Glutamine-Arginine 192 Polymorphism of Paraoxonase 1 Gene in Coronary Artery Disease Patients Compared to Healthy Controls in a Study from Kerala

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ABSTRACT

BACKGROUND

Coronary artery disease is multifactorial in origin. Coronary artery disease predisposition is attributed to genetic factors also. Many gene polymorphisms are implicated out of which paraoxonase 1 (*PON 1*) gene is an important one. The product of paraoxonase gene is paraoxonase enzyme which is seen in serum associated with high density lipoprotein (HDL). This enzyme is mainly synthesised by the liver. The protective effect of HDL is attributed to the presence of such enzymes on it. Gln to Arg polymorphism at position 192 confers a risk of developing atherosclerosis and coronary artery disease (CAD). This study is done to assess the genotype distribution of *PON 1* gene in CAD patients compared to healthy controls in a population from Kerala.

METHODS

The case group consists of 100 angiographically proven CAD patients with no history of hypertension, diabetes mellitus, hepatic disease or smoking. The control group had 100 healthy controls from the general population. *PON 1* gene was amplified by a polymerase chain reaction (PCR) technique already reported and restriction fragment length polymorphism by the restriction enzyme AlwI was done to assess the polymorphism.

RESULTS

In this study, the frequency of heterozygous genotype QR was 86 % in control and 76 % in cases. Though there was no significant difference in allele distribution of Q or R, RR genotype was significantly higher in the case group (χ^2 = 8.82; p value = .012). With binary logistic regression model, adjusting for age and sex, RR genotype is independently associated with CHD. Adjusted odds ratio of RR was 5.24 with 95 % confidence interval (CI) 1.41 - 19.47 for developing CHD ($p < 0.05$).

CONCLUSIONS

The RR genotype is more frequently seen in CAD patients than in controls. The QR genotype is more frequent than QQ or RR in both cases and controls.

KEYWORDS

Coronary Artery Disease, Paraoxonase, Gene Polymorphism

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BACKGROUND

Cardiovascular diseases are increasing at an alarming pace in India. Around 32 % of all deaths in 2000 was attributed to this disease and the World Health Organisation (WHO) estimates that India will have 60 % of the world's cardiac patients by 2020. Coronary artery disease is defined as impairment of heart function due to inadequate blood flow to the heart compared to its needs, caused by obstructive changes in the coronary circulation to the heart.[1] In India the prevalence of coronary artery disease among adults is more in urban population compared to rural population. Coronary artery disease is multifactorial in origin with modifiable and non-modifiable risk factors for the development of atherosclerosis. Atherosclerosis of the epicardial coronary arteries is the most common cause of myocardial ischemia. CAD is manifested clinically in the fourth decade of life. But atherosclerosis is a process that begins early in life and progresses silently for decades. In the International Atherosclerotic Project, atherosclerotic lesions on aorta were found at the age of 3 years and on the coronaries at the age of ten.

Many candidate genes for the development of CAD have been identified and studied in different populations like the *APOA1, APOE, TNFa* and *ACE* etc. Paraoxonase 1 gene whose location is on chromosome 7 is a potential candidate gene for the development of CAD. The protein product of the gene, the paraoxonase enzyme is a calcium dependent esterase which can hydrolyse organophosphates to relatively harmless substances. Any oxon which escapes hepatic detoxification in mammals can be hydrolysed in the blood by serum paraoxonase before it reaches the brain, which is the site of organophosphate action. Since these oxons are synthetic, their hydrolysis may be an accident of their structure and they are not the natural substrate for this enzyme. The natural substrate for serum paraoxonase is thought to be lipid peroxides that accumulate on low density lipoprotein (LDL). The paraoxonase enzyme shows substrate activity polymorphism. R allele is more active with paraoxon, methyl paraoxon etc and Q allele is active with sarin, soman, diazoxon. But the activity towards phenyl acetate substrate is similar.

LDL has been recognised by the National Cholesterol Education Program as the major atherogenic lipoprotein. The functions of the normal vascular endothelium are disturbed by the high plasma LDL levels. LDL being heterogeneous in both physical properties and chemical composition, the tendency for inducing atherosclerosis differs for different forms of LDL. There is increasing evidence that oxidised LDL exists in atherosclerotic lesions of human and experimental animals. Oxidised LDL activate immune and inflammatory responses, promote coagulation and can also regulate vascular tone. Moderately oxidised LDL interact with endothelium and stimulate the production of MCP–1[2], induce the expression of a monocyte–specific binding protein on the cell surface[3] & injure the endothelial cells in the DNA synthesis phase of cell cycle.[4] Paraoxonase enzyme in serum is seen exclusively located on HDL. Studies have shown that HDL can decrease lipid peroxide accumulation on LDL incubated under oxidising conditions by an enzymatic mechanism. Paraoxonase is one of the components of HDL responsible for this activity.[5,6] Paraoxonase enzyme activity was low in subjects who had a myocardial infarction than in control subjects in some studies.[7]

