at 94°C for 2 minutes followed by 35 cycles at 94°C for 30 seconds, 61°C for 30 seconds and at 72°C for 30 seconds. The final extension step was prolonged to 5 minutes. Genotyping the polymorphism 894 G→T was performed by amplification in exon 7 with two primers: 5'-AGGCAGGA-GACAGTGGATGGA-3' (sense) and 5'-CCCAGTCAA-TCCCTTTGGT-GCTCA-3' (antisense). Then amplified PCR products were digested using the restriction endonuclease enzyme Eco 241 (Ban II) (MBI Fermentas) at 37°C for 2 hours, and visualized by 2% agarose gel electrophoresis. The 248 bp PCR product, was cleaved into two fragments of 163 bp and 85 bp in the presence of a G at nucleotide 894, which corresponds to wild type Glu298 (GG homozygote genotype). In the presence of SNP of 894 G→T, the restriction endonuclease enzyme could not digest the 248 bp PCR product. So, the presence of 248 bp PCR product, without split products of 163 bp and 85 bp was accepted as homozygote polymorphism (TT homozygote genotype). The presence of 248 bp together with split products of 163 and 85 bp products were accepted as GT heterozygote genotype. Each of the three DNA specimens were confirmed by direct DNA sequencing method. PCR products and fragments were visualized by UV transluminator.

Statistical analysis

Statistical analysis was made using the statistical package program (SPSS 7.5). The frequencies of the alleles and genotypes among the patient and control groups were determined and were compared by chi-square and Fischer's exact tests. Odds ratios were calculated together with their 95% confidence interval (CI). Corrected p values were calculated by multiplying p by the number of alleles compared. The distribution of the control and Behçet group genotypes was checked for Hardy-Weinberg equilibrium. Formal power calculations were made using Power and Precision Analysis 2 program (trial version).

Table I. Allele and genotype frequencies of the Glu298Asp polymorphism in exon 7 of the eNOS gene in patients with Behçet's disease and healthy controls.

	Alleles % (n)		Genotypes % (n)		
ENOS +894	G	T	GG	GT	TT
Patients (n=132)	62 (163)	38 (101)	44 (58)	36 (47)	20 (27)
Controls (n=91)	80 (145)	20 (37)	65 (59)	29 (27)	6 (5)

Results

The allele and genotype frequencies of the 894 G→T (Glu298Asp) polymorphism in exon 7 of the eNOS gene both in BD patients and healthy controls are reported in Table I. The statistical power was found to be 89% and 88% for the allele and genotype analysis, respectively. The allelic distribution in healthy controls was in Hardy-Weinberg equilibrium; however Hardy-Weinberg equilibrium was not detected in BD patients. The overall distribution of the Glu298Asp polymorphism genotypes differed significantly between the BD group and healthy controls ($p = 0.000001$). This was reflected in increases in the frequency of the T allele, the homozygote TT genotype and the heterozygote GT genotype in BD group ($p = 0.001$). The frequency of the T allele in BD group (101/264) was significantly higher than in healthy controls (37/182) (OR 1.88, %95 CI 1.27-2.49, $p < 0.001$). The frequency of the homozygote Glu298Asp polymorphism in BD (27/132) was significantly higher than in healthy controls (5/91) (OR 3.72, %95 CI 3.44-4.0, $p < 0.001$). Similarly, the frequency of the heterozygote Glu298Asp polymorphism in BD (47/132) was significantly higher than in healthy controls (27/91) (OR 1.2, %95 CI 0.55-1.85, $p < 0.001$).

The frequency of Asp298 was significantly higher in BD patients (74/132) than in controls (32/91).The frequency of Glu298 was significantly lower in BD patients (105/132) than in controls (86/91) ($p = 0.000001$).

No significant association was found between the Glu298Asp polymorphism and the presence of major organ involvement in BD. Similarly, the frequencies of the Glu298Asp polymorphism between different subgroups of Behçet's patients, or between the patients with and without any particular manifestation of the disease, were

not significantly different. The distribution of the genotype frequencies of Glu298Asp polymorphism in exon 7 of the eNOS gene in association with clinical characteristics of the Behçet's patients were given in Table II.

Discussion

In this study we found that Glu298Asp polymorphism in exon 7 of the eNOS gene was significantly associated with BD in a randomly selected group of Turkish patients. Differently from healthy controls, the lack of Hardy-Weinberg equilibrium in allelic distribution of BD patients also supports the finding of a BD-related allele in our study. To our knowledge, our study is the fourth one investigating the eNOS gene polymorphisms in BD (10-12). In the previous studies, Salvarini *et al.* (11) and Kim *et al.* (12) found that Glu298Asp polymorphism of the eNOS gene was associated with BD susceptibility in Italian and Korean patients, respectively. However, Karasneh *et al.* (10), could not confirm this association in Turkish Behçet's patients. They found no significant difference in the distribution of the allele and genotype frequencies of the Glu298 Asp polymorphism between BD cases and controls, even when the family history was taken into consideration. So, with regard to Glu298Asp polymorphism of the eNOS gene, our findings supported Salvarini *et al.* and Kim *et al.*, while disagreed with the findings of Karasneh *et al.* In consistent with the previous studies (11, 12), we also could not find any association between Glu298Asp polymorphism and clinical parameters in BD. However, we do accept that this lack of association may be due to the relatively limited number of our cohort.

