

Association of DNA Polymorphism at the Apolipoprotein B-100 Gene Locus with Plasma Lipid Concentration and Coronary Artery Disease among North Indians

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Abstract: The aim of this study was to investigate the association between apolipoprotein B gene polymorphisms and coronary artery disease and lipid levels in north Indians. Two hundred patients of angiographically proven atherosclerotic CAD and two hundred age and sex matched control subjects were included in the study. Serum lipids including cholesterol, triglycerides, HDL, LDL, VLDL and ApoB were analyzed. PCR based genotyping was done for ApoB 3' Hypervariable region (HVR). RFLP analysis was carried out to genotype regions carrying XBA1, EcoR1 and Msp1 restriction sites in the ApoB gene. Biochemical analysis of lipid parameters revealed that Total cholesterol, triglycerides, VLDL, LDL and ApoB were significantly high ($p < 0.001$) in patients while HDL was found elevated in controls. ApoB 3' HVR polymorphism analysis revealed a total of 17 alleles where HVE34 ($p < 0.001$), HVE43 ($p < 0.001$) and HVE47 ($p < 0.01$) were found significantly higher in CAD patient. Comparison based on three and five allelic model revealed that group of high repeat alleles (> 37) were significantly ($p < 0.001$) high in patients. RFLP analysis of three cutting sites namely EcoR1, MspI and XbaI revealed that combined genotypes E+E+M+M+X+X+ and E+E+M+M-X-X- were significantly more prevalent ($p < 0.002$) in CAD patients. Haplotype analysis revealed that two locus E+X- and three locus E+M-X- and E-M+X- were significantly higher ($p < 0.01$) in CAD patients. Complete four locus analysis revealed that high repeat haplotype E+M+X-/ 43 was significantly higher ($p = 0.0002$) in patients. A significant association ($p = 0.03$) was also observed between the higher repeats HVE39-49 alleles and higher levels of Apo B. RFLP haplotype E+/X- also depicted significant differences for total cholesterol, triglycerides, LDL-cholesterol and Apolipoprotein B levels. The study elucidates that ApoB gene polymorphisms are associated with CAD in north Indian patients. High repeat alleles (> 37) of ApoB3'HVR and E+/X- RFLP haplotype is strongly associated both with elevated lipid levels and CAD occurrence. Therefore, ApoB gene polymorphisms can be helpful in delineating the high risk group for CAD and may be of use in genetic screening of CAD patients from north India.

Key words: Apolipoprotein B, RFLP, Hyper variable region, polymorphism, coronary artery disease, hyperlipidaemia

INTRODUCTION

Coronary deaths in India are expected to be double over 20 years and reach two million by 2010^[1]. Both overseas and resident Indians have two to four times' higher prevalence of coronary artery disease at the age of < 40 years as compared to Whites, Black and other Asians^[2]. Cardiovascular risk factors have been traditionally divided into 2 categories: modifiable risk factors (obesity, smoking, alcohol, hypertension, elevated cholesterol, reduced high density lipoprotein and diabetes and non-modifiable risk factors (age, gender and hereditary factors)^[3]. Although heritability estimates, genetic models and modes of transmission vary between different studies, a considerable portion

of the variability of serum levels of these lipids and lipoproteins are believed to be genetically determined^[4].

Numbers of candidate genes have been implicated in the pathogenesis of CAD and myocardial infarction. The most studied and well-characterized genetic variants in this respect are located in the Apolipoprotein-B (ApoB) gene. Apolipoprotein-B exists in human plasma as two isoforms, Apo-48 and ApoB-100 for the LDL receptor. It is the largest monomeric protein sequenced so far, containing 4536 amino acid residues^[5]. Its gene has been mapped on the short arm of chromosome 2 with an approximate length of 43 kilobase and 29 exons^[6]. Apolipoprotein (Apo) B100 is the principal protein component of LDL. The interaction of ApoB100 with LDL receptors mediates

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the uptake of LDL from liver and peripheral cells and hence it plays an important role in cholesterol homeostasis. The LDL-binding domain of the molecule is proposed to be located between the residues 3129 and 3532^[7].

