ORIGINAL ARTICLE

RELATIONSHIP BETWEEN *PON1*L55M AND Q192R GENE POLYMORPHISMS AND HIGH APO B/APO A-I RATIOS

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ABSTRACT

Elevated Apolipoprotein B/Apolipoprotein A-I ratio is a risk factor for predicting coronary artery disease (CAD). Paraoxonase 1 (PON1) is a high density lipoprotein (HDL) associated serum enzyme. PON1 protects low-density lipoproteins (LDLs) from oxidative modifications and thus has a protective effect against CAD progression. There are two common polymorphisms, Q192R and L55M, in PON1 gene. There may be a relationship between these polymorphisms and elevated ApoB/ApoA-I ratios. Therefore, we decided to evaluate effect of these polymorphisms on individuals with high and normal ApoB/ApoA-I ratios. To evaluate Q192R and L55M polymorphisms in Iranian case group (n=75) with high ApoB/ApoA-I ratio, and control group (n=75) with normal ApoB/ApoA-I ratio, we carried out PCR using specific primers. Then, we digested PCR products by RFLP. ApoB and ApoA-I levels were determined by immunoturbidimetry method. Genotype frequencies for Q192R were determined: 49.3%QQ, 44%QR, 6.7%RR in case group, and 53.3%QQ, 33.3%QR, 13.4%RR in controls ($P=0.236$). Genotype frequencies for L55M were determined: 21.3%LL, 68%LM, 10.7%MM in case group and 42.7%LL, 52%LM, 5.3%MM in controls ($P=0.016$). A significant relationship between L55M polymorphism and familial history of cardiovascular disease was found ($P=0.011$). In our study *PON1*L55M polymorphism was associated with high ApoB/ApoA-I ratios in case group. Thus, L55M polymorphism may be an independent risk factor for cardiovascular disease. Since L55M polymorphism was associated with familial history of cardiovascular disease, it is better to evaluate L55M polymorphism in younger ages even in the absence of high ApoB/ApoA-I ratios.

KEY WORDS

Apolipoprotein B, Apolipoprotein A-I, Paraoxonase 1 (*PON1*) gene.

INTRODUCTION

Apolipoprotein B plays a crucial role in the intracellular assembly and secretion of triglyceride-rich lipoproteins and is a ligand for the LDL receptor. Apolipoprotein A-I is important for HDL functions. High plasma levels of apoB are directly correlated with the risk of atherosclerotic coronary artery

disease (CAD) (1-3). The apolipoprotein B/apolipoprotein A-I ratio represents the balance of pro-atherogenic and anti-atherogenic lipoproteins. Walldius et al reported the ApoB/ApoA-I ratio is better than the cholesterol indices to estimate the balance between plasma pro-atherogenic and anti-atherogenic lipoproteins and to predict coronary heart disease (CHD) (2). Human paraoxonase/arylesterase (*PON1*:aryldialkylphosphatase [E.C.3.1.8.1]) is an enzyme synthesized in the liver and secreted into the plasma where it is associated with high-density lipoproteins (HDL); *PON1* gene is a member of a multigene family conformed also by *PON2* and *PON3* genes (4).

Two polymorphisms in the coding region of *PON1* gene have been reported: at position 55 (Leu [L]/Met [M]) that is related

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Table 1: PCR and RFLP conditions

Polymorphisms	Primers	Tannealing (°C)	Restriction enzyme	PCR fragment	RFLP fragments
Q192R	F: 5'TATTGTTGCTGTGGGACCTGAG3' R: 5'CACGCTAAACCCAAATACATCTC3'	56	<i>Bsp</i> PI	99 bp	Q Allele: 99 bp R Allele: 66+33 bp
L55M	F: 5' CCTGCAATAATATGAAACAACCTG3' R: 5' TGAAAGACTTAACTGCCAGTC 3'	53	<i>Hin</i> 1II	172 bp	L Allele: 172 bp M Allele: 103+69 bp

with differences in PON1 activity (5), and polymorphism at position 192 (Gln [Q]/Arg [R]) has been described as substrate-dependent (6). The physiologic role of PON1 as an antioxidant of lipoproteins has been established. As an HDL-associated enzyme, it was initially shown that PON1 prevents lipid-peroxide accumulation in low density lipoproteins (LDL) that are involved in the initiation of atherosclerosis and constitute a major risk factor for the development of coronary heart disease (7). Epidemiological evidence suggests that *PON1*Q192R polymorphism may represent a risk factor to develop CHD, in particular the 192RR genotype (8), but some studies have failed to find this relationship (9, 10).

The association between *PON1*L55M polymorphism and lipid profile is scarcely studied. Deakin et al reported that this polymorphism may be of particular relevance for vascular disease and impaired glucose metabolism (11). This highlights the need to evaluate the association between *PON1* polymorphisms and lipid profile, particularly ApoB/ApoA-I ratio, which is one of the risk factors to develop heart diseases, in general populations.

