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Association of common genetic polymorphisms with plasma lipid and coronary artery disease in West Bengal population

Saurav Bhattacharya[a], Swagata Roy Chowdhury[a], Pradip Ghoshal[b], Kajal Ganguly[b],
Nirmalendu Bhattacharya[b], Nandan Kumar Jana[a]*

[a]Department of Biotechnology, Heritage Institute of Technology, 994, Chowbagha Road, Anandapur, East Kolkata Township, Kolkata-700107, West Bengal, India

[b]Department of Cardiology, Nilratan Sirkar Medical College and Hospital, 138, Acharya Jagadish Chandra Bose Road, Kolkata-700014, West Bengal, India

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Both apolipoprotein B (APOB) and low-density lipoprotein receptor (LDL-R) play crucial role in LDL uptake by cells. The association of *apob Msp*I polymorphism and *ldl-r Ava*II polymorphism with coronary artery disease (CAD) has already been reported in other populations. Genetic variations in these two gene locus is associated with CAD in West Bengal population was investigated here. Blood samples were collected from angiographically proven 254 CAD patients and age matched 246 healthy people (controls) from different districts of West Bengal, India. Serum lipids including total cholesterol (TC), triglycerides (TG), high density lipid-cholesterol (HDL-C), low density lipid-cholesterol (LDL-C) and very low density lipid-cholesterol (VLDL-C) were evaluated in all the subjects. Genotyping was performed by PCR-RFLP combined with gel electrophoresis. The lipid-profile analysis revealed that TC, TG, LDL-C and VLDL-C were significantly high ($p < 0.001$) in patients than controls. Genotyping study showed that homozygous A^+A^+ genotype was significantly more prevalent (22% vs 10%, $p = 0.0011$) among patient group in this population than control. This genotype was also associated with higher LDL-C and TC levels. But there was no significant association of genotypes with serum lipid concentration was evident in APOB gene. The A^+A^+ genotype could be a genetic marker for CAD.

Keywords: APOB, LDL-R, CAD, PCR-RFLP, West Bengal

Introduction

Coronary artery disease (CAD) is the most common form of cardiovascular diseases (CVD)[1]. World Health Organisation (WHO) has already declared that CVD is the major reason of death in the planet. An estimated 17.3 million people died from CVDs in 2008, representing 30% of all global deaths and over 80% of the world's deaths from CVDs occur in low- and middle - income countries like India. Moreover, among these deaths, an estimated 7.3 million were due to CAD. So, we need to devise the highly potential diagnostic and therapeutic techniques which will efficiently help to reduce the number of death worldwide. In order to do that we first need to understand the pathology of the disease. CAD occurs when coronary arteries are narrowed which actually reduces or blocks the blood supply through the narrow arteries to the heart. The main cause of narrowing of the coronary arteries is the formation of atherosclerotic plaque[3]. There are several mechanisms in the body that leads to the plaque formation inside arteries. As they help to develop atherosclerotic plaques, these mechanisms are considered as risk factors for CAD. Retention of lipoproteins especially low-density lipoprotein (LDL) in the blood is one of the risk factors for developing CAD[3,4]. Among other reasons, improper clearance of LDL-C from blood is reported as one of the major causes of LDL retention[5,6]. Moreover, it has been also documented that the genetic polymorphisms at candidate gene locus actually modulate the LDL retention in blood as well as the formation of atherosclerotic plaque[7,8]. In this study we have focused on the genetic polymorphism at gene locus of two very well known proteins responsible for cellular uptake of LDL. They are low density lipoprotein receptor (LDL-R) and apolipoprotein B (APOB).

