



ORIGINAL ARTICLE

Coronary artery disease and the frequencies of *MTHFR* and *PON1* gene polymorphism studies in a varied population of Hyderabad, Telangana region in south India



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Abstract Coronary artery disease (CAD) also known as coronary heart disease is one of the leading causes of death and disability worldwide. Genetic and environmental factors play an important role in the pathogenesis and progression of CAD. The aim of this study was to evaluate the combined contribution of 3 gene polymorphisms to the risk of CAD and gene–gene interaction in the south Indian population. In this case-control study, 200 cases of CAD and 200 healthy controls were recruited. We studied 3 well known genetic polymorphisms of *MTHFR* (C677T; rs1801133), *PON1* (Q192R; rs662) and *ACE* (I/D; rs4646994) in relation to CAD in South Indian population. Polymerase chain reaction (PCR) was carried out and followed by the restriction fragment length polymorphism and agarose gel electrophoresis. Genotypes of *MTHFR* C677T, CT and CT+TT, and *PON1* Q192R QR were associated with the risk of CAD (C677T CT+TT vs CC: odds ratio [OR] = 3.3, 95% confidence intervals [CI] = 1.8–6.2; $p = 0.00001$), (CT vs CC: OR = 3.2, 95%CI = 1.8–5.6; $p = 0.00003$), and (Q192R QQ vs QR: OR = 2.1, 95%CI = 1.1–3.9; $p = 0.03$). The allele frequencies for T vs C: OR = 3.1, 95%CI = 1.8–5.3; $p = 0.00001$ and R vs Q: OR = 1.3, 95% CI = 1.0–1.7 $p = 0.03$. Multifactor dimensionality reduction (MDR)

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analysis was carried out with the combination of three genes and the results indicate that MDR analysis showed that, *PON1* gene polymorphism formed a significant model in predicting the CAD risk in south Indian population.

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1. Introduction

Atherosclerosis plays a key role in the pathogenesis of coronary artery disease (CAD), a leading cause of death, accounting for approximately 30% of deaths worldwide and has become a priority health problem in India. Currently, genetic and environmental factors play an important role in the pathogenesis and progression of CAD (Liu et al., 2014; Ahmed, 2012). Clinical manifestations of CAD comprise a host of acute and chronic conditions such as stable angina, acute coronary syndromes and heart failure. Various lines of evidence have suggested that genetic factors contribute significantly to the risk of CAD (Liu and Qiu, 2013) as conventional risk factors are insufficient to explain the etiology of CAD in all cases (Vijaya Lakshmi et al., 2011). The formation of atheromatous plaques and plaque rupture that leads to thrombotic occlusion of the coronary arteries that are multifactorial with multiple processes contributes to this pathologic cascade. These include inflammation, lipid disorders, renin angiotensin system, diabetic vasculopathy, hyperhomocysteinemia and others. Genetic epidemiologic studies have suggested certain genetic variants, including polymorphisms in the genes like methylenetetrahydrofolate reductase (*MTHFR*), paraoxonase 1 (*PON1*) and angiotensin converting enzyme (*ACE*) genes to have a strong association with CAD and related outcomes.

MTHFR is one of the metabolic pathways for CAD and is a key regulatory gene of the remethylation pathway. The C677T polymorphism (rs1801133) in *MTHFR* has been implicated in vascular disease (Masud and Qureshi, 2011). *MTHFR* catalyzes the conversion of 5', 10'-methylene tetrahydrofolate reductase to 5'-methylene tetrahydrofolate reductase, the major circulating form of folate, which is required for a number of metabolic pathways, including the methylation of homocysteine to methionine (Wang et al., 2013). Individuals with C677T polymorphism (rs1801133) have reduced *MTHFR* activity and elevated homocysteine levels, which is associated with CAD (Danesh and Lewington, 1998). Controversy exists regarding the association of C677T polymorphism and CAD (Klerk et al., 2002; Lewis et al., 2005). Human *PON1* is a calcium-dependent glycoprotein that is synthesized in the liver and secreted into the plasma associated with high density lipoproteins. *PON1* prevents LDL lipid peroxidation and protects against atherosclerosis and coded by the *HUMPPONA*, located on 7q 21.3, along with two adjacent genes, *PON2* and *PON3* (Macharia et al., 2012). The Q192R (rs662) polymorphism in the coding region of *PON1* gene is an amino acid exchange from glutamine (Q) to arginine (R) in the A575G region and is associated with variation in its hydrolysis activity (Macharia et al., 2012). *PON1* activity has been consistently reported to be significantly diminished in atherosclerosis and other cardiovascular diseases (Mohamed et al., 2013; Agrawal et al., 2009). *ACE* gene is present on the surface of vascular endothelial cells, generates the potent vasoconstrictor

