ACTA ABP BIOCHIMICA POLONICA

Vol. 61, No 2/2014
271–274
on-line at: www.actabp.pl

Regular paper

The relation of *PON*1-L55M gene polymorphism and clinical manifestation of Behcet's disease

Ahmet Dursun[1]✉, Salih Cicek[1], Fatih M. Keni[2], Sevim Karakas-Celik[1], Tuna Sezer[3] and Cevdet H. Altinyazar[4]

[1]Bulent Ecevit University, Medical Faculty, Medical Genetics Dept., Zonguldak, Turkey; [2]Istanbul Training and Research Hospital, Medical Genetics Department, Istanbul, Turkey; [3]Bulent Ecevit University, Medical Faculty, Dermatology Department, Zonguldak, Turkey; [4]Selcuk University, Selcuklu Medical Faculty, Dermatology Department, Konya, Turkey

Purpose: Behçet's disease is a multisystem disease characterized by recurrent oral and genital ulcers, relapsing uveitis, mucocutaneous, articular, gastrointestinal, neurologic, and vascular manifestations. Paraoxonase is believed to play an important role in protection of LDL and HDL particles from oxidation, in antioxidant effect against lipid peroxidation on cellular membranes, and in anti-inflammatory process. Lipid peroxidation and free oxygen radicals have been thought to play a role in pathogenesis of BD. The association of paraoxonase gene polymorphisms with Behçet's Disease in a group of Turkish patients with clinical manifestations and healthy controls has been investigated. Patients and Methods: Paraoxonase (*PON*-1-L55M) gene polymorphism was investigated in 50 Behcet patients and 50 healthy individuals with a PCR/RFLP method. Results: There were significant differences between patients and the control group in allele frequencies of the *PON*1 L55M polymorphism (p=0.04). Also, when patients were compared with the control group according to clinical manifestations, this statistical significance was getting sharper. Compared with the *PON*55 L allele, the M allele was associated with greater than 3.5 fold (OR 3.5, 95% CI 1.3–8.9) increased risk of ocular (OR 2.4, 95% CI 1.1–5.3), 2.4 fold joint and 3.1 fold (OR 3.1, 95% CI 1.1–8.4) central nervous system manifestations of BD. Conclusion The *PON* L55M gene polymorphism seemed to play a role in the pathogenesis of BD.

Key words: *PON*1 gene, Polymorphism, Behçet's disease

Received: 25 June, 2013; revised: 02 April, 2014; accepted: 10 April, 2014; available on-line: 16 June, 2014

INTRODUCTION

Behçet's disease (BD) is described as a chronic unclassified systemic vasculitis. Although it was originally established with recurrent oral and genital ulcers and uveitis, it is now accepted as a multisystem disorder also affecting all types and sizes of blood vessels, joints, lungs, central nervous system, and intestines (Sakane *et al*., 1999). In about 20–35% of cases of BD, diverse vascular complications, such as a deep vein thrombosis, myocardial infarction, arterial aneurysm, and arterial thrombus formation have been diagnosed (Kural-Seyahi *et al*., 2003). Vascular and cardiac events are partly due to atherosclerosis (Oztürk *et al*., 2006). In patients with BD, the mechanism of atherosclerosis may be attributed to lipid abnormalities. Lipids and lipoprotein peroxidation are considered to be important in the pathogenesis of atherosclerosis (Loeper *et al*., 1983). Their profiles and relation with atherogenesis was described in patients with BD (Orem *et al*., 1995; Mitamura *et al.,* 1988).