LDL-R is a trans-membrane protein that modulates plasma levels of LDL-C by regulating the uptake of LDL-C particles by cells of liver and delivers

*Author for correspondence:
Tel: 91-033-2443-0454; Fax: 91-033-2443-0455
nandankumar.jana@heritageit.edu; nandanjana@gmail.com

cholesterol to the adrenal glands and gonads for steroid hormone synthesis and to the liver for bile acid synthesis[9]. The occurrence of mutation in *ldl-r* gene may disrupt this normal function and increases the chance of elevated LDL-C level in blood and premature CAD[10,11]. The human *ldl-r* gene consists of 18 exons with the length of approximately 45 kb, and was mapped to chromosome 19p13.2[12,13]. A common *Ava*II polymorphic site is present in exon 13, has been found to be associated with variation of serum lipid levels in previous studies[14-17]. Another gene that has been studied the most and is responsible for CAD and myocardial infarction is *apob*. It encodes Apolipoprotein-B100 (ApoB100). The interaction of ApoB100 with LDL-R, mediates the uptake of LDL-C from liver and peripheral cells and hence it plays an important role in cholesterol homeostasis[18]. It was mapped to 2p24.1 with an approximate length of 43 kb and 29 exons. A common *Msp*I polymorphism in the exon 26 causes the change of arginine to glutamine at codon 3611 of the mature ApoB protein[19]. The association of this polymorphism with CAD was studied in previous studies[19,20]. So, both these genes (*ldl-r* and *apop)* are reported for their association with CAD and/or high lipid levels in different populations round the globe, but not in case of West Bengal population.

The present study was designed to evaluate the association of both *Ava*II DNA polymorphism at *ldl-r* gene locus and *Msp*I DNA polymorphisms at *apob* gene locus with plasma lipid concentrations and CAD in West Bengal population. We have tried to identify a potential genetic marker that can be used to infer the abnormal lipid levels and predict the occurrence of CAD.

Method

Subjects for the Case-Control Study

Angiographically proven 254 CAD patients were included in this study. Patients less than 6 weeks after a myocardial infarction were excluded. Age and ethnicity matched 246 healthy subjects were selected as ‘control’. All the subjects were included after their submission of the dully signed consent form. 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. The blood samples were collected from the Department of Cardiology, NRS Medical College and Hospital, Kolkata as well as a health examination camp organised by the Department of Biotechnology, Heritage Institute of Technology, Kolkata, India. The study was approved by the Institutional Ethical Committee. Blood samples for measuring serum biochemical and lipid profiles were obtained in the morning. Both patients and controls were fasting for 12 h prior to blood collection. Three ml of venous blood sample was also collected in EDTA vials for the extraction of genomic DNA.

Lipid Profile Analysis

Serum lipids including total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL) were determined by enzymatic methods with commercially available kits ERBA Mannheim cholesterol liquid stable reagent CHOD-PAP end point, Triglycerides estimation kit (ENZOPAK) and ERBA Mannheim HDL direct liquid stable reagent, respectively. The VLDL cholesterol and LDL cholesterol were calculated using the William Friedewald’s formulae[21].

DNA Analysis

The genomic DNA was isolated from using HiPura^{TM} Blood Genomic DNA Miniprep Purification Spin Kit (MB505) of (HIMEDIA, Mumbai, India). The quality of DNA was checked on 1% agarose (Sigma, USA) gel electrophoresis with 0.5 µg/mL ethidium bromide, and quantification was done on a UV spectrophotometer (Specgene Ltd, UK). All the polymorphisms were detected after DNA amplification by the polymerase chain reaction (PCR), restriction isotyping and size separation by agarose gel electrophoresis (2.5% with 0.5 µg/mL ethidium bromide). Primer sequences and PCR conditions for the *Ava*II polymorphism at the LDL-R gene were reported by Long *et al*[17]. APOB gene fragments containing the *Msp*I site was amplified according to Singh *et al*[22]. Primers were procured from Chromous Biotech, Bangalore, India) and restriction enzymes were purchased from New England Biolabs (UK).

