Associations of ApoE gene polymorphism with Coronary Artery Disease in patients from North Indian State Haryana

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Abstract
Apolipoprotein (Apo) E gene is associated with the regulation of human lipid metabolism. The genetic variations in ApoE gene constitute three isoforms (ε2, ε3, ε4) and six genotypes (ε2/2, ε3/3, ε4/4, ε2/3, ε2/4, ε3/4). These isoforms differ in one or two amino acids and may confer higher risk for coronary artery disease (CAD). Hence, the first study was conducted to assess the ApoE gene polymorphism as CAD risk from Haryana. The study comprised of 97 CAD patients (56 males, 41 females) and 93 healthy controls (males 51, females 42). The lipid profile and risk factors were recorded. DNA was isolated from blood, ApoE gene was amplified and genotyped using PCR-RFLP.

ApoE ε3/3 has been observed as the most prominent genotype in CAD and healthy controls. ApoE ε3 is having highest allelic frequency followed by ε4 and ε2. The incidence of ApoE ε4 has been found to be significantly higher ($p<0.05$) in CAD patients than controls. Young CAD patients having ApoE ε4 and positive family history were observed on high risk. However, no gender based difference was observed in distribution of ApoE genotypes. Significant difference in the VLDL- cholesterol and triglycerides level was observed in patients having ApoE ε4 compared to other genotypes. Data revealed the significant association of ApoE ε4 with the CAD. Result suggests the need of screening of ApoE polymorphism in young CAD patients with positive family history in Haryana. Significant increase in VLDL-cholesterol and triglycerides level in CAD patients having ApoE ε 4 confers higher risk of the disease.

Keywords: ApoE gene polymorphism, Coronary artery disease, Haryana, Lipid profile.

Introduction

Coronary artery disease (CAD) is one of the major causes of death and disability in India and worldwide.[1-4] According to WHO, an estimated 17 million people die of cardiovascular disease (CVD), particularly heart attack and stroke every year across the globe and in most of the developing country. Among them, 7 million die due to coronary artery disease, 6 million due to stroke and 4 million because of the other form of heart diseases.[5-7] The prevalence of CAD is consistently increasing from last 60 years in India, among adults over 20 years of age, the estimated prevalence of CAD is around 3-4% in rural areas and 8-10% in urban areas.[8-10]

Coronary artery disease is caused by atherosclerosis that is narrowing and/ or blockage of the arteries. Atherosclerosis can happen throughout the vasculature. In atherosclerosis, the inner walls of arteries become thick and stiff because of the plaque buildup which restrict the flow of blood. Atherosclerosis occurs over a period of time and its consequences can be serious which includes angina, heart attack and stroke.

CAD is a multifactorial disorder, associated with genetic and environmental factors. The well determined risk factors of CAD included hypertension, obesity, diabetes, smoking, dyslipidemia and family history.[9,11] Nearly 60 % of heritability of CAD demonstrates the strong genetic associations with the progression of CAD.[12,13] Therefore, numerous studies had been conducted on range of candidate genes (e.g., ApoE, ApoB, LPL, iNOS, ACE, COX2, CD14, CETP, FH, P-Selectin, E-Selectin, MTHFR, PON1, TNFα etc.) to establish their connection with the progression of the CAD.[14-18]

The candidate genes which abnormally metabolize the lipoprotetins always remain at prime importance for investigation in CAD as possible risk factors. The ApoE is a polymorphic gene on chromosome 19q13.2 coding for three isoforms: ApoE ε2, ApoE ε3 ApoE ε4 resulting in six different genotypes (ε2/2, ε3/3, ε4/4, ε2/3, ε2/4 ε3/4).[19] These isoforms differ from one another by one or two amino acid substitutions (Cys and Arg) which may have profound functional consequences at both the cellular and molecular levels because of isoform-specific functional properties and affinities with LDLR.[19-21]

Lipoproteins play a major role in the development of atherosclerotic cardiovascular disease in humans and the levels of lipoproteins in plasma are determined by apolipoproteins present on their surface.[19] The role of ApoE polymorphism is well established in the metabolism of plasma lipoprotein and metabolism/transportation of

cholesterol.[21] Due to the in-depth involvement of ApoE in the metabolism of lipoproteins, the study is focused on ApoE polymorphism in CAD patients.