PON and related genes are even present in invertebrates such as *Caenorhabditis elegans*. Closely related genes are divided into three subfamilies: *PON1, PON2* & *PON3.* Paraoxonase gene polymorphisms are very common. In human *PON 1* gene alone there are over two hundred single nucleotide polymorphisms (SNPs) reported.[8] The role of *PON2* and *PON3* in the regulation of atherosclerosis is not known. Expression of *PON1* is primarily in the liver. *PON2* shows wide expression in a number of tissues, including, brain, liver, kidney, and testis and may have multiple mRNA forms. There is no evidence for the expression of *PON3* in human tissues.

Two most common polymorphisms in *PON1* coding region are R192Q and L55M. A ten–fold decrease in paraoxonase activity and higher susceptibility to paraoxon is shown by R192Q polymorphism.[8] The R192Q variant shows a significantly reduced activity to *PON 1*'s physiological substrate, the lipid peroxides accumulated on LDL. Thus, the susceptibility to atherosclerosis is enhanced. A lot of studies have been conducted all over the world to determine whether people with *PON1* 192 R allozyme are more prone to CAD than are those with the Q allozyme. A statistically significant overall association between the *PON1* 192 R allele (high activity towards paraoxon) and the presence of CAD is found in meta-analysis of case-control studies.[9] The *PON1* R allele is reported to increase the likelihood of occurrence of CAD by increasing susceptibility to other established risk factors, such as diabetes mellitus, cigarette smoking and age. As various studies around the world reported correlation between Gln–Arg polymorphism & CAD, this study was undertaken to probe the distribution of *PON1* genotype in our population and any association of a particular *PON 1* genotype with coronary artery disease.

METHODS

Sample size was calculated using the formulae

$$N = \frac{\{z_{1-\alpha/2}\sqrt{2p(1-p)} + z_{1-\beta}\sqrt{p_1(1-P_1) + p_2(1-P_2)}\}^2}{(p_1 - p_2)^2}$$

Where, $p = \frac{p_1+p_2}{2}$

P_1: Proportion in the first group
P_2: Proportion in the second group
α : Significance level
$1 - \beta$: Power

According to similar study, genotype R in controls was 10 % and that in CAD was 25 %. Substituting in the formulae P_1: 10 %, P_2: 25 %, α : 5 %, $1 - \beta$: 80 %; N = 100 in each group.

The case group was selected from 100 consecutive adult patients with angiographically proven CAD attending the Cardiology Department of Government Medical College, Thiruvananthapuram, Kerala. Exclusion criteria were patients with hypertension, diabetes mellitus, hepatic disease and smokers. The study was approved by the human ethical committee of the institution and informed consent for genetic study was obtained from both cases and controls.

The healthy controls (n = 100) were selected from the general population, spouse or bystanders of the patients, medical, paramedical staff of the institution. With a standard proforma, history of coronary artery disease, diabetes mellitus, hypertension, hepatic disease and any other serious illness which needed hospital admission was excluded from this group. Smokers were excluded from the control group also.

2 ml of whole blood was collected into an ethylenediaminetetraacetic acid (EDTA) tube. Isolation of DNA was done with GenElute™. Blood Genomic DNA kit as per the manufacturer's instructions. DNA quality and concentration were determined using spectrophotometer using the following procedure.

DNA was diluted using TE buffer (10mMTrisHCl, 2mMEDTA, p H 8.0 - 8.5) and the absorbance at 260 nm, 280 nm and 320 nm were measured using a quartz microcuvette. The absorbance of the isolate at 260 nm should be between 0.1 - 1.0. Background absorbance was corrected using 320 nm. At 260 nm, an absorbance of 1.0 corresponds to approximately 50 µg / mL of double stranded DNA. The A260 - A320 ratio should be 1.6 - 1.9.

Polymerase chain reaction was done with Forward Primer 5'TATTGTTGCTGTGGGACCTGAG3' and Reverse Primer 5'CACGCTAAACCCAAATACATCTC3'. The DNA amplification was done in the MJ Research Thermal cycler using the following program:

Step 1: 95° C for 4 min; Step 2: 94° C for 1 min; Step 3: 61° C for 45 seconds; Step 4: 72° C for 45 seconds; Step 5: Steps 2 - 4 is repeated for 39 cycles; Step 6: 72° C for 5 min; Step 7: 4° C for 2 hours; Step 8: end. The amplified PCR product is 99 base pair length identified by 1 % agarose gel electrophoresis with ethidium bromide staining and 50 base pair ladder.