Paraoxonase 1 (PON1) is an enzyme exclusively located on high-density lipoprotein (HDL) in the serum (Ruiz *et al*., 1995). PON1 hydrolyzes organophosphate substrates and metabolizes lipid peroxides leading to protection against accumulation of low-density lipoprotein (LDL) that otherwise might lead to atherosclerotic plaque formation (Mackness *et al*., 1997). Major polymorphisms of *PON*1 include the replacement of Gln (Q) by Arg (R) at position 192, and that of Leu (L) by Met (M) at position 55. For the L55M *PON*1 polymorphism, the L allele carriers were found to have higher mRNA levels (Adkins *et al*., 1993; Humbert *et al*., 1993) and accordingly, the L allele carriers have significantly higher enzyme concentrations (Eckerson *et al*., 1983). When compared to PON55M isoform, PON55L is associated with higher serum activity, higher stability and resistance to proteolysis. Furthermore PON55L plays an important role in the packing of the protein correctly (Harel *et al*., 2007). A relationship between *PON*1 genotypes and the antioxidant activity of HDL has also been demonstrated (Kuremoto *et al*., 2003).

Functional polymorphisms in the *PON*1 gene are attractive candidates due to their impact on antioxidant activity of HDL and subsequently on inter-individual vascular disease susceptibility. Common polymorphisms in paraoxonase 1 gene are described as risk factors in a variety of vascular disorders including coronary artery disease and carotid artery stenosis. However, to our knowledge, no investigation has been undertaken on the association between the *PON*1 L55M single nucleotide polymorphism and BD. In this study, the possible associations between PON1 L55M polymorphism and Turkish BD patients and its clinical manifestations have been investigated.

MATERIALS AND METHOD

Fifty patients with Behçet's disease and fifty healthy, unrelated control subjects who were all Turkish were included in this study. Patients with Behçet's disease were

✉e-mail: ahmet_dursun@hotmail.com

Abbreviations: BD, Behçet's disease; PON1, Paraoxonase 1; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ROS, reactive oxygen products

Table 1. Distribution of the cases and the controls by sex and age, and clinical manifestations of BD

	Cases n (%)		Controls n (%)	*P* value
Total	50 (100)		50 (100)	
Age, years mean ± S.D.	38.5 ± 10.16		36.3 ± 10.03	p=0.27
Sex				p=0.50
Female	27 (52.0)		26 (54.0)	
Male	23 (48.0)		24 (46.0)	
Clinical Manifestation	Positive n (%)	Negative n (%)		
Oral Aphthae	50 (100)	–		
Genital Ulcers	47 (94)	3 (6)		
Positive Pathergy test	23 (46)	27 (54)		
Ocular Manifestations	16 (32)	34 (68)		
Joint Manifestations	34 (68)	16 (32)		
Central Nervous System Manifestations	14 (28)	36 (72)		
Cardiovascular Manifestations	8 (16)	42 (84)		
Deep Vein Thrombosis	8 (16)	42 (84)		
Gastrointestinal Manifestations	19 (38)	31 (62)		

all fulfilling three or more of the International Study Group criteria for BD and were clinically diagnosed by Department of Dermatology. Clinical characteristics of both populations are shown in Table 1.

Genomic DNA was extracted from peripheral blood leucocytes (200 μl of total blood) by using Macherey-Nagel Nucleospin blood® DNA extraction kit (Cat no. 740.951.250) according to manufacturer's instructions. The DNA purity and concentration were determined by spectrophotometric measurement of absorbance at 260 and 280 nm. The PCR product was amplified with primers *PON*1-F 5'-CCT GCA ATA ATA TGA AAC AAC CTG 3' and *PON*1-R 5' TGA AAG ACT TAA ACT GCC AGTC-3'. Amplifications were performed in 0.2 ml thin-wall tubes of 50 μl aliquots containing 50 mM KCl, 10 mM Tris/HCI, 1.5 mM $MgCl_2$, 0.5 μM of each of the four deoxynucleotides, 50 pmol of each primer (*PON*1-F and *PON*1-R), 1 U of *Taq* DNA polymerase and 20 ng genomic DNA. After an initial 4 min denaturation step at 94°C, 32 PCR cycles were run, each consisting of: 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. A 72°C elongation was performed for 4 min at end of the PCR cycles. The PCR products of subjects and positive/negative controls were checked on a 1.5% agarose gel for the assay completion and then the PCR products of 172 bp were digested with restriction enzyme *Nla*III by overnight incubation at 37°C.