The association of genetic variations in the CAD patients has been reported in different population of the world and India. ApoE ε3 has been reported as the most common in controls and CAD patients from Punjab, India whereas ApoE ε3/ε4 genotype is strongly associated with the incidence of myocardial infarction in young South African Indians and South Indian patients.[22-27] Data from European populations suggests that the low frequency of the ApoE ε4 allele in Southern Europeans was partly responsible for the low incidence and mortality of CAD as compared to the northern populations.[28]

Previously, we have reported the need of screening of genetic risk factors in the young CAD patients from Haryana.[29] It is of importance to investigate the ApoE polymorphism as associated risk factors of CAD. The patients from various part of Haryana (Rohtak, Jhajjhar, Jind, Bhiwani, Sonepat and Panipat etc.) come to cardiology department of the institution for the treatment which provides us the scope to conduct this study. In view of the above, a study has been designed to investigate the association of ApoE gene polymorphisms as possible marker/risk factor for CAD in this study population.

Material and Methods

Study group: The 97 CAD patients were recruited in the present study out of 641 CAD patients screened who attended the Cardiology OPD and ward of Pt. B. D. Sharma PGIMS, Rohtak. The details of CAD patient's recruitment with the details of exclusion criteria and study designed had been depicted in figure 1.

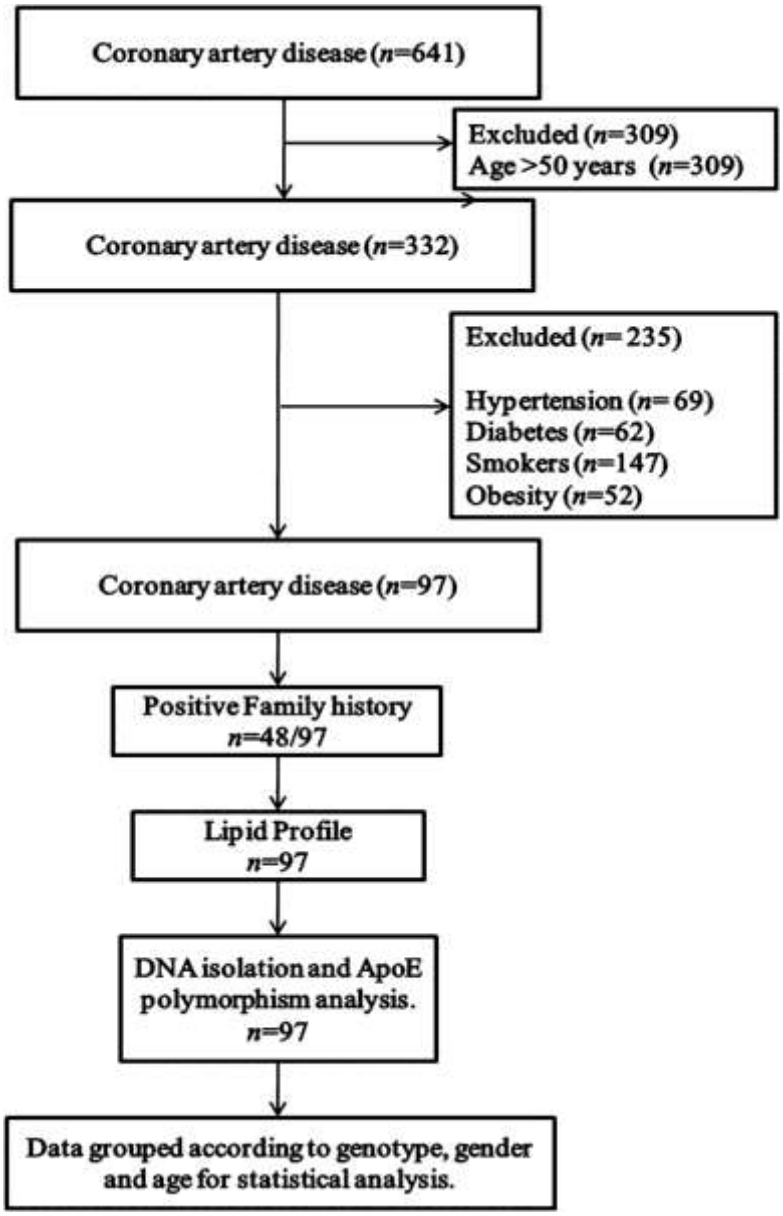

Fig. 1: Flow diagram showing the details of the recruitment of coronary artery disease patients along-with exclusion criteria and study designed

Similarly, 93 healthy sex/ age matched control (With normal ECG) were recruited in the study. The clinical, biochemical parameters and exposure to risk factors of the patients and controls were recorded in the pre-designed proforma with prior consent of the patients. The risk factor was considered as positive as per the criteria illustrated in our previous study.[29] One ml of blood sample from the patients and controls was drawn for DNA isolation and molecular investigations. The DNA isolation and ApoE polymorphism were done in the Department of Biotechnology and Molecular Medicine, PGIMS Rohtak and Centre for Medical Biotechnology, M.D. University, Rohtak.

