

Original Article

Association of Cys 311 Ser Polymorphism of Paraoxonase-2 Gene with the Risk of Coronary Artery Disease

Ali Jalilian MS*, Ebrahim Javadi PhD**, Mohamad Akrami MD PhD**,
Hossin Fakhrzadeh MD**, Ramin Heshmat PhD**, Mazaher Rahmani BS**,
Fatemeh Bandarian MD**

Background: Recently another member of the paraoxonase gene family designated paraoxonase-2 has been identified. Paraoxonase-2 has antioxidant properties similar to paraoxonase-1 and paraoxonase-3. However, in contrast to paraoxonase-1 and paraoxonase-3, paraoxonase-2 is not associated with high-density lipoprotein and may only exert its antioxidant function at the cellular level.

Methods: We assessed the frequency and genotype distribution of cys 311 ser paraoxonase-2 polymorphism in 300 subjects (>40 years old) with angiographic documentation of coronary artery disease (150 patients with >50% stenosis served as cases and 150 individuals with <20% stenosis served as controls) to determine the possible association between this mutation and susceptibility for coronary artery disease. The paraoxonase-2 genotypes were determined by polymerase chain reaction and Ddel restriction enzyme digestion.

Results: The cases (coronary artery disease positive patients) showed significant differences in the distribution of cys 311 ser paraoxonase-2 genotypes as compared with the controls (coronary artery disease negative subjects, $P=0.015$). The analysis of paraoxonase-2 genotypes distribution showed higher percentage of CC genotype among coronary artery disease positive compared with coronary artery disease negative ($P=0.008$). After controlling for other risk factors, the cys 311 ser polymorphism had not correlation with age, body mass index, gender, smoking, diabetes, level of high-density lipoprotein, low-density lipoprotein, triglyceride, and total cholesterol.

Conclusion: Our data indicate a major effect of the paraoxonase-2 polymorphism on coronary artery disease risk in patients referred to Shariati Hospital in Tehran.

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Keywords: Coronary artery disease • paraoxonase-2 • polymorphism

Introduction

The development of coronary artery disease (CAD) is a complex process whose central elements include the entrapment of low-density lipoprotein (LDL) in the vessel wall, its subsequent oxidative modification, and the stimulation of proinflammatory gene

expression leading to inflammatory cell recruitment, infiltration, and activation.¹ Therefore, gene variants influencing LDL oxidation are potential factors for CAD.² The paraoxonase (PON) gene family in mammals includes at least three members: PON1, PON2, and PON3.³ The three 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 and chromosome 6 in mice.⁴ Both PON2 and PON3 possess antioxidant properties and lactonase activity, but unlike PON1, they lack the paraoxon or phenyl acetate-hydrolyzing activity.⁵ Like PON1, PON3 is associated with high-density lipoprotein (HDL). It not only prevents the formation of mildly modified LDL (MM-LDL), but

Authors' affiliations: *Department of Medical Biochemistry, **Endocrinology and Metabolism Research Center, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran.
•Corresponding author and reprints: Ebrahim Javadi PhD, Endocrinology and Metabolism Research Center, Shariati Hospital, Tehran 14114, Iran. Tel: +98 21 802 690 23, Fax: +98 21 802 93 99
E-mail: javadieb@tums.ac.ir; emrc@sina.tums.ac.ir
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also inhibits MM-LDL-induced monocyte chemotactic activity.⁶ PON2 is absent in plasma and plays an antiatherogenic role by reducing the oxidation of LDL and or by reducing the production of intracellular hydroperoxides.⁷ A common polymorphism at codon 311 (cys→ser) in the PON2 gene has been reported to be associated with the risk of CAD in many studies.⁸ The present study evaluated the role of PON2 (cys 311 ser) polymorphism on the risk of CAD in an Iranian population.

