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PON192 Polymorphism and LDL-Oxidation As Risk Factors For Coronary Artery Disease in Young Turkish Population

[Genç Türk Popülasyonunda Koroner Arter Hastalığı Risk Faktörü Olarak PON192 Polimorfizmi ve LDL-Oksidasyonu]

[1]Eser Y. Sözmen,
[2]Meral Kayıkçıoğlu,
[1]Ebru Sezer,
[3]Bilal İlanbey,
[1]Yasemin D. Akçay,
[2]Hakan Kültürsay

[1] Dept.of Medical Biochemistry, Ege University Faculty of Medicine, Izmir, TURKİYE
[2] Dept.of Cardiology, Ege University Faculty of Medicine, Izmir, TURKİYE
[3] Specialist, Dept .of Biochemistry, Devlet Hastanesi, Yozgat, TURKİYE

Yazışma Adresi
[Correspondence Address]

Eser Yıldırım Sözmen, Prof, M.D., Ph.D.

Ege Universitesi Tıp Fakültesi
Biyokimya Anabilim Dalı
Bornova-Izmir/ TÜRKİYE
Te: 90 232 390 4098
Fax: 90 232 373 9477
E-mail:eser.sozmen@ege.edu.tr, esersoz@yahoo.com

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ABSTRACT

Purpose: There are conflicting reports on paraoxonase1 (PON1) polymorphism as a risk factor for cardiovascular disease. It's been proposed that there is relationship between coronary artery disease and PON1-55L or PON1-192R alleles. Nevertheless recent publications showed major differences of PON1 activity and concentration in different populations. We aimed to study low-density-lipoprotein (LDL) oxidation and PON1 activities (paraoxonase/arylesterase) regarding to PON192 polymorphism, in order to determine if they might be useful markers especially for young patients with coronary artery disease in Turkish population.
Methods: 60 patients (38.1±5.0 years) with coronary artery disease and 52 healthy control subjects (32.5±6.1 years) were taken into study. Paraoxonase192 polymorphism, paraoxonase activity and oxidation status of all patients were analyzed.
Results: Total cholesterol, triglyceride, basal-LDL-diene and stimulated-LDL-TBARS levels were higher, paraoxonase and arylesterase activities were lower in patients with coronary artery disease compared to controls($p<0.001$). While there was no difference in any parameters within control group in regard to polymorphism, stimulated-LDL TBARS levels were higher in patients with RR polymorphism (5,27±2,4nmol/mg LDL protein for RR; 3,64±1.28nmol/mg LDL protein for QR and 4,95±2,8nmol/mg LDL protein for QQ). RR polymorphism was more common in patient group than controls but not statistically significant.
Conclusion: Our data suggest that presence of RR polymorphism might be a predictive marker for determination of atherosclerosis in early ages.
Key Words: atherosclerosis, paraoxonase, arylesterase, PON192 polymorphism, LDL oxidation, Turkish population

