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Original Article

Endothelial Nitric Oxide Synthase Enzyme Gene Polymorphism (Exon 7, 894 G→T, Glu298Asp) in Behçet's Patients: a Case-Control Study

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Abstract

Background The etiopathology of Behçet's disease (BD) remains partially unknown and the eNOS gene polymorphism indicated a particular characteristic of vasculitis in the disease progress.

Methods The study was planned as a case-control study. The BD group included 36 patients, 22 (61.1%) female patients and 14 male (38.9%), aged 20-70 years while the control group consisted of 36 volunteers, 20 (55.6%) female volunteers and 16 (44.4%) male with similar age distributions. Polymerase Chain Reaction (PCR) - restricted fragment length (RFL) polymorphism analysis was used to determine Glu298Asp variants in all samples.

Results In the RLP analysis conducted in the BD group, the TT (5.6%) genotype was detected in 2 patients, the GG (55.6%) in 20, and the GT genotype in 14 (38.9%). While the genotype TT was not found in the control group, the GG genotype was detected in 30 individuals (83.3%) and the GT genotype in 6 individuals (16.7%). The GG genotype was higher in the control group significantly and the rate of GT genotype was significantly higher in the GH group (p= 0.027). While 18 (25.0%) T alleles and 54 (75.0%) G alleles were detected in the BD group, 6 (8.3%) T alleles and 66 (91.7%) G alleles were detected in the control group (p=0.007). The risk of having the T allele in patients with Behçet's disease was 3.7 times higher when compared to that of the control group (OR=3.7, 95% Cl for OR: 1.36-9.88, p<0.05).

Conclusion A significant association of the eNOS gene Glu298Asp polymorphism with BD found in the Turkish population.

Key words
Behçet's disease; eNOS; Glu298Asp; Polymerase Chain Reaction.

Introduction

Behçet's disease (BD) was first defined in 1937 by a Turkish dermatologist, Professor Hulusi Behçet, as a range of symptoms characterized by recurrent oral aphthae, genital ulceration and hypopyon iritis. BD is an autoinflammatory disease with multisystemic neutrophilic perivasculitis that first occurs with multiple clinical manifestations and continues with inflammatory exacerbations. Although clinically, the diagnosis of BD is solely based on the clinical manifestations determined by the International Criteria for Behçet's Disease (ICBD). Genetically the disease has historically been associated to the human leukocyte antigen-(HLA-) B5 serotype, and then to the HLA-B51 allele. Historically, BH has been associated with the famed Silk Road, and its prevalence is widespread among peoples settled along trade routes connecting Southern Europe, East Africa, East Asia, and the Mediterranean.[1-4]

Various subsets of BD have been described over the years, each with a different genetic origin

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and the involvement of many organs such as the lungs, and kidneys, as well as the joints, gastrointestinal, and central nervous systems.[5,6] BD is accepted as an autoinflammatory disease caused by infection and environmental factors in genetically predisposed individuals, but its etiology remains partially unknown.[4,7]

Nitric oxide (NO) is synthesized by nitric oxide synthase (NOS) after the oxidation of L-arginine amino acid.[5,6] The NOS enzyme, which mediates NO synthesis, has three isoforms: neuronal (nNOS, I), inducible (iNOS, II) and endothelial (eNOS, III).[8-10] NO, which is produced in endothelial cells as a result of eNOS activity, is an important molecule of endothelial functions (in response to hypoxia, and angiogenesis) that plays an important role in the reduction of vascular tone and the regulation of cardiovascular hemostasis.[11,12] The eNOS gene contains 26 exons of 21 kb and approximately 23,000 DNA sequences located on chromosome 7q36.1, and approximately 4 thousand variations have been described. Variations in this gene cause a decrease in NO, and diseases associated with defined variations of this gene have been identified in the literature. Many vascular disorders, including coronary arteries and myocardial infarction (MI), higher blood pressure, cerebrovascular event, and kidney disease, have been linked to eNOS gene polymorphism to date. Since these genetic variations result from factors such as race and geography, specific genetic variants are referred to as specific populations.[13-15]

Presented study aimed to compare the polymorphism of the eNOS gene in the exon 7 region (Glu298Asp) in a group of patients with BD with that of a healthy control group.