Materials and Methods

Subjects

A total of 300 Iranian individuals (>40 years old) were investigated. The study participants were recruited from the patients who referred to Catheterization Laboratory of Shariati Hospital because of chest pain or positive exercise test. Overall, 150 patients were classified as having CAD (at least one coronary vessel with >50% stenosis; CAD⁺ group). The patients were categorized as having one-, two-, or three- vessel disease according to this definition. Those with renal or hepatic insufficiency were excluded from the study, and 150 individuals were classified as having negative coronary angiograms (<20% stenosis; CAD⁻ group). A structured questionnaire was used to characterize both groups. Data collection included height and weight (to assess body mass index [BMI]), age, diabetes mellitus, and smoking. The study protocol was approved by the Ethics Committee of Endocrinology and Metabolism Research Center of Tehran University of Medical Sciences and informed consent was

taken from all the participants.

Methods

Total cholesterol (TC) and triglyceride concentrations in plasma were measured by automated enzymatic methods (Hitachi 902 Auto Analyzer, Japan) .HDL-C was determined in the whole plasma after precipitation of apolipoprotein B (apoB)-containing lipoproteins with phosphotungstic acid/MgCl₂. LDL-C was estimated according to Friedewald's formula.⁹

The blood samples were collected in 10-mL tubes containing Na-EDTA and kept frozen at -20°C. DNA was extracted by the phenol-proteinase K method.¹⁰ PON2 cys 311 ser genotypes were detected by a combination of polymerase chain reaction (PCR) and digestion with restriction enzyme with some modifications. PCR amplification was performed using the following primers:

5'ACATGCATGTACGGTGGTCTTATA3' and 5'AGCAATTCATAGATTAATTGTTA3'

(Cinagene, Iran). The PCR product (262bp, shown in Figure 1) was digested overnight with 2U DdeI restriction enzyme and diagnosed in a 5% agarose gels. Fragment size of 142bp and 120bp corresponds to cys 311 (C allele), whereas 75bp and 67bp are diagnostic bands for ser 311 (S allele). Note that a 120bp band is present in all genotypes (Figure 2), because of the presence of a common DdeI site.¹⁰ The PCR mixture contained 100 – 200 ng DNA template, 0.2 mmol/L dNTPs, 1.5 mmol/L MgCl₂, 0.3mmol/L of each primer, and 1.25 IU Taq DNA polymerase with 5% dimethyl sulfoxide (Cinagene, Iran) in a final volume of 25μL.

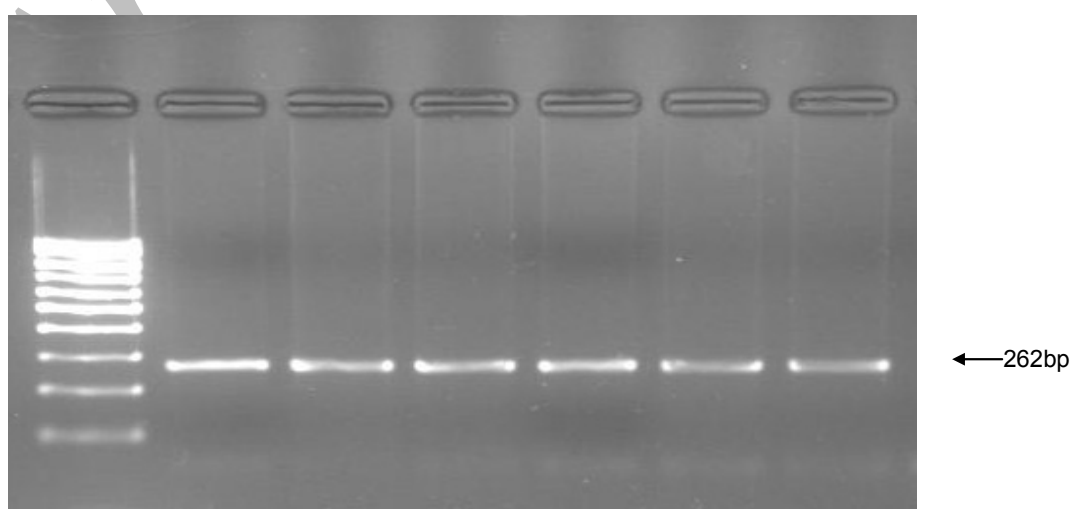


Figure 1. PCR product (262-bp) related to the PON2 polymorphism.