This study has been limited in the patients/controls in the age between 21-50 years to exclude the old age related risk factor for the development of CAD. The data has been grouped according to genotype, gender and age (21-30, 31-40 and 41-50 years) for analysis.

Inclusion and Exclusion criteria: The CAD patients between the age of 20-50 yrs were included in the study. The patients with normal ECG were recruited as healthy control. The possible risk factors and biochemical parameters were recorded. The CAD patients of more than 50 yrs of age were excluded. The CAD patients and the healthy controls having past history of hypertension, diabetes, smoking and obesity were excluded to limit the study for identification of genetic risk factor.

Biochemistry: Biochemical investigations including blood sugar and lipid profile (Total-cholesterol, LDL-cholesterol, HDL-cholesterol, VLDL-cholesterol and triglycerides) were done in all patients and controls at Pt. B. D. Sharma PGIMS, Rohtak. The values of blood sugar and lipid profile have been represented in mg/dL.

DNA Extraction from blood: The DNA was isolated from the blood according to the methods described elsewhere.[30] Briefly, the RBCs were lysed in RBC lysis buffer (Ammonium Chloride and Potassium bicarbonate) and centrifuged. Supernatant was removed and the RBC lysis buffer treatment was repeated till no hemoglobin remains. The nuclear pellet was resuspended in DNA lysis buffer (Tris, NaCl, EDTA) with proteinase K followed by incubation at 37^0C for 60 min. The lysate was treated with equal amount of phenol/chloroform and centrifuged. The upper aqueous layer was precipitated using sodim acetate and isopropanol and centrifuged. The pellet was washed with 70% ethanol, air dried and dissolved in nuclease free water.

Detection of ApoE polymorphism by Restriction fragment length polymorphism (RFLP): The polymorphism in the ApoE gene was done by RFLP as described elsewhere.[31] Briefly, the DNA isolated from whole blood was amplified by PCR using Forward primer (5'-ACAGAATTCGCCCCGGCCTGGTACAC-3') and Reverse Primer (5'-TAAGCTTGGCA CGGCTGTCCAAGGA-3') which gave PCR product of 244 bp on agrose gel electrophoresis. The PCR product was digested with *Hha1* restriction enzyme (Figure 2) and the ApoE genotypes were assigned as per the restriction pattern observed in 12% polyacrylamide gel electrophoresis (Table 1).