Methods

The study was planned as a case-control study. Patients, who applied to the Cumhuriyet University Faculty of Medicine, Dermatology Clinic and were diagnosed with BD were included. The study began following receipt of approval from the ethics committee and was conducted in accordance with the Helsinki principles.

Eligibility criteria Definitive diagnosis of BD was made according to the criteria of the ICBD (16). The BD group included 36 patients, 22 (61.1%) female patients and 14 male (38.9%), aged 20-70 years, while the control group consisted of 36 volunteers, 20 (55.6%) female volunteers and 16 (44.4%) male with similar age distributions. Before blood sampling, none of the BD patients or healthy control group members had received systemic or local therapy for at least 3 months.

DNA Isolation and Glu298Asp Polymorphism Analysis 5 ml of venous blood from patients and members of the control group was placed in 15 ml centrifuge tubes containing 1 ml of EDTA (2%) and refrigerated at -20°C until isolation. DNA isolation was performed using the NucleoSpin Blood DNA Isolation Kit (leukocytes).[17]

Polymerase chain reaction (PCR) was performed using a PCR Master Mix Kit (Fermentas). The reaction was performed using a Thermal Cycler apparatus (Techne Progene, Cambridge, UK). On the same device, the exon 7 gene region of the eNOS gene was amplified. PCR-restricted fragment length (RFL) polymorphism analysis was used to identify Glu298Asp variants in all samples.[18] In order to multiply the fragment, a set of primers was used, including the Glu298Asp mutation site. The replicated PCR products were digested with the restriction enzyme Ban-II in accordance with the manufacturer's instructions (Fermentas International, Ontario, Canada). DNA fragments

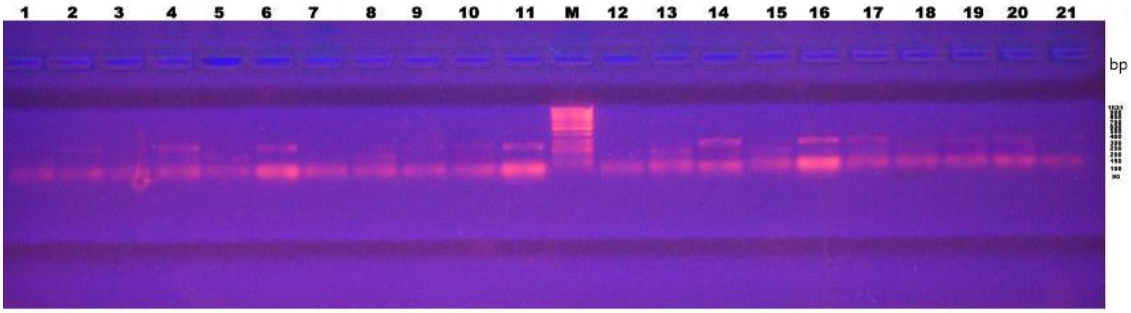

Figure 1 Behçet's patients enos exon 7, 2% agorose gel, Ban 2 RFL profiles. TT (homozygous Asp/Asp); columns 9, 11. GG (homozygous Glu/Glu); columns 1, 3, 5, 7, 12, 13, 15, 18. GT (heterozygous Glu/Asp); columns 2, 4, 8, 10, 14, 16, 17, 19, 20. Uncut PCR product of Behçet's patients. columns 6, 21.