angiotensin II from angiotensin I and inactivates the vasodilator bradykinin. Angiotensin II modulates NO synthesis in cardiovascular tissue, and NO modulates the action of angiotensin II (Abdelhedi et al., 2013). A polymorphic variant in intron 16 (*ACE-I/D*) of the *ACE* gene, characterized by an insertion (I) or deletion (D) of a repeat sequence of 287 non-coding base pairs (Zintzaras et al., 2008), has been shown to be associated with hypertension and other cardiovascular risk factors (Abdelhedi et al., 2013). The aim of the present case-control study was to examine the association of C677T *MTHFR*, A575G *PON1* and *ACE I/D* polymorphisms individually and in combination with coronary artery disease in South Indian population.

2. Materials and methods

2.1. Study design

This is a case-control study carried out on 400 subjects from South India region. The study cohort consists of 200 patients with documented CAD and 200 unrelated healthy control subjects. The cases were recruited between January 2007 and January 2012 from the patients, who visited Kamineni hospitals in Hyderabad/Telangana region. Healthy controls ($n = 200$) were recruited from the general population without any complications.

2.2. Angiographic assessment and participant recruitment

All patients were admitted with acute coronary syndrome and underwent coronary angiogram (CAG). Patients with $\geq 70\%$ stenosis in one or more coronary arteries were considered to have CAD. Our study recruited 200 patients with age ranging from 50 to 70 years who have triple vessel disease and underwent coronary artery bypass graft surgery (CABG). We excluded patients with septicemia, liver cirrhosis, renal failure, colitis, cardiomyopathy, congenital heart disease, rheumatic heart disease, neurological and cancer problems.

The control group consisted of 200 (139 males and 61 females) age and gender-matched healthy subjects from the same region, and with prior normal lipid profile. Clinical data were obtained from all patients using a standard case report form (CRF). Our study complied with the Declaration of Helsinki and was approved by the institutional review board, Kamineni hospitals, Hyderabad and informed consent was obtained from all the patients and controls.

2.3. Anthropometric measurements

Body mass index (BMI) was calculated as weight in kg and height in m^2 (kg/m^2). Height was measured using a wall-mounted stadiometer, and body weight was measured using a digital scale. BMI of each subject was calculated.

2.4. Blood samples

Peripheral blood (5 mL) was collected from all the patients and controls ($n = 400$) in EDTA tubes. The collected blood was divided into two portions; 2 mL for DNA extraction and molecular analysis and 3 mL for lipid profile in biochemical analysis.

2.5. Lipid profile

Serum sample was collected in the plain tube to measure the total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) and very low density lipoprotein cholesterol (VLDL-C) by using commercially available kits.

2.6. Molecular analysis

Genomic DNA was extracted using the conventional salting out extraction method (Khan et al., 2013) and quantified by using NanoDrop 2000 (Thermo Fisher Scientific, MA, USA).

