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Relationship of *PON2* gene Ser311Cys polymorphism and serum paraoxonase activity with coronary artery disease in Turkish population

[Türk popülasyonunda koroner arter hastalığı ile serum paraoksonaz aktivitesi ve *PON2* Ser311Cys polimorfizminin ilişkisi]

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ABSTRACT

Objective: Paraoxonase-2 (PON2) is a cellular enzyme that has antioxidant properties similar to PON1, an HDL-associated serum enzyme playing an important role in the prevention of atherosclerosis. 1. The aim of this study is to investigate whether serum paraoxonase activity and PON2 Ser311Cys polymorphism are related to coronary artery disease and the extent of atherosclerosis.

Methods: Blood specimens were collected from 131 individuals who had no coronary artery lesions angiographically and 110 individuals who had angiographically-documented coronary artery disease at several degrees. The extent and severity of arterial lesions were evaluated by Gensini scoring system. Paraoxonase and arylesterase activities were measured in serum using a spectrophotometric method. PON2 Ser311Cys polymorphism was evaluated using PCR-RFLP after DNA isolation from samples.

Results: Serum paraoxonase and arylesterase activities were significantly lower in the patient group than controls (respectively, 135.6±55.4 U/ml vs 152.9±61.2 U/ml, $p<0.05$ and 104.7±15.6 U/ml vs 121.6±25.7 U/ml, $p<0.01$). There were negative correlation between serum paraoxonase, arylesterase activities and Gensini score (respectively, $r=-0.183$, $p=0.005$, $r=-0.210$, $p=0.017$). The distribution of PON2 Ser311Cys polymorphism between groups was not significant. There was no significant difference in Gensini scores according to genotypes, either.

Conclusion: These results indicated that serum paraoxonase activity might be a more important marker than PON2 Ser311Cys polymorphism in evaluating the cardiovascular disease risk and the extent and severity of atherosclerosis.

Key words: Serum paraoxonase activity, arylesterase activity, PON2, atherosclerosis, polymorphism

Conflict of interest: The authors declared no competing interest with any group.

ÖZET

Amaç: Paraoksonaz 1 (PON1) aterosklerozun önlenlenmesinde önemli fonksiyonları olan, HDL ile ilişkili antioksidan bir enzimdir. PON2 ise, antioksidan özellikleri PON1'e benzeyen hücresel bir enzimdir. Bu çalışmanın amacı, serum paraoksonaz aktivitesi ve PON2 Ser311Cys polimorfizminin, koroner arter hastalığı ve ateroskleroz yaygınlığı ile ilişkisini araştırmaktır.

Yöntem: Anjiyografik olarak koroner damarlarında tıkanıklık olmayan 131 birey kontrol grubu, çeşitli derecelerde tıkanıklığı olan 110 birey hasta (ateroskleroz) grubu olarak tanımlanmıştır. Aterokleroz yaygınlığı ve derecesi Gensini skoru ile değerlendirilmiştir. Paraoksonaz ve arilesteraz aktivitesi spektrofotometrik olarak ölçülmüştür. PON2 Ser311Cys polimorfizmi, örneklerden DNA izole edildikten sonra RFLP yöntemi ile incelenmiştir.

Sonuçlar: Koroner arter hastalığı olan kişilerde serum paraoksonaz ve arilesteraz aktivitesi kontrol grubuna gore anlamlı derecede düşük bulunmuştur (sırasıyla, 135,6±55,4 U/mL vs 152,9±61,2 U/mL, $p<0,05$ ve 104,7±15,6 U/ml vs 121,6±25,7 U/ml, $p<0,01$). Serum paraoksonaz, arilesteraz aktivitesi ile Gensini skoru arasında negatif korelasyon olduğu görülmüştür ($r=-0,183$, $p=0,005$ ve $r=-0,210$, $p=0,017$). Hasta ve kontrol gruplarında, PON2 Ser311Cys polimorfizminin dağılımları açısından anlamlı bir farklılık bulunamamıştır. Genotiplere göre de Gensini skorlarında anlamlı bir farklılık yoktur.