*Nla*III recognizes the CATG sequence corresponding to the Met allele. The Leu/Leu homozygote was identified by the presence of an uncut 172 bp band, whereas the Leu/Met heterozygote produces all three bands (172, 106, and 66 bp) following restriction digestion. The digestion products were electrophoresed on 3.5% agarose gel and visualized by staining with ethidium bromide and evaluated using a gel documentation system (Syngene, Genegenius Bio Imaging System).

Statistical analysis. A case-control study was performed and allelic frequency of the polymorphism was calculated both in case and control samples. The χ^2 test was used to compare allele frequency of the *PON55* gene polymorphism between BD patients and controls. 95% confidence interval (CI) was calculated to compare BD risk around genotypes and alleles. P value less than 0.05 was considered as statistically significant. The software used for the calculations was SPSS version 18 (SPSS Inc., Chicago, IL).

RESULTS

Age and gender matched 50 subjects with Behçet's disease (26 women and 24 men), and 50 healthy control subjects (27 women and 23 men) were genotyped for the *PON55* (rs854560) SNP. The distribution of the genotypes in the controls was in Hardy-Weinberg equilibrium. The mean age (±S.D.) was 38.5±10.16 in patients, and 36.3±10.03 in control subjects (Table 1).

The frequencies of L and M alleles were 75.0% and 25.0% in cases, and 87.0 % and 13.0% in controls, respectively (Table 2) and the difference in allele frequency was significant (p=0.04). The relative risk for BD patients was more than 2.23 times higher (OR 2.23, 95% CI 1.07–4.66) in individuals with the *PON*55 M allele compared to the L allele.

Also, patients were compared with the control group according to clinical manifestations (Table 3 and 4). Frequency of the L allele was 65.6%, 73.5%, 67.9% and of the M allele was 34.4%, 26.5%, 32.1% in ocular, joint and central nervous system manifestations of BD patients, respectively. In the ocular, joint and central nervous system manifestations of BD patients, the frequency of the *PON*55 M allele was higher in comparison with that of the control group and the difference was significant (Table 3, p=0.01, p=0.04, p=0.02, respectively). Compared with the *PON*55 L allele, the M allele was associated with greater than 3.5 fold (OR 3.5, 95% CI 1.3–8.9) increased relative risk of ocular, 2.4 fold (OR 2.4, 95% CI 1.1–5.3) joint and 3.1 fold (OR 3.1, 95% CI 1.1-8.4) central nervous system manifestations of BD. The statistical significance was not present when other clinical manifestations of Behçet patients were compared with the control group (p>0.05).

Table 2. Genotypes and allele frequencies of PON55 and the risk of developing Behçet's disease

	Cases (N=50)	Controls (N=50)		
Variable	n (%)	n (%)	OR ‡	95% CI
Genotype				
LL	39 (62.0)	31 (78.0)	1 (reference)	
LM	13 (26.0)	9 (18.0)	1.817	0.688–4.803
MM	6 (12.0)	2 (4.0)	3.774	0.712–20.016
Allele				
L	75 (75.0)	87 (87.0)	1 (reference)	
M	25 (25.0)	13 (13.0)	2.23	1.07–4.66

Table 3. The distribution of PON55 genotype frequency in healthy controls and patients according to clinical manifestations.

	PON55 Genotype n (%)			*p*
	LL	LM	MM	
Control	39 (78.0)	9 (18.0)	2 (4.0)	
Genital Ulcers	31 (66.0)	11 (23.4)	5 (10.6)	0.3
Positive Pathergy test	14 (60.9)	7 (30.4)	2 (8.7)	0.3
Ocular Manifestations	8 (50.0)	5 (31.3)	3 (18.8)	0.07
Joint Manifestations	20 (58.8)	10 (29.4)	4 (11.8)	0.1
Central Nervous System Manifestations	7 (50.0)	5 (35.7)	2 (14.3)	0.1
Cardiovascular Manifestations	4 (50.0)	4 (50.0)	0 (0)	0.1
Deep Vein Thrombosis	4 (50.0)	4 (50.0)	0 (0)	0.1
Gastrointestinal Manifestations	11 (57.9)	5 (26.3)	3 (15.8)	0.1

Table 4. Distrubition of the PON55 allele frequency in healthy controls and patients according to clinical manifestations.