Statistical Analyses

Epidemiological data were recorded on a pre-designed form and managed with Excel software. All statistical analyses were done with the Graphpad Prism 5.0. For each variable, the values were expressed as mean ± SD. Differences among lipid and lipoprotein concentrations in different groups of individuals were compared using the Student’s *t*-test. Allele frequencies and genotype distribution for each

polymorphic site were estimated by gene counting. Chi-square (χ^2) analysis was used to test Hardy–Weinberg equilibrium, and for comparison of allele frequencies and genotype distribution between the studied groups. The significance was considered to be at the 5% level ($p < 0.05$).

Results & Discussion

Biochemical Characterization of Study Population

As lipid retention in blood is one of the major risk factors for development of CAD, we first investigated the serum-lipid levels of both control and patient group. As illustrated in Table 1, there is no significant difference in the mean age of the patient and control group included into the study. This finding indicates that we have included age-matched subjects into both the groups of our study. Table 1 also reveals that low density lipoprotein cholesterol (LDL-C) and total cholesterol (TC) were significantly higher ($p<0.0001$) in patient than control. This result confirms the association between CAD and higher lipid concentrations. This situation probably works as one of the risk factor for developing the pathogenesis of the disease CAD[23-25]. On the other hand the high density lipoprotein cholesterol (HDL-C) reported for its ability to drive reverse cholesterol transport. A series of reactions by which HDL is able to interact with cells in the systemic vasculature and deliver excess cholesterol back to the liver for disposal as bile salts[26]. So, low HDL is known for an independent risk factor for CAD[26-28]. When we checked the HDL-C levels of the subjects included in our study (Table 1), we observed that HDL-C concentration was significantly ($p<0.0001$) lower in CAD patient group than control. All these findings indicate that high LDL-C and low HDL-C concentrations in patient may be responsible for the development of CAD in this population. But in case of control, LDL-C and HDL-C concentrations were normal. It can be said that these differential concentration among patient and control might be because of some genetic polymorphisms of the proteins which are involved in the regulation of LDL-C and HDL-C. So, next we attempted to find out the association of DNA polymorphism with the disease CAD as well as lipid concentration.

Association of Polymorphisms with CAD

Based on the presence or absence of the *Ava*II cut-site in LDL-R (designated as “A”) and *Msp*I cut-site in APOB (designated as “M”) gene locus in the amplified products, different band patterns were observed (Fig. 1). The presence or absence of the cut-sites were indicated with ‘+’ or ‘-’ sign, respectively. The genotypic and allelic frequencies of two polymorphisms in West Bengal population are presented in Table 2. From Table 2 we observed that both genotypic and allelic frequencies of *Ava*II polymorphism at LDL-R gene but not *Msp*I polymorphism at APOB gene were in Hardy-Weinberg equilibrium which implied that they are not significantly different from the expected values. It was also found that heterozygotes for both the gene locus were predominant in the genotypic distribution. In genotypic frequencies, A^+A^+ genotype was significantly (p = 0.0011) more frequent in patient than control (22% vs 10%). The allele frequency of A^+ among patient group was significantly higher (P = 0.0475) than control group (58.5% vs 45%). On the other hand, the allele frequency of A^- in control group was significantly higher (P < 0.0001) than patients (55% vs 41.5%). But surprisingly in case of APOB gene heterozygous M^+M^- was the only genotype found in both patient and control. These results tempted us to hypothesise that *Ava*II polymorphisms at LDL-R gene locus may be associated with the disease CAD in this population, but no association is evident with *Msp*I polymorphisms at APOB gene locus.