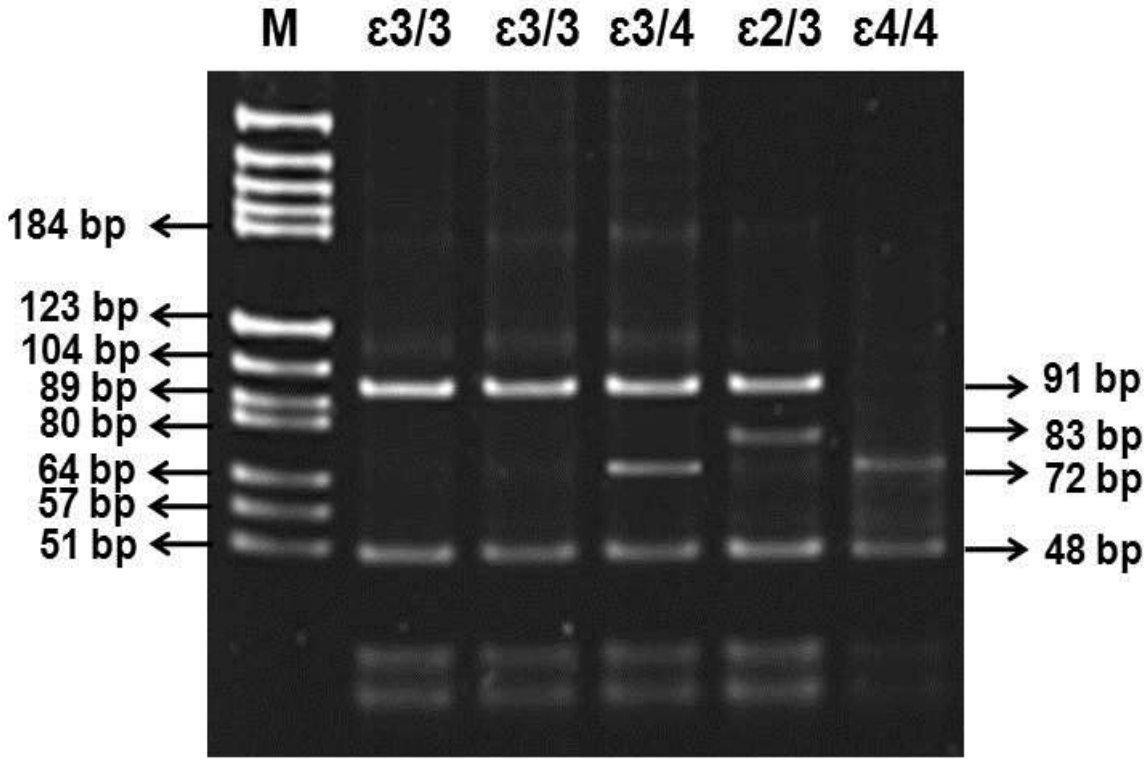

Fig. 2: Polyacrylamide gel electrophoresis (PAGE) showing the various ApoE genotypes as detected on the basis of restriction pattern of *Hha1*digested ApoE gene. M is the DNA marker -*pBR322 DNA/BsuR1 (HaeIII)*

Statistical analysis: The categorical variables have been presented as number and percentage and the continuous variables as Mean ± S.D. Statistical analysis was performed using Chi-square between percentage, Chi-square, Chi-square trend and ANOVA test, wherever applicable, using SPSS software. The p value < 0.05 was considered as statistically significant.

Results

Distribution of ApoE genotypes and allelic frequency: The ApoE ε3/3 homozygous genotype was observed to be the most prevalent in CAD patients and healthy controls. The comparable incidence of ApoE ε2/3 genotypes was observed among CAD patients and healthy controls respectively. The incidence of ApoE ε3/3 genotype was significantly higher ($p< 0.05$) in healthy controls whereas incidence of ApoE ε3/4 and ε4/4 genotype was significantly higher ($p< 0.05$) in CAD patients in comparison to each other (Table 2).