2.7. Primers

Genotyping for *MTHFR* (rs1801133) and *PON1* (rs662) was performed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). *ACE* (rs4646994) I/D polymorphism was screened by direct PCR amplification. The details of the primers and PCR products are documented in Table 1. Successful amplification was confirmed by electrophoresis on an ethidium bromide impregnated 2% agarose gel. PCR reactions were performed in a thermal cycler with an initial denaturation at 95 °C for 5 min followed by 35 amplification cycles with each cycle consisting of denaturation at 95 °C for 30 s, 68 °C for rs1801133 (*MTHFR* gene) polymorphism (50 °C for rs662 polymorphism in *PON1* gene and 59 °C for *ACE* gene) and 30 s, 72 °C for 45 s and the final extension with 72 °C for 5 min. PCR products were digested for 2 h with *Hinf*I for *MTHFR* gene (Fermentas, USA) at 37 °C (2.5 µL of distilled water with 10 units of enzyme for 15 µL PCR product and 2 µL buffer in a final volume of 20 µL). For *PON1* gene, PCR products were digested for 16 h with *Alw*I (Fermentas, USA) at 37 °C (2.5 µL of distilled water with 10 units of enzyme for 15 µL PCR product and 2 µL buffer in a final volume of 20 µL).

2.8. Statistical analysis

Data were analyzed using the statistical software for windows (SPSS version 19.0, Chicago, IL, USA) and are represented as mean \pm standard deviation (SD). Differences in baseline characteristics between CAD patients and healthy subjects were assessed by χ^2 test for categorical variables and independent sample *t*-test was used to test for continuous variables. Allele and genotype frequency differences between patients and subjects were tested for each SNP using a chi-square test. Odds ratios (ORs) and 95% confidence intervals were calculated by multinomial logistic regression for the allele and genotype frequencies. A *p* value < 0.05 was considered statistically significant. Multifactor dimensionality reduction (MDR) analysis was carried out with the selected genes to find the correlation.

Table 1 List of multiple polymorphisms of DNA examined in this study.

Gene	Mutation	rs number	Amino acid substitution	Forward primers	Reverse primers	PCR fragments	Annealing temperature	Enzyme	Digested fragments
<i>MTHFR</i>	Exon 4	rs1801133	Ala-Val	TGAAGGAGAAGGTGTCTGAGGGA	AGGACGGTGCGGTGAGAGTG	198bp	68 °C	<i>Hinf</i> I	C: 198bp T: 175/23bp
<i>PON1</i>	Exon 6	rs662	Glu-Arg	AAACCCAAATACATCTCCCAGAAT	GCTCCATCCCACATCTTGATTTTA	214bp	60 °C	<i>Alw</i> I	A: 214bp G: 190/24bp
<i>ACE</i>	Intron 16	rs4646994	–	CTGGAGACCACCTCCCATCCTTCT	GATGTGGCCATCACATTTCGTCACGAT	490bp	59 °C	–	I: 490bp D: 190bp

Table 2 Demographic details involved in this study.

Profile	Cases (<i>n</i> = 200)	Healthy controls (<i>n</i> = 200)	<i>p</i> -Value
Age (mean ± SD)	56.4 ± 2.6	55.4 ± 2.8	<i>p</i> = 0.29
Sex (Male: Female)	140:60	136:64	<i>p</i> = 0.36
BMI (kg/m ²)	27.2 ± 3.6	26.3 ± 3.4	<i>p</i> = 0.42
Hypertension	88 (44%)	12 (6%)	<i>p</i> < 0.0001
Diabetes	90 (45%)	0 (0)	<i>p</i> < 0.0001
Smoking	65 (32.5%)	21 (10.5%)	<i>p</i> < 0.0001
Alcoholism	42 (21%)	18 (9%)	<i>p</i> < 0.0001
Family history of MI	48 (24%)	0 (0)	<i>p</i> < 0.0001
TC (mg/dL)	235.0 ± 13.7	162.9 ± 16.2	<i>p</i> < 0.0001
TG (mg/dL)	173.5 ± 12.5	135.1 ± 10.5	<i>p</i> < 0.0001
HDL-C (mg/dL)	33.1 ± 4.2	38.3 ± 5.4	<i>p</i> < 0.0001
LDL-C (mg/dL)	167.5 ± 14.5	93.7 ± 17.9	<i>p</i> < 0.0001
VLDL (mg/dL)	34.2 ± 2.5	26.8 ± 2.0	<i>p</i> < 0.0001

