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Research Article

Clinical Implications of *PON1* Gene Polymorphism in Patients with Coronary Artery Disease

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ABSTRACT
Genetic and environmental factors play an importantrole in the pathogenesis and progression of coronary artery disease (CAD).*PON1 Q192R* polymorphism has been analyzed extensively, but data on associationand role of its polymorphism in the etiology of CAD are conflicting.We aimed to test the genetic association between *PON1 Q192R* polymorphism and CAD among Egyptian populations. In this preliminary study 288 CAD and 160 healthy controls were enrolled. DNA was extracted from whole blood and*PON1* polymorphism were studiedin relation to CAD using polymerase chain reaction(PCR) followed by restriction length polymorphism. *PON1-192* polymorphism reported significant differences between patients and control in RR genotype and lipid profiles. Smoker CAD patients showed significant RR genotype as compared to non-smokers. PON1 enzyme activity was lower in patients as compared to control individuals and was significantly correlated with TC, HDL-C, LDL-C and QR genotype of *PON1-192* gene polymorphism. Thus *PON1-192*R allele and RR genotypewere significantly associated with CAD patients. This association was stronger in smokers, supporting the conclusion that an interaction between *PON1*polymorphism and smoking augments CAD risk. Further studies with larger sample size are warranted to confirm these associations in different risk factors.

Keywords: Coronary artery disease, *PON1* gene polymorphism, PCR, RFLP.

INTRODUCTION

Coronary artery disease (CAD) is the principal threat to health in countries in Africa and theMiddle East, as elsewhere. The prevalence of coronary heart disease is promoted in turn by a high prevalence ofcardiovascular risk factors, particularly smoking, hypertension and dyslipidemia[1].Various lines of evidencehave suggested that genetic factors contribute significantly tothe risk of CAD[2] as conventional risk factorsare insufficient to explain the etiology of CAD in all cases[3]. Genetic epidemiologic studies have suggested certain geneticvariants, including polymorphisms in some genes among them; paraoxonase1 (*PON1*) gene to have astrong association with CAD and related outcomes. Human *PON1* is a calciumdependentglycoprotein that is synthesized in the liver thensecreted into the plasma and associated with high density lipoproteins.*PON1* prevents LDL lipid peroxidation and protectsagainst atherosclerosisand coded by the *HUMPONA*, locatedon 7q 21.3, along with two adjacent genes, *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). The *Q192R* (rs662) polymorphism inthe coding region of *PON1* gene is an amino acid exchange glutamine (Q) to arginine (R) substitution at position 192 in the A575G region andis associated with variation in its hydrolysis activity[4]. *PON1* activity has been consistentlyreported to be significantly diminished in atherosclerosis andother cardiovascular diseases[5,6]. More recently,there have been many studies investigating theassociation between the *PON1* gene polymorphismsand CAD. However, the results are controversial. As number of studies reported that the*PON1* c.575A>G polymorphism does not representa risk factor for CAD[7-9]. In parallel,others report that same polymorphic site is animportant risk factor for CAD[6,10,11]. Paraoxonase 1 (PON1) is an HDL-associated enzyme esterasewhich appears to contribute to the antioxidant and anti-atheroscleroticcapabilities of HDL-C. Several prospectivestudies have shown that low PON1 is an independent riskfactor for atherosclerotic events. Although this finding isnot universal, low PON1 is a general feature of people whodevelop atherosclerosis[12,13]. Hence, the present case control study was undertaken to examine the association of A575G *PON1* polymorphismwith coronary artery disease (CAD) and to evaluate the PON1 enzyme activity and their relations to lipid profile and risk factors for CAD