The most prominent allelic frequency of ApoE ε 3 was observed both in CAD patients and healthy controls. The allelic frequency of ApoE ε 3 was significantly higher ($p<0.05$) in healthy controls while the frequency of ApoE ε 4 was significantly higher ($p<0.05$) in CAD patients compared to each other (Table 2).

Associations of ApoE genotypes with family history in CAD patients: The positive family history was observed in nearly 50% of the CAD patients of the study. The utmost incidence of positive family history was observed in the CAD patients having the presence of ApoE ε 4/4 genotype followed by ApoE ε 3/4, 3/3 and 2/3 genotype. The statistical significant difference ($p<0.05$) in the trend of positive family history was observed among various ApoE genotypes in CAD patients (Table 3).

Associations of ApoE genotypes with gender and age in CAD patients: The difference in the distribution of ApoE genotypes in male and female among the CAD patients and healthy controls was found to be statistically not significant ($p>0.05$) (Table 4).

Table 1
Restriction pattern of *HhaI* digested Apo E gene (244 bp) to detect the polymorphism.

Apo E ε 2/2	Apo E ε 3/3	Apo E ε 4/4	Apo E ε 2/3	Apo E ε 2/4	Apo E ε 3/4
91 bp	91 bp	-	91 bp	91 bp	91 bp
83 bp	-	-	83 bp	83 bp	-
-	-	72 bp	-	72 bp	72bp
-	48 bp	48 bp	48 bp	48 bp	48 bp
-	35 bp	35 bp	35 bp	35 bp	35 bp

Table 2
Distribution of Apo E genotypes and allele frequency in CAD patients and healthy controls.

Genotype	CAD n=97 (%)	Healthy controls n=93 (%)	*P Value*
Apo E ε2/3	8 (8.25%)	9 (9.68%)	>0.05
Apo E ε3/3	61 (62.88%)	78(83.87%)	**<0.05**
Apo E ε3/4	21 (21.65%)	6 (6.45%)	**<0.05**
Apo E ε4/4	7 (7.22%)	0	**<0.05**
Allele Frequency			
Apo E ε2	4.12 (4.25%)	4.84 (5.2%)	>0.05
Apo E ε3	77.84 (80.28%)	91.93 (98.85%)	**<0.05**
Apo E ε4	18.04 (18.6%)	3.23 (3.47%)	**<0.05**

Table 3
Family history of the Coronary artery disease in the study subjects as provided by the patients.

Genotype	Family history Yes/No	Family history (%)	*P Value*
Apo E ε2/3 (n=08)	3/5	37.5%	
Apo E ε3/3 (n=61)	26/35	42.62%	
Apo E ε3/4 (n=21)	13/8	61.9%	
Apo E ε4/4 (n=07)	6/1	85.71%	**<0.05**
CAD (n=97)	48/49	49.47%	

The distribution of ApoE genotype varies in the CAD patients of various age groups. The difference in the distribution of ApoE genotype in various age group was found to be statistical significant ($p<0.05$). The incidence of ApoE ε2/3 and 3/3 genotypes was lower in the age group of 21-30 years than the frequency in age group of 31-40 years and 41-50 years in CAD patients. However, the frequency of ApoE ε 3/4 and 4/4 genotypes was higher in the age group of 21-30 years than the frequency in age group of 31-40 years and 41-50 years in CAD patients (Table 5).

Associations of CAD patients and healthy controls lipid profile: The difference in the total cholesterol and LDL-cholesterol of the CAD was not found to be statistically significant comparison to the healthy controls ($p>0.05$). However, the difference in the HDL-cholesterol, VLDL-cholesterol and triglycerides of the CAD patients was statistically significant to the healthy controls ($p<0.05$) (Table 6).