Cloning and sequencing of the ApoB gene have identified number of genetic polymorphisms in the ApoB gene which may exert some impact on lipid metabolism and contribute to the susceptibility in developing Coronary artery disease (CAD). These polymorphisms are of interest they have a putative effect on the physiological function of the ApoB protein like receptor binding and recognition. Four common polymorphisms, MspI and XbaI (exon 26), EcoRI (exon 29) and 3' HVR, have been associated with variation in lipid levels, CAD and myocardial infarction. MspI polymorphism in codon 3611 of the mature ApoB protein, results in an amino acid change from arginine to glutamine. On the other hand, the polymorphic region of XbaI is caused due to a base substitution (A→T) in the threonine codon, resulting in a silent mutation. The EcoRI polymorphism of the ApoB gene appears in exon 29 (GAA→AAA; 4,154th nucleotide), resulting into an amino acid change (Glu→Lys).

The ApoB 3'VNTR polymorphism (variable number of tandem repeats) or 3'HVR (hypervariable region) consists of a multiallelic locus with tandem repeats 11 to 16 bp AT-rich DNA sequences beginning 73 bp 3' to the second polyadenylation signal^[8]. It has been suggested that DNA variability in this region is associated with myocardial infarctions^[9]. Some of the earlier studies have shown that these 3' VNTR locus alleles of apolipoprotein B (ApoB) are associated with coronary artery disease (CAD) and / or variation in plasma lipid concentrations^[10,11]. However, this association of Apolipoprotein B3' hypervariable repeat genotype with plasma lipid concentration and coronary artery disease has not found such a correlation^[12]. The 3'VNTR was originally defined as a simple length polymorphism, resulting from differing numbers (from 21 to 57) of repeated sequences and it is most often genotyped based on their size variation by polymerase chain reaction-based methods in ordinary agarose^[13] or denaturing acrylamide electrophoresis gels^[6]. To date, 26 different-sized 3'VNTR alleles have been characterized in humans^[14]. The most widely studied polymorphism of ApoB gene is the Xba-I polymorphism in exon 26. In some populations the presence of the Xba -I cutting site is associated with hypercholesterolemia^[15]. Interestingly, the association of ApoB100 VNTR and RFLPs with plasma lipid concentration or coronary artery disease varies in different ethnic groups^[16] and has not always been found to be associated with CAD^[17].

The present study has been designed to analyze six different lipid parameters and genotyping of four polymorphic sites (Apo3'HVR and three RFLP-EcoRI, MspI and XbaI locus) in CAD patients and

normal healthy controls from north India in order to assess their association with each other and with coronary artery disease. We have tried to identify a potential molecular marker that can be used to infer the abnormal lipid levels and predict the occurrence of CAD.

MATERIALS AND METHODS

Subjects: Two hundred patients of angiographically proven coronary artery disease were evaluated at the Cardiology Department of Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, (Uttar Pradesh), India were included in the study. Patients less than 06 weeks after a myocardial infarction were excluded from the study. Two hundred age, sex and ethnicity matched control subjects were also selected in the study. The controls were subjected to treadmill test to be sure that they were not suffering from any coronary disease. Further, all control subjects with hypertension, diabetes and endocrine or metabolic disorders were excluded from the control group. Informed consent was taken from both patient and control groups before blood collection. The study was approved by the ethical committee of SGPGIMS.

Blood collection: Blood samples for measuring serum biochemical and lipid profiles were obtained in the morning. Both patients and controls were fasting for 12 hrs prior to blood collection. 3 ml of venous blood sample was also collected in EDTA vials for the extraction of genomic DNA.

Lipid analysis: Serum lipids including cholesterol, triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) were analyzed according to methods previously described^[18]. Apolipoprotein B levels were assessed by the immunoturbidimetric immunoassay using commercial kit (Randox Laboratories Ltd. UK).