Table 1 — Lipid-profile analysis was grouped according to patient and control

Parameter	Patient[a] (n = 254)	Control[a] (n = 246)	Significance level (P^c-value)
Age (years)	54.02 ± 16.24	58.04 ± 20.05	*NS*
TC[b] (mg/dl)	201.6 ± 44.14	130.6 ± 21.56	< 0.0001
TG[b] (mg/dl)	156.3 ± 48.19	115.3 ± 37.98	< 0.0001
HDL-C[b] (mg/dl)	38.13 ± 9.115	42.12 ± 8.929	< 0.0001
LDL-C[b] (mg/dl)	128.40 ± 39.73	68.43 ± 19.18	< 0.0001
VLDL-C[b] (mg/dl)	31.06 ± 9.572	23.03 ± 7.559	< 0.0001

[a]Values are mean ± S.D. [b]TC = Total cholesterol; TG = Triglyceride; HDL-C = High density lipoprotein cholesterol; LDL-C = Low density lipoprotein cholesterol; VLDL-C = Very low density lipoprotein cholesterol. [c] *P values* from Student’s *t*-test. NS = non-significant.

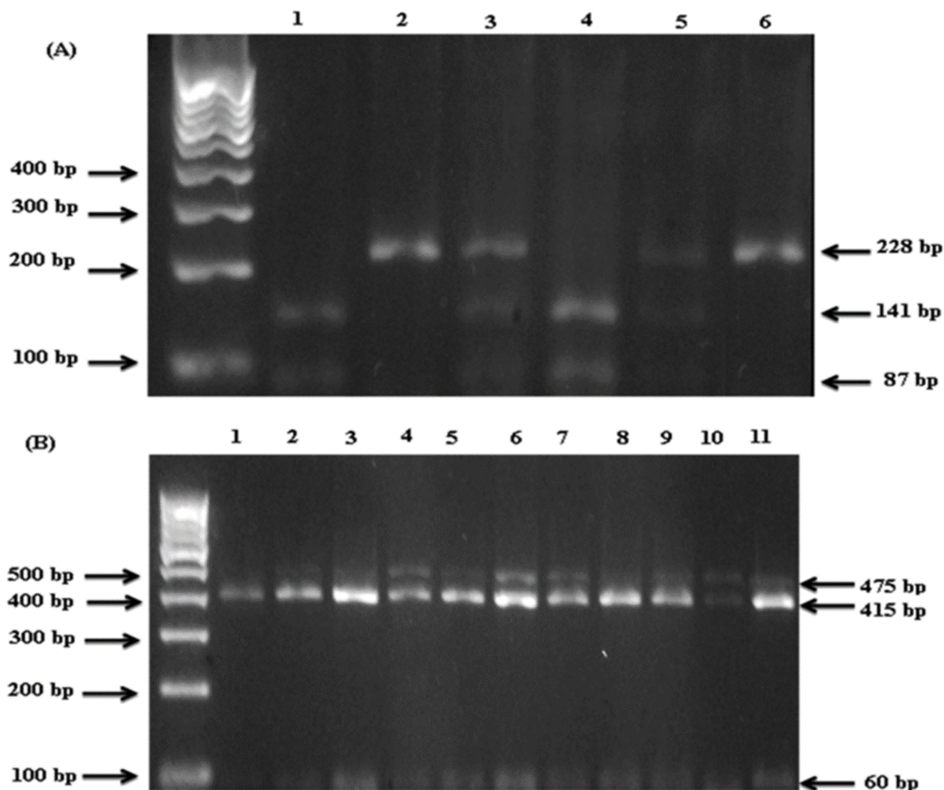

Fig. 1 — 2.5% AGE containing polymorphism bands. (A) *Ava*II polymorphism at LDL-R gene. The first lane from left contains 100 bp DNA ladder; other lanes contain six samples. Lane 1 & 4 show A^+A^+ genotype (141- and 87-bp); lane 3 & 5 show A^-A^+ genotype (228-, 141- and 87-bp) and lane 2 & 6 show A^-A^- genotype (228-bp). (B) *Msp*I polymorphism at APOB gene. The first lane from left contains 100 bp DNA ladder; other lanes contain ten samples, Only M^-M^+ genotype (475-, 415- and 60-bp) could be seen in all lanes.