Tartışma: Aterosklerozun yaygınlığı ve derecesi ile ilişkili olarak serum paraoksonaz ve arilesteraz aktivitesinin, PON2 Ser311Cys polimorfizminden daha anlamlı olduğu sonucuna varılmıştır.

Anahtar Kelimeleri: Paroksonaz/arilesteraz aktivitesi, ateroskleroz, PON2, polimorfizm

Çıkar çatışması: Yazarlar hiçbir grupla çıkar çatışması bulunmadığını beyan eder.

Introduction

The paraoxonase (PON) family of enzymes are structurally related calcium-dependent lactonases. (1-3). PON1 is a high density lipoprotein (HDL) -associated protein that was first defined as an organophosphate-hydrolysing enzyme and later it has been shown that PON1 protected low density lipoprotein (LDL) particles from oxidative injury by hydrolysing lipoperoxides (1,2). Therefore, PON1 was defined as a protective factor against atherosclerosis. PON1 is part of a gene cluster located on chromosome 7q21.3–22.1, which contains two other members, PON2 and PON3, with approximately 65%similarity at the amino acid level (1,2). Like PON1, PON3 is associated with HDL and participates in the prevention of LDL oxidation (3).

PON2 also has antioxidant properties, but unlike PON1 and PON3, which are expressed primarily in the liver, it is ubiquitously expressed, especially in endothelial and human aortic smooth muscle cells (2). Although the physiological role of the PON2 gene product is unknown, its tissue distribution suggests a role different from that of PON1 in the atherosclerotic process [4].

Two polymorphisms in the coding region of PON2 have been identified: 311 (Ser→Cys) and 148 (Ala→Gly) (5). Either one or both of these polymorphisms have been shown to be associated with variations in lipoprotein metabolism (6), risk of coronary artery disease (CAD) (7), nephropathy in Type II diabetes (8) and Parkinson disease (9). However, to date, little is known about the functional role of PON2 in the pathogenesis of these diseases (3,4). *In vitro* studies suggest that PON2 may have an anti-atherogenic function. PON2 is absent in plasma and may have an antiatherogenic role by reducing the production of intracellular hydroperoxides and/or by preventing the oxidation of LDL (2-4).

The present study evaluates the role of PON2 Ser311Cys polymorphism and serum paraoxonase activity in the extent and severity of CAD in Turkish population.

Methods

Subjects and the evaluation of coronary arteries

Two hundred fourty one subjects presenting with chest pain who did not have acute coronary syndrome, underwent diagnostic coronary angiography at the Department of Cardiology. Only non-smoking patients were recruited. All subjects were in a stable condition and none of them had sustained myocardial infarction within 6 months before taking part in the study. The patients with hepatic disorders, endocrinological or renal disorders, diabetes mellitus or impaired glucose tolerance, inflammatory or malignant diseases were excluded. The study was approved by the Hacettepe University Faculty of Medicine Ethics Committee (04.11.2004, FON 04/26-14) and all subjects had given informed consent. Diagnostic coronary angiography by Judkins method was performed at the Department of Cardiology. Gensini scoring system is used to assess the extent and severity of atherosclerosis (10,11). In this system, scores are calculated by evaluating the functional importance and luminal narrowing percentage of arteries in the coronary arterial tree. Higher Gensini scores represent more severe disease. One hundred thirty one of the subjects had no evidence of coronary arterial lesions (Gensini score = 0, control group), while 110 had lesions at different degrees of severity (coronary artery disease group).

Lipid profile was evaluated at the Laboratory of Clinical Pathology in Hacettepe University Hospital. Cholesterol and HDL-cholesterol (HDL) levels were measured spectrophotometrically using Roche Modular Systems. Cholesterol levels were determined by an enzymatic method using cholesterol esterase and cholesterol oxidase. HDL was also determined by an enzymatic method using cholesterol esterase and cholesterol oxidase coupled with PEG to the amino groups.

Sample preparation

Blood samples were obtained during coronary angiography and collected either without any additives or into EDTA-containing tubes. Samples for activity assay were centrifuged at 3500 x*g* for 10 minutes. The serum was removed and stored at -20°C. Blood for DNA isolation was collected into EDTA-containing tubes and DNA was extracted from peripheral blood leukocytes using a commercial kit (Promega, ABD). Isolated DNA samples were stored frozen at -80°C.