DNA extraction: DNA was extracted from blood by salting out method using phenol-chloroform as described by Coomey *et al.*^[19] and was purified by ethanol precipitation. DNA was used as a template for ApoB-gene polymorphism analysis.

Analysis of ApoB3'HVR: ApoB3'HVR was amplified using primers described by Boerwinkle *et al.*^[13], encompassing the entire ApoB 3' VNTR sequence. The sequence of primer used was 5' ATGCAAACGGAGAAATTATG 3' and 5' CCTTCTCACTTGCCAAATAC 3'. The polymerase chain reaction was performed in M. J Research.Inc. Thermocycler, with 26 cycles of denaturation at 94°C for 1min and annealing & extension at 58°C for 6 min. The amplified product was then electrophoresed on 5%

PAGE and allele scoring was done with the help of ApoB3'HVR allelic ladder and commercial ladder (Invitrogen Ltd.) as shown in Fig. 1a.

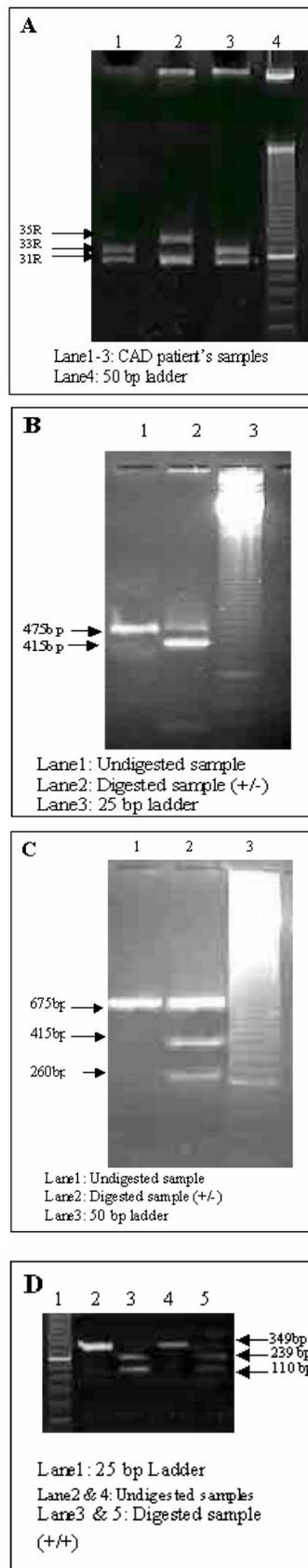
Analysis of RFLP: Regions of ApoB gene carrying EcoR1, Msp1 and Xba1 restriction sites were amplified separately using their respective primers: 5'CTG,GCT,TGC,TAA,CCT,CTC, TG and 3'GAG,AAG,CTT,CCT,GAA,GCT,CG for EcoR1, 5'TCT,CGG,GAA,TAT, TCA,GGA,ACT,ATT,G3' and 5'CTA,AGG,ATC,CTG,CAA,TGT,CAA,GGT 3' for MspI; and 5'GGA,GAC,TAT,TCA,GAA,GCT,AA3' and 5'GAA,GAG,CCT,GAA,GA C,TGA,CT3' for Xba1. The amplified product was digested with the respective enzymes separately as previously described^[20]. Alleles of each polymorphic site were classified as (+) or (-) according to the presence or absence at the cutting site of each restriction enzyme respectively (Fig. 1b-d).

Statistical analysis: All the statistical calculation for the continuous data of biochemical factors were performed using SPSS version 10 statistical software packages. For each variable, the values were expressed as mean ± S D. Data was evaluated by student's t test and one-Way Analysis of Variance (ANOVA) followed by Tukey's multiple comparison tests. Allele and genotypic frequencies for ApoB 3'HVR and three RFLP loci alleles were calculated with the gene counting method. Assumption of HWE was calculated from POPGENE-v16 software. Comparison of the categorical data i.e. different ApoB genotypes among controls and patients was done by Fischer's exact test and χ^2 test. Odd's ratios were calculated with a 95% confidence interval limit using 2X2 contingency table. "p" value <0.05 was considered significant.