3. Results

3.1. Characteristics of the study group

Clinical and baseline characteristics of CAD patients and healthy controls are shown in Table 2. The mean age of the CAD patients and the controls was similar. The mean age was not different between the two groups and was 56.4 ± 2.6 for the patients and 55.4 ± 2.8 for the controls. Male formed 70% of the patients and 68% 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; diabetes 45% vs 0% (*p* < 0.0001), hypertension 44% vs 6% (*p* < 0.0001), smoking 32.5% vs 10.5% (*p* < 0.0001) and family history of CAD 24% vs 0% (*p* < 0.0001). Lipid profile analysis showed significantly higher TC, TG, LDL-C and VLDL-C and lower HDL-C in patients compared to control subjects (*p* < 0.0001) (Figs. 1 and 2, Table 2).

3.2. Genotype frequencies

The genotype distribution of the three variants was in Hardy-Weinberg equilibrium in both groups. The genotype and allele distribution of *MTHFR* (rs1801133), *PON1* (rs662) and *ACE* (rs4646994) variants are summarized in Table 3. There was a statistically significant difference in the genotype distribution in the rs1801133 polymorphism between the patients and controls (*p* = 0.00003). Similarly, the allele frequencies were significantly different (*p* = 0.00001). The risk of developing the disease was found to be 3.1-fold for T allele carriers, signifying a potential association. The genotype frequency for *PON1* (rs662) polymorphism was also found to be significantly different between the patients and controls with more prevalent GG (*p* = 0.01), and AA+GG (*p* = 0.01) genotypes, and the G (*p* = 0.03) allele of rs662 polymorphisms in CAD patients.

Our results could not find a significant association between *ACE* I/D polymorphism and CAD in our study population. Table 4 represents the genotype and allele distribution of *MTHFR* (rs1801133), *PON1* (rs662) and *ACE* (rs4646994) variants based on gender. In male subjects, the odds ratios for having CAD with T allele and CT genotype in *MTHFR* were 3.4 (CI = 1.2–9.7, *p* = 0.00008) and 2.7 (CI = 1.4–5.3,

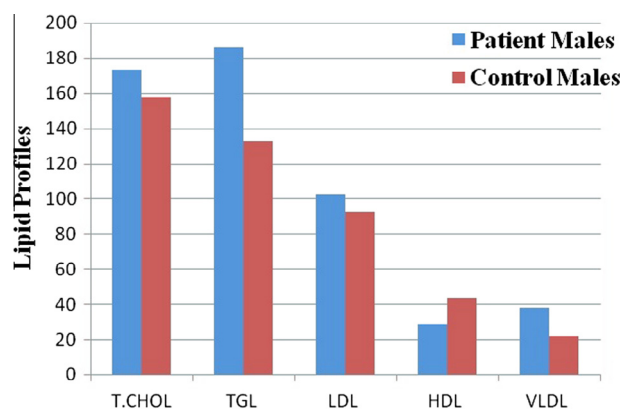


Figure 1 Lipid profile for CAD and control male subjects. T.CHOL – Total Cholesterol; TGL – Triglycerides; LDL – Low Density Lipoprotein Cholesterol; HDL – High Density Lipoprotein Cholesterol and VLDL – Very Low Density Lipoprotein Cholesterol.