Restriction fragment length polymorphism was done with the restriction enzyme AlwI (New England Biolabs) 250 units, 5000 U / ml and NE buffer with 20 mM tris acetate, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol of pH 7.9 at 25° C. 20 µL PCR product, 0.2 µL restriction enzyme and 2 µL buffer is taken in PCR tube. Overnight incubation (16 hours) at 37° C is done. The digested product is separated by electrophoresis using 3 % agarose gel. The adenine (A) to guanine (G) transition creates a unique AlwI site in the amplified fragment.

QQ Genotype: 1 band of 99 base pairs as no restriction site is there.

RR Genotype: 2 bands of 69 & 30 base pairs as the polymorphism create a restriction site.

QR Genotype: 3 bands of 99, 30 & 69 base pairs, as both alleles are present.

Statistical Analysis

SPSS for windows version 15 was used for statistical analysis. To compare the differences in the percentage of genotype frequencies between groups, chi-square test or Fisher's exact test were used. A significant difference between groups was assigned when P value was < 0.05.

RESULTS

The baseline characteristics of the study population are given in Table 1. There is no significant difference in mean age when age group was divided into ≤ 50 and > 50, but there is a significant difference in gender in case and control group (p value 0.00008). There is no significant difference in mean BMI between cases and controls (p value 0.744).

	Characteristic	CAD	Control
Age	≤50	40	47
	> 50	60	53
Gender	Female	25	53
	Male	75	47
BMI	Mean / Standard Deviation	24.28 (3.54)	24.13 (3.05)

Table 1. Baseline Characteristics of Cases & Controls

Characteristic	Control n = 100	Cases n = 100
Genotype*QQ	11 (11 %)	9 (9 %)
QR	86 (86 %)	76 (76 %)
RR	3 (3 %)	15 (15 %)
Allele**Q	0.54	0.47
R	0.46	0.53

Table 2. PON 1 Genotype Frequencies and the Frequency of Allele

*χ^2 = 8.82, P value = .012, **χ^2 = 1.960, p value = 0.161

Characteristic	P	Adjusted OR	95 % C.I. for OR Lower	Upper
Age > 50	0.265	1.41	0.77	2.58
Sex – Male	< 0.001	3.47	1.87	6.46
Genotype -RR	0.014	5.24	1.41	19.47
Constant	0.001	0.34		

Table 3. Binary Logistic Regression Model

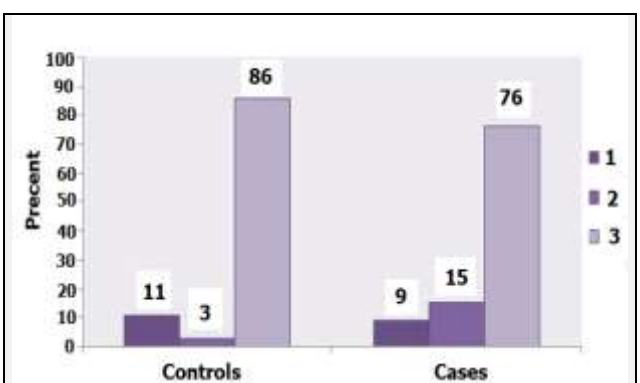

Figure 1. Percentage Distribution of Genotype Frequency in the Study Groups

1 = QQ Genotype, 2 = RR Genotype, 3 = QR Genotype

The genotype distribution and frequency of alleles in 100 CAD patients and 100 healthy controls is given in table II. The QR (heterozygous) genotype is more common in both control (86 %) and case (76 %) groups. The QQ homozygous genotype was found in 11 % of controls and 9 % of cases. The RR homozygous variant was seen only 3 % in controls but 15 % in case group.

