Endothelial Nitric Oxide Synthase Gene Polymorphisms in Behçet's Disease

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ABSTRACT. ***Objective.*** To analyze potential associations of Glu-Asp298 polymorphism in exon 7 and 4 a/b polymorphism in intron 4 of the endothelial nitric oxide synthase (eNOS) gene with susceptibility for Behçet's disease (BD).

Methods. Seventy-three consecutive Italian patients who satisfied the International Study Group criteria for BD and 135 healthy blood donor controls from the same geographic area were genotyped by polymerase chain reaction and allele-specific oligonucleotide techniques for eNOS polymorphisms in exon 7 and in intron 4.

Results. The distribution of the Glu-Asp298 genotype differed significantly between BD patients and controls ($p_{corr} = 0.00009$). Allele Asp298 was significantly more frequent in BD patients than in controls ($p_{corr} = 0.0006$, OR 2.1, 95% CI 1.5–3.3). The distribution of 4 a/b genotype was similar in patients and controls.

Conclusion. Our findings show that Glu-Asp298 polymorphism of eNOS gene is associated with BD susceptibility. (J Rheumatol 2002;29:535–40)

Key Indexing Terms:
BEHÇET'S DISEASE
NITRIC OXIDE PRODUCTION
ENDOTHELIAL DYSFUNCTION
eNOS POLYMORPHISMS

Behçet's disease (BD) is a multisystemic inflammatory disease of unknown cause characterized by recurrent oral aphthous ulcers, genital ulcers, uveitis, and skin lesions. Vasculitis is the pathological lesion common to most of the clinical manifestations of BD. Vascular injuries with thrombotic tendency are important pathological features of BD[1,2].

Abnormalities related to endothelial cell functions have been described in BD. Prostanoid synthesis in endothelial cells or vessel walls is impaired in BD[3,4], whereas plasma levels of thromboxane[3-5], von Willebrand factor antigen[6-8], and thrombomodulin[5,9,10] are higher compared to those of healthy controls. Endothelial cell dependent vasodilatation is significantly impaired in BD as shown by high resolution ultrasound imaging[11]. Nitric oxide (NO) production was found to be decreased in patients with active disease compared to the inactive period and the control group[12,13]. Therefore endothelial dysfunction may have a central role in the pathophysiology of BD.

Constitutive endothelial NO synthase (eNOS) is expressed in the endothelium, where it produces NO from L-arginine[14,15]. Studies suggest that the basal release of NO by endothelium mediates local vasodilatation[16], antagonizes platelet aggregation[17], and inhibits vascular smooth muscle cell proliferation[18] and platelet and leukocyte adhesion[19-21]. A genetically determined dysfunction of this important mechanism may explain many of the endothelial function abnormalities observed in BD and may be implicated in its pathogenesis.

The Glu-Asp298 polymorphism in exon 7 and the 4 a/b polymorphism in intron 4 of the eNOS gene are the most studied polymorphisms of the eNOS gene in those conditions where endothelial dysfunction plays a key pathogenic role, such as coronary atherosclerosis and thrombosis[22-27] and other ischemic/thrombotic conditions[27-31]. Interestingly, a functional role has been postulated for both polymorphisms[32-34].

We assessed the role of these 2 eNOS gene polymorphisms in the susceptibility to BD. We also investigated possible associations of these polymorphisms with different clinical features of BD.

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MATERIALS AND METHODS

Study population. Case patients were consecutive patients with BD who were followed at the Bologna, Ferrara, Milan, Potenza, Prato, Reggio Emilia, and Trento rheumatology, ophthalmology, and neurology units over a 3 year period (1997–99) who satisfied the International Study Group criteria for BD[35].

Seventy-three Italian patients with BD were identified and studied. The control group consisted of 135 healthy subjects who were unrelated blood donor volunteers. All the study subjects were Caucasians of Italian descent residing in Italy for at least one generation. No ethnic differences were present between patients and controls; none were of Jewish background. Informed consent was obtained from all patients and controls.

HLA class I typing. Serological HLA class I typing was performed by a standard microlymphocytotoxicity technique, using peripheral blood lymphocytes[36]. Fifty-six of the 73 patients with BD were typed for HLA-B51 allele. The control group consisted of 130 healthy blood donors.