RESULTS

Two hundred patients with angiographically proven coronary artery disease (169 males, 31 females) and 200 normal healthy controls (163 males and 37 females) were evaluated for six different biochemical parameters providing the lipid profile of all four hundred subjects. The mean age of the patients was 47.31 ± 12.11 while that of the controls was 44.58 ± 13.36 . All the samples were genotyped for four ApoB-gene polymorphisms namely, ApoB3'HVR, EcoR1, Xba1 and Msp1.

Biochemical characteristics of study population: Total six biochemical parameters namely, total cholesterol, triglyceride, high density lipoprotein, low density lipoprotein; very-low density lipoprotein and Apolipoprotein B were estimated in patients and controls (Table 1). It was observed that total cholesterol



A: ApoB3'VNTR analysis; B: MspI RFLP analysis; C: XBAI RFLP analysis; D: EcoR1 RFLP analysis

Fig. 1: ApoB-Gene polymorphism

(193 ± 85), triglycerides (177 ± 48), was higher ($p=0.0001$) in CAD patients as compared to controls (142 ± 65), triglycerides (177 ± 48), low-density lipoprotein (105 ± 45) and Apolipoprotein B were elevated significantly ($p=0.0001$) in CAD patients (193 ± 85 ; 177 ± 48 ; 105 ± 45 and 149 ± 44 respectively) in comparison to the controls (142 ± 65 ; 144 ± 31 ; 80 ± 24 and 103 ± 41 respectively). On the contrary, high density lipoprotein was significantly higher ($p=0.0001$) in controls (35 ± 9.4) as compared to CAD patients (30 ± 9.0). Very-low density lipoprotein (38 ± 17) were found higher in patients as compared to controls (35 ± 12), however, the difference was not significant.

Allele and genotype frequency distribution at ApoB 3' HVR: ApoB 3' HVR polymorphism was studied in both control and CAD patients using polyacrylamide gel electrophoresis. HVR region begins 73 bp downstream from the second putative polyadenylation signal of the gene. Total 17 different alleles were observed in the 400 studied subjects. The alleles varied according to the number of repeats i.e. from 21 to 49 (HVE 21- HVE 49). The frequency distribution of Apo B VNTR alleles in normal controls and CAD patients is shown in Fig. 2. Genotype frequencies calculated for both the groups were utilized for testing the conformity of the assumption of HWE. After applying Bonferroni correction to the 'p' value obtained by the Fischer's exact test based on 1000 Markov-Chain algorithm steps no significant departures were observed from HWE.

The allele distribution in our population is unimodal in nature as the relative frequency of the most common allele i.e. HVE 35 occurred with a highest frequency in both the groups i.e. 33 % and 27% in control and CAD patients (Table 2). HVE 29 and HVE 49 were found only in patient group, while HVE 36 was present only in control group. HVE 34 ($p < 0.001$), HVE 43 ($p < 0.001$) and HVE 47 ($p < 0.01$) were found significantly higher in CAD patient as compared to control group (Fig. 2). Interestingly, it was observed that alleles smaller than HVE 35, i.e. HVE 23, HVE 27, HVE 31, HVE 33, were more frequent in the control group as compared to patient group while alleles larger than HVE 35, i.e. HVE 38, HVE 39, HVE 41, HVE 43, HVE 47 were more frequent among patients (Fig. 2).

A total of 60 genotypes were observed in 400 subjects (both controls and CAD patients) at ApoB 3'HVR. Genotypic frequency distribution was in accordance to the allele frequency as the higher repeats (39-49) genotypes were significantly higher ($P < 0.001$) in patients than lower repeats genotypes (21-33) and median repeats (34-37) genotypes.