	PON55		P	OR (% 95 CI)
	L ALLELE N (%)	M ALLELE N (%)		
Control	87 (87.0)	13 (13.0)		
Genital Ulcers	73 (77.7)	21 (22.3)	0.09	1.9 (0.9–4.1)
Positive Pathergy test	35 (76.1)	11 (23.9)	0.14	2.1 (0.8–5.1)
Ocular Manifestations	21 (65.6)	11 (34.4)	0.01*	3.5 (1.3–8.9)
Joint Manifestations	50 (73.5)	18 (26.5)	0.04*	2.4 (1.1–5.3)
Central Nervous System Manifestations	19 (67.9)	9 (32.1)	0.02*	3.1 (1.1–8.4)
Cardiovascular Manifestations	12 (75.0)	4 (25.0)	0.25	2.2 (0.6–7.9)
Deep Vein Thrombosis	12 (75.0)	4 (25.0)	0.25	2.2 (0.6–7.9)
Gastrointestinal Manifestations	27 (71.1)	11 (28.9)	0.25	2.2 (0.6–7.9)

DISCUSSION

The etiology and pathogenesis of Behçet's disease (BD) are not yet well understood. Histopathologic studies have established that vasculitis is the predominant lesion, affecting both the *vessel* wall and perivascular tissues (Sakane *et al*., 1999). Growing evidence indicates that oxidative stress is increased in BD, relating to overproduction of reactive oxygen products (ROS) and decreased efficiency of antioxidant resistance (Kose *et al*., 1951; Orem *et al*., 1997; Niwa *et al*., 1982). It has been demonstrated in *in vitro* studies that activated leucocytes form a large number of free oxygen radicals and this in turn causes endothelial cell damage. ROS can attack and damage a variety of critical biological molecules, including lipids, essential cellular proteins and DNA. Under oxidative stress, LDL and other serum lipoproteins, including HDL, are prone to lipid peroxidation. Recent studies show that lipid peroxidation in the serum of patients with BD was increased (Kose *et al*., 1951; Orem *et al*., 1997; Kose *et al*., 2001).

PON-1 is a serum enzyme bound with high density lipoproteins (HDL) and has been closely linked to the control of oxidative stress and inflammation, mainly at the circulation level (NgD *et al*., 2008). PON 1 protects lipoproteins against oxidative stress and makes possible to metabolize lipid peroxides that are largely distributed among tissues such as the liver, kidney, and intestine; but it is also present in plasma. There is a 10- to 40-fold inter-individual variability in serum PON1 activity (Hum-

bert *et al*., 1993). *PON1* gene polymorphism is one of the sources of this variability. When compared to *PON*1-55M allele, *PON*1-55L is correlated with higher PON1 activity and mRNA levels (Leviev *et al*., 1997; Li *et al*., 2000; Leviev *et al*., 2001). Karakucuk and coworkers (2004) and Mungan (2006) and coworkers found a decreased serum PON1 activity in BD patients in comparison with healthy controls. Decreased PON1 could explain the increased lipid peroxidation and oxidative stress observed in BD. This suggests a pathogenic mechanism that is supported by our study, where we show a significant correlation between the *PON*1 gene polymorphism and BD patients. Our study shows that carriers of the *PON*1 M allele have a 2.23 fold increased relative risk for developing BD. To our knowledge, this is the first study that investigates the possible associations between *PON* 55 polymorphism with Behçet's patients in a Turkish population.