ÖZET

Amaç: Kardiyovasküler hastalık risk faktörü olarak paraoksonaz1 (PON1) polimorfizminin rolüyle ilgili çok sayıda çelişkili rapor bulunmaktadır. Koroner arter hastalığı ile PON1-55L ya da PON1-192R alelleri arasında ilişki olduğu öne sürülmüştür. Diğer yandan son yayınlarda farklı popülasyonların PON1 aktivitesi ve konsantrasyonları arasında büyük farklılıklar olduğu gösterilmiştir. Bu çalışmada PON192 polimorfizmine göre düşük-dansiteli-lipoprotein (LDL) oksidasyon belirteçleri ve PON1 aktivitelerinin (paraoksonaz ve aril esteraz) özellikle Türk popülasyonunda koroner arter hastalığı bulunan gençlerde bu hastalık açısından yararlı belirteçler olup olmadıklarının araştırılması amaçlandı.
Gereç ve Yöntem: Koroner arter hastalığı olan 60 hasta (38.1±5.0 yaşında) ve 52 sağlıklı kontrol (32.5±6.1 yaşında) çalışmaya alındı. Tüm hastalarda PON192 polimorfizmi, paraoksonaz aktivitesi, serum oksidasyon düzeyi belirlendi.
Bulgular: Koroner arter hastalarında total kolesterol, trigliserid, bazal-dien ve stimüle-LDL tiobarbitürik asidle reaksiyon veren yapıların (TBARS) düzeyleri kontrollere göre yüksek bulunurken PON1 ve arilesteraz aktivitelerinin düşük olduğu tespit edildi ($p<0.001$). Kontrol grubu içinde bakılan hiçbir parametrede polimorfizm açısından bir farklılık saptanmazken, RR polimorfizmlilerde stimüle LDL-TBARS düzeylerinin daha yüksek olduğu görüldü (RR için 5,27 ± 2,4 nmol/mg LDL protein, QR için 3,64 ± 1.28 nmol/mg LDL protein for QR ve QQ için 4,95 ± 2,8 nmol/mg LDLprotein). RR polimorfizmine hasta grubunda daha sık rastlanmakla birlikte istatistiksel anlamlılık saptanamadı.
Sonuç: Bulgularımız RR polimorfizminin erken yaşlarda aterosklerozun belirlenebilmesi için yararlı bir gösterge olabileceğini düşündürmektedir.
Anahtar Kelimeler: ateroskleroz, paraoksonaz, arilesteraz, PON192 polimorfizmi, LDL oksidasyonu, Türk popülasyonu

Introduction

Coronary artery disease (CAD) is the most important cause of mortality in developing countries. It has been clearly shown that Turkish population has low HDL cholesterol levels and PON1 activity therefore they have higher risk for CAD (1,2) and early diagnosis of CAD has great importance in prevention of CAD. Since recent data showed that there may not be direct interaction between the well-known risk factors and the occurrence of CAD in young patients, many investigations have focused on the various genetic markers such as CETP, PON and etc (3-14). There is great deal of conflicting reports on the role of PON1 polymorphism on the risk of cardiovascular disease (4-6, 8-18).

It has been clearly known that atherogenesis is initiated by oxidation of the low-density lipoprotein (LDL) (19-21) and this modification plays a central role in further propagation of atherogenesis (21-23). In accordance to the "oxidative stress theory of aging"; aging, is primary risc factor, strongly correlated with CAD caused by increase in oxidative stress as well as lipid peroxidation and inhibition of antioxidant enzymes. Although numerous studies suggested an elevation of ox-LDL (TBARS and diene levels) according to the stage of atherosclerosis in middle aged (>50 years old) and/or elderly patients with CAD (6,8,9,12,15,17,24), there is limited data related on young patients.

It has been also known that PON1, an HDL-associated enzyme, protects LDL against oxidation by removing oxidized phospholipids from LDL. Previously we determined a depletion of Paraoxonase activity in patients with CHD (24) and also patients with cardiac syndrome-x (with a typical anginal chest pain, noninvasive stress tests indicative of myocardial ischaemia, angiographically normal epicardial coronary arteries with no inducible coronary artery spasm, it's related to endothelial dysfunction, representing of early stage of atherosclerosis) (25). Although numerous reports demonstrated depletion in PON1 activity in older CAD patients (26-28), recent publications showed that there are major differences in PON1 activity and concentration depends on the populations as well as genetic polymorphism (29). There is limited literature on paraoxonase/arylesterase activities and LDL oxidation markers in young patients (<50 years old) with CAD to establish the markers for the disease.

In the light of this data, we have planned to investigate the markers of LDL oxidation and PON activities (paraoxonase and arylesterase) in regard to PON192 polymorphism, in order to determine if they might be useful markers for coronary artery disease in especially young patients (<50 years old) with CAD in Turkish population.

Materials and Methods

Patients

The study group consisted of 60 consecutive patients (between 22 and 50 years old) who were hospitalized for coronary artery disease with complaint of typical angina pectoris and/or AMI and positive exercise test. All patients were examined by a cardiologist and their medical histories, habits, medications were obtained via a questionnaire. Patients with known renal, hepatic, malignant or immunologic disorders including diabetes mellitus were excluded.