DNA extraction and genotyping. Genomic DNA was extracted from samples of whole blood by standard method with phenol:chloroform:isoamyl alcohol (25:24:1)[37].

Genotyping of Glu-Asp298 was carried out using a polymerase chain reaction (PCR) restriction fragment length polymorphism technique[24]. PCR was performed at a total volume of 50 μl: 250 ng of genomic DNA, 1.5 U AmpliTaq DNA Polymerase (Perkin-Elmer), 20 pmol of each primer, 200 nmol of each dNTP on a Perkin-Elmer 9600 Thermal Cycler under the following conditions: an initial denaturation at 94˚C for 2 min, followed by 35 cycles at 94˚C for 30 s, 61˚C for 30 s, and 72˚C for 30 s. The final extension step was prolonged to 5 min. Genotyping of this polymorphism was investigated by amplification in exon 7 with 2 primers: 5′-AAGGCAGGAGACAGTGGATGGA-3′ (sense) and 5′-CCCAGTCAATCCCTTTGGTGCTCA-3′ (antisense) followed by Eco 24I (Ban II) (MBI Fermentas) restriction endonuclease digestion for 2 h at 37˚C and resolution by 2% agarose gel electrophoresis. The 248 bp PCR product was cleaved into 163 bp and 85 bp fragments in the presence of a G at nucleotide 894, which corresponds to wild-type Glu298 (Figure 1A).

Genotyping of variable number of tandem repeat (VNTR) was executed by PCR in a total volume of 50 μl: 250 ng of genomic DNA, 1.5 U AmpliTaq DNA Polymerase (Perkin-Elmer), 20 pmol of each primer, 200 nmol of each dNTP on a Perkin-Elmer 9600 Thermal Cycler under the following conditions: initial denaturation at 94˚C for 2 min, followed by 35 cycles at 94˚C for 1 min, 54˚C for 40 s, and 72˚C for 1 min. The final extension step was prolonged to 5 min. Genotyping of VNTR was performed by amplification in intron 4 with 2 primers: 5′-GGGAACCTCAGCCCAGTAGTGAA-3′ (sense)[38] and 5′-TCTCTTAGTGCTGTGGTCAC-3′ (antisense)[22] and visualized by 2.5% agarose gel electrophoresis. The 454 bp product (allele b) was associated at 5 tandem repeat and 427 bp (allele a) was associated at 4 tandem repeat (Figure 1B).

Statistical analysis. Statistical analysis was with the SPSS statistical package (SPSS Inc., Chicago, IL, USA). The frequencies of the alleles and genotypes among the patient and control groups were determined and were compared by chi-square test. Odds ratios were calculated together with their 95% confidence interval. Corrected p values were calculated by multiplying p by the number of alleles compared.

RESULTS

Table 1 shows the clinical and demographic characteristics of 73 Italian patients with BD.

The allele and genotype frequencies of eNOS 4 a/b and Glu-Asp298 polymorphisms are shown in Table 2. The distribution of the Glu-Asp298 genotype differed significantly between patients and controls (p = 0.00003, p_{corr} = 0.00009). Interestingly, Glu-Asp298 genotype distribution showed that the difference in allele distribution was related to a reduced frequency of Glu/Glu homozygosity in BD patients compared to controls, whereas Asp/Asp homozygosity and Glu/Asp heterozygosity were both higher in patients with BD. Allele Asp298 was significantly more frequent in patients with BD than in controls (p = 0.0003, p_{corr} = 0.0006).

Table 1. Demographic and clinical features of 73 Italian patients with Behçet's disease.

	N	%
Female/male	29/44	40/60
Mean age at disease onset ± SD, yrs	32 ± 12	
Mean disease duration ± SD, yrs	12 ± 8	
Oral ulcers	73	100
Papulopustular lesions	58	79.5
Eye lesions	52	71.2
Genital ulcers	42	57.5
Arthritis	34	46.6
Positive pathergy test*	7	33.3
Erythema nodosum	25	34.2
Central nervous system involvement	20	27.4
Vein thrombosis	21	28.8
Epididymitis	3	4.1

* Pathergy test was performed on 21 patients.