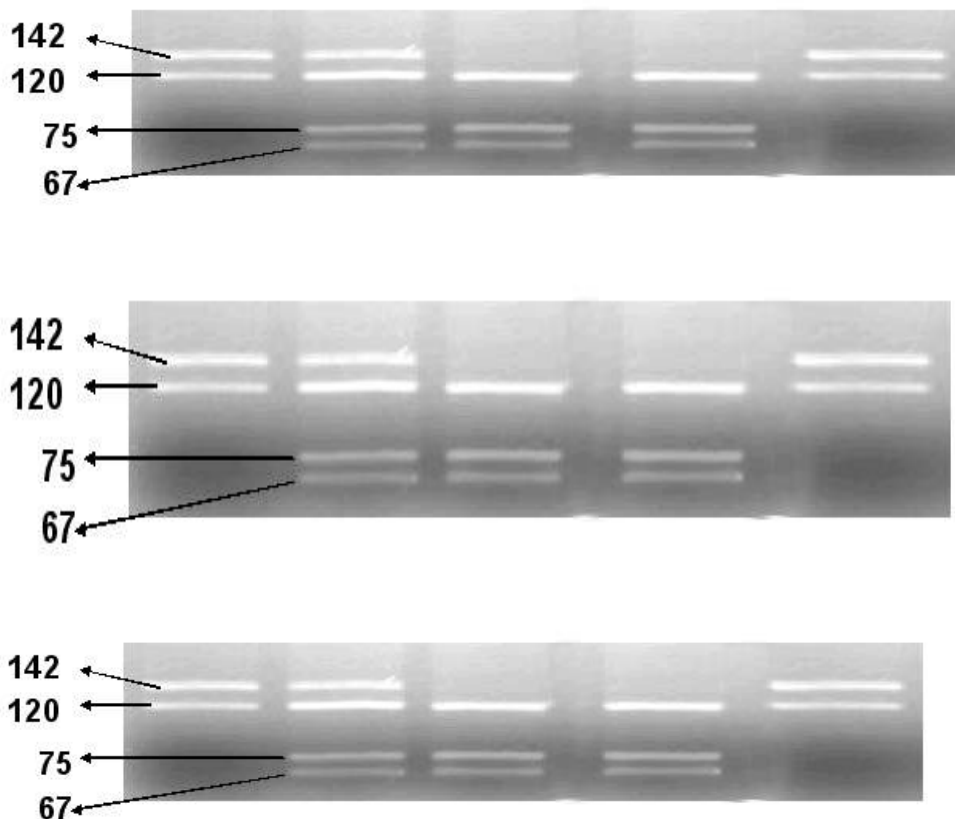


Figure 2. Restriction patterns of the PCR-amplified 262-bp fragment after digestion with DdeI. Lanes 3 and 4 SS genotype, Lanes 1 and 5 CC genotype, and Lane 2 CS genotype.

After initial denaturation (3 min at 95°C), 35 cycles were run of 1 min at 95°C, 1.5 min at 50°C, and 1 min at 72°C with a final extension time of 8 min.¹¹

Statistical analysis

We used the STATA 8.0 for data analysis. A value of $P < 0.05$ was considered significant. To compare CAD⁺ patients with subjects without CAD for quantitative data (age, BMI, and lipid levels), we used *t*-test. Differences with respect to qualitative data (gender, smoking, and diabetes) were analyzed using the χ^2 test (Chi-square test). The χ^2 test was also used to compare paraoxonase-2 genotype distributions and allele frequencies between the studied groups. One-way analysis of variance (ANOVA) was used to analyze the relationship between genotypes and clinical and laboratory findings in the patients group.¹² Odds ratios and their 95% confidence intervals were calculated for estimating the risk of CAD positivity in each genotype comparing with homozygote S (SS). Logistic regression modeling was performed for adjustment of age, sex, and BMI effects.