Associations of ApoE genotype with lipid profile in CAD: The difference in the total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides of the CAD was found to be statistically not significant on comparison between the various ApoE genotypes ($p>0.05$). However, the difference in the VLDL-cholesterol of the CAD was found to be statistically significant ($p<0.05$) in comparison between the various ApoE genotypes (Table 7a).

On comparison of the lipid profile among the various ApoE genotypes, the difference in the total cholesterol, LDL-cholesterol, HDL-cholesterol was found to be statistically non-significant ($p>0.05$). The difference in the triglycerides level among ApoE ε2/3 *vs* ε 4/4 was found to be statistically significant ($p<0.05$) whereas statistically non-significant in comparison of various other group of ApoE genotypes. The difference in VLDL-cholesterol level among ApoE ε2/3 *vs* ε4/4, ApoE ε3/3 *vs* ε3/4, ApoE ε3/3 *vs* ε4/4 was found to be statistically significant ($p<0.05$) (Table 7a and b).

Discussion

A large number of studies using candidate genes and genome-wide association analysis have shown some promising signals and a number of studies confirm the genetic factors may confer the increased CAD risk.[12,15-18] The ApoE gene polymorphism/ variations had been reported as significant risk factor for progression of the Alzheimer's disease, vascular dementia cardiovascular disease.[21,32-34]

Table 4
Gender wise distribution of Apo E genotypes in CAD patients and healthy control.

Genotype	CAD		*P Value*	Healthy control		*P Value*
	Male n=56 (%)	**Female n=41(%)**		**Male n=51(%)**	**Female n=42(%)**	
Apo E ε2/3	4 (7.13%)	4 (9.76%)		4 (7.84%)	5 (11.9%)	
Apo E ε3/3	33 (58.93%)	28 (68.29%)		44 (86.28%)	34 (80.96%)	
Apo E ε3/4	13 (23.22%)	8 (19.51%)		3 (5.88%)	3 (7.14%)	
Apo E ε4/4	6 (10.72%)	1 (2.44%)	>0.05	-	-	>0.05

Table 5
Association of Apo Eε genotypes with age in CAD patients.

Age	Genotypes				*P Value*
	Apo E ε2/3 n =8 (%)	**Apo E ε3/3 n =61 (%)**	**Apo E ε3/4 n =21 (%)**	**Apo E ε4/4 n =7 (%)**	
21-30 (Years)	0	11(18.04%)	10 (47.62%)	5 (71.43%)	
31-40 (Years)	4 (50%)	26 (42.62%)	9 (42.86%)	2 (28.57%)	
41-50 (Years)	4 (50%)	24 (39.34%)	2 (9.52%)	0	**<0.05**

Table 6
Association of lipid profile between CAD and healthy controls.

Parameters	CAD (n=97)	Healthy Controls (n=93)	*P Value*
Total -C	183.39 ± 36.09	175.83 ± 29.93	>0.05
LDL- C	109.35 ± 28.97	104.67 ± 26.7	>0.05
HDL-C	35.84 ± 5.96	40.91 ± 5.59	**<0.05**
VLDL-C	38.45 ± 11.98	29.92 ± 8.07	**<0.05**
Triglycerides	162.61 ± 53.66	120.38 ± 24.1	**<0.05**

Table 7 (a)
Association of Apo E genotypes with lipid profile in CAD patients.