PON1 is an HDL-associated enzyme which is able to hydrolyze organophosphates. Due to its functions in protecting LDL against oxidation, PON1 is also an antioxidant. Decrease in the levels of paraoxonase enzyme is a great risk for patients with cardiovascular diseases, rheumatoid arthritis, gout, and age-related macular degeneration (Ekinci *et al*., 2009; Jiang *et al*., 2011). Karakucuk et al. also found a decreased serum PON1 activity in BD patients with ocular involvement in comparison with healthy controls. PON is also important in metabolism as an organophosphate hydrolyser. Thus, PON1 protects the nervous system against organophosphate toxicity. Therefore, when patients with ocular, joint and central nervous system involvement were compared to the control group, this statistical significance was getting sharper.

Although the result of this study is statistically significant, because of the rarity of the Behçet's disease the sample size is considered as a limitation. In addition, further studies with different ethnic populations should be performed to validate whether there is a relationship between Behcet's disease and PON1 L55M gene polymorphisms.

Our findings suggest that *PON*1 L55M polymorphism is associated with an increased relative risk for BD. This finding was getting sharper when ocular, joint and central nervous system involvement was considered separately. Polymorphism in the *PON*1 gene might contribute to the reduced PON activity that causes increased lipid peroxidation and oxidative stress and inflammatory endothelial changes observed in patients with BD.

REFERENCES

Abuja PM, Albertini R, Esterbauer H (1997) Simulation of the induction of oxidation of low-density lipoprotein by high copper concentrations: evidence for a nonconstant rate of initiation. *Chem Res Toxicol* **10**: 644–651.

Adamson DC, Wildemann B, Sasaki M, Glass JD, McArthur JC, Christov VI, Dawson TM, Dawson VL (1996) Immunologic NO synthase: elevation in severe AIDS dementia and induction by HIV-1 gp41. *Science* **274**: 1917–1921.

Adkins S, Gan KN, Mody M, et al (1993). Molecular basis for the polymorphic forms of human serum paraoxonase/arylesterase: glutamine or arginine at position 191, for the respective A or B allozymes. *Am J Hum Genet* **52**: 598–608.

Eckerson HW, Wyte CM, La Du BN (1983). The human serum paraoxonase/ arylesterase polymorphism. *Am J Hum Genet* **35**: 1126–38.

Ekinci D, Beydemir S (2009) Evaluation of the impacts of antibiotic drugs on PON 1; a major bioscavenger against cardiovascular diseases. *Eur J Pharmacol* **617**: 84-89.

Freitas JP, Filipe P, Yousefi A, Emerit I, Guerra Rodrigo F (1998) Oxidative stress in Adamantiades-Behcet's disease. *Dermatology* **197**: 343–348.

Harel M, Brumshtein B, Meged R, Dvir H, Ravelli RB, McCarthy A, et al (2007). 3-D structure of serum paraoxonase 1 sheds light on its activity, stability, solubility and crystallizability. *Arh Hig Rada Toksikol* **58**: 347–353.

Humbert R, Adler DA, Disteche CM, et al (1993). The molecular basis of the human serum paraoxonase activity polymorphism. *Nat Genet* **3**: 73–76.

Jiang XL, Li M, Zhou JG, Yang QB, Du LJ, Du J (2011). Plasma paraoxonase-1, oxidized low-density lipoprotein and lipid peroxidation levels in gout patients. *Cell Biochem Biophys*. **61**: 461–466.

Karakucuk S, Baskol G, Oner AO, Baskol M, Mirza E, Ustdal M (2004) Serum paraoxonase activity is decreased in the active stage of Behçet's disease. *Br J Ophthalmol* **88**: 1256–1258.

Kose K, Dogan P, Ascioglu M, Erkilic K, Ascioglu O (1951) Oxidative stress and antioxidant defenses in plasma of patients with Behcet's disease. *Tohoku J Exp Med* **76**: 239–248.

Kose K, Yazici C, Assioglu O (2001) The evaluation of lipid peroxidation and adenosine deaminase activity in patients with Behcet's disease. *Clin Biochem* **34**: 125–129.

Kural-Seyahi E, Fresko I, Seyahi N, Ozyazgan Y, Mat C, Hamuryudan V *et al.* (2003) The long-term mortality and morbidity of Behcet syndrome: a 2-decade outcome survey of 387 patients followed at a dedicated center Medicine (Baltimore) **82**: 60–76.