MATERIALS AND METHODS

Study Population: A case-control study was performed in North-west of Iran, Zanjan, during March to December 2008, to evaluate the association between *PON1*Q192R and *PON1*L55M polymorphisms and serum ApoB/ApoA-I ratio. Eligible participants were 150 unrelated individuals aged 25 to 85 years, include men and women without coronary heart disease who had diagnosed by angiography and admitted to Bu-Ali clinical laboratory of Zanjan. 75 subjects who had elevated ApoB/ApoA-I ratio selected as case group and 75 subjects who had normal ApoB/ApoA-I ratio selected as control group. Some information asked from participants by trained personnel. Structured questionnaire included age, sexuality, smoking habits, familial history of cardiovascular disease, history of refer to cardiology clinic, history of hospitalization due to heart problems, history of bypass, history of angioplasty, hypertension and drug usage especially anti lipid and anti oxidant drugs. Participants with liver diseases, renal /and

thyroid problems, diabetes mellitus and participants who had taken anti lipid /and anti oxidant drugs during one month before sampling, deposited from study.

Sampling: Blood samples were collected after overnight fasting in EDTA coated tubes to analyze *PON1* genotypes and without anticoagulant for serum lipid profile, apoB and apoA-I determinations. Serum was isolated by 1600g during 10 minute centrifugation. Collected samples were stored at -20°C until analysis.

***PON1*Q192R and L55M polymorphisms:** Genomic DNA was extracted using Bioneer AccuPrep® Genomic DNA Extraction Kit (Bioneer, South Korea) according to the protocol of the manufacturers. To prevent contamination, all PCR procedures were performed under an ultraviolet-irradiated hood. *PON1*Q192R and L55M polymorphisms were determined by PCR (ASTEC thermal cycler, PC-818), lyophilized premix PCR tubes (Bioneer AccuPower™ PCR Premix; lot number: K-2013) and followed by restriction fragment length polymorphism analysis (RFLP). Primers, annealing temperature and length of PCR products for each polymorphism explored in this study are given in Table 1. Each restriction endonuclease mixture (total volume 24 µl) contained 15 µl amplified fragment, 5 µl distilled water, 2 µl appropriate buffer for each restriction endonuclease (Fermentas Life Science) and 4 unit of *Bsp*PI for Q192R and 4 unit *Hin*1II for L55M polymorphism (1 unit of restriction enzyme is the amount of enzyme required to digest 1 ig of lambda DNA in 1 hour at 37°C for *Hin*1II or at 55°C for *Bsp*PI). Restriction products were separated by electrophoresis on a 4 % agarose gel in TBE buffer (1x) and stained with ethidium bromide (0.5 µg ml⁻¹).

Lipid profile, ApoB and ApoA-I determination: Serum total cholesterol (TC) and triglycerides (TG) were determined by enzymatic methods using cholesterol oxidase-p-aminoantipyrin and glycerol phosphate oxidase-p-aminoantipyrin (Pars-Azmoon®, Iran/Hitachi 717), respectively. HDL-C and LDL-C were measured using the cholesterol-esterase and cholesterol-oxidase enzymes

(Randox[®], England/Hitachi 902). Apolipoprotein B and Apolipoprotein A-I determined on basis of the reaction of the sample containing human apo B or apo A-I and a specific antiserum to form an insoluble complex which can be measured turbidimetrically at 340 nm (Randox[®], England).

Statistical analysis: SPSS software (version 11.5) was used for statistical analysis. Comparisons of allele and genotype frequencies between case and control groups were analyzed using the Chi-squared test. In addition to, the incidence probability of Q192R, L55M and other variables in lipid profile were analyzed by Odd ratio. *P* values lower than 0.05 were considered statistically significant.

RESULTS

The general characteristics of the studied population are shown in Table 2. Participants mean ages in case and control groups were 56.2 ± 11.5 and 49.6 ± 14.2 , respectively. As we had expected, the ApoB/ApoA-I ratio mean in case and control groups were 1.5 ± 0.43 and 0.83 ± 0.12 , respectively.

PON1 Q192R and L55M genotypes: Exchange of codon CAA to CGA (exon 6) results in substitution of amino acid glutamine

with arginine at position 192 (Q192R) and introduces the restriction site for *Bsp*PI endonuclease, while exchange of codon TTG to ATG (exon 3) results in leucine to methionine substitution at position 55 (L55M) and introduces the restriction site for *Hin*1II endonuclease (SNP ID for Q192R: rs662, SNP ID for L55M: rs854560). Restriction products were separated on 4% agarose gel and genotypes for each polymorphism were determined as described below.