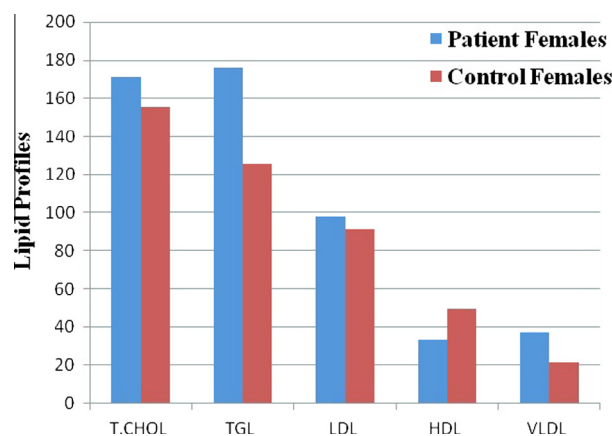


Figure 2 Lipid profile for CAD and control female subjects. T.CHOL – Total Cholesterol; TGL – Triglycerides; LDL – Low Density Lipoprotein Cholesterol; HDL – High Density Lipoprotein Cholesterol and VLDL – Very Low Density Lipoprotein Cholesterol.

Table 3 Genotype distribution of C677T, A575G and I28005D polymorphisms in the individuals with CAD and controls enrolled in the South Indian population.

SNP Genotype/allele	CAD patients (n = 200)	Healthy controls (n = 200)	Odds ratio (95%CI)	p-Value
677C→T				
CC	148 (74%)	181 (90.5%)		
CT	50 (25%)	19 (9.5%)	3.2 (1.8, 5.6)	0.00003
TT	2 (1%)	0 (0)	0.1 (0.04, 0.5)	0.002*
CT+TT	52 (26%)	19 (9.5%)	3.3 (1.8, 6.2)	0.00001
C	346 (0.87)	381 (0.95)		
T	54 (0.13)	19 (0.05)	3.1 (1.8, 5.3)	0.00001
192Q→R				
QQ	56 (28%)	67 (33.5%)		
QR	103 (51.5%)	110 (55%)	1.1 (0.7, 1.7)	0.61
RR	41 (20.5%)	23 (11.5%)	2.1 (1.1, 3.9)	0.01
QR+RR	144 (72%)	133 (66.5%)	1.2 (0.8, 1.9)	0.23
Q	215 (0.54)	244 (0.61)		
R	185 (0.46)	156 (0.39)	1.3 (1.0, 1.7)	0.03
I→D				
II	65 (32.5%)	60 (30%)		
ID	91 (45.5%)	105 (52.5%)	0.8 (0.5, 1.2)	0.33
DD	44 (22%)	35 (17.5%)	1.1 (0.6, 2.1)	0.60
ID+DD	135 (67.5%)	140 (70%)	0.8 (0.5, 1.3)	0.58
I	221 (0.55)	225 (0.56)		
D	179 (0.45)	175 (0.44)	1.0 (0.7, 1.3)	0.77

Bold values indicates the significantly associated when we perform the odds ratio.

* Indicates Yates correction.

Table 4 Genotypic distribution of C677T, A575G and I/D polymorphisms in male and female individuals.