PATIENTS AND METHODS

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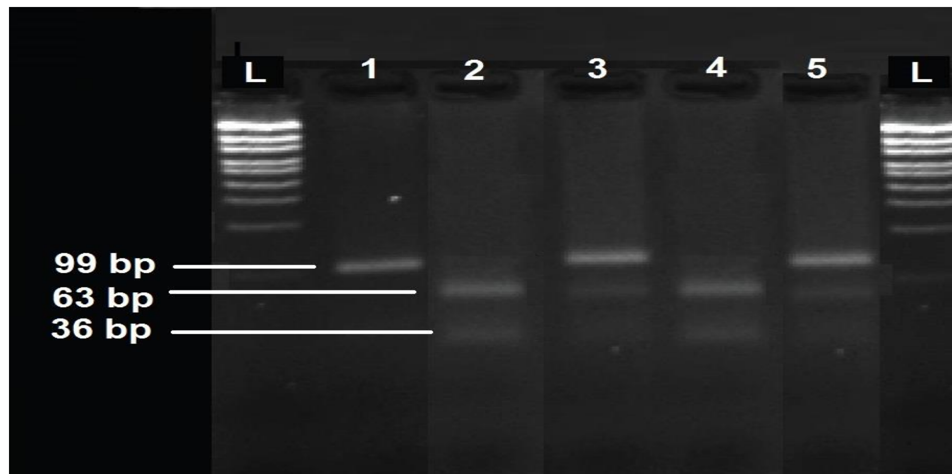

Figure 1: Polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) of *PON1* gene. Lane (L) DNA molecular marker (50-1000), Lane (1). undigested PCR product; Lane (2) lane 2: QQ genotype (homozygous, wild-type); lane 3: QR genotype (heterozygous); lane 4: RR genotype (homozygous, polymorphic).

Table 1: Demographic profile and clinical characteristics of coronary artery disease patients and controls.

Parameters	Controls =160 (Mean ± SD)	Patients =288 (Mean ± SD)	*P*-value
Age (Years)	53 ± 5.5	55 ± 5.6	0.042
Gender (Male/Female)	108/52	184/104	0.836
Smokers (Yes/No)	124/36	168/120	0.04
Hypertension (Yes/No)	0/160	172/116	< 0.0001
S. TG (mg/dL)	94 ± 13	180 ± 19	< 0.0001
S. TC (mg/dL)	162 ± 16	301 ± 58	< 0.0001
S. HDL (mg/dL)	57.8 ± 7.4	35 ± 7	< 0.0001
S. LDL (mg/dL)	82 ± 13	190 ± 17	< 0.0001
S. VLDL (mg/dL)	37 ± 6.5	59 ± 7.8	< 0.0001
Apo-A1 (mg/dL)	100 ± 10	67 ± 13	< 0.0001
S. PON1 activity (U/ml)	263 ± 70	153 ± 49	< 0.0001

Study population and blood collection

This study was approved by the Ethical Committee of Faculty of Medicine, Al-Azhar University. Accordingly, two hundred-eighty eight four patients proven chronic artery disease (CAD) were enrolled in this study from October 2013 to September 2014. These patients were evaluated at the Internal Medicine Department, Faculty of Medicine, Al-Azhar University, Egypt. Complete medical examination, ECG, Echo and doppler were done to confirm diagnosis and all of them were having a history of myocardial infarction, repeated chest pain which diagnosed as angina. One hundred-sixty individuals were selected as controls after a detailed evaluation of history;clinical features; and investigations, they were free from any chronic disease, their lipid profile was normal, no history of smoking or chest pain, had normal blood pressure and free E.C.G. to exclude presence of CAD.Controls with risk factors like family historyof CAD, diabetes mellitus, hypertension andhyperlipidemia were excluded from the study. After signing informed consent from all participants, 5ml blood were collected from them after overnight fasting and then divided into two portions; the first was taken on EDTA the other portion was left until clotting. Serum and plasma samples were stored in -80°C till further examinations.

Lipid profile

Lipid profile was measured in serum samples includedtotal cholesterol[14]; HDL-cholesterol(HDL-c)[15],LDL-cholesterol (LDL-c)[16]; and triglycerides (TG)[17] using commercially available kits. Apo-A1 was measured by Immunturbidometric using DADE Behring Kits, Marburg/ Germany.Paraoxnase (PON1) enzyme activity, using phenylacetate as the substrate, wasanalyzed in serum samples as described previously[18].

Molecular analysis

Genomic DNA was extracted from whole blood using Biospin whole blood genomic DNA extraction kit (Bioer Technology , Binjiang, Hanchuan, Hubei, China) and quantified by using NanoDrop Q5000 (Quawell Technology, Inc. USA)

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

The c575A>G (g.34650T>C, g.21439A>G, *PON1*192) polymorphic site of the *PON1* (rs662) (NM-000446.5;GI: 5444) gene was investigated using the PCR-RFLPtechnique[19]. The primers used were provided by Bio Basic Inc. (Ontario, Canada)(sense primer 5\-TATTGTTGCTGTGGGACCTGAG-3\, and anti-sense 5\-CACGCTAAACCCAAATACATCTC-3\)[20]which amplify a PCR product of 99–bp.PCR was performed in a

Table 2: Distribution of genotype and allele frequency of *PON1-192* polymorphism in patients and controls.