Table 2 — Comparison of the genotype and allele frequencies of two genetic polymorphisms in West Bengal Population

Polymorphism	Genotype/Allele[a]	Patient[b] (n=254)	Control[b] (n=246)	P^c- value
*LDL-R Ava*II	A^+A^+	22 (56)	10 (25)	χ^2 = 13.56, d f= 2, p = 0.0011
	A^+A^-	73 (185)	70 (172)	
	A^-A^-	5 (13)	20 (49)	
	A^+	58.5 (297)	45 (221)	χ^2 = 3.926, d f = 1, p = 0.0475
	A^-	41.5 (211)	55 (271)	
*APOB Msp*I	M^+M^+	0	0	NS
	M^+M^-	100 (254)	100 (246)	
	M^-M^-	0	0	
	M^+	50 (254)	50 (246)	NS
	M^-	50 (254)	50 (246)	

[a] +/- refers to the presence and absence of cutting site for restriction endonuclease. A = *Ava* II; M= *Msp* I. [b] Values are in % of total number of subjects. Original numbers are presented inside the parenthesis. [c] *P values* from Chi-square (χ^2) test. NS= non-significant.

Genotypes and Serum Lipid Levels

Next, we attempted to explore the relation between the genotypes and lipid concentrations of the subjects. The result is illustrated in Table 3. It reveals that the levels of TC and LDL-C in West Bengal population were significantly different among the three genotypes (p = 0.0070 and p = 0.0002 respectively). Interestingly, it was found that subjects with A^+A^+ genotype had both higher serum TC and LDL-C levels than other two genotypic groups. But HDL-C levels were significantly lower ($p = 0.0001$) among A^+A^+ genotype than that of the other genotypes. These data clearly indicates the association of *Ava*II polymorphism at LDL-R gene locus with higher TC and LDL-C concentration as well as lower HDL-C concentration. On the other hand, there was only one

Table 3 — Comparison of lipid profile along with age of different individuals in West Bengal populations among different genotypic groups for two genetic polymorphisms

*ldl-r Ava*II polymorphism

Parameter	A^+A^+ [a]	A^+A^- [a]	A^-A^- [a]	Significance level (P^c-value)
Age (years)	57.84 ± 11.46	58.96 ± 9.992	59.06 ± 11.34	NS
TC[b] (mg/dl)	203.4 ± 48.43	187.2 ± 42.62	181.0 ± 64.27	0.0070
TG[b] (mg/dl)	148.3± 36.84	158.6 ± 69.14	159.3 ± 60.59	NS
HDL-C[b] (mg/dl)	38.63 ± 7.266	41.13 ± 9.529	42.61 ± 7.547	0.0001
LDL-C[b] (mg/dl)	132.2 ± 42.28	128.7 ± 36.88	107.2 ± 54.24	0.0002
VLDL-C[b] (mg/dl)	28.84 ± 7.373	30.06 ± 9.964	31.22 ± 11.49	NS

*apob Msp*I polymorphism

Parameter	M^+M^+ [a]	M^+M^- [a]	M^-M^- [a]	Significance level (P^c-value)
Age (years)	-	58.620 ± 0.68	-	NS
TC[b] (mg/dl)	-	190.53 ± 11.57	-	NS
TG[b] (mg/dl)	-	154.10 ± 8.41	-	NS
HDL-C[b] (mg/dl)	-	40.79 ± 2.01	-	NS
LDL-C[b] (mg/dl)	-	122.70 ± 13.54	-	NS
VLDL-C[b] (mg/dl)	-	30.04 ± 1.19	-	NS

[a] Values are mean ± S.D. [b] *P values* from One way ANOVA. NS = non-significant. [c] TC = Total cholesterol; TG = Triglyceride; HDL-C = High density lipoprotein cholesterol; LDL-C = Low density lipoprotein cholesterol; VLDL-C = Very low density lipoprotein cholesterol.

genotype was found so *Msp*I genotype didn't show any significant result. So, considering all these results it can be assumed that homozygous A^+A^+ genotype is associated with the disease CAD in this population. All the above-described results cumulatively signify that homozygous A^+A^+ genotype at LDL-R gene locus is clearly associated with the disease CAD and higher LDL-C in West Bengal population.