Q192R polymorphism: As Figure 1 shows undigested fragment (99 bp) was detected in homozygotes for Q192 allele (genotype QQ), digested fragments (66 and 33 bp) were detected in homozygotes for R192 allele (genotype RR) and both digested and undigested fragments (99, 66 and 33 bp) were detected in heterozygotes (genotype QR). **L55M polymorphism:** according to Figure 2, undigested fragment (172 bp) was detected in homozygotes for L55 allele (LL genotype), digested fragments (103 and 69 bp) were detected in homozygotes for M55 allele (genotype MM) and digested and undigested fragments (172, 103 and 69 bp) were detected in heterozygotes (genotype LM).

Table 2: General characteristics and lipid profile from participating subjects

Parameter	Case (n=75)	Control (n=75)	P-value
Age (years) ^a	56.2 ± 11.5	49.6 ± 14.2	0.002
Sex (men) (%)	33.9	66.1	0.0001
Smokers (%)	56.2	43.8	0.792
Hypertension (%) ^c	61.1	38.9	0.014
Total Cholesterol (mg/dL) ^{a,b}	243 ± 40.7	172.9 ± 30.5	0.0001
Triglycerides (mg/dL) ^{a,b}	262 ± 215.7	147.6 ± 60.3	0.0001
HDL-C (mg/dL) ^{a,b}	40.3 ± 4.1	43.6 ± 4.4	0.0001
LDL-C (mg/dL) ^{a,b}	174.6 ± 40	112.7 ± 24.2	0.0001
Total Cholesterol/HDL-C ^{a,b}	6.1 ± 1.4	4.0 ± 0.8	0.0001
LDL/HDL ^{a,b}	4.4 ± 1.2	2.6 ± 0.7	0.0001
ApoB (mg/dL) ^{a,b}	171.2 ± 35.5	105.4 ± 13.7	0.0001
ApoA-I (mg/dL) ^{a,b}	119 ± 18.3	127.7 ± 12.7	0.003
ApoB/ApoA-I ^{a,b}	1.5 ± 0.43	0.83 ± 0.12	0.0001

^a Data are expressed as arithmetic mean $\pm$ SD. ^b Lipid profile normal values: Total Cholesterol = 200; Triglycerides = 190; HDL = 40; LDL = 129; total cholesterol/HDL = 4.7; LDL/HDL = 3.5; ApoB = 114; ApoA-I = 120; ApoB/ApoA-I: 0.35-0.96. ^c SBp=140; DBp=90.

Fig 1: Investigation of Q192R genotypes by Agarose electrophoresis. QQ (Normal), QR (polymorphism in one allele) and RR (polymorphism in two alleles)

Association between PON1 Q192R and L55M polymorphisms and ApoB/ApoA-I ratios: Frequencies for PON1Q192R one allele polymorphism (QR) in case and

Fig 2: Investigation of L55M genotypes by Agarose electrophoresis. LL (Normal), LM (polymorphism in one allele) and MM (polymorphism in two alleles)

control groups were 44% and 33.3%, respectively. Frequencies for *PON1*Q192R two allele polymorphism (RR) in case and control groups were 6.7% and 13.4% ($P = 0.236$). Frequencies for *PON1*L55M one allele polymorphism (LM) in case and control groups were 68% and 52%, respectively. Frequencies for *PON1*L55M two allele polymorphism (MM) in case and control groups were 10.7% and 5.3%, respectively ($P = 0.016$). Table 3 shows the *PON1* genotype frequencies in case and control groups. In investigation of polymorphisms odd ratios in case group, as table 4 shown, the odd ratios of Q192R and L55M polymorphisms in one or two allele were 1.55 and 3.12, respectively. However, the effect of L55M polymorphisms in

Table 3: PON1 genotype frequencies in case and control groups. Q192R genotypes include: QQ (Normal), QR (Polymorphism in one allele), RR (Polymorphism in two allele); L55M genotypes include: LL (Normal), LM (Polymorphism in one allele), MM (Polymorphism in two allele)

Studied groups	PON1Q192R and L55M polymorphism genotypes					
	QQ	QR	RR	LL	LM	MM
Case (ApoB/ApoA-I ratio > 0.96)	49.3%	44%	6.7%	21.3%	68%	10.7%
Control (ApoB/ApoA-I ratio: 0.35-0.96)	53.3%	33.3%	13.4%	42.7%	52%	5.3%

Table 4: Q192R and L55M polymorphisms odd ratios in case group

Polymorphisms in one or two allele	Odd ratio	P- value	(95%) CI
Q192R (QR/ RR)	1.55	0.212	0.78 – 3.10
L55M (LM/ MM)	3.12	0.003	1.47 – 6.63

side of the other cardiovascular risk factors come small (data not shown).