Genotype *PON1-192*Q/R	Controls	Patients	*P*-value	OR (95% CI)
Genotype Frequency				
n	68	284		
QQ	12	96	0.228	1.723 (0.708 – 4.201)
QR	20	32	0.806	0.876 (0.266 – 2.789)
RR	36	156	< 0.0001	14.566 (4.115 – 51.633)
Allele Frequency				
Q Allele	44	224	0.138	1.577 (0.875 – 2.842)
R Allele	92	344	< 0.0001	9.301 (4.535 – 19.074)
Allele Carriage Frequency				
Q Allele Carriage	32	128	0.234	1.49 (0.667 – 3.303)
R Allele Carriage	56	188	< 0.0001	7.5 (3.02 – 18.76)

Table 3: The clinical profile with and without polymorphic genotype for *PON1-192* gene in patients with CAD (n=252).

	PON1-192 gene		
Genotypes	QQ	RR	*P*-value
Subjects (n)	96	156	
Age (years)	54.2 ± 5.7	53.3 ± 6	0.983
S. TG (mg/dL)	167 ± 11	187 ± 8	< 0.0001
S. TC (mg/dL)	262 ± 19	317 ± 20	< 0.0001
S. HDL (mg/dL)	34 ± 4	41.6 ± 6	< 0.0001
S. LDL (mg/dL)	182 ± 14	202 ± 12	< 0.0001
S.VLDL (mg/dL)	57.8 ± 6	58 ± 8	0.876
Apo-A1 (mg/dL)	64 ± 11	71 ± 12	0.014
S. PON1 activity	142 ± 53	155 ± 31	0.281

final volume of 50 µl that contained 100ng of DNA template (6 µl), 0.5 µM of each primer(6µl of each primer), *Taq* PCR MasterMix containing: 1.5 mM $MgCl^2$, 200µM of each deoxynucleotidetriphosphate (dNTPs; Fermentas, St. Leon-Rot,Germany)and 2.5 units of Taq DNA polymerase (25 µl) (QiagenGmbh, Hilden, Germany) and DdH_2O(7µl).PCR reactions were performed in a thermal cycler (Agilent Surecycler 8800) for the amplification of the polymorphic region with an initial denaturation at 95ºC for 5min, followed by 35 cycles, each consisting of three 1-min steps: denaturation at 95ºC, annealing at 61ºC and exetension at 72ºC. The reaction was completed with a final extension at 72ºC for 10 min. The *PON1* 192 (99-bp) PCR product was digested with 8 U of *AIWI* (New England Biolabs, Cambridge, MI U.S.A) at 37ºC for 5 hours. Digestion with *AIWI* resulted in 63-bp and 36-bp fragment for 192Arg(arginine) allele and a non-digested 99-bp fragment for the PON1 192 GLn (glutamine) allele, respectively. Successful amplification was confirmed by electrophoresis of the digested fragments on an ethidium bromide impregnated 4% agarose gel, then visualized on UV transilluminator (G BOX F3, Syngene UK).The digested *PON1*c.575 Qallele yielded 1 fragment of 99 bp, and the R alleleyielded 2 fragments of 63 and 36 bp(Figure 1).

Statistical analysis

Analysis of variance (ANOVA) was used to compare the means for continuous variables. Genotype andallele frequency of *PON1* c.575A>G was tested for Hardy-Weinberg equilibrium byemploying the chi-square test also 95% confidence intervals were calculatedusing standard epidemiological/ associationmethods,. Genotype and allelefrequencies of these polymorphisms were analyzedwith Fisher's exact test.Statistical significance was defined as $P< 0.05$.All analyses were performed using Statistical Package for the Social Sciences software (SPSS Inc., Chicago, IL)(SPSS version 11.0).

RESULTS

Characteristics of the study groups

Clinical and baseline characteristics of CAD patients andhealthy controls are shown in Table 1. Patients with CAD were older than control individuals (P=0.043). Male formed 92% of the patients and 54% of the control group. There was no age difference between males and females in the two groups. Rate of risk factors for CAD was markedly higher in the patient group than control individuals; hypertension 86% *versus*0% ($P< 0.0001$), smoking 84% *versus*62% ($P= 0.041$).Lipid profile analysis showed significantly higherTC, TG, LDL-C and VLDL-C and lower HDL-C and Apo-A1in patientscompared to control ones ($P<0.0001$).

Genotype and allele frequencies among investigated groups

Table 2 describes the distribution of *PON1*genotypes and allele frequencies and associated odds ratios with confidence intervals. It is clear that *PON1*-192 RR genotype and *R allele are significantly associated with CAD risk among Egyptian patients as compared to control individuals.

Demographic and clinical profiles with and without PON1 gene polymorphic genotype in CAD patients

As reported in Table 3, significant differences were

Table 4: The effect of *PON1* genotypes on risk of CAD among different risk factors

PON1 genotypes	Controls (%)	Patients (%)	*P*-value	OR (95% CI)
Smokers				
n	56	164		
QQ	32 (57.2)	52 (31.7)	0.641	1.5 (0.5 – 4.3)
QR	12 (21.4)	24 (14.6)	0.654	1.4 (0.34 – 6.5)
RR	12 (21.4)	88 (53.7)	< 0.0001	9.9 (2.6 - 37.7)
Allele Q	76	128	0.076	2.7 (0.92 – 8.06)
Allele R	36	200	0.821	2.7 (0.33 – 2.04)
Non-Smokers				
n	12	180		
QQ	4 (33.3)	44 (36.7)	0.155	2.316 (0.415 – 12.9)
QR	8 (66.7)	8 (6.7)	0.187	0.286 (0.035- 2.360)
RR	0	68 (56.6)	0.004	0.5 (0.49 – 0.809)
Allele Q	16	96	0.6873	1.45 (0.25 – 8.3)
Allele R	8	144	0.56	0.64 (0.15 – 2.8)

reported between CAD patients with (RR) or without (QQ, wild – type) the polymorphic genotype in terms of TG, TC, HDL-C, LDL-C at ($P<0.0001$), and Apo-A1at ($P= 0.014$). Authors also assessed if there was any interaction between smoking and *PON1* polymorphism, accordingly, individuals were classified as smoking and non-smoking status and genotypes were assessed (Table4).

Association between PON1 activity with lipid profile and PON1-192 polymorphism

PON1 activity was significantly correlated with all lipid profile markers when considering enrolled individuals (n=448), while significance was reported between PON1 activity and TC, HDL-C and LDL-C among CAD patients as reported in Table (5).

Also the association of PON1 activity and *PON1* gene polymorphism was investigated. PON1 activity was significantly detected in genotypes QR and QQ (F=22.5, 27.27, $P<0.0001$ respectively) when considering all individuals. For CAD patients, PON1 activity was significantly detected in genotype QR (F=8.44, P=0.005).

DISSCUTION

In this case-control study, 288 cases with CAD and 160 healthy control individuals were enrolled. Despite its moderate sample size, this study has the merit of enabling assessment of the risk associated with *PON1* gene polymorphism and CAD. Accordingly the frequencies of *PON1* genotype and allele were significantly higher in patients compared with controls, indicating the association of *PON1* gene Q192R polymorphism with the disease. These results were in accordance with the observations of earlier investigators, which support that RR genotype is associated with CAD[21,22]. Moreover, regarding all enrolled individuals (n=448) the RR genotype was the most common in our population, followed by QQ and then QR. This distribution in agreement with Asian population and disagreed with others[23]. For the allele frequency; our study is in disagreement with other populations[23] as our results showed predominance of R allele in *PON1* 192 over the Q allele. Moreover, significant association was detected between *PON1-192* R (Arg) allele and CAD risk (Table 2), these results has been agreed with previously detected in Asian Indians[6] and North American Caucasians[24] but not in Korean[25] Spanish[26] and British Caucasian[27] and Polish[28] populations. This ethnic variability in allele frequency may explain variations in the activity of paraoxonase in different populations, resulting in differences in the toxicity of organophosphates such as paraoxon, diazoxon, and sarin, and hence in morbidity and mortality, as well as in susceptibility to CAD[29]. The *PON1-192* polymorphism may be associated with an increased CAD risk due to the R (Arg) allele (high-activity R isoform) being less significant than the Q (Gln) allele (low-activity Q isoform) in reducing the oxidative modification of LDL, decreasing lipid peroxide hydrolysis[30] However, it has also been suggested that the quality of the PON1 enzyme may be a better predictor of coronary heart disease (CHD) risk than the *PON1* genotype; because as compared to controls, patients consistently demonstrate lower PON1 activity level, regardless of their *PON1* genotype.

