

ROLE OF THE *APOB* GENE POLYMORPHISM (c.12669G>A, p.Gln4154Lys) IN CORONARY ARTERY DISEASE IN THE INDIAN PUNJABI POPULATION

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ABSTRACT

High concentration of apolipoprotein B (apoB) is a risk factor for coronary artery disease (CAD). The association of the *APOB* gene polymorphism c.12669G>A, p.Gln4154Lys with the risk of CAD varies considerably in different populations. The present study represents the first investigation regarding the role of this *APOB* gene polymorphism with CAD in the Indian Punjabi population. We have studied the *APOB* gene polymorphism c.12669G>A, p.Gln4154Lys and its relationship with lipid, apoB, low-density lipoprotein (LDL) heterogeneity and oxidation in subjects suffering from CAD. The study was conducted on 87 patients with CAD; 75 healthy subjects served as controls. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to determine the DNA polymorphism in the *APOB* gene. Frequency of R- (mutant) allele was significantly high ($p < 0.05$) in CAD patients when compared to controls. Variations in serum lipid levels in the R+R+ and R+R- *APOB* genotypes were insignificant ($p > 0.05$). However, serum apoB levels were significantly raised ($p < 0.05$) in CAD patients with the R+R- genotype as compared to those with the R+R+ *APOB* genotype.

Coronary artery disease patients had raised significantly raised ($p < 0.01$) Log triglyceride/high density lipoprotein-cholesterol (HDL-C) ratio, apoB carbonyl content and increased malondialdehyde-low density lipoprotein (MDA-LDL levels, irrespective of *APOB* genotype as compared to controls. Carriers of the R- allele are at higher risk of CAD, probably because of elevated serum apoB levels in the Indian Punjabi population. Overall, it may be concluded that the R- allele might be associated with increased susceptibility towards CAD development in the Indian Punjabi population, and one of the linking factor is the elevation in serum apoB levels. However, this association needs further evaluation in a larger population. Secondly, the robust mechanism behind the positive association of the R- allele with raised serum apoB levels needs to be explored, which might be helpful in the strengthening the observed results.

Key words: Apolipoprotein B (apoB), Polymorphism, Coronary artery disease (CAD)

INTRODUCTION

Apolipoprotein B (apoB) is the predominant apolipoprotein found in atherogenic low-density lipoprotein (LDL). Raised serum apoB levels are associated with increased risk of coronary artery disease (CAD) [1-3]. Similar results have also been reported from our laboratory [4,5]. The gene encoding for the *APOB* has been cloned and sequenced. It is 45 kb in length with 29 exons [6]. The *APOB* gene is known to have several

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sequences which are the recognition sites for various restriction enzymes. Polymorphism in the *APOB* gene with respect to the *EcoR*I restriction site exists due to a change in guanidine to adenine at exon 29, leading to the substitution of glutamine for lysine at position 4154 in the apoB polypeptide product [7]. This nucleotide change leads to loss of the *EcoR*I restriction site. The wild type and the mutated alleles are designated as R⁺ and R⁻, respectively. The frequency of the R⁻ allele has been reported to be more prevalent in CAD patients as compared to normal subjects in some but not all populations, thus suggesting that subjects having the R⁻ allele are probably more susceptible to CAD development [8-13]. Some investigators reported the positive association of the R⁻ allele with raised serum lipid levels [12], while others reported no such association [11]. The available reports indicate considerable variation in linking *APOB* gene polymorphism (c.12669G>A,p.Gln4154Lys) with CAD risk in different populations. The present study aimed to investigate the role of this *APOB* gene polymorphism with CAD in the Indian Punjabi population. Punjabi's are known to consume a fat-rich diet and are genetically more prone to CAD development. The present study would help in delineating a subset population at higher or lower risk of CAD. To the best of our knowledge, no such study has so far been done on the native Indian Punjabi population.

MATERIAL AND METHODS

We recruited 87 CAD patients from the Out Patient Departments and Medical Wards of the Employee's State Insurance (ESI) and Guru Nanak Dev hospitals, Amritsar, India, diagnosed with CAD by the clinician on the basis of clinical symptoms, echocardiogram (ECG), changes, stress test and angiography (if required). Seventy-four healthy individuals without any evident symptoms of CAD and any past history of the disease, were taken as controls from the general population. Subjects were segregated into males and females and divided into three age groups: 35-45, 46-55 and 56-65 years. A written informed consent was signed by each individual. A detailed questionnaire was prepared for all the necessary details of both patients and control subjects to answer. Coronary artery disease patients suffering from diabetes mellitus, renal or thyroid disease, rheumatoid arthritis, acute infections, on lipid lowering medications or women on oral

contraceptives or who had undergone hysterectomy were excluded.

Venous blood samples were taken after 12-hour overnight fasting. Serum was used for various biochemical investigations. High-density lipoprotein (HDL) was isolated from serum by precipitating LDL and very low-density lipoprotein (VLDL) with magnesium chloride and sodium phosphotungstate reagent [14]. Total cholesterol and HDL cholesterol levels were estimated by extracting it quantitatively into acetone-ethanol mixture. The cholesterol residue was dissolved in glacial acetic acid and allowed to react with concentrated sulphuric acid and ferric chloride reagent [15]. Serum triglycerides were estimated using a commercially available kit from Biotech, Baroda, Gujrat, India [16]. The LDