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The paraoxonase 1 (*PON1*) gene polymorphisms in coronary artery disease in the southeastern Turkish population

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Aim: To investigate the association between the paraoxonase 1 (*PON1*) c.163T>A and c.575A>G polymorphisms and coronary artery disease (CAD), and whether specific polymorphisms in the *PON1* gene are associated with CAD in the southeastern Turkish population. Many different genetic and clinical factors have been identified as causes or contributors to atherosclerosis. Complex diseases such as CAD, hypertension, and diabetes are usually caused by an individual's susceptibility to various genes, environmental factors, and the interactions between them. The *PON1* enzyme has been implicated in the pathogenesis of atherosclerosis and CAD.

Materials and methods: Enrolled in this study were 61 patients with CAD and 103 healthy individuals; their DNA was isolated. A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was used to determine the frequencies of the *PON1* gene (NM_000446.5, GI: 5444) c.163T>A (*Hsp*92IIT>A) and c.575A>G (*AlwI*A>G) polymorphisms in CAD cases.

Results: Our data indicated that the body mass index (BMI) (27.5 ± 5.7), low-density lipoprotein (LDL-C) values (127.0 ± 45.8), and frequencies of diabetes (13.0) and hypertension (35.0) were significantly higher in CAD patients than in the control subjects (25.9 ± 4.1; 105.6 ± 26.3; 2.0; and 19.0, respectively) ($P < 0.05$). No significant differences were observed in the frequencies of the c.163T>A and c.575A>G genotypes of the *PON1* gene in CAD patients compared to healthy individuals ($P > 0.05$).

Conclusion: The *PON1* gene c.163T>A and c.575A>G polymorphisms did not represent an important risk factor for this disease in the southeastern Turkish population.

Key words: *PON1* gene, polymorphism, coronary artery disease, PCR, RFLP

Güneydoğu Türk toplumunda koroner arter hastalığında paraoxonaz (*PON1*) geni polimorfizmleri

Amaç: Arteriyoskleroze sebep veya katkı yapan birçok farklı genetik ve kliniksel faktör, tanımlanmıştır. Koroner arter hastalığı (KAH), hipertansiyon ve diyabet gibi karmaşık hastalıklar, çoğunlukla çeşitli genler, çevresel faktörler ve bunlar arasındaki etkileşimlerin bireysel duyarlılığı sonucu oluşurlar. Paraksonaz 1 (*PON1*) enzimi, aterosklerozis ve KAH'ın patojenezinde rol oynar. Bu çalışmanın amacı, *PON1* geni c.163T>A ve c.575A>G polimorfizmleri ile KAH arasındaki ilişkiyi araştırmak ve Güneydoğu Türk toplumunda *PON1* geni özel polimorfizmleri ile KAH arasında ilişkinin olup olmadığını belirlemektir.

Yöntem ve gereç: Çalışmaya KAH'lı 61 hasta ile sağlıklı 103 birey dahil edildi ve bunların DNA'ları elde edildi. KAH vakalarında *PON1* geni (NM_000446.5, GI: 5444) c.163T>A (*Hsp*92IIT>A) ve c.575A>G (*AlwI*A>G) polimorfizm sıklıklarının belirlenmesinde polimeraz zincir reaksiyonu-restriksiyon parça uzunluk polimorfizmi (PCR-RFLP) tekniği kullanıldı.

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Bulgular: Sonuçlarımız, vücut kitle indeksi (BMI) (27,5 ± 5,7), düşük-yoğunluklu lipoprotein (LDL-C) (127,0 ± 45,8), diyabet (13,0) ve hipertansiyon (35,0) sıklıklarının KAH'lı hastalarda kontrol bireylerine (sırasıyla 25,9 ± 4,1; 105,6 ± 26,3; 2,0; ve 19,0) göre anlamlı şekilde yüksek olduğunu gösterdi (P < 0,05). KAH'lı hastaların *PON1* geni c.163T>A ve c.575A>G genotip sıklıkları, sağlıklı bireylerle karşılaştırıldığında herhangi bir anlamlı farklılık bulunmadı (P > 0.05).

Sonuç: *PON1* geni c.163T>A ve c.575A>G polimorfizmleri, güneydoğu Türk toplumunda bu hastalık için önemli bir risk faktörü göstermedi.

Anahtar sözcükler: *PON1* geni, polimorfizm, koroner arter hastalığı, PCR, RFLP

Introduction

Coronary artery disease (CAD) is a common and complex disorder, and it has become a major source of morbidity and mortality in different parts of the world (1). Among genetic factors, the gene encoding human paraoxonase 1 (*PON1*) has been implicated in conferring genetic susceptibility to CAD (2).

Genetic polymorphisms that affect *PON1* could be predisposing risk factors both in environmental toxicology and in cardiovascular diseases. Human *PON1* has 2 genetic polymorphisms giving rise to amino acid substitutions at positions 55 (c.163T>A) and 192 (c.575A>G). The c.575A>G polymorphism is the major determinant of the *PON1* activity against organophosphates. The c.163T>A polymorphism also modulates its activity (3).

The paraoxonase (*PON*) gene family in mammals includes at least 3 members: *PON1*, *PON2*, and *PON3* (4). The 3 *PON* genes share about 65% similarity at the amino acid level and are located adjacent to each other on chromosome 7 (7q21.3) in humans (5). Both *PON2* and *PON3* possess antioxidant properties and lactonase activity, but unlike *PON1*, they lack the paraoxon or phenyl acetate-hydrolyzing activity (6). The *PON1* gene has 2 common polymorphisms in the coding region, which lead to a glutamine-to-arginine substitution at position c.575A>G and a leucine-to-methionine substitution at position c.163T>A. Both independently influence *PON1* activity and have been referred to as the molecular basis for this interindividual variability (7).

CAD has a multifactorial etiology involving physiological, environmental, and genetic factors, leading to increased susceptibility (8). More recently, there have been many studies investigating the association between the *PON1* gene polymorphisms and CAD. However, the results are controversial. A great number of studies suggest that there is not an association between the genotype and allele of the *PON1* gene c.163T>A polymorphic site and CAD, as observed in different populations (9-16). On the other hand, some other studies show an association between CAD and the c.163T>A polymorphisms of the *PON1* gene in various populations (17-22).

Additionally, some studies reported that the *PON1* c.575A>G polymorphism does not represent a risk factor for CAD (9,13-17,19-21,23). In parallel, others report that this same polymorphic site is an important risk factor for CAD (10,11,18,24-26).

Ex vivo, the *PON1* polymorphisms are important in determining the capacity of high-density lipoprotein (HDL) to protect low-density lipoprotein (LDL) against oxidative modification in vitro, which may explain the relationship between the *PON1* alleles and coronary heart disease in case-control studies (3). The coding region polymorphisms c.163T>A and c.575A>G of the *PON1* gene showed no association with HDL (27).

The aim of this study was to investigate whether *PON1* polymorphisms are important risk factors of CAD in the southeastern Turkish population.

Materials and methods

Subjects and DNA extraction

This study was designed in Şanlıurfa Province in the southeastern Anatolia region of Turkey between September 2008 and August 2009.

Enrolled in this study were 61 patients with CAD (18 females, mean age: 62.8 ± 8.4; and 43 males, mean age: 62.6 ± 9.4) who had 1 or more coronary lesions causing ≥50% stenosis, from the Regional Referral Hospital of Harran University. Randomly selected from blood donors were 103 healthy controls (50

females, mean age: 33.0 ± 10.3; and 53 males, mean age: 32.5 ± 9.9) who did not have any diseases or clinical evidence of CAD or other disorders in the family history of their first-degree relatives.

Detailed demographic information, including anthropometric measurements, cardiovascular risk factors, and medication use, were recorded for all participants. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters (kg/m^2). The levels of triglyceride (TG), total cholesterol (TC), HDL-cholesterol (HDL-C), and LDL-cholesterol (LDL-C) were determined using commercially available assay kits with an Abbott Aeroset autoanalyzer (Abbott®, Illinois, USA).

We matched the CAD patients and healthy controls for age and sex. EDTA-blood was taken from these individuals, and genomic DNA was extracted from whole blood leukocytes using the standard salting-out procedure (28).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique

The c.163T>A (g.12801T>A, rs854560, *PON1* 55) and c.575A>G (g.34650T>C, g.21439A>G, *PON1* 192) polymorphic sites of the *PON1* (NM_000446.5; GI: 5444) gene were investigated using the PCR-RFLP technique. The PCR reactions were performed in 10 mL of reaction volume, including 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate (dNTPs; Fermentas, St. Leon-Rot, Germany), 0.2 µM of each primer (Table 1) (29) (Bio Basic Inc., Ontario, Canada), 30 ng of DNA, and 0.5 U of Taq DNA polymerase (Fermentas). The PCR program for these 2 polymorphic sites was performed at 94 °C for 3 min (initial denaturation), 30 cycles, with 94 °C for 30 s, 58 °C and 63 °C (c.163T>A and c.575A>G, respectively) for 30 s, 72 °C for 40 s, and 72 °C for 5 min (final extension).

Separately digested was 10 µL of PCR product in a 30-µL volume for c.163T>A and c.575A>G with 2 U of *Hsp*92II and *Alw*I (Fermentas), respectively, at 37 °C for 2 h.

The digested PCR products were separated on 3% agarose gel and analyzed using the Alpha Imager System (Alpha Innotech, San Leandro, California, USA). The digested *PON1* c.163 T allele yielded 1 fragment of 171 bp, and the A allele yielded 2 fragments of 127 and 44 bp (Figure 1). The c.575 A allele yielded 1 fragment of 99 bp, and the G allele yielded 2 fragments of 63 and 36 bp (Figure 2).

Statistical analysis

Student's t-test and chi-square test were used to determine differences in the means of the demographic and clinical profiles. Genotype and allele frequencies of *PON1* c.163T>A and c.575A>G were tested for Hardy-Weinberg equilibrium by employing the chi-square test. Genotype and allele frequencies of these polymorphisms were analyzed with Fisher's exact test. Statistical significance was defined as $P < 0.05$.

Ethics

Informed consent was obtained from all patients before they were recruited to the study. The study was approved by the Institutional Ethics Committee of Harran University.

Results

The baseline characteristics of the patients and controls are presented in Table 2. The patients in the CAD group were older than the healthy controls (P = 0.001). The BMI, LDL-C, and prevalence of diabetes and hypertension were significantly higher in the CAD group than in the healthy subjects (P = 0.039, P

Table 1. Primer sequences used in this study.

PON1 gene SNPs	Primer sequences	PCR products (bp)
c.163T>A	5'-GAAGAGTGATGTATAGCCCCAG-3' 5'-TTTAATCCAGAGCTAATGAAAGCC-3'	171
c.575A>G	5'-TATTGTTGCTGTGGGACCTGAG-3' 5'-CACGCTAAACCCAAATACATCTC-3'	99

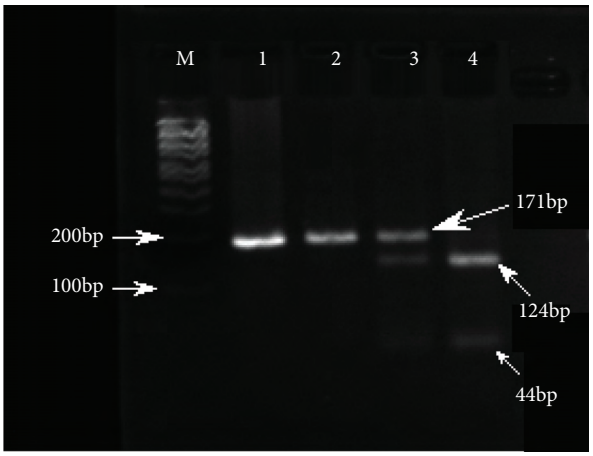

Figure 1. The restriction profile of the *PON1* gene *Hsp*192II (c.163T>A). Lane M: DNA marker (100-1500 bp, Bio Basic Inc., Canada); lane 1: undigested PCR product; lane 2: TT genotype (homozygous, wild-type); lane 3: TA genotype (heterozygous); lane 4: AA genotype (homozygous, polymorphic).

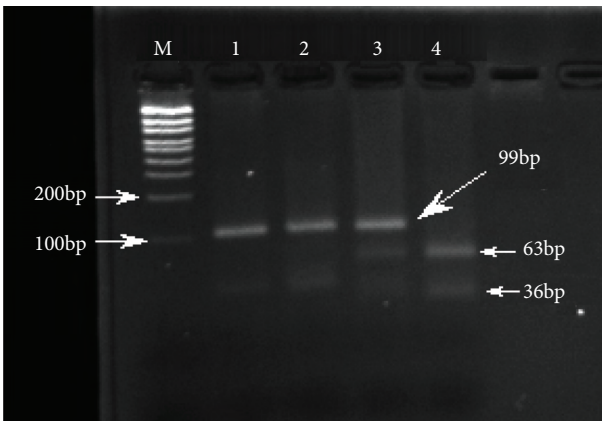

Figure 2. The restriction profile of the *PON1* gene *Alw*I (c.575A>G). Lane M: DNA marker (100-1500 bp, Bio Basic Inc., Canada); lane 1: undigested PCR product; lane 2: AA genotype (homozygous, wild-type); lane 3: AG genotype (heterozygous); lane 4: GG genotype (homozygous, polymorphic).

Table 2. The demographic parameters in the patients with CAD and the control group.

	CAD patients (n = 61)	**Controls (n = 103)**	**P-value**
Gender (M/F)	43/18	50/53	0.017
Age (years)	62.6 ± 9.1	32.7 ± 10.0	0.001
BMI (kg/m^2)	27.5 ± 5.7	25.9 ± 4.1	0.039
TG (mg/dL)	153.2 ± 63.5	168.7 ± 47.5	0.079
HDL-C (mg/dL)	37.6 ± 12.7	38.0 ± 9.3	0.797
LDL-C (mg/dL)	127.0 ± 45.8	105.6 ± 26.3	0.001
Diabetes	13	2	0.001
Smoking	10	17	0.985
Hypertension	35	19	0.001

Values are mean ± standard deviation, M: male, F: female.

= 0.001, P = 0.001, and P = 0.001, respectively), while there were no statistically significant differences in TG, HDL-C, or prevalence of current cigarette smoking (P = 0.079, P = 0.797, and P = 0.985, respectively).

The genotype frequencies observed for the c.163T>A and c.575A>G polymorphisms among this study population did not differ significantly from those expected under the Hardy-Weinberg equilibrium (P > 0.05). For the c.163 polymorphism, although the AA genotype (16.4%) and A allele (43.4%) frequencies in the CAD individuals were higher than those in the healthy individuals (12.6%, and 36.4%, respectively), these differences were not significant (P = 0.501). Furthermore, there was no significant difference between CAD patients and healthy controls for the c.575 GG genotype (13.1% vs. 12.6%) and G allele (36.1% vs. 36.9%) frequencies (Table 3).

Table 3. *PON1* gene polymorphisms in a Turkish population with CAD and the control group.

SNP genotype/allele	CAD patients (n = 61)	Healthy controls (n = 103)	X^2	OR (95% CI)	P-value
c.163T>A					
Genotypes					
TT	18 (29.5%)	41 (39.8%)		Reference	
TA	33 (54.1%)	49 (47.6%)	0.653	1.299 (0.688-2.451)	0.419
AA	10 (16.4%)	13 (12.6%)	0.452	1.357 (0.556-3.316)	0.501
Alleles					
T	69 (56.6%)	131 (63.6%)		Reference	
A	53 (43.4%)	75 (36.4%)	1.594	1.342 (0.850-2.119)	0.126
c.575A>G					
Genotypes					
AA	25 (41.0%)	40 (38.8%)		Reference	
AG	28 (45.9%)	50 (48.5%)	0.107	0.899 (0.477-1.697)	0.435
GG	8 (13.1%)	13 (12.6%)	0.008	1.045 (0.407-2.685)	0.553
Alleles					
A	78 (62.3%)	130 (63.1%)		Reference	
G	44 (36.1%)	76 (36.9%)	0.023	0.865 (0.606-1.537)	0.488

Abbreviations: CAD = coronary artery disease, X^2 = chi-squared, OR = odds ratio, CI = confidence interval, SNP = single nucleotide polymorphism.

On one hand, there was no significant difference between CAD patients with (AA) or without (TT, wild-type) the polymorphic genotype in terms of age, BMI, TG, HDL-C, LDL-C, diabetes, current cigarette smoking, and hypertension for the c.163T>A polymorphism ($P > 0.05$). On the other hand, there was a statistically significant difference between CAD patients with (GG) or without (AA, wild-type) the polymorphic genotype in terms of hypertension prevalence for the c.575A>G polymorphism ($P < 0.05$), except for age (Table 4).

Table 4. The demographic and clinical profiles with and without polymorphic genotype for the *PON1* gene in patients with CAD.

	c.163T>A			c.575A>G		
Genotype	TT	AA	P-value	AA	GG	P-value
Subjects (n)	18	10		25	8	
Age (years)	63.4 ± 7.7	63.8 ± 8.1	0.895	59.9 ± 8.6	68.7 ± 4.8	0.011
BMI (kg/m^2)	26.7 ± 6.0	29.0 ± 9.0	0.788	27.9 ± 6.2	28.1 ± 5.8	0.962
TG (mg/dL)	159.9 ± 69.9	145.3 ± 58.8	0.580	148.4 ± 62.1	115.0 ± 52.9	0.182
HDL-C (mg/dL)	40.2 ± 15.0	38.6 ± 15.2	0.758	37.9 ± 13.6	41.3 ± 22.7	0.618
LDL-C (mg/dL)	124.8 ± 42.4	119.5 ± 27.3	0.724	132.5 ± 52.3	107.0 ± 29.3	0.201
Diabetes	2	1	0.927	6	1	0.429
Smoking	3	2	0.825	7	0	0.076
Hypertension	12	5	0.387	15	4	0.447

Values are mean ± standard deviation; TT (*Hsp*92II) and AA (*Alw*I) genotypes: wild-types, not polymorphic; AA (*Hsp*92II) and GG (*Alw*I) genotypes: polymorphic.

Discussion

CAD is the leading cause of mortality worldwide (2). A great number of previous studies have provided evidence to propose that the *PON1*-c.163T>A (at position 55) and *PON1*-c.575A>G (at position 192) polymorphisms may be associated with CAD risk in the general population, due to the PON1 enzyme's role in lipid metabolism (11). Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries (6). However, the genetic factors underlying this form of cardiovascular disease are complex and not clearly established (17).

Upon investigating the effects of 2 exonic polymorphic sites of the *PON1* gene on CAD risk in the southeastern Turkish population, we did not establish any association of the *PON1* c.163T>A and c.575A>G genotypes and alleles with CAD. Although the frequencies of the c.163 AA genotype (16.4%) and A allele (43.4%) in CAD patients were higher than those in healthy subjects (12.6% and 36.4%, respectively), we did not find any significant difference when CAD patients were compared with healthy individuals ($P > 0.05$). Similarly, we did not observe any association between CAD and the frequencies of the *PON1* c.575 GG genotype and G allele ($P > 0.05$). Although several studies on its association with cardiovascular diseases have been performed, inconsistent results have been obtained from different populations. We here, however, did not determine an association with sex in either study group (data not shown).