RFLP analysis depicting allele frequency and genotyping for EcoR1, Xba1 and Msp1 RFLP polymorphism: Genomic DNA from all subjects was digested with three restriction enzymes and alleles were designated according to the presence or absence (+/-) at

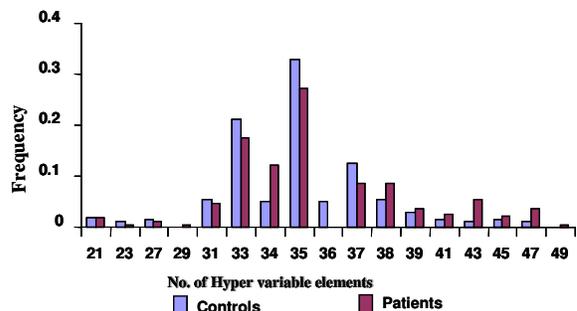


Fig. 2: Allele frequencies of ApoB3'HVR alleles in CAD patients and controls

Apo B cutting site of EcoR1 (R), Msp1 (M), or Xba1 (X). It was clearly observed that the frequencies of homozygotes at R^+ , M^+ and X^- is higher in both CAD patients and controls (Table 2). When individually different alleles were compared between patient and control groups it was seen that the frequency of R^- allele was present in a frequency of 15% in the patient group but it was remarkably lower in the control group i.e. on 6.3% ($p = 0.0001$). Similarly, R^+/R^+ genotype was found significantly higher ($p < 0.05$) in controls as compared to CAD patients (Table 2) but the frequency of other genotypes were almost same in both the groups. Furthermore, when the combined genotyping by three RFLP loci revealed that $E+E+M+M+X+X+$ and $E+E+M+M-X-X-$ were significantly more prevalent ($p < 0.002$) in CAD patients (Table 2) while $E+E-M+M+X-X-$ and $E+E-M+M-X-X-$ were more common in controls ($p < 0.01$ and $p < 0.003$ respectively).

Haplotype analysis of four ApoB gene polymorphisms: Two, three and four locus haplotype analysis was carried out for three RFLP and one HVR polymorphism of ApoB gene. Two locus haplotypes based on the presence or absence of cutting sites for EcoR1, Msp1 and Xba1 revealing four combination $+/+$, $+/-$, $-/+$ and $-/-$ were evaluated. It was observed that only $E+X-$ was significantly higher ($p < 0.01$) in CAD patients as compared to controls (Table 2). Three locus haplotypes comprising of all the three RFLP loci revealed that haplotype $E+M+X-$ and $E+M-X+$ were significantly higher ($p < 0.0001$ and $p < 0.01$) in controls and haplotype $E+M-X-$ and $E-M+X-$ were significantly higher ($p < 0.002$) in patients (Table 2). Finally, four locus haplotypes were constructed by combining alleles at three RFLP loci (EcoR1, Msp1, Xba1) with VNTR alleles (21-49). This analysis revealed 56 different haplotypic combinations where $E+M+X-/ 33$ and $E+M+X-/ 35$ were significantly higher ($p=0.002$) in controls and as expected a high repeat haplotype $E+M+X-/ 43$ was significantly higher ($p=0.0002$) in patients as compared to controls (Table 2).

Correlation of lipid parameters with ApoB gene polymorphisms: All the subjects were subdivided into three groups on the basis of HVR genotypes as of

Table 1: Biochemical characteristics of CAD patients and normal healthy controls

	Patients (n= 200)	Control (n = 200)	P value
Lipid profile (mg/dl)			
T. Cholesterol	193 ± 85	142 ± 65	0.0001***
Triglycerides	177 ± 48	144 ± 31	0.0001***
HDL	30 ± 9.0	35 ± 9.4	0.0001***
LDL	105 ± 45	80 ± 24	0.0001***
VLDL	38 ± 17	35 ± 12	NS
Apo B	149 ± 44	103 ± 41	0.0001***

***p value < 0.0001, NS= not significant

Table 2: Allele, genotypes and haplotypes of four loci having significant differences among patients and controls