Genotype/allele	CAD N (%)		Healthy controls N (%)		Odds ratio (95% CI)		p-Value	
	Males	Females	Males	Females	Males	Females	Males	Females
677C→T								
CC	102 (73.9)	46 (74.2)	124 (89.2)	56 (91.8)	Reference	Reference	Reference	Reference
CT	34 (24.7)	16 (25.8)	15 (10.8)	5 (8.2)	2.7 (1.4, 5.3)	3.8 (1.3, 11.4)	0.002	0.009
TT	2 (1.4)	0 (0)	0(0)	0 (0)	6.0 (0.2, 127.9)	1.2 (0.02, 62.5)	0.18	0.92
CT+TT	36 (26.1)	16 (25.8)	15 (10.8)	5 (8.2)	2.9 (1.5, 5.6)	3.8 (1.3, 11.4)	0.001	0.009
C	238 (0.86)	108 (0.87)	263 (0.95)	117 (0.96)	Reference	Reference	Reference	Reference
T	38 (0.14)	16 (0.13)	15 (0.05)	5 (0.04)	2.7 (1.5, 5.2)	3.4 (1.2, 9.7)	0.0008	0.01
192Q→R								
AA	35 (25.4)	21 (33.9)	45 (32.4)	22 (36)	Reference	Reference	Reference	Reference
AG	70 (50.7)	33 (53.2)	78 (56.1)	32 (52.5)	1.1 (0.6, 1.9)	1.0 (0.4, 2.3)	0.6	0.8
GG	33 (23.9)	8 (12.9)	16 (11.5)	7 (11.5)	2.6 (1.2, 5.5)	1.1 (0.3, 3.8)	0.009	0.7
AG+GG	103 (74.6)	41 (66.1)	94 (67.6)	39 (64)	1.4 (0.8, 2.3)	1.1 (0.5, 2.3)	0.19	0.7
A	140 (0.51)	75 (0.60)	168 (0.60)	76 (0.62)	Reference	Reference	Reference	Reference
G	136 (0.51)	49 (0.40)	110 (0.40)	46 (0.38)	1.4 (1.0, 2.0)	1.0 (0.6, 1.8)	0.02	0.7
I→D								
II	45 (32.6)	20 (32.3)	40 (28.8)	20 (32.8)	Reference	Reference	Reference	Reference
ID	61 (44.2)	30 (48.4)	75 (53.9)	30 (49.2)	0.7 (0.4, 1.2)	1 (0.4, 2.2)	0.2	1
DD	32 (23.2)	12 (19.3)	24 (17.3)	11 (18)	1.1 (0.6, 2.3)	1.0 (0.3, 3.0)	0.6	0.8
ID+DD	93 (67.4)	42 (67.7)	99 (71.2)	41 (67.2)	0.8 (0.5, 1.3)	1.0 (0.4, 2.1)	0.4	0.9
I	151 (0.55)	70 (0.56)	155 (0.56)	70 (0.57)	Reference	Reference	Reference	Reference
D	125 (0.45)	54 (0.44)	123 (0.44)	52 (0.43)	1.0 (0.7, 1.4)	1.0 (0.6, 1.7)	0.8	0.8

$p = 0.002$), and with G allele and AG genotype of *PONI* 1.4 (CI = 1.0–2.0, $p = 0.02$) and 2.6 (CI = 1.2–5.5, $p = 0.009$). While in female subjects only the T allele and CT genotype in *MTHFR* were associated with CAD [OR 3.4 (CI = 1.2–

9.7); $p = 0.001$] and [OR 3.8 (CI = 1.3–11.4); $p = 0.009$]. Power analysis revealed that power of 76% can be achieved by genotyping only 24% of the total number of samples used in our study.

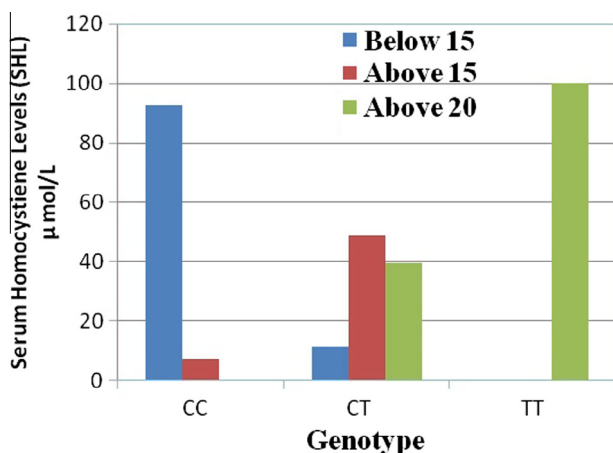


Figure 3 Serum homocysteine levels of *MTHFR* genotypes in CAD patients.