Our results show that PON1 activity was lower in patients with CAD as compared to control individuals. Activity of PON1 can vary up to 40-fold in human populations[31]. Part of this variability is explained by the polymorphism of *PON1* gene because of an amino acid substitution at 192[32]. The R allele (arginine at position 192) displays several-fold higher activity toward paraoxon hydrolysis than the Q allele (glutamine at position 192), and may have a greater capacity to protect against copper ion-induced LDL oxidation[33]. In contrast, phenylacetate hydrolysis is largely unaffected by the polymorphism, and has been used as a surrogate for protein concentrations[34]. Thus the use of phenylacetate, as in our study, provides a better overall indication of PON1 activity levels. However, which PON1 substrates should be used in correlative studies with cardiovascular disease is unresolved[35]. Paraoxonase 1 (PON1) is an HDL-associated enzyme esterase which appears to contribute to the antioxidant and antiatherosclerotic capabilities of HDL-C, according to the results of this study, PON1 activity was significantly correlated with HDL-C. It has been reported earlier that factors modulating the HDL-associated PON1 enzyme activity are the same for endothelial modulation, encouraging the proposal that HDL-associated PON1 enzyme activity is the cornerstone for endothelial

Table 5: Association between PON1 activity and lipid profile.

Lipid profile	PON1 activity in all individuals (n=448)		PON1 activity in CAD patients (n=288)	
	R	*P*	R	*P*
S. TG (mg/dL)	-0.542	< 0.0001	0.209	0.077
S. TC (mg/dL)	-0.405	< 0.0001	0.394	0.001
S. HDL (mg/dL)	-0.558	< 0.0001	0.443	< 0.0001
S. LDL (mg/dL)	-0.443	< 0.0001	0.294	0.012
S. VLDL (mg/dL)	-0.614	< 0.0001	-0.105	0.889

function[36]. Thus assays of HDL-associated PON1 enzyme action on endothelium may increase our knowledge to assign atherosclerotic disease risk, and they may boost our understanding of the outcomes of future trials testing HDL associated PON1 enzyme targeted therapies. There appears to be a strong gene environment interaction in relation to the *PON1-192* gene polymorphism and CAD. The environmental factors may mask or induce the atherogenic potential of the *PON1-192* R (Arg) allele, thus modifying the effect of the *PON1* gene on CAD risk[36]. It is conceivable that the *PON1-192* R allele has a more exaggerated effect on CAD risk for those living in high-risk environments of enhanced oxidative stress, such as smokers. In relation to smoking, in the present study, a larger proportion of patients were smokers in comparison to controls (*P*= 0.041). Cigarette smoke contains many free radicals, and smokers generally have a lower antioxidative capacity than nonsmokers. Therefore, oxidized LDL particles from smokers generate more lipid peroxidation products than LDL from nonsmokers. Thus, it is possible that PON1 activity has a more important protective role of lipid peroxidation in smokers than in nonsmokers. Dyslipidemia is a major risk factor for CAD, which is associated with oxidative stress[37]. The analysis of *PON1* 192 QR polymorphism has revealed significant difference between the two genotypes (QQ *vs*RR) among TG, TC, HDL, LDL and Apo-A1 thus confirm the association between high lipid profile and CAD. This study is, to our knowledge, the first to present data on both PON1 enzyme activity and the polymorphism of *PON1-192* gene in Egyptian patients with CAD population. We believe that the methodology used in this study was appropriate and that our results may contribute to understanding the far from simple genetic aspects of complex and multifactorial diseases such as CAD. However, further research is warranted on larger populations to clarify if the PON loci are independent risk factors for CAD.

CONFLICT OF INTEREST

Authors have nothing to disclose

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