Hong et al. in a Japanese population (9), Allebrandt et al. in a Euro- and an Afro-Brazilian population (10), Agrawal et al. in an Indian population (11), Arca et al. in an Italian population (12), Mackness et al. in a British population (13), Cascorbi et al. in a German population (14), Mendonça et al. in a Portuguese population (15), and Thyagarajan et al. in an American population (16) showed that genotype distributions and allele frequencies of the c.163T>A polymorphism were not significantly different between control subjects and patients. However, Oliveira et al. in a Brazilian population (17); Özkök et al. (18), Taşkiran et al. (19), and Kaman et al. (20) in Turkish populations; Chi et al. in a Chinese population (21), and Malin et al. in a Finnish (23) population showed that genotype distributions and allele frequencies of the c.163T>A polymorphism were significantly different between control subjects and patients.

On the other hand, Hong et al. in a Japanese population (9); Mackness et al. in British population (13); Cascorbi et al. in a German population (14); Mendonça et al. in a Portuguese population (15); Thyagarajan et al. (16) in an American population; Oliveira et al. in a Brazilian population (17); Taşkiran et al. (19), Kaman (20), and Aynacioglu (22) in Turkish populations; and Malin et al. in a Finnish population (23) showed that genotype distributions and allele frequencies of the c.575A>G polymorphism were not significantly different between control subjects and patients. Additionally, Allebrandt et al. in a Euro- and Afro-Brazilian population (10), Agrawal et al. in an Indian population (11), Özkök et al. in a Turkish population (18), Gluba et al. in a Polish population (24), and Mohamed et al. in an Egyptian (26) population showed that genotype distributions and allele frequencies of the c.575A>G polymorphism were significantly different between control subjects and patients.

When the allele frequencies were analyzed separately according to gender, age, cigarette smoking, and hyperlipidemia, there was still no statistically significant difference for either polymorphism. Although there has been some controversy about the relationship between *PON1* polymorphisms and CAD, our findings were similar to those of several previous reports (9-17,19-21,23). The inconsistent association of *PON1* with CAD may be attributed to factors that are different between studies, including ethnic and environmental factors, and methodological factors such as sampling scheme and trial size. Among these factors, the most notable is the fact that allele frequencies of the 2 polymorphisms do vary between different ethnic groups.

In the comparison of CAD patients without (TT, wild-type) and with (AA) polymorphic genotypes for the c.163T>A polymorphism, there was no significant difference in terms of age, BMI, TG, HDL-c, LDL-c, diabetes, smoking, and hypertension ($P > 0.05$). However, we found a statistically significant difference between patients without (AA genotype, wild-type) and with (GG) the polymorphic genotype

in terms of average age (59.9 ± 8.6 vs. 68.7 ± 4.8) for the c.575A>G polymorphism in the southeastern Turkish population. The ages of the patients with the GG genotype (Arg) were higher than in patients with AA genotypes (Glu). A number of studies have shown that the c.575 GG homozygotes have been found to have further loss in enzyme activity with age (30,31). In order to confirm this result, it would be useful to investigate the difference between the PON1 gene expression levels in young and old CAD patients with the c.575 GG genotype. Additionally, we did not correlate between healthy controls with or without polymorphic genotypes for the c.163T>A and c.575A>G (data not shown).

The PONs prevent LDL-C from peroxidation, thereby preventing atherosclerosis. The PON1 is exclusively associated with HDL-C, and its antioxidant activity is largely attributed to the *PON1* located on it (6). The *PON1* genotype clearly determined the oxidative modification of lipoproteins and may have played a role in the pathogenesis of atherosclerosis via its protective effect against lipoprotein oxidation in Japanese subjects (32). It has been shown that PON1 activity inversely relates to CAD risk, but is not independent of HDL due to its close association with HDL particles. These data strongly suggest that low PON1 activity is not a causal factor for atherogenesis (33).

Our data indicated that gender, age, BMI, LDL-C, diabetes, and hypertension were significantly higher in the CAD group than in the healthy subjects, while there were no statistically significant differences in TG, HDL-C, and the prevalence of current cigarette smoking. Our results were consistent with the data reported previously (19,22).

We conclude that the *PON1* gene c.163T>A and c.575A>G polymorphisms do not represent an important risk factor for this disease in the southeastern Turkish population. However, studies with higher numbers of patients and controls in various populations will be required in order to determine the influence of these and other candidate genes and single nucleotide polymorphisms on the risk of CAD.

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