Hypertension was defined as resting systolic blood pressure≥140 mmHg and/or diastolic blood pressure ≥90 mmHg or current use of antihypertensive medications for blood pressure control. Family history of CAD was defined as symptomatic CAD occurring in first degree male relatives aged ≤ 55 years or first degree female relatives aged ≤ 65 years.

Age matched 52 volunteers constituted the control group. None of them had prior history of coronary artery disease (CAD) and all of them had normal resting ECG.

The investigation conforms with the principles outlined in the Declaration of Helsinki.

Materials and Methods

All reagents were purchased from Sigma Chemical Co (St. Louis, MO,USA) and Merck (Darmstadt, Germany) and materials for genotyping from Fermantase.

Blood samples with and without heparin were obtained after an overnight fasting (between 8:30-9:30). While serum and plasma were immediately separated and blood samples were kept for DNA isolation.

Serum samples were stored at –80°C until analysis. Serum parameters (total cholesterol, triglyceride, LDL-cholesterol, HDL-cholesterol, HbA1c, Apo-A1, Apo-B) were determined by routine laboratory methods. Paraoxonase and arylesterase activities were measured using paraoxon and phenyl acetate as substrates, respectively (30) .

For LDL isolation plasma samples were incubated at room temperature for 30 minutes with a commercial precipitant reagent (Merck (Darmstadt, Germany),as proposed by Taus et al (31).

After centrifugation at 1600 g x 10 minutes, LDL samples were solubilized by 0.15 N NaOH. LDL oxidation was determined by using TBARS and conjugated diene levels directly, in LDL samples containing 200 mgr protein. Protein measurements were done according to Lowry's method. TBARS measurements were performed by incubation the LDL with TBARS solution (0.12 M TBA in 15% TCA and 1% HCl) for 30 min at 95° C(32).

In vitro oxidation of LDL was induced by incubating the isolated lipoproteins with 5mM $CuSO_4$ and conjugated diene formation was analysed by monitoring the absor-

bance change at 234 nm during 3 hours within 5 minute intervals. The peak of diene conjugation was reached at 110th minute after the initiation of in vitro oxidation of LDL with $CuSO_4$ and TBARS levels at this point were also determined by incubation the LDL with TBARS solution as described before (32). For conjugated dien levels calculations were performed using the extinction coefficient of 29 500 L/mol-cm and the results were given as mmol/mg protein units. LDL-TBARS (basal) levels were given in nmol/mg protein units.

PON192 QR polymorphism: The amino acid polymorphism at position-192 (glutamine to arginine) results in two allozymes (Q and R) which differ in their hydrolytic activity towards paraoxon. Genomic DNA was isolated from peripheral leukocytes by using phenol-ethanol extraction method. PON gene was amplified by polymerase chain reaction in thermal cyler. The sense primer 5_TATTGTTGCTGTGGGACCTGAG3 and the antisense primer 5_CACGCTAAACCCAAATACATCTC3, which encompass the polymorphic region of the human PON1 gene was used. The 99 bp PCR products were then digested with 8U of Alw 1 restriction endonuclease, which results in 66 and 33 bp products. Digested products were separated by electrophoresis on 3% agarose gel and visualised by ethidium bromide(30).

All statistical analyses were performed by the statistical package SPSS for Windows, version 11.0 (SPSS, Chicago, IL). Correlation was calculated as Spearman correlation coefficients. Analysis of variance followed by the Newman-Keulls multiple range tests and for inter-group comparisons, student t test for parametric variables were used. Kruskall Wallis and Mann-Whitney U test were used for non-parametric analysis.

Results

Table 1 presents the baseline characteristics of the groups. BMI, triglyceride, Apo-B levels ($p<0.01$) and LDL-basal diene, total cholesterol, LDL-stimulated TBARS levels ($p<0.05$) of patients were higher than the controls, while HDL-cholesterol, Apo-A levels ($p<0.01$), arylesterase and paraoxonase activities ($p<0.05$) of patients were lower than the controls. When the data were evaluated based on gender; there was no statistically significant difference in all parameters between the male and female.