	Cases (n=200) Frequency (n)		Controls (n=200) Frequency (n)		p value
ApoB3'HVR					
Three allelic model					
34 - 37	0.479	(192)	0.556	(222)	0.04*
38 - 49	0.262	(105)	0.137	(55)	0.0001***
Five allelic model					
V (>37)	0.262	(105)	0.137	(55)	0.0002 ***
Restriction fragment length polymorphism					
Allele distribution					
EcoR1 (-)	0.15		0.06		0.001***
Genotypes					
R+ / R+	0.75		0.88		0.05*
Three locus genotype					
E+E+M+M+X+X+	30	(15)	06	(03)	0.001**
E+E+M+M-X-X-	54	(27)	20	(10)	0.001**
E+E-M+M-X-X-	02	(01)	22	(11)	0.001**
Two locus haplotype					
E ⁺ X ⁻	0.6400		0.5164		0.05*
Three locus haplotype					
E+M+X-	0.5959		0.3858		0.0001***
E+M-X+	0.0692		0.0153		0.01*
E+M-X-	0.0440		0.1394		0.002**
E-M+X-	0.0107		0.1097		0.002**
Four locus haplotypes					
E+M+X-/ 43	0.0219		0.1232		0.0002***

+, - refers to the presence and absence of cutting site for restriction endonuclease. E = EcoR1; M= MspI; X = XbaI

* p = < 0.05, **p = <0.001 and *** p = < 0.0001

Table 3: Comparison of the genotypic and haplotypic profile of four ApoB-gene polymorphism with different lipid parameters

	Cases (n=200)	Controls (n=200)	P Value
HVR genotype groups			
Total cholesterol			
HVE 36-49/ HVE 36-49	205 ± 88 (n=47)	150 ± 59 (n=30)	0.07
Triglycerides			
HVE 36-49/ HVE 36-49	107 ± 48 (n=47)	85 ± 26 (n=30)	0.06
Apolipoprotein B			
HVE 36-49/ HVE 36-49	150 ± 47 (n=47)	119 ± 37 (n=30)	0.003 *
Restriction fragment length polymorphism			
Apolipoprotein B			
X++	145 ± 40	113 ± 22	0.05*
X+-	130 ± 29	109 ± 24	
X--	128 ± 35*	116 ± 22	
Xba-EcoR1 genotypes			
T. cholesterol	181± 54	152 ± 48	0.0001
Triglycerides	165 ± 42	150 ± 35	0.0005
LDL-cholesterol	104 ± 30	90 ± 27	0.0001
Apolipoprotein B	132 ± 35	120 ± 31	0.001

+, - refers to the presence and absence of cutting site for restriction endonuclease. E = EcoR1; M= MspI; X = XbaI

* p = < 0.05, **p = <0.001 and *** p = < 0.0001

higher repeats (39-49), lower repeats (alleles (21-33) and median repeats (34-37) genotypes. It was observed that in extension of the genotype analysis, a significant association (p = 0.03) was observed between the higher repeats HVE 39-49 alleles and higher levels of Apo B

(Table 3). Additionally, higher levels of cholesterol, triglycerides and LDL were also found associated with higher repeat group (HVE 36-49 alleles), however, the association did not reach statistical significance. The RFLP genotype correlation with different lipid

parameters demonstrated that there was no association between lipid parameters and different genotypes of EcoR1 and Msp1. However, the mean value of total serum cholesterol and Apo B were found significantly higher ($p < 0.04$) in CAD patients carrying homozygous X+ genotypes as compared to controls, while, levels of HDL cholesterol was lower in X+X+ genotype but differences were not significant (Table 3).

DISCUSSION

Several genetic polymorphic variants of Apo B have been described, many of which have been associated with elevated serum cholesterol, triglyceride, LDL-cholesterol or Apo B levels in different populations^[21]. Based on the analysis of six lipid biochemical parameters and genotyping of four important polymorphic sites (ApoB 3' HVR and EcoR1, Msp-I, Xba-1 RFLP loci) of ApoB-100 gene, present study has revealed a significantly elevated lipid profile in all the patients and strong correlation of high repeat HVR alleles and high repeat combined haplotypes with occurrence of CAD in general and with elevated lipid levels in particular.