Determination of PON2 genotypes

Standard PCR protocols, followed by restriction enzyme digestion, were used to genotype the PON2 Ser311Cys polymorphism, as previously described (12). Briefly, the primer pairs used for Ser311Cys were: forward 5'-ACA TGC ATG TAC GGT GGT CTT ATA-3' and reverse 5'AGC AAT TCA TAG ATT AAT TGT TA-3'. Each PCR reaction contained 200 µM dNTP, 0.2 µM primer, 10 mM Tris-HCl, 500 mM KCl, %5 dimethyl sulfoxide, 1.5 mM $MgCl_2$, and 1.25 U *Taq* polymerase (Promega, ABD). The PCR conditions were 94°C for 4 min, 30 cycles of 94°C for 60 min, 50°C for 90 min, 72°C for 2 min, followed by 72°C extension for 7 min. PCR products were digested with *DdeI* and digested products were resolved by gel electrophoresis.

Paraoxonase Activity

Serum PON1 activity was measured according to Gan et al. (13), using paraoxon (*O*,*O*-diethyl-*O*-*p*-nitrophenylphosphate; Sigma) as substrate at a final concentration of 1 mM. Formation of *p*-nitrophenol at 37°C was monitored at 412 nm, in the presence of 100 mM Tris-HCl (pH 8.0) containing 1 mM $CaCl_2$ and 2 mM NaCl. Enzymatic activity was calculated from the molar extinction coefficient of *p*-nitrophenol (ϵ_{412} = 18290

$M^{-1}cm^{-1}$) and corrected for the non-enzymatic hydrolysis. One unit of paraoxonase activity is defined as 1 nmol of substrate hydrolyzed per min, under the defined assay conditions.

Arylesterase Activity

Arylesterase activity was measured spectrophotometrically using phenyl acetate (13). Formation of phenol was monitored at 270 nm. Reaction mixtures contained 50 mM Tris/HCl (pH 8.0), 1 mM $CaCL_2$, 1 mM substrate. Enzymatic activity was calculated from the molar extinction coefficient of phenol (ϵ_{270} = 1310 $M^{-1}cm^{-1}$) One unit of arylesterase activity is equal to 1 µmol of phenyl acetate hydrolyzed per min, under the defined assay conditions.

Statistics

All analyses were performed using SPSS software, version 11.0. Continous variables with normal distribution were expressed as mean ±SD, variables with skew distribution are expressed as median (minimum-maximum), categorical variables are expressed as percentage. Correlation between two numeric variables was calculated by either Pearson's correlation coefficient test or Spearman's rho correlation. The Hardy-Weinberg equilibrium, allele frequency and genotype distributions were tested by the chi-square test. The relationship between PON1 genotype and Gensini score was evaluated using Kruskal-Wallis analysis. $p \leq 0.05$ was considered statistically significant.

Results

In this study, we evaluated the genotype distribution of PON2 Ser311Cys polymorphism and serum paraoxonase activity in 241 subjects. The severity and extent of coronary artery disease of these patients were evaluated according to the Gensini scoring system. One hundred thirty one participants who had normal coronary arteries had score 0 and were defined as the control group.

The biochemical and demographic features of the subjects are shown in Table 1. Serum paraoxonase arylesterase activities of CAD patients was significantly lower than the control subjects (respectively,135.6 ±55.4 U/ml vs 152.9 ±61.2 U/ml, $p < 0.05$ and 104.7 ±15.6 U/ml vs 121.6 ±25.7 U/ml, $p < 0.01$). No significant correlation was determined between Gensini scores and plasma levels of HDL, the protective lipoprotein against cardiovascular disease. The ratio of total cholesterol to HDL is considered to be a better marker than HDL alone for the prediction of CAD risk. No significant difference was observed between total cholesterol/HDL ratios of patients with coronary artery disease and controls. There was a weak correlation found between Gensini scores and total cholesterol/HDL ratios ($r = 0.133$, $p = 0.040$). On the other hand, as shown in Table 2, there were slight negative correlation between Gensini scores and serum paraoxonase, arylesterase activities (respectively, $r = -0.183$, $p = 0.005$ and $r = -0.210$, $p = 0.017$).