When we evaluated the distribution of polymorphisms between the groups, we determined that RR polymorphism was more common in patient group (n=7) than the controls (n=2), with a likelihood ratio= 3.58 but not statistically significant ($p=0.167$). Only paraoxonase activities changed in both control and patient group according to PON192 polymorphism, they were lower in subjects who had QQ polymorphism than those with RR polymorphism ($p<0.05$). On the other hand arylesterase activities of patients were lower than control's in all polymorphism group (Figure 1).

The patient group was evaluated in regard to the risk factors (smoking, hypertension, obesity, family history) for CAD. The risk factor numbers were calculated by giving a value for each risk factor. The summation of risk factors was used to compare the effects of risk factors on oxidative stress and paraoxonase, arylesterase activities. It was noticed that TBARS levels raised with the increase in number of risk factors for coronary artery disease (Figure 2). On the contrary, arylesterase activity is decreased as the number of risk factors increased.

Table 1. All data from controls and patients. *$p<0.01$ and **$p<0.05$ compared to controls.

	Control (Mean±SD)	Patient (Mean±SD)
Age	33±5,8	38±5,2
BMI	22±2,8*	27±3,8
Waist cm	77±10,4	97±10,9
Waist/hip ratio	0,82±0,14	1,19±1,52
T. cholesterol mg/dL	180±31	198±49**
Triglyceride mg/dL	118±50	234±236
HDL-cholesterol mg/dL	51±8,9	40±9,1*
LDL-cholesterol mg/dL	118±26	113±42
Apo-A1	151±25	116±21*
Apo-B	82±19	97±26*
Basal LDL-dien	158±51	184±61**
Simulated LDL-dien	202±48	204±64
Basal LDL TBARS	0,33±0,11	0,36±0,19
Stimulated LDL TBARS	3,66±1,48	4,52±2,40
Paraoxonase	76,7±41,9	60,9±39,9**
Arylesterase U/L	120±33	102±27**

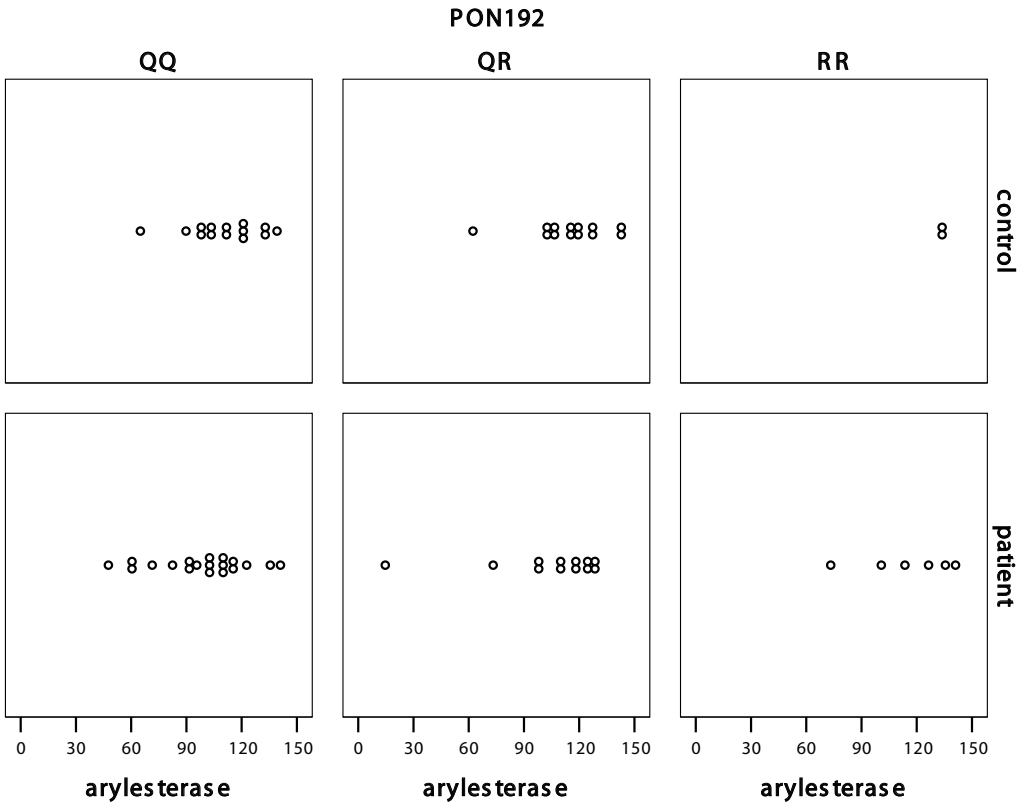

Figure 1. Arylesterase activities (U/L) of controls and patients according to PON192 polymorphism. The amino acid polymorphism at position-192 (glutamine to arginine) results in two allozymes Q and R.

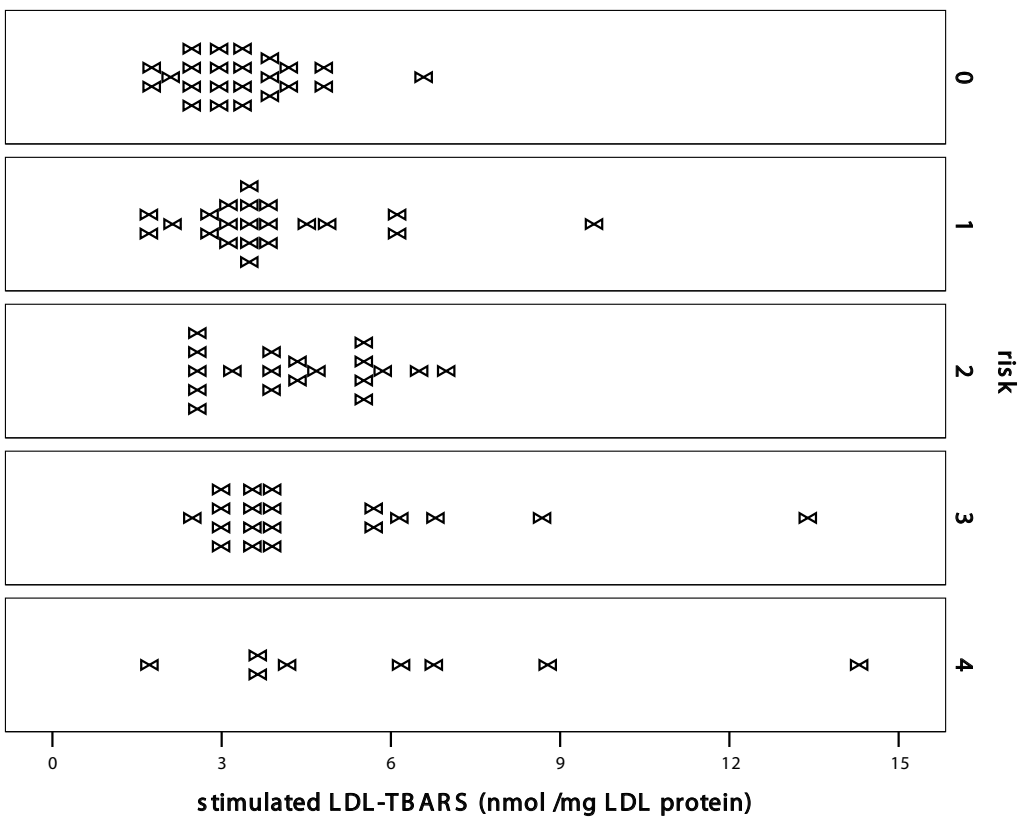

Figure 2. Copper-stimulated LDL-TBARS levels according to the number of risk factors the patients have for CAD. ,00 represents patients with no risk factors, and =>4 represents patients with 4 or more risk factors.