Results

A total of 150 CAD⁻ and 150 CAD⁺ participants were included in the study. No differences were found in smoking habits between the groups. CAD⁺ patients were older ($P=0.000$) and they showed significantly higher levels of risk factors such as male sex, diabetes, higher BMI, and LDL-C compared with CAD⁻ participants. No differences in total cholesterol, HDL-C, and triglycerides were observed between the groups. The restriction patterns indicating the presence of three genotypes due to the existence of two common alleles, (S and C) are shown in Figure 2. The genotype distributions and allele frequencies of the studied polymorphism (cys 311 ser PON2) are given in Table 1. The genotype distribution was significantly different between the two groups ($P=0.015$). The CAD⁺ patients had a significantly higher frequency of the CC genotype compared with the controls ($P=0.008$, OR=3.19, 95%CI: 1.22 – 8.96; adjusted OR: 2.64, 95%CI: 1.31 – 3.69). The frequency of the PON2*C allele was significantly higher in the cases than in the controls

Table 1. Genotype and allele frequency of the participants with or without coronary artery disease.

| Genotype | CAD ⁺ (n=150) | CAD ⁻ (n=150) | P value | OR (95%CI) | Adjusted OR (95%CI) |
|------------------|-----------------------------|-----------------------------|---------|------------------|---------------------|
| SS | 61 | 82 | — | 1(REF) | 1 (REF) |
| CS | 70 | 60 | 0.065 | 1.57 (.95-2.60) | 1.42 (0.84 – 2.40) |
| CC | 19 | 8 | 0.008 | 3.19 (1.22-8.96) | 2.64 (1.31 – 3.69) |
| CC+CS | 89 | 68 | 0.015 | 1.76 (1.11-2.78) | 1.66 (1.05 – 2.59) |
| allele frequency | | | | | |
| C | 108 | 76 | 0.005 | 1.66 (1.15-2.39) | 1.53 (1.03 – 2.28) |

P=0.015; CAD=coronary artery disease, OR=odds ratio, CI=confidence interval; REF=reference.

(*P*=0.005, OR: 1.66, 95%CI: 1.15 – 2.39; adjusted OR: 1.53, 95%CI: 1.03 – 2.28). When clinical and laboratory values were compared among genotype in the patients' group (CAD⁺), no significant differences were noted with regard to BMI, age, male sex, smoking, diabetes, or triglyceride, TC, HDL-C, and LDL-C levels (Table 2). The genotype frequencies were significantly different between one-, two-, and three- vessel diseased groups (*P*=0.018, Table 3). These data showed a significant association with an increasing number of diseased vessels for the PON2 codon 311 polymorphism.

Discussion

Oxidation of LDL trapped in the arterial subendothelial space is a key process in CAD.¹³ Therefore, mechanisms preventing LDL oxidation appear to be antiatherogenic.¹⁴ Studies performed during the last 10 years indicate that PON family has multiple important functions. PONs have received increasing attention in the field of CAD prevention for their presumed antioxidant and anti-inflammatory role.

PON1 was the first of the proteins to be identified and it is thus the most studied.¹⁵ Unlike PON2 and PON3, it is an efficient esterase towards many organophosphate compounds, including paraoxon (from which it takes its name), the insecticides parathion and chlorpyrifos as well as

the nerve agents sarin and soman.¹⁶

HDL-PON1 is able to hydrolyze long-chain oxidized phospholipids, cholesterol esters hydroperoxides that were isolated from oxidized LDL, and also hydrogen peroxide.¹⁷ Most of the studies to evaluate the association between PON1 gene polymorphism and CAD, have revealed significant association, although some have not.¹⁸

Carey and coworkers demonstrated that PON2, although being widely expressed in many different human tissues, was not detectable by western analysis in either HDL or LDL (unlike PON1 and PON3, PON2 is not associated with HDL). They found that PON2 was constitutively expressed in both primary and immortalized human endothelial cells and human aortic smooth muscle cells.¹⁹

PON2 overexpression lowers the intracellular oxidative state of cells that have been treated by either hydrogen peroxide (a major reactive oxygen species produced under oxidative stress during atherogenesis) or oxidized phospholipid. MM-LDL that has been incubated with cells which over-express PON2 shows lower levels of lipid hydroperoxides and is less able to induce monocyte chemotaxis than MM-LDL incubated with control cell.

Their data suggest that PON2 has antioxidant properties and is capable of preventing the peroxidation of LDL.¹⁹ One genetic polymorphism in the PON2 (ser 311 cys) gene have been reported to be associated with the risk of CAD in some

Table 2. Classical coronary risk factors and PON2 polymorphism in patients with CAD.

| Variable | S/S (n=61) | C/S (n=70) | C/C (n=19) | P value |
|--------------------------|---------------|---------------|---------------|---------|
| Age (yr) | 56.67±10.35 | 58.37±9.95 | 59.00±10.57 | 0.543 |
| BMI (kg/m ²) | 26.85±2.09 | 26.58±2.17 | 26.66±1.94 | 0.751 |
| Sex, male (%) | 48(78.7%) | 49(70.0%) | 14(73.7%) | 0.527 |
| Diabetes mellitus (%) | 8(13.1%) | 17(24.3%) | 5(26.3%) | 0.214 |
| Smoking (%) | 24(39.3%) | 22(31.4%) | 8(42.1%) | 0.538 |
| Cholesterol (mg/dL) | 177.69±39.63 | 179.30±42.33 | 179.02±36.50 | 0.974 |
| Triglycerides (mg/dL) | 147.41±64.74 | 169.12±128.79 | 181.73±87.71 | 0.321 |
| LDL cholesterol (mg/dL) | 102.67±23.88 | 102.40±23.48 | 101.40±25.04 | 0.980 |
| HDL cholesterol (mg/dL) | 53.54±13.94 | 51.95±14.59 | 54.26±13.31 | 0.736 |

Table 3. PON2 cys 311 ser polymorphism by number of diseased vessels.

| Genotype | No. of diseased vessels | | |
|----------|-------------------------|---------------|-----------------|
| | One (n=65) | Two (n=44) | Three (n=41) |
| SS | 26 (40%) | 18 (40.9%) | 17 (41.46%) |
| CS | 35 (53.84%) | 22 (50%) | 13 (31.7%) |
| CC | 4 (6.15%) | 4 (9.09%) | 11 (26.8%) |

$P=0.018$.

studies.⁸

In our study, we assessed the frequency and genotype distribution of PON2 (cys 311 ser) polymorphism in 300 participants with angiographic documentation of CAD to determine the possible association between this mutation and susceptibility for CAD. Several points should be considered when interpreting these results. The study design was case-control, and to avoid potential misclassification, only subjects without evidence of CAD (< 20 % stenosis) were selected as control subjects (CAD⁻), and younger subjects (< 40 years old) were not considered.

Our results were consistent with those of Martinelli et al.'s in genotype, allele frequency, and LDL, but different in term of TG, TC, and HDL-C measures. This inconsistency would result from the different sample size, where in Martinelli et al.'s study the sample size consisted of 890 individuals and all the variables (risk factors) were shown to differ significantly.⁷ Leus et al. demonstrated that PON2 ser 311 carriers were at risk for CAD, and that subjects with homozygous cys genotype were probably protected against the development of CAD.¹¹ Previously, the PON2 ser 311 allele had been shown to increase the risk of CAD in an Indian sample.¹⁰ However, in the present study, we found that PON2 cys 311 allele was associated with the risk of CAD. The frequencies of both the cys allele ($P=0.005$) and cys/cys genotype ($P=0.008$) were higher in the CAD⁺ group than in the control group. The cys/cys genotype was also higher in the group with three-vessel disease than in the groups with one-vessel or two-vessel disease. This is in agreement with a recent observation by Chen et al.⁸ Boright et al. reported that the individuals with PON2 cys/cys had the highest mean plasma TC compared with individuals with both cys/ser and/or ser/ser.²⁰ Another observation in our study was the lack of significant difference in the mean TC and LDL-C levels between the individuals with PON2 cys/cys, cys/ser, and ser/ser genotype. This is not in

agreement with Boright et al.'s results. This result indicates that the association between the PON2 codon 311 polymorphism and the risk of CAD may differ among specific subgroups.

In summary, the results of our analyses of data suggest that common genetic variation in the PON2 gene may affect the risk of CAD.

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