Among the previous three studies investigating eNOS gene polymorphisms in BD, the study of Karasneh *et al.* differed from the others not only with the larger number of Behçet's patients included (193 Turkish patients versus 73 Italian and 65 Korean patients), but also with the number of the eNOS gene polymorphisms studied (10). In this study, in addition to missense SNP of 894 G→T in exon 7 (Glu298Asp), another SNP of -786 T→C in the promoter region and a variable number of tandem repeats (VNTR) polymorphism in intron 4 of the eNOS gene were also studied. As mentioned above, they found no association with Glu298Asp polymorphism. However, unlike the previous reports (11, 12), they found that the overall distribution of the 27 bp insertion/deletion VNTR polymorphism genotypes in intron 4 was significantly higher in BD cases than in controls (χ^2 = 10.1, P = 0.006) (10). Unfortunately, (due to technical and financial problems), we could not investigate intron 4 27 bp VNTR polymorphism in our study group, and we accept that this is an important limitation factor in our study. Another limitation of our study was the lack of HLA B51 analysis in BD group. Although in previous studies it has already been shown that Glu298Asp polymorphism was independent of HLA-B51 status in different populations (10-12), we could not investigate whether the same holds true or not in our study group.

eNOS gene polymorphisms may be functionally significant only if they cause altered function of this gene. Glu298Asp polymorphisms in exon 7 and VNTR polymorphism in intron 4 may lead to a change in eNOS expression and enzymatic activity, causing impairment of endothelial NO dependent flow mediated vasodilatation, which in turn contributes to the ECD seen in BD (14-17). Furthermore, the impaired production of basal NO and associated ECD may predispose to thrombosis or atherosclerosis related disorders (18). Recently, carotid artery intima-media thickness and plaque frequency in BD patients, as assessed by ultrasonography, were found to be increased compared with healthy controls (19). Although increased carotid IMT does not invariably mean atherosclerosis, together with the increased plaque frequency, mild to moderate atherosclerosis may develop during the course of BD (19).

With respect to impaired NO production, the significant association of Glu298Asp polymorphism with BD in Turkish patients, in parallel to the Italian and Korean BD patients, may have a clinical implication, as suggested by Tesauro *et al.* (20). These authors showed that this polymorphism generates protein products with differing susceptibility to cleavage. They demonstrated that the Asp298 variant was more susceptible to proteolytic cleavage than eNOS Glu298, and this might have contributed to abnormally low NO generation in carriers of the Asp variant

Table II. The distribution of the genotype frequencies of the Glu298Asp polymorphism in exon 7 of the eNOS gene in association with clinical characteristics of the patients with Behçet's disease.

Clinical Features	n (%)	Genotypes n (%) GG	GT	TT
Oral ulcer	132 (100)	58 (43.9)	47 (35.6)	27 (20.5)
Genital ulcer	119 (90.6)	51 (42.9)	43 (36.1)	25 (21)
Acneiform lesions	114 (86.4)	48 (42.1)	40 (35.1)	26 (22.8)
Erythema nodosum	94 (71.2)	46 (48.9)	26 (27.7)	22 (23.4)
Positive pathergy test	56 (42.4)	29 (51.8)	21 (37.5)	6 (10.7)
Eye involvement	55 (41.7)	21 (38.2)	24 (43.6)	10 (18.2)
Arthritis	40 (30.3)	15 (37.5)	15 (37.5)	10 (25)
Deep vein thrombosis	34 (25.8)	16 (47.1)	14 (41.2)	4 (11.8)
Superficial thrombophlebitis	32 (24.2)	14 (43.8)	14 (43.8)	4 (12.5)
Large vessel involvement	17 (12.9)	9 (52.9)	6 (35.3)	2 (11.8)
Epididymitis *	15 (20.5)	7 (46.7)	3 (20)	5 (33.3)
Central nervous system involvement	9 (6.8)	4 (44.4)	4 (44.4)	1 (11.1)
Major organ involvement **	76 (57.6)	32 (42.1)	30 (39.5)	14 (18.4)

*The calculations for epididymitis were made on 73 male Behçet's patients only.

**The presence of at least one of the following clinical findings including eye involvement, deep vein thrombosis, large vessel involvement and central nervous system involvement was accepted as major organ involvement as indicated in the "materials and methods" section.

(20). However, since glutamate and aspartate are conservative substitutions, another possibility would be that this polymorphism serves as a marker for a functional effect elsewhere in the eNOS gene or in its vicinity (10, 11). So, we do accept that, 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. On the other hand, based on their findings of increased frequency of the promoter region -786*T and intron 4 VNTR*b haplotype in BD patients, Karasneh *et al*. suggested that the regulatory polymorphism may be located on this particular haplotype (10).

The controversial findings of the studies investigating the possible association of eNOS gene polymorphisms with BD, may be explained in several ways (14-17, 21, 22). Firstly, the characteristics of the haplotype having the regulatory polymorphism(s) may show differences between different ethnic populations. Secondly, 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 (10). Thirdly, despite the proposed pathogenic mechanisms, the functional significance of eNOS polymorphisms is still unclear. Further studies connecting the eNOS gene polymorphisms in BD with the transcription of eNOS enzyme and function and activity of NO, as well as with the ECD, are clearly needed.

In conclusion, polymorphisms in genes encoding for host effector molecules, including eNOS may have a supplementary role in disease susceptibility and/or severity in BD (23). Although the type of SNP might be different, eNOS gene polymorphism in BD has been a common finding in all the studies including our study. Since the functional relevance of these SNP's could not be proven yet, the real functional gene polymorphism may simply be in linkage disequilibrium with the previously found SNP's. Our study shows that, despite the presence of genetic differences, eNOS gene polymorphism seems to be conserved in another randomly selected group of Turkish BD patients.

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