Genotype distribution and allele frequencies of PON2 Ser311Cys polymorphism in both groups are shown in Table 3. In the polymorphism analysis, PON2 311 genotype frequencies were as follows: Ser/Ser homozygotes 132 (55%), Cys/Ser heterozygotes 94 (39%) and Cys/Cys homozygotes 15 (6%). The Ser and Cys allele frequencies were 0.74 and 0.26 in the control group and 0.75 and 0.25 in the patient group, respectively. The allele distribution for PON2 Ser311Cys was consistent with the Hardy-Weinberg equilibrium in both patients ($p = 0.406$) and controls ($p = 0.660$). There was no significant difference in the gene frequencies of PON2 Ser311Cys polymorphism between groups (Table 3). The distribution of Gensini scores according to genotypes in the patient group was not significant, either ($p = 0.495$) (Fig. 1).

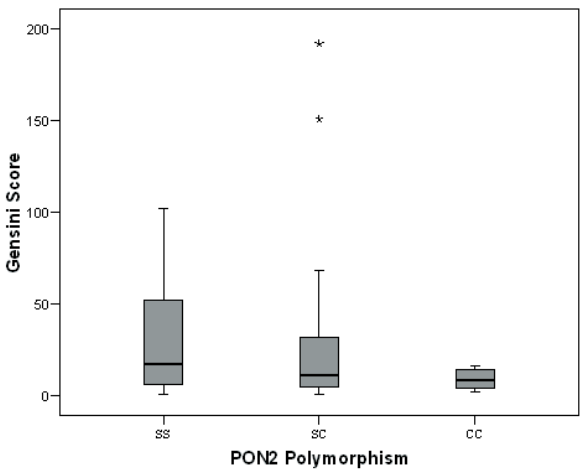

Figure 1. Distribution of Gensini scores according to genotypes. SS: Ser/Ser homozygote, SC: Ser/Cys heterozygote, CC: Cys/Cys homozygote. Extreme values (*) are indicated.

Discussion

Atherosclerosis is an inflammatory process that develops as a result of several factors and it has been revealed that oxidative stress has a pivotal role in its pathogenesis (3,4,14). In atherosclerotic patients, increased oxidative stress is present in blood, as well as in arterial wall cells, including macrophages (14,16). In early atherogenesis, LDL particles accumulate in the subendothelial space and oxidatively modified, probably as a result of exposure to reactive oxygen species produced by vascular endothelial cells. HDL-associated PON1 reduces oxidative stress in lipoproteins, in macrophages, and in the atherosclerotic lesion, whereas PON2 acts as an antioxidant at the cellular level and protects cells, tissues and lipoprotein particles by reducing intracellular oxidative stress, and attenuates the inflammatory response (16). Thus, PON2 appears to be beneficial for the prevention of atherogenesis.

Table 1. Clinical and biochemical features of the CAD patient group compared to controls.

	Control (*n*=131)	CAD (*n*=110)
Sex (male/female)	57/74	77/33
Age (years)	58.1±11.9	61.3±10.8
BMI (kg/m^2)	28.5±3.7***	26.2±3.3***
Glucose (mg/dL)	103.3±37.9	110.2±35.5
Total Cholesterol (mg/dL)	194.9±40.1	197.8±48.3
HDL (mg/dL)	51.7±15.4	48.4±12.5
LDL (mg/dL)	117.6±33	117.2±41.6
Triglyceride (mg/dL)	143.6±69.5	159.5±82.9
VLDL (mg/dL)	26.0 [10-90]	29.4 [2-105]
Total Cholesterol/HDL	4.02±1.23	4.3±1.3
Paraoxonase Activity (U/mL)	152.9±61.2*	135.6±55.4*
Arylesterase Activity (U/mL)	121.6±25.7**	104.7±15.6**

CAD: Coronary artery disease. *: p<0.05; ** p<0.01, ***: p< 0.001.