DISCUSSION

Atherosclerosis is a complex and multi-factor illness, in which changes in serum lipid levels, towards low HDL-C and increased LDL-C, TG and TC, among other changes, are believed to be determinant to its pathogenesis (12). It has been proposed that the contribution of genetic factors on lipid profile must be considered along with environmental factors (13). Several studies have focused on HDL as a protective factor against LDL lipoperoxidation. Mackness et al first reported the antioxidant activity of HDL by decreasing the accumulation of lipidperoxides on LDL, due in part, by the HDL-associated enzymes, such as PON1 (14). PON1 destroys the active LDL-derived oxidized lipids once they are formed and also prevents their formation (14, 15). On the other hand, *PON1*Q192R polymorphism has been controversially associated with the capacity to protect LDL lipoperoxidation; HDL containing the 192Q alloenzyme better retained the protective antioxidative effect than the 192R alloenzyme (14).

Thus, we evaluated the association between lipid profile and two *PON1* polymorphisms that may modulate serum lipids in a Iranian population with no diagnosis of cardiovascular illness in two groups: cases with high ApoB/ApoA-I ratio and controls with normal ApoB/ApoA-I ratio. Frequencies of *PON1* alleles

Fig 3: PON1 Q192R and L55M polymorphisms in individuals with familial history of cardiovascular disease. Normal genotypes include: QQ and LL, Polymorphic genotypes include: QR and RR in Q192R region, LM and MM in L55M region

widely vary across human populations. The susceptibility to CAD is a complex trait (16, 17) and recently one ¹⁹²Arg variant of the *PON1* gene has been reported to be associated with the incidence of CAD in the French (18) and US (12) populations of whites. However, another study in the Finnish population failed to show any correlation between this variant and CAD (19). We observed that *PON1*Q192R genotype frequencies in our population were similar to those reported in several Caucasian populations (9, 20), but different from those in some Asian, Afro-American and Mexican populations (21-25).

The association between *PON1*L55M polymorphism and lipid profile has been less studied. Norma et al didn't observe an association between *PON1*L55M polymorphism and HDL-C, but observed significant higher levels of LDL-C in 55LM heterozygote subjects (26). Fanella et al and Deakin et al reported a trend of higher LDL-C and lower HDL-C levels according to *PON1*L55M polymorphism, 55MM subjects had the less favorable profile (27, 28). *PON1*L55M genotype frequencies in our population were different from those reported in Mexican and Japanese populations (21, 26). In this study, we observed a significant association between *PON1*L55M polymorphism and higher LDL-C levels (data not shown). Also, we found a significant relationship between L55M polymorphism and high ApoB/ApoA-I ratio in case subjects ($P = 0.016$).

In contrary of some studies, there were 8% MM genotype in our population, but this genotype was not seen in Japanese and Mexican population (21, 26). Additionally, according to Figure 3, the 55LM and 55MM genotypes was associated with the familial history of cardiovascular disease ($P = 0.011$). This study was conducted in healthy individuals with no clinical diagnosis of cardiovascular disease, while most evidence has been obtained from case- control study that the cases were subjects with cardiovascular disease. In our study, strong linkage disequilibrium was detected between Q192R and L55M ($P = 0.016$). Although we are aware that the low number of participants may be an inherent limitation of our study, the estimated power was 80%, that similar to the standard (80%) (29). According to Walldius et al studies, the HighApoB/ApoA-I ratio is the best predictor for CoronaryArtery Disease in future (1-3). In the present study, the two *PON1* genotypes were determined in 75 control subjects with normal ApoB/ApoA-I ratio and 75 patients with High ApoB/ApoA-I ratio. We have found a significant association between the L55M polymorphism and High ApoB/ApoA-I ratio.

Further studies are needed to identify the molecular

mechanisms relevant to the association between the *PON1* gene and susceptibility to CAD. In contrast of van Himbergen's study on the *PON1* and familial combined hyperlipidemia (30) who found the 55LL was associated with lower HDL-C levels and higher triglycerides, and Q192R genotypes were not associated with any of the lipid or lipoprotein traits. In present study we found that there was a significant difference between 192QR and lower HDL-C levels and higher TG, but L55M genotypes was not associated with this trait (data not shown). The *PON1* gene is a member of a multigene family including *PON2* and *PON3* located at the same locus on chromosome 7 (4). Recently, Hegele et al (31) and Sanghera et al (32) described two polymorphisms present in the *PON2* gene with possible clinical relevance. However, function of the *PON2* gene product is still unknown, making the estimation of the importance of these findings difficult.

In summary, this is the first study on the relationship between *PON1*Q192R and L55M polymorphisms and ApoB/ApoA-I ratio as the best predictor for cardiovascular disease. Independent of other cardiovascular risk factors, LM and MM genotypes due to *PON1*L55M polymorphism were associated with high ApoB/ApoA-I ratios and we suggest that investigation of *PON1*L55M polymorphism is better for individuals with familial history of cardiovascular disease, even though they have normal ApoB/ApoA-I ratio.

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