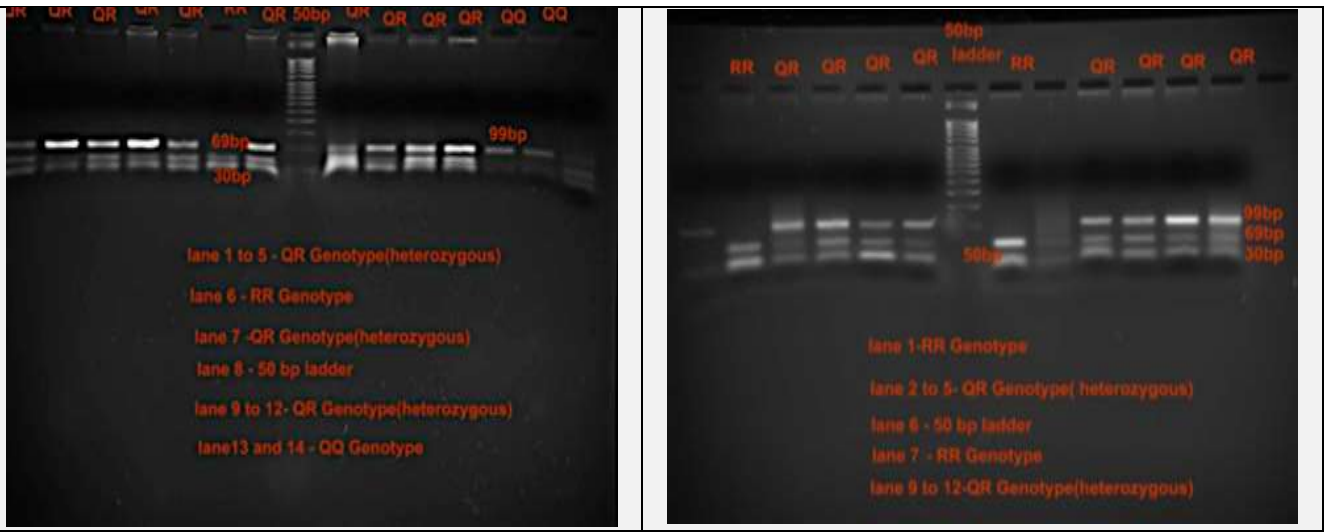

Figure 2. RFLP of Selected Samples

P value was found to be 0.012 suggesting a significant difference in genotype distribution between control and cases.

The allelic distribution is in accordance with Hardy-Weinberg equilibrium. Q allele is more frequent (0.54) in controls than cases (0.47). The frequency of R allele is more in cases (0.53) compared to controls (0.46). But there is no significant difference in allele distribution when P value was calculated (P value 0.161).

DISCUSSION

In the present study the case group is angiographically proven CAD with no history of hypertension, diabetes mellitus, hepatic disease or smoking. The control was a group of healthy individuals among the staff and bystanders of patients. Only the genotype was assessed for cases and controls to know about the variants that exist in our population for the Gln 192 Arg alleles. This makes the study design in itself simple. On statistical analysis of baseline data there is no significant difference in the mean age between cases and controls when the age is grouped into less than or equal to 50 and above 50 (P value 0.392, Table I). But there are studies that have reported age difference between cases and controls.[10] There was a higher number of males in the case group (75) compared to females (25) in the case group. In the control group there is not much of difference in the number of males (53) compared to females (47). But men are more susceptible to CAD especially in South Asian countries like India.

Regarding the genotype distribution, our population has the heterozygous QR genotype more frequent than the homozygous QQ or RR (86 % in controls and 76 % in cases). The frequency of RR genotype (15 %) is more in cases compared to control group (3 %) (P value .012). This is comparable to the studies reported by Serrato et al in Caucasian population,[11] Takaru Zama et al in Japanese population[10] and Pati N et al in Indian subjects[12] that RR genotype is positively associated with the presence of CAD. But negative associations are also reported from an Italian study[13] and Finnish study.[14] Binary logistic regression model was used to identify the association of *PON1* genotype to CAD. Adjusting for age and sex, RR genotype is independently associated with CHD. Adjusted odds ratio of RR was 5.24 with 95 % confidence interval 1. 41 - 19.47 for developing CHD ($p < 0.05$). So, from our study *PON1* RR allele can be considered as a risk factor for development of coronary artery disease.

There have been many case-control studies to test the hypothesis that the 192R allele is associated with CAD.[15,16] A meta-analysis of these studies found that the R allele is significantly related to the presence of CAD, but there was evidence of publication bias. The R allele is highly active against the substrate paraoxon, but it is this allele that is positively associated with CAD. But it is proved that paraoxonase showed substrate polymorphism.[17] The activity polymorphism of paraoxonase enzyme is due to has the amino acid substitution at position 192. The capacity of *PON1* QQ allele to protect LDL from oxidation is the complete reverse of that of paraoxon hydrolytic activity. The L55M polymorphism has also been shown to have an effect on *PON1* activity. Although this is much smaller than that of the 192 polymorphisms, it is independent of the 192 polymorphisms. Thus, *PON155MM / PONI192QQ* individuals have HDL and PON1 associated with the greatest protective capacity.[9]

CONCLUSIONS

With the available data it is possible to state that Gln 192 Arg polymorphism is an independent risk factor for CAD in our population from the adjusted odds ratio. The heterozygous variant is more frequent than the protective QQ allele. CAD patients have significantly higher distribution of RR genotype compared to controls.

Limitations of the Study

Our sample size is small, and the study did not take into consideration other risk factors included in CHD like

hypertension or diabetes mellitus. Paraoxonase concentration or activity was also not assayed to study any association between those parameters and genotype due to financial constraints of the project.

Scope of the Study

This can be taken as a pilot study to explore the association of CAD & PON gene polymorphism in our population where the disease is prevalent so that larger studies[18] can be done.

Data sharing statement provided by the authors is available with the full text of this article at jebmh.com.
Financial or other competing interests: None.
Disclosure forms provided by the authors are available with the full text of this article at jebmh.com.

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