Table 2. The relationship between lipid profile and PON1 activity with the severity and extent of atherosclerosis (Gensini score).

	r	p
Paraoxonase Activity	-0.183	0.005
Arylesterase Activity	-0.210	0.017
Total Cholesterol/HDL	0.133	0.040
HDL	-0.048	0.462
Total Cholesterol	0.102	0.115
LDL	0.065	0.314

Table 3. Genotype distribution and allele frequencies of PON2 Ser311Cys gene polymorphisms.

Covariates	Control (n:131)	CAD (n:110)
Genotype		
Ser/Ser	0.54	0.57
Cys/Ser	0.41	0.36
Cys/Cys	0.05	0.07
χ^2	0.687	0.192
p	0.406	0.660
Allele Frequency		
Ser	0.74	0.75
Cys	0.26	0.25

A number of previous studies investigated the role of PON2 polymorphism in CAD risk. Sanghera (5) et al. and Leus (7) et al. demonstrated that PON2 Ser311 allele was associated with a higher risk of CAD in Indian and Caucasian populations, respectively. In contrast, PON2 Cys311 allele has been shown to be associated with a higher risk of CAD in the Iranian population (15). However, Gluba et al. (14) did not find any relationship between PON2 polymorphisms and CAD risk. It has been suggested that conflicting results should be considered in the light of the fact that the gene-gene and gene-environment interactions may modulate the risk of CAD associated with different polymorphisms (14).

In this study we aimed to evaluate the role of PON2 polymorphism in both CAD risk and the extent of the disease, compared to serum paraoxonase activity. We found

that serum paraoxonase activity was significantly lower in CAD patients than the controls. Moreover, paraoxonase activity was negatively correlated with the extent and severity of CAD. In a previous study with Turkish population, serum paraoxonase activity of patients with coronary heart disease was found to be lower than control subjects but the difference was not statistically significant (17). However, their study group was relatively small (51 controls and 57 patients) and the patients were not precisely defined whereas a fairly larger group (110 controls and 131 patients) with angiographically documented coronary artery status was enrolled in our study.

Regarding the PON2 Ser311Cys polymorphism, it was associated neither with CAD nor with the extent and severity of atherosclerosis in Turkish population. Serine-coding allele is ancestral and it is strongly conserved in evolution (SNP database). In agreement with that, Ser/Ser genotype was the most common one in our CAD group. These findings are consistent with the HapMAP-CEU Europan SNP database showing 57%of individuals homozygous for Ser/Ser, 38%heterozygous for Ser/Cys, and 5%homozygous for Cys/Cys (18)

Recombinant PON2 has been shown to inhibit the cell-mediated oxidation of LDL and reduce cellular lipid hydroperoxides in cultured macrophages (19) whereas PON2 overexpressed in cultured cells prevented the oxidation of LDL added to the culture medium (20). Moreover, PON2-overexpressing cells inhibited the ability of mildly oxidized LDL to induce monocyte chemotaxis (20). In transgenic mice models, overexpression of PON2 increased cholesterol efflux (21) while PON2 deficiency increased the size of atherosclerotic lesions (22). PON2 has an enzymatic lactonase activity that is considered to be irrelevant to its antioxidant action (4). Ser311Cys mutation has been shown to alter the glycosylation pattern and impair the lactonase activity of PON2 (18) but does not affect the antioxidant action of the enzyme (4). This is in accordance with our results demonstrating a relatively low frequency for Cys/Cys genotype in CAD patients and no correlation between Ser311Cys polymorphism and Gensini scores.

The present study indicated that the circulating PON1 which is associated with HDL is effective in protecting LDL from oxidation as shown by the negative correlation between serum paraoxonase activity and the impact of atherosclerosis. In addition to PON1, HDL has several other components that have beneficial effects in the prevention of atherosclerotic lesions. Although not significant, a negative correlation was found between Gensini scores and HDL levels in our study, and it has been shown that loss of PON1 activity has been linked with reduced HDL capacity in the prevention of LDL oxidation, leading to CAD (23-27). These observations suggest that PON1 is the major component of HDL in controlling the extent and severity of atherosclerosis. Taken together, our results indicated that serum PON1 activity might be a more important marker than PON2 Ser311Cys polymorphism in evaluating the extent and severity of atherosclerosis as well as the CAD risk.

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