In the present study we have found total 17 alleles for ApoB 3'HVR (Fig. 2). The alleles varied according to the number of repeats i.e. from 21 to 49 (HVE 21-HVE 49). The allele distribution in the present study revealed that alleles smaller than HVE 35, were more frequent in the control group as compared to patient group. On the other hand alleles larger than HVE 35 were more frequent among patients. Overall, we found a strong correlation between HVR alleles HVE 34, HVE 43 and HVE 47 and coronary heart disease. Moreel *et al.*^[22] found an association of VNTR-34 in patients but has not reported any positive correlation with HVE 43 and HVE 47. The differences in the allelic association with CAD patients in different studies conducted by various workers on different ethnic groups may be due to ethnic variation at this polymorphic site. In Koreans, a unimodal distribution with a peak on HVE35^[23] and in African populations, unimodal peaks on alleles 35 or 37 and with a considerable number of both large and small alleles as well as the presence of unique alleles were not detected in Caucasians^[24]. In our population we have seen that VNTR 35 was most common allele followed by HVE 33 and HVE 37. Most of the Caucasian populations have also depicted modal frequency of HVE 37 and HVE 35.

The Apo B VNTR alleles with higher repeat numbers were reported to occur more frequently in CAD patients than in the control group^[9]. To test the hypothesis we have used three allelic and five allelic models of Apo B 3'HVR alleles. In three allelic model of Apo B the alleles were subdivided into three groups. It was found that higher repeats (38-49) were most frequent ($p < 0.0001$) in patients as compared to controls.

This observation indicates that although minor difference were observed at allele frequency level when individual alleles were considered, but the differences became more prominent when we grouped the alleles into three or five allelic model, indicating that higher repeat allele were more among patients and hence are more associated with the defective genotypes.

Similarly, in five allelic model our results demonstrated that the higher repeats of VNTR allele (>37) were more frequent ($p < 0.0001$) in patients as compared to controls. As mentioned in the earlier section that grouping of the alleles into different allelic model like five-allelic model proposed by Frossard and Lestringant,^[25] it was clear that group comprising of higher repeat alleles are more frequent in patients as compared to controls, an observation which was not so clearly visible when individual alleles were compared. Probable reason behind this trend lies in the high degree of allelic differentiation observed at ApoB3'HVR which might have hid the association signals among the two groups that belongs to same ethnicity, which became pronounced once these numerous alleles were grouped into three or five allelic structure.

Further, we have tried to correlate the difference in the lipid profile of the two groups with Apo B 3'HVR alleles. A small number of studies have been conducted so far to correlate the single ApoB 3'VNTR alleles with serum lipid levels and a study has shown an association of ApoB 3'VNTR allele 35 with essential hypertension^[26]. To test the hypothesis that HVE alleles were associated with serum levels of cholesterol, triglyceride, or Apo B, we grouped the HVR genotypes as low, median and higher repeat homozygous genotypes. Interestingly, a significant association ($p = 0.03$) was observed between the HVE 36-49 alleles and higher levels of Apo B and a non-significant but marked association was observed between the HVE 36-49 alleles and higher levels of cholesterol, triglycerides and LDL. Similar to our results, some other workers have also found an association of Apo B gene variations with elevated lipid levels^[27]. The mechanism for the influence of the 3'HVR polymorphism on serum lipids is not clearly elucidated. Possibly, these genetic variations are segregated together with functional mutations, which predispose the individuals to the development of lipid profile alterations. Our results revealed that the plasma HDL and Apo B concentration abnormalities were the major risk factor for CAD in individuals with high repeat numbers.