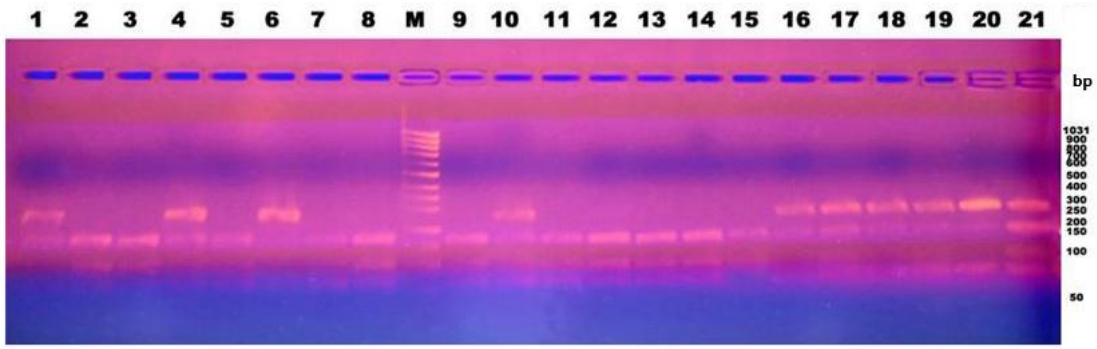

Figure 2 Enos exon 7, 2% agarose gel, Ban II RFLP profiles of the control group. GG (Homozygous Glu/Glu); columns 9, 11, 12, 13, 14, 15. GT (Heterozygous Asp/Glu): columns 6, 10, 16, 17, 18, 19, 20, 21.

were discreted by electrophoresis (Midicell EC-350) on a 2% agarose gel for 45 minutes at an 80 Volt potential voltage. DNA fragments were discreted by electrophoresis (Midicell EC-350) on a 2% agarose gel for 45 minutes at an 80 Volt potential voltage. At the end of the processes for the RFL polymorphism in exon 7 of the eNOS gene, in the imaging system, genotyping was performed as TT (homozygous Asp/Asp) when there was a single band at 248 base pairs (bp), as GG (homozygous Glu/Glu) when there were two bands at 163 bp and 85 bp and as GT (heterozygous Glu/Asp) when there were three bands at lines 248, 163 and 85.

The data was analyzed with SPSS (Ver:10.0) program. The Chi-Square test, Odds ratio (OR), and significance test of the difference between the two means were used to evaluate the data. The data in the tables were stated as the number and percentage of individuals, and the margin of error was set at 0.05.

Results

The BD group's mean age was 34.02±10.49 (range: 20-70), while the mean age was 37.59±9.82 (range: 19-65) in control group (t=1.62; p=0.107). Sex distribution was similar in both the BD and control groups (p=0.633). Individuals in the patient group had a mean age of disease onset of 26.02±8.86. Family history was present in 11 patients (30.6%). Oral aphtha and genital ulceration were among the clinical findings of BD in all 36 patients at every stage of their disease. Eye stiffness was detected in 19 (52.8%) patients, a positive pathergy test in 14 (38.9%), gastrointestinal tract involvement in 5 (13.9%), arthritis in 5 (13.9%), neurological involvement in 2 (5.6%), pulmonary

involvement in 2 (5.6%), skin lesions in 35 (97.2%), and cardiovascular involvement in 5 (13.9%). In the RLP analysis conducted in the BD group, the TT (5.6%) genotype was detected in 2 patients, the GG (55.6%) in 20, and the GT genotype in 14 (38.9%). While the TT genotype was not found in the control group, the GG genotype was detected in 30 individuals (83.3%) and the GT genotype in 6 individuals (16.7%). According to the genotype distribution of both groups, a significant difference was found in the frequency of both the GG and GT genotypes, while the difference was insignificant in terms of the TT genotype (p= 0.027). While 18 (25.0%) T alleles and 54 (75.0%) G alleles were detected in the BD group, 6 (8.3%) T alleles and 66 (91.7%) G alleles were detected in the control group (p=0.007). The risk of having the T allele in patients with BD had 3.7 times higher risk compared to the control group (OR=3.7, 95% Cl for OR: 1.36-9.88, p<0.05).

Discussion

The age of onset of BD in patients was approximately in the middle of the third decade. The most frequent type of involvement in patients was found to be skin involvement, while neurological and pulmonary involvement were the least common. The genotype GT was higher in the BH group significantly and the GG genotype was significantly higher in the control group. While the T allele was more often in the BD group significantly, the risk of having the T allele was found to be 3.7 times higher in patients with the disease than in the control group.