The frequency of Asp298 was significantly higher in BD patients than in controls (98.6% vs 74.1%; p = 0.00001, p_{corr} = 0.00002, OR 25.2, 95% CI 3.4–181.3). The frequency of Glu298 was significantly lower in patients (71.2 vs 83.7%; p = 0.04, OR 0.5, 95% CI 0.2–0.9); however, significance was lost after the correction of p value (p_{corr} = 0.08).

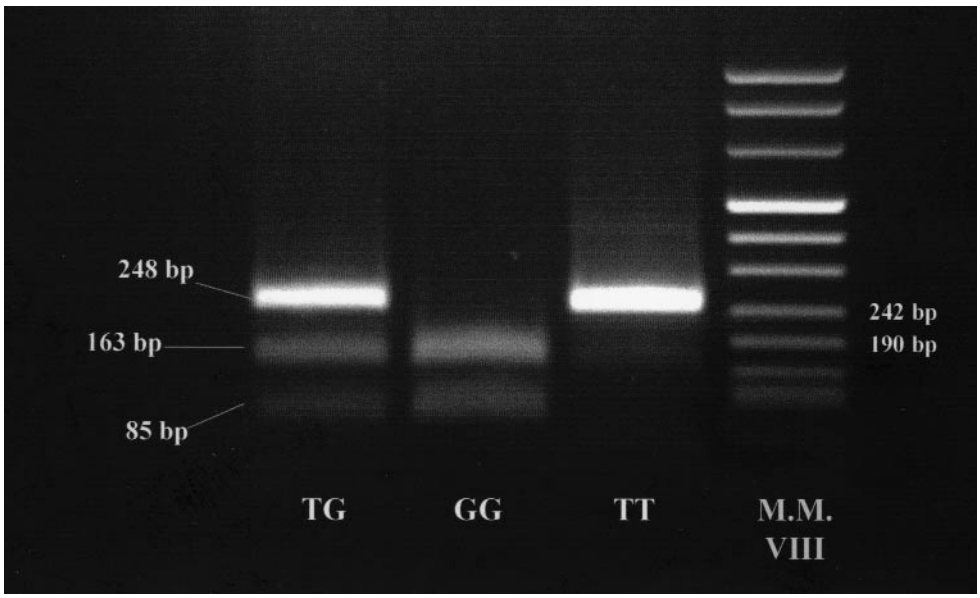
The distribution of allele and genotype frequencies of 4 a/b polymorphism did not differ significantly between BD patients and controls when the p value was corrected (Table 2).

We investigated eNOS 4 a/b and Glu-Asp298 polymorphism associations with BD stratifying on HLA-B51. HLA-B51 allele was available in 56 patients with BD. HLA-B51 was significantly higher in patients compared to controls (58.9 vs 19.2%; p = 0.0001, OR 6.0, 95% CI 3.0–12.0). The significant association with Asp298 was preserved in both HLA-B51+ patients (97.0 vs 74.1%; p = 0.003, p_{corr} = 0.006) and HLA-B51–patients (100 vs 74.1%; p = 0.002, p_{corr} = 0.004).

The associations between eNOS 4 a/b and Glu-Asp298 polymorphisms and clinical manifestations of BD defined in Table 1 were evaluated in the 73 BD patients, comparing patients with and without manifestations. No significant association was found, in particular within the subgroup with vein thrombosis (data not shown).

DISCUSSION

BD is a polygenic disease whose multiple genetic factors, in combination with environmental risk factors such as infectious agents, are probably of importance in determining