Associations of DNA markers at the candidate genes for cardiovascular disease, such as LDL-R or APOB, with disease itself or with established risk factors, such as high cholesterol levels, seems to be population-specific as a result of (i) variations in different subsets of genetic and environmental factors predisposing individuals to CAD from different populations and (ii) interpopulation differences in genetic background[23]. In this study, we examined the possible relationship between genetic markers at two candidate loci with CAD patients and controls from West Bengal population. The presence of homozygous A^+A^+ genotype (at LDL-R gene locus) was significantly higher in patient than control. So, it could be assumed that this genotype might be associated with CAD in West Bengal population. But how this genotype worked in disease pathogenesis was not clear. The analysis of genotypes with the serum lipid concentration of all the subjects in this study gave some idea about association of homozygous A^+A^+ genotype with CAD. It was seen that A^+A^+ homozygotes had higher serum LDL-C, TC but lower HDL-C levels than both A^-A^+ heterozygotes and A^-A^- homozygotes of this population. So, it can be said that higher LDL-C and lower HDL-C in A^+A^+ homozygotes might play a huge role to develop CAD here. Long *et al*[17] reported that in two Chinese populations homozygous A^+A^+ genotype was associated with higher LDL-C level. Salazar *et al*[15] also reported that the A^+A^+ genotype was also associated with higher LDL-C level and could be a potential genetic marker for coronary heart disease in Brazilian population. Ahn *et al*[14] investigated the effect of LDL-R gene *Ava*II polymorphism on plasma lipid profiles in 385 normolipidemic Hispanics and 543 non-Hispanic whites from the San Luis Valley, Colorado. They showed that the frequency of A^+ allele was higher in Hispanics than in non-Hispanic whites (56% vs. 43%; P <0.001). Pongrapeeporn *et al*[16] showed that LDL-C level was slightly higher in the A^+A^+ genotype than the other *Ava*II genotypes in Thai population. So, all these previous works are supporting our hypothesis that homozygous A^+A^+ genotype is related with high LDL-C levels in this population. As it was also associated with CAD, it could be a genetic marker of CAD in West Bengal population. On the other hand there is no association was found in *Msp*I DNA polymorphism at APOB

gene locus. Only occurrence of heterozygous M^+M^- in both control and patient group was visible in West Bengal population. Genest *et al*[19] reported that the frequency of a rare allele M^- of the *Msp*I polymorphism was increased in patients with CAD in Caucasian subjects. Delghandi *et al*[20] showed the significant association of *Msp*I genotypes with the patients with the risk of atherosclerosis. The heterozygous M^+M^- genotype had the highest levels of serum TC, LDL-C and APOB. But non-association of *Msp*I genotypes with CAD was reported by Sing *et al*[22] in North-Indian population and Pan *et al*[32] in Chinese population. So, we need to find other polymorphisms in APOB gene that is associated with CAD in this population. However, in this study, quite clearly, homozygous A^+A^+ genotype showed significant association with CAD and increased LDL-C levels but no such association was evident in case of *Msp*I polymorphism at APOB gene locus. This finding could be useful to develop a potential genetic marker for CAD in this population. As they have a wide spectrum of activities, both the gene and the protein might be promising therapeutic targets for the management and control of CAD. Currently, we are trying to find out the regulatory role and the information regarding transcriptional expression, transcriptional regulatory mechanisms of the gene and proteomic data to delineate the entire molecular mechanisms of the gene to develop the disease CAD.

In conclusion the association of *Ava*II polymorphism at LDL-R gene locus was evident with CAD which implied that LDL-R gene could be one of the useful genetic markers to detect CAD in West Bengal population.

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