3.3. Correlation of *MTHFR* genotypes with serum homocysteine levels (SHL)

Forty two patients exhibited CC genotype while 45 had CT genotype and 2 were TT genotype patients. The Mean ± SD of SHL for CC genotype was 12.95 ± 1.83 μmol/L, for CT genotype 19.34 ± 3.13 μmol/L, for TT genotype 27 ± 1.41 μmol/L. The comparisons of SHL levels with the genotypes in patients are presented in Fig. 3. The levels of SHL were abnormal in both TT and CT genotype patients and were within normal limits in CC genotype patients. Furthermore, SHL were higher in the TT than CT genotype patients. These results indicate that C677T polymorphism might possibly be associated with increased homocysteine levels and may therefore influence individual susceptibility to CAD.

3.4. Interaction of nuclear genes by MDR analysis

The effect of interaction of three nuclear genes are analyzed in the pathogenesis of CAD by MDR. The list of attributes included three genes (*ACE*, *MTHFR* and *PON1*). All attributes in these datasets are categorical. Graphical representation of MDR is shown in Fig. 4. By MDR model analysis *MTHFR* can be used as a disease marker because of its high sensitivity value. Further radial graphic representation by

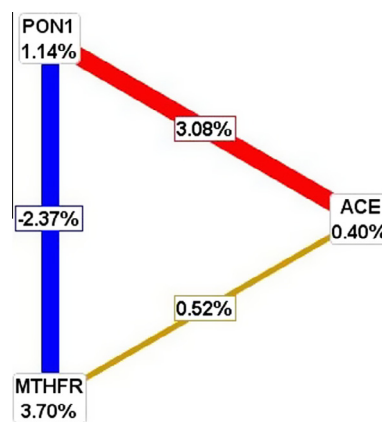


Figure 5 Radial graphic representation of interaction of *ACE*, *MTHFR*, *PON1* genes by MDR analysis.

MDR analysis was carried out between three genes and the interaction appears to be between *PON1* and *ACE* (Fig. 5).

4. Discussion

We have carried out a case-control study involving 200 cases and 200 controls with CAD in south Indian population. Thus, two of three studied polymorphisms of the *MTHFR* and *PON1* genes were significantly associated with the risk of CAD. Both the alleles and genotypes were found to be significantly associated. This study shows lack of association with *ACE* I/D polymorphism but in combination of MDR analysis it is associated with the combination of *PON1* and *ACE* gene, with CAD, is a complex disease with the contribution of multiple genetic and environmental risk factors. A number of studies on the impact of genetic polymorphism in *ACE* on the risk of coronary heart disease (CHD) have been conducted in various ethnic populations (O'Malley et al., 1999; Oei et al., 2005). In this study, no significant difference was observed in the distribution of genotype of *ACE* in CAD cases and controls. This confirms previous finding of Lindpaintner et al. (1995), Sobti et al. (2010) and Pandey et al. (2011). The other studies carried out on *ACE* and CHD risk among Indians have shown association of D allele with the risk of CHD (Ashavaid et al., 2002; Mishra et al., 2012; Dhar et al., 2012). The present results have shown the susceptible effect of polymorphism in *ACE* on CAD