Parameters	Apo E ε2/3 (n=8)	Apo E ε3/3 (n=61)	Apo E ε3/4 (n=21)	Apo E ε4/4 (n=7)	*P Value*
Total -C	174.12 ± 19.82	179.62 ± 37.64	195.14 ± 39.92	191.57 ± 9.44	>0.05
LDL- C	104.5 ± 17.29	108.14 ± 30.97	113.85 ± 31.16	111.85 ± 11.21	>0.05
HDL-C	37.87 ± 4.08	35.96 ± 6.35	35.38 ± 5.21	33.85 ± 6.59	>0.05
VLDL-C	35.5 ± 6.52	35.85 ± 11.2	44.66 ± 14.57	45.85 ± 2.47	**<0.05**
Triglycerides	126.37 ± 37.82	158.91 ± 48.7	169.66 ± 61.02	188.57 ± 27.31	>0.05

Table 7 (b)
Statistical analysis of lipid profile between various Apo E genotypes among CAD patients.

Parameters	ε2/3 vs ε3/3	ε2/3 vs ε3/4	ε2/3 vs ε4/4	ε3/3 vs ε3/4	ε3/3 vs ε4/4	ε3/4 vs ε4/4
Total -C	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
LDL- C	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
HDL-C	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
VLDL-C	>0.05	>0.05	**<0.05**	**<0.05**	**<0.05**	>0.05
Triglycerides	>0.05	>0.05	**<0.05**	>0.05	>0.05	>0.05

In this study, the comparable incidence of ApoE ε2/3 polymorphism and ApoE ε2 allelic frequency in CAD patients and healthy controls rules out the probability of CAD risk because of the ApoE ε2/3 polymorphism and ApoE ε2 allele. Moreover, the significantly higher incidence of ApoE ε3/3 polymorphism and ApoE ε3 allelic frequency among healthy controls compared to CAD patients is suggestive of no CAD risk due to the presence of ApoE ε3/3 polymorphism and ApoE ε3 allele in this study. No gender associated difference was observed in the distribution of ApoE ε polymorphism among CAD patients and controls group.

The significantly higher incidence of ApoE ε3/4, ε4/4 polymorphism and ApoE ε4 allele in the CAD patients compared to healthy control suggests that the ApoE ε4 confers a genetic risk factor for the CAD in both heterozygous and homozygous state. These results were in accordance with the previous reports from India that the subjects carrying ApoE ε 4/4 or ε4 allele confer susceptibility to CAD risk.[22,34,35] The case control studies from different ethnicity reported that the patients having ApoE *ε*2 allele appear to decreased risk while ApoE *ε*4 allele increased risk of CAD disease.[23,34-36]

Results are nearly in agreement with previous reports from India that ApoE ε4 allele confers higher risk of CAD disease.[23, 34,35] The low affinity of ApoE with the low-density lipoprotein (LDL) receptor delays the clearance of chylomicron and remnants VLDL cholesterol. The studies assessing the role of ApoE gene on plasma lipids indicate that the ApoE ε4 is associated with elevations while ApoE ε2 is associated with reduction in LDL cholesterol level.[22-24,34]

It was interesting to note that nearly half of the CAD patients have the positive family history with utmost (>85%) among CAD patients having ApoE *ε*4/4 polymorphism. The positive family history was recorded in more than 60% of CAD patients having ApoE *ε*4 alleles. The significantly higher positive family history in the patients with ApoE *ε*4 /alleles than *ε*2/3 and *ε*3/3 suggests the contribution of ApoE ε4 for the positive family history. The presence of ApoE ε4 allele has also been reported to be associated with an increased risk for development of atherosclerosis in children, middle age and school children with positive family history of CAD.[37-39]

Similar observation regarding the age associated distribution of ApoE ε polymorphism among CAD patients has been made in this study. The higher incidence of ApoE ε4/4 and ε3/4 polymorphism was observed in young CAD patients suggesting the presence of ApoE ε 4/4 and allele 4 contributing significantly as risk for CAD risk and contributing to the early onset of the CAD. The identification of the individual on higher risk may be helpful in delaying the progression of CAD by proper management of lifestyle and food habits along with therapy.[40]

There was no significant difference in the level of total cholesterol and LDL- cholesterol whereas significant difference in the level of HDL- cholesterol, VLDL-cholesterol and triglycerides was observed in CAD patients compared to controls in this study. Among the CAD patients, the higher level of total cholesterol, LDL- cholesterol, VLDL- cholesterol and triglycerides in the patients having ApoE ε4/4 or ε4 carriers than ε3/3 or ε2/3. However, the level of HDL- cholesterol was lower in patients was having ApoE ε4/4 homozygote or ε4 carriers.