Kuremoto K, Watanabe Y, Ohmura H, Shimada K, Mokuno H, Daida H (2003) R/R genotype of human paraoxonase (PON1) is more protective against lipoprotein oxidation and coronary artery disease in Japanese subjects. *J Atheroscler Thromb* **10**: 85–92.

Leviev I, Deakin S, James RW (2001) Decreased stability of the M54 isoform of paraoxonase as a contributory factor to variations in human serum paraoxonase concentrations. *J Lipid Res* **42**: 528–535.

Leviev I, Negro F, James RW (1997) Two alleles of the human paraoxonase gene produce different amounts of mRNA. An explanation for differences in serum concentrations of paraoxonase associated with the (Leu-Met54) polymorphism. *Arterioscler Thromb Vasc Biol* **17**: 2935–2939.

Li WF, Costa LG, Richter RJ, Hagen T, Shih DM, Tward A, Lusis AJ, Furlong CE (2000) Catalytic efficiency determines the *in-vivo* efficacy of PON1 for detoxifying organophosphorus compounds. *Pharmacogenetics* **10**: 767–79.

Loeper J, Ement J, Goy J, et al (1983) Lipid peroxidation during human atherosclerosis. *IRCS Med Sci* **11**: 1035–43.

Mackness B, Mackness MI, Arrol S, Turkie W, Durrington PN (1997). Effect of the molecular polymorphisms of human paraoxonase (PON1) on the rate of hydrolysis of paraoxon. *Br J Pharmacol* **122**: 265–268.

Mitamura T, Ohmo S, Ariga H, et al (1988) Lipoprotein cholesterol concentrations in patients with Behcet's disease. *Clin Chim Acta* **175**: 277–284.

Mungan AG, Can M, Açikgöz S, Eştürk E, Altinyazar C (2006) Lipid peroxidation and homocysteine levels in Behçet's disease. *Clin Chem Lab Med* **44**: 1115–1118.

NgD S, ChuT, Esposito B, et al (2008) Paraoxonase-1 deficiency in mice predisposes to vascular inflammation, oxidative stress, and thrombogenicity in the absence of hyperlipidemia. *Cardiovasc Pathol* **17**: 226–322.

Niwa Y, Miyake S, Sakane T, Shingu M, Yokoyama M (1982). Autooxidative damage in Behcet's disease: endothelial cell damage following the elevated oxygen radicals generated by stimulated neutrophils. *Clin Exp Immunol* **49**: 247–255.

Orem A, Efe H, Deger O, Cimsit G, Uydu HA, Vanizor B (1997) Relationship between lipid peroxidation and disease activity in patients with Behcet's disease. *Dermatol Sci* **16**: 11–16.

Orem A, Deger O, Cimsit G, et al (1995) Plasma lipoprotein (a) and its relationship with disease activity in patients with Behçet's disease. *Eur J Clin Chem Clin Biochem* **33**: 473–478.

Orem A, Deger O, Memis O, et al (1995) Lp(a) lipoprotein levels as a predictor of risk for thrombogenic events in patients with Behçet's disease. *Ann Rheum Dis* **54**: 726–729.

Oztürk MA, Oktar SO, Unverdi S, Ureten K, Göker B, Haznedaroğlu S, *et al.(2006)* Morphologic evidence of subclinical atherosclerosis obtained by carotid ultrasonography in patients with Behçet's disease. *Rheumatol Int* **26**: 867–872.

Ruiz J, Blanche H, James RW, Garin MC, Vaisse C, Charpentier G, Cohen N, Morabia A, Passa P, Froguel P (1995). Gln-Arg192 polymorphism of paraoxonase and coronary heart disease in type 2 diabetes. *Lancet* **346**: 869–872.

Sakane T, Takeno M, Suzuki N, and Inaba G (1999) Behçet's disease. *The New England Journal of Medicine* **341**: 1284–1291.