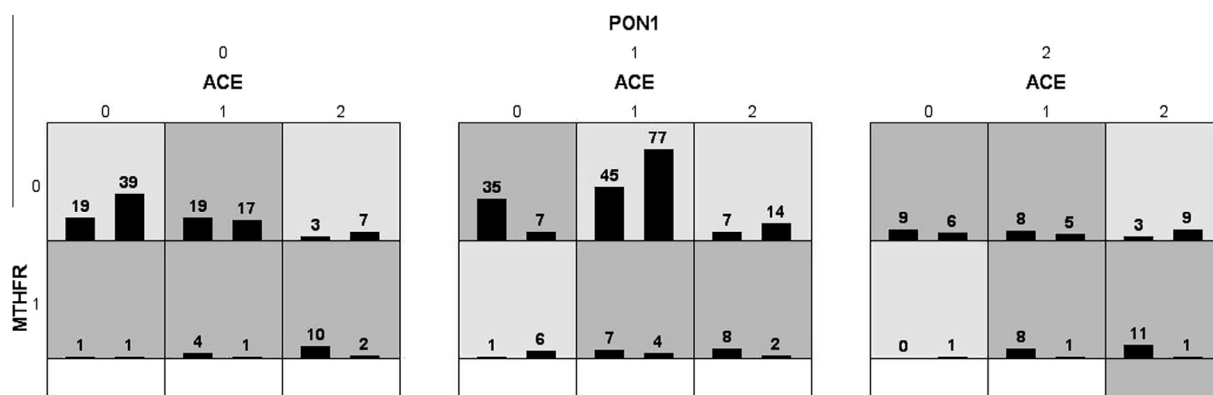


Figure 4 Interaction of *MTHFR*, *PON1* and *ACE* genes between cases and controls by MDR analysis by graphical representation.

among south Indians to be involved with factors like smoking, alcohol, diabetes and hypertension.

Diabetes and dyslipidemia are two major risk factors for CAD, which are associated with oxidative stress (Ganesan et al., 2011). The analysis of *PON1* 192 QR polymorphism has revealed that the high 10 activity allele (R) is associated with a more atherogenic lipid profile than the low-activity allele (Q). In this study, *PON1* 192R allele frequency in CAD patients was found to be higher when compared with the controls ($p = 0.03$). RR genotype was found to be associated with two fold increase in the risk of CAD development. In our earlier study *PON1* gene was proposed to be a candidate biomarker for T2DM and CAD patients (Ganesan et al., 2011). The present study investigated the role of this polymorphism in the development of CAD. The frequencies of the genotypes and alleles were significantly higher in patients compared with controls, indicating the association of *PON1* gene Q192R polymorphism with the disease in both the formats (i.e., gene association study and the MDR analysis). The results of the present study were in accordance with observations of earlier investigators, which support that RR genotype is associated with CAD (Ahmed, 2012; Ganesan et al., 2011; Hassan et al., 2013).

MDR analysis was performed to interact the *MTHFR*, *PON1* and *ACE* genes by the pathogenesis of CAD. Our study concludes that, *PON1* can be used as a disease marker because of its high sensitivity value. *ACE* alone does not play any role in the disease risk but *PON1* and *ACE* interaction increase the risk by 3.08%. There is a negative interaction between *PON1* and *MTHFR* genes, but *MTHFR* alone has a risk of 3.70%. MDR analysis reveals that in overall entropy (randomness of disorder) *MTHFR* is showing 3.70%, *PON1* is showing 1.14% and *ACE* is showing 0.4% which is negligible indicating *MTHFR* and *PON1* are showing a major effect. Our study indicated that gene–gene interaction showed a significant model in predicting the CAD risk.

The significance of homozygous polymorphisms considered to be risk factors for CAD when analyzed: *MTHFR* and *PON1*. The genotypes with statistically significant higher frequencies in the CAD patients were *MTHFR* and *PON1*. Unlike many other studies, our analysis was not limited to examining whether a single polymorphism presents increased risk, but evaluated various polymorphisms and some of the most important gene–gene associations, some of them never studied before (Mendonca et al., 2009). Sanghera et al. (1997), Sanghera et al. (1998) reported that the *PON1*-192*R allele is a risk factor for CHD among Asian Indians in Singapore. Pati and Pati (1998) reported a higher frequency of the QR and RR genotypes among Indian CAD patients.

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. Our results suggest that the association of polymorphisms in the same gene does not increase the risk of the isolated polymorphism, but that the association of polymorphisms in genes belonging to different enzyme systems is linked to increased risk compared to the isolated polymorphisms. The study may explain the difficulty of interpreting the risk of different polymorphisms in CAD, and may open up new possibilities in this field.

In conclusion, our results suggest that the D allele of the *ACE* (rs4646994) may not be associated, whereas T allele of

MTHFR (rs1801133) and R allele of *PON1* (rs662) gene polymorphisms were associated with an increased risk of CAD in south Indian population. Our data strongly suggest that *PON1* gene is associated with genotype analysis as well as in the combination of MDR analysis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jksus.2014.09.002>.

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