Along with ApoB3'HVR, various restriction fragment length polymorphism (RFLPs) in the Apo B gene have been frequently studied^[28]. We have tried to correlate the RFLP pattern at EcoR1, Msp1 and Xba1 loci with CAD. We have observed that the frequency of E⁻ allele was 15% in patient group but it was remarkably lower in the control group i.e. on 6.3%. The differences were highly significant ($p = 0.0001$), but the frequency of other alleles was almost same in both the

groups. Our observations are in agreement with other report^[29] of myocardial infarction, where the frequency of the E- was significantly higher in patients. The frequency of M- allele (absence of Msp1 cleavage site) in control group (0.15), was similar to that found in Europeans, but higher than the frequencies seen in US (0.06) and China^[10]. The frequency of X+ allele in control group (0.30) was similar to that found in South Asian descent (0.29) and lower than Caucasians (0.52), Switzerland (0.55). Overall, the frequency profiles of North Indians have resemblance with that of Caucasians for all the three loci analyzed by RFLP. It was observed that the alleles found in a high frequency among Caucasians like E- (0.24), M- (0.11) and X+ (0.45) was also found raised in our study group i.e. E- (0.15), M- (0.18) and X+ (0.36). Incidentally, human genetic variation studies based on autosomal, Y-chromosomal and mt-DNA markers have suggested that north Indians carry high frequency of Caucasian specific mutations and haplotypes^[30,31]. Our result demonstrate that the genotypes of Xba1 when compared with the lipid parameters it was observed that the mean value of serum total cholesterol and Apo B were significantly higher ($p < 0.04$) in CAD patients carrying homozygous X+ genotypes as compared to controls, while, levels of HDL cholesterol was lower in X+X+ genotype but differences were not significant. Similar observations have been reported by, Law *et al.*^[5], have reported a positive association between the X+ allele and total cholesterol, Apo B concentration and triglyceride levels, whereas others have not shown such correlation. Overall, RFLP analysis showed that EcoR1 with R+/R+ genotype was significantly raised in controls and three locus genotypes E+E+M+M+X+X+ and E+E+M+M-X-X- were significantly more prevalent ($p < 0.002$) in CAD patients.

Finally, the haplotype analysis revealed that frequency of E+/X- haplotype was significantly different between CAD patients and controls and the frequency of the X-X-/E+E+ genotype was significantly higher in the CAD patients group when compared to the control group, suggesting that this genotype may be one of the risk factor for CAD. The combined analysis of all four polymorphic sites identified 56 different haplotypic combinations where haplotype carrying both X- and higher ApoB3'HVR alleles E+M+X-/ 43 were significantly higher ($p = 0.0002$) in patients as compared to controls.

Alternatively, changes in the amino acid sequence associated with the Xba1 polymorphic site may be responsible for the differences in mean serum cholesterol levels that are being observed. Since the accurate molecular weight of both LDL and Apo B is known, it is evident that there is only one molecule of Apo B on each LDL particle. Hence, genetic variation affecting either the level or the structure of Apo B is most likely to act in a co-dominant fashion. Our results on total cholesterol and Apo B levels associated with

different Xba1 genotypes are in agreement with this model, since individuals heterozygous for the alleles of the polymorphism have intermediate levels of total and LDL- cholesterol.

Conclusively, present study identifies high risk alleles, genotypes and haplotypes of four different polymorphisms located in ApoB3'HVR for CAD. Different alleles at these polymorphic sites might alter the Apo-B levels and could prove to be a better risk marker. Both high repeats allele at ApoB3'HVR and Xba (-) haplotypes could be regarded as molecular risk factor for abnormal lipid parameter and as potential risk factor for CAD. However, all the studies in the past reflect the genetic heterogeneity in the Apo-B gene. As CAD is a multifactorial disease the Apo-B gene alone may not have a direct effect on the lipid profile or CAD severity. However, it does emphasize the importance of such studies which may in future help to delineate the high risk group for CAD and may be of use in genetic screening of CAD patients of different genetic / ethnic background.

ACKNOWLEDGEMENTS

Supported by grant from the Uttar Pradesh Council for Science and Technology (UPCST), Lucknow, India.

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