Although the etiology of BD is not fully understood, genetic predisposition, microbial factors, environmental factors, and immunoregulation disorders are all mentioned.[2,4,6,7] BD is always accompanied by vasculitis histopathologically. The disease is characterized by vasculitic lesions that encompass a wide spectrum, and include small vassal vasculitis, large arterial/ venous involvement, superficial thrombophlebitis, and deep vein thrombosis.[19] The vasculitis event mainly develops secondary to endothelial destruction and endothelial functions. Although the pathogenic mechanism of vascular lesions in the development of GH is not fully understood, it is suggested that endothelial dysfunction plays an important role.[20,21] Recently, it has been shown that brachial artery flow-mediated dilation decreases in Behçet's patients because current-mediated dilation is largely dependent on endothelial function and is performed by endothelial NO release.[22] NO production is reduced in both the active and inactive periods of the disease compared to that in the control group, according to studies in these fields.[18,23,24]

Variations in the eNOS gene (7q36.1) cause NO reduction and are involved in the etiology of a group of disease. Functional DNA variants in the eNOS gene may cause changes in eNOS expression and thus enzymatic activity, and may play a role in a variety of cardiovascular diseases and vasculitis caused by abnormal vasodilation resulting from impaired endothelial NO secretion. Many polymorphisms in the exon, intron and promoter regions of the eNOS gene have been found in various populations. The Glu298Asp polymorphism in exon 7 and the VNTR polymorphism in intron 4 have been the most studied ENOS gene polymorphisms so far. Both of these can result in the development of a different vascular pathologies, including coronary artery disease, coronary spasm, MI, hypertension, renal diseases and stroke.[13,15,19,25-27]

Kim *et al*.[19] studied the Glu298Asp polymorphism in 65 Korean BD patients, 27 connective tissue patients with vasculitis, and 80 control group members for the Glu298Asp

polymorphism in the eNOS gene. For the indicated variation, they discovered a significant risk with OR=3.2 in BD and OR=5.9 in patients with rheumatoid vasculitis.[19] The Glu298Asp polymorphism of the eNOS gene was investigated in a study conducted by Salvarani *et al*.[18] in Italy, which included 135 healthy individuals and 73 BD patients from the same geographical region. The study found that the Glu298Asp genotype distribution altered significantly between the BD and control groups. Asp/Asp homozygous and Glu/Asp heterozygous genotypes were found to be high in BD. They also found that the number of Asp298 alleles was significantly higher in the BH group compared to control group.[18] In their study of 193 BD patients and 106 healthy participants in Türkiye, Karasneh *et al*. demonstrated no difference for allele or genotype frequencies of the Glu298Asp polymorphism between the BD and control groups, even positive family history.[28] Similarly, a meta-analysis with a total of 13 case-control studies found no association between the eNOS Glu298Asp polymorphism, although this meta-analysis included data from other vasculitis syndromes. Still, the majority were patients with BD-related vasculitis.

In our study, the b allele (Glu/Glu) was lower and, the c allele (Glu/Asp) was higher in BD compared to the control group. The frequency of Asp298 was higher in Behçet's group compared to that in the control group. Differences in the literature could be attributed to the method used (MboI and BanII restriction enzymes or different primers), in homogeneous patient groups caused by BD subsets, different races from different geographies, and sample numbers. In the Turkish population, the eNOS gene Asp 298 polymorphism is linked to BD. However, this relationship may differ among races. Finding the responsible genes in Behçet's disease can enable for personalized management to early diagnosis and treatment and a better clarification of the disease's pathophysiology and clinical course. Increased eNOS activity may aid in the elimination of pathologies in Behçet's disease. Administration of substrate or cofactors such as L-arginine or BH4 can increase NO production. It can also provide some degree of insight into potential drug resistance. Another way to ensure NOS expression is gene transfer. Transferring the eNOS gene to specific regions can locally increase NO levels and activity.

There are some limitations in present study. These are the small sample size, the inability to analyze the findings based on potential BD subsets or the clinical course of the disease. Also, the possible non-homogeneous study group is another limitation because of the study is from a geographical area with diverse ethnic identities, such as Turkey.

Conclusion

The Glu298Asp eNOS gene polymorphism is associated with BD in the Turkish population. This relationship will allow for early diagnosis, and individual treatment approaches, in addition to allowing for an understanding of the disease. However, studies in more homogeneous groups with larger sample sizes, including the BD clinic, are required.

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