These results are consistent with previous reports that the subject carrying ApoE ε4 alleles is having higher cholesterol level while ApoE ε2 alleles carrier have lower than those of ε3 alleles carrier.[36] The meta-analysis of the studies has also reported the lower level of HDL in ApoEε4 carriers in

comparison with ε3/3 homozygotes and ε2 carriers.[41] The relatively higher total-cholesterol, LDL-cholesterol low HDL cholesterol in patients with ApoE ε4 are markers of early onset of CAD.

The significant difference in the level of VLDL- cholesterol was observed among CAD patient of various ApoE polymorphism (ε 2/3 vs ε 4/4, ε 3/3 vs ε 3/4, ε 3/3 vs ε 4/4) on independent comparison. The significant difference in the triglyceride level was observed on comparison of CAD patient with Apo ε 2/3 vs ε 4/4 polymorphism. The ApoE is a LDL receptor-binding protein ligand of liver which mediates cholesterol and triglyceride metabolism by clearing the chylomicron and remnants of VLDL-cholesterol from plasma[21]. The decrease in the activity of receptors binding ligands leads to the over synthesis of LDL/ VLDL/ triglyceride and confers the CAD risk as observed in the CAD patients with ApoE ε4 of the study.

In summary, no gender associated difference in the distribution of ApoE genotype was observed in this study. The ApoE ε 3/3 is the most prominent genotype with highest allelic frequency of ε3 in the CAD patients and controls. The incidence of Apo ε 2/3 and ε2 allele in the CAD patients and healthy control was comparable and the higher incidence of ApoE ε 3/3 and ε3 allele in healthy controls rules out the probability of significant role of ApoE ε 2/3, ε 3/3 and ε2 and ε3 allele as CAD risk factor. The ApoE ε4/4 and ε4 allele look to be genetic risk factor for CAD in the present study. The significant association of positive family history with the young age was observed with the presence of ApoE ε 4/4 genotype and ε4 alleles in CAD patients of this study. Hence, there is a need of screening of ApoE ε4/4 genotype and ε4 alleles in the young ones among the families having positive history of CAD.

Our study demonstrate that there was insignificant difference of total cholesterol, LDL- cholesterol levels but significant difference of HDL-cholesterol, VLDL-cholesterol and triglycerides levels between CAD and controls. The relatively higher total-cholesterol, LDL-cholesterol and low HDL cholesterol levels among ApoE ε4 genotype CAD patients suggest it as marker of higher CAD risks. On independent comparison, the VLDL- cholesterol level significantly differs amongst ε 2/3 vs ε 4/4, ε 3/3 vs ε 3/4, ε 3/3 vs ε 4/4 and triglycerides among ε 2/3 vs ε 4/4, these findings demonstrate the influence of ApoE ε 4 with VLDL-cholesterol and triglycerides level.

Conclusion

The presence of ApoE ε4/4 genotype and ε allele 4 appears to be a genetic risk factor confering higher CAD in this region of India. The ApoE ε4/4 genotype and allele was associated with positive family history and age. Therefore, this study revealed the need of screening of ApoE genotypes in the young CAD patients who had positive family history. The presence of ApoE ε4/4 genotype and allele looks to be contributing for the prognosis of CAD in such patients.

The studies revealed that the occurrence of ApoE ε4 is associated with high level of VLDL- cholesterol, triglycerides and low level of HDL- cholesterol and deserve to be screened. Hence, ApoE ε4 genotype may be an important marker for higher risk of CAD. However, the findings of the current study need to be validated with the large sample study in future.

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