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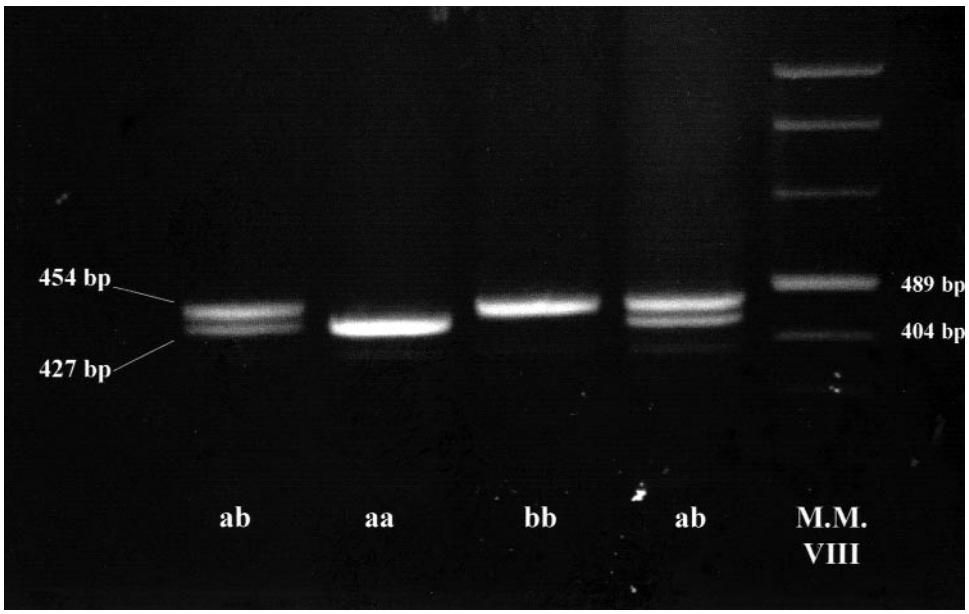

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Figure 1. A. PCR analysis to detect the Glu298Asp mutation G to T substitution at nucleotide position 894 of the eNOS cDNA. The size of the fragments produced by digestion with Ban II are shown: heterozygote (TG), homozygote (GG), and homozygote (TT). B. PCR polymorphisms of the 27 bp repeat in intron 4 of the eNOS gene. The band of 454 bp (allele b) indicating 5 repeats, the band of 427 bp (allele a) indicating 4 repeats: heterozygote ab, homozygote aa, and homozygote bb.

susceptibility. Although the pathological mechanism is not well understood, vasculitis is the pathological lesion common to most of the clinical manifestations. Thrombotic tendency is also an important pathological feature of BD, prominent in 20–30% of patients[1,2].

Various abnormalities related to endothelial cell functions have been described in BD. Prostanoid synthesis in endothelial cells or vessel walls was found to be impaired[3,4]. Chambers, *et al* evaluated endothelial function in BD, measuring brachial artery flow-mediated, endothelium-dependent vasodilatation (EDV). EDV was significantly impaired in patients with BD compared to healthy controls[11]. Brachial artery flow-mediated vasodilatation is exclusively related to shear stress-induced NO production[39]. NO is the major endothelium-derived vasodilator.

These observations indicate that endothelial NO activity

Table 2. Allele and genotype frequencies of 4 a/b and Glu-Asp298 polymorphisms of the eNOS gene in Italian patients with BD and controls.

Variable	Healthy Controls, n = 135 (%)	Behçet's Disease, n = 73 (%)	p	OR (95% CI)
Alleles				
4a	59/270 (21.9)	23/146 (15.8)	NS	0.7 (0.4–1.1)
4b	211/270 (78.1)	123/146 (84.2)	NS	1.5 (0.4–2.5)
Asp	122/270 (45.2)	93/146 (63.7)	0.0003	2.1 (1.5–3.3)
Glu	148/270 (54.8)	53/146 (36.3)	(p_{corr}= 0.0006)	0.5 (0.3–0.7)
Genotypes				
4a/a	13/135 (9.6)	1/73 (1.4)	0.04	
4b/a	33/135 (24.4)	21/73 (28.8)	(p_{corr} = NS)	
4b/b	89/135 (65.9)	51/73 (69.9)		
Asp/Asp	22/135 (16.3)	21/73 (28.8)	0.00003	
Glu/Asp	78/135 (57.8)	51/73 (69.9)	(p_{corr}= 0.00009)	
Glu/Glu	35/135 (25.9)	1/73 (1.4)		

is impaired in BD. Consistent with these findings, NO production was found to be decreased in patients with active BD compared to those with inactive disease and to controls[12,13].