Discussion

We investigated if PON192 polymorphism might be used as a genetic indicator of risk for future CAD in young adults and proposed that subjects with RR polymorphism might be more prone to CAD than other people.

There are various reports on the association between the PON192 polymorphism and coronary heart disease in literature, however they present conflicted data (4-6, 8-18). Baum et al (15) proposed that these discrepancies in the literature might be associated with various factors such as differences in age distributions, ethnicity, diet, exercise, prevalence of hypertension, diabetes, smoking etc. In the literature searched only one report was conducted on young patients with arterial ischemic stroke, our study seems as first report which investigates the importance of PON192 polymorphism in young adults (18). Recently, Baum et al has shown that paraoxonase1 gene 192R polymorphism is associated with young (<60 years old) MI (15), our data also showed that subjects (<50 years old) with RR polymorphism are more prone to CHD. Interestingly, Senti et al (8) investigated the impact of PON polymorphism on myocardial infarction among individuals stratified by tertiles of age distribution and they determined that the risk of MI increases with advancing age principally in subjects with QQ genotype; patients aged over 62 have a four-fold increased risk for MI compared with younger (<50 years old). However their data has no clear explanation on the role of PON192 polymorphism on MI in young patients and low paraoxonase activity in controls compared to patients with RR.

The PON1 genotype has a direct effect on serum PON1(paraoxonase) activity and also on endothelial functions. Previously, we demonstrated low activities of paraoxonase in patients with both angiographically proven coronary artery disease and syndrome x, associated with impairment in microvascular endothelial functions which is one of the first finding during atheroclerotic process (24,25). Numerous reports demonstrated a depletion in PON1 activity in CAD patients (26-28) speculating on different mechanisms (such as attack by hydroxyl radicals, direct oxidation by peroxides and negatively charged lysophospholipids, alkylation by a,b-unsaturated lipid aldehydes, etc). Since it has been shown that PON1 activities (paraoxonase) were affected by risk factors (hypertension, aging and gender) (24, 33), and the lactonase/arylesterase activities of PON1 have been suggested more important than the paraoxonase activity in the enzyme's physiological role (in oxidation protection and cholesterol efflux) by Rosenblat et al (34), in this study we also determined arylesterase activities together with paraoxonase activities. Arylesterase activities of patients were lower than control's in each polymorphism group (Figure 1), and TBARS levels increased as the number of risk factors increased (Figure-2). Since Watson et al. (35) previously proposed that PON1 does not only have a role in inactivation of LDL-oxidation during the initiation phase of atherosclerosis, but also in the prevention of monocyte-endothelial interaction during the propagation phase of atherosclerosis, we proposed that paraoxonase/arylesterase activities of patients with CAD are lower than healthy controls in the beginning of atherosclerotic process and/or subjects who has low paraoxonase/arylesterase activities might be more susceptible to atherosclerosis and/or paraoxonase/arylesterase activities might be an indicator for future CAD in early ages of life.

It has been known that increase in oxidative stress and impairment of antioxidant status during aging are crucial processes playing an important role in progress of atherosclerosis by leading to modification of LDL, modification of arterial wall, inhibition of antioxidant enzymes etc. Our previous data suggested an elevation of oxidized-LDL (TBARS and diene levels) according to the stage of atherosclerosis (24), in patients aged between 25-80 years old, current data also showed increase in LDL-basal diene, LDL-stimulated TBARS levels ($p<0.05$) of young patients. Cherki M et al (36) determined a significant increase in LDL susceptibility to lipid peroxidation as a function of aging and they proposed this elevation is strongly associated with the high incidence risk of atherosclerosis with aging. Since our current data showed an elevation in the susceptibility of LDL to oxidation (increase in stimulated TBARS levels) in young patients compared to age matched controls we might consider that it's a reliable marker for further CAD in young adults independent from aging process.

In conclusion, our findings suggest that presence of RR polymorphism and low paraoxonase/arylesterase activities as well as increase in LDL oxidation might be useful predictive marker for determination of future atherosclerosis in early ages.

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