A significant impairment of EDV was also found by Raza, *et al* in patients with primary systemic vasculitis (Wegener's granulomatosis, polyarteritis nodosa, and Churg-Strauss syndrome). A lack of difference between EDV in the subgroups with active and inactive disease was observed, although EDV improved after the induction of remission in the longitudinal study on a limited number of patients. These authors suggested that endothelial dysfunction mediated by NO production was also present in vasculitis and could be secondary to vasculitic process[40].

Endothelial dysfunction, as evaluated by EDV studies, occurs early in the development of atherosclerosis, even before the formation of plaque, and persists in the later stages. The constitutive eNOS is expressed in the endothelium, where it produces NO from L-arginine[14,15]. Several studies suggest that the basal release of NO by endothelium mediates local vasodilatation[16], antagonizes platelet aggregation[17], and inhibits vascular smooth muscle cell proliferation[18] and platelet and leukocyte adhesion[19-21]. These processes are relevant in the pathophysiology of vascular damage in both atherosclerosis and vasculitis.

As noted, the Glu-Asp298 polymorphism in exon 7 and the 4 a/b polymorphism in intron 4 of eNOS gene are the most studied polymorphisms of the eNOS gene in coronary atherosclerosis and thrombosis[22-27] and other thrombotic/ischemic conditions[27-31]. Asp298 and/or 4a polymorphisms have been found to be associated with angiographic evidence of coronary artery disease and myocardial infarction[22-27]. However, discordant results were found studying the association between ischemic cerebrovascular disease and these polymorphisms in different populations[29-31].

The 894 G/T substitution in exon 7 results in a glutamate or aspartate, respectively, at position 298 in the eNOS protein. Because glutamate and aspartate are conservative substitutions, it has been postulated that the polymorphism serves as a marker for a functional effect elsewhere in the eNOS gene or in its vicinity. Recently, Tesauro, *et al* showed that the eNOS gene with polymorphism at nucleotide 894 generates protein products with differing susceptibility to cleavage, suggesting that this polymorphism has a functional effect on the eNOS protein[32]. These authors demonstrated that the Asp298 variant is more susceptible to proteolytic cleavage than eNOS Glu298, and this might contribute to abnormally low NO generation in carriers of the Asp variant. Philip, *et al* recently showed that the pressure response to a systemic infusion of phenylephrine in patients undergoing cardiopulmonary bypass differed according to eNOS genotype[33]. Asp298 homozygotes had the most marked blood pressure increase. These findings would be consistent with carriers of the Asp298 allele having a reduced basal release of vasodilator NO. A functional role may be postulated also for 4 a/b polymorphism. Tsukada, *et al* found a strong association between this polymorphism and plasma NO levels; in particular the mean plasma NO level in the subjects homozygous for the allele a was nearly 20% lower than in the subjects with allele b[34].

If the Asp298 and 4a polymorphisms are associated with altered NO synthesis, this could provide a mechanism for promoting vessel wall damage not only in atherosclerosis but also in vasculitis. No study has evaluated if a genetic alteration related to a reduction in the amount or activity of eNOS could have a potential role in predisposing to vasculitis. We have studied both these polymorphisms in Italian patients with BD. We found that Asp298 was associated with susceptibility to BD, while no association was shown with 4 a/b polymorphism. We also evaluated if these polymorphisms were associated with specific clinical findings. However, no association with specific clinical manifestations, in particular with venous thrombosis, was found.

The strongest genetic association identified in Italian

patients with BD has been with HLA-B51 allele[41]. We investigated eNOS polymorphism associations with BD stratifying on HLA-B51. The association between BD and Asp298 seemed to be independent of the status of HLA-B51 allele.

In conclusion, Asp298 polymorphism of eNOS gene was found to be associated with BD. This finding is potentially important, but requires confirmation in other populations. Interestingly, the same polymorphism is also an identified genetic risk factor of coronary atherosclerosis. Our results imply that a similar genetically determined endothelial dysfunction, related to basal NO production, may predispose to both atherosclerotic and vasculitic process. This is possible because the reduced release of NO from the endothelium not only affects vascular tone, but also predisposes to coagulation and inflammation by promoting platelet aggregation and leukocyte adhesion. As a consequence, drugs that upregulate the eNOS enzyme could improve endothelial function in Behçet's disease and have a potential therapeutic effect, not only in atherosclerosis, but also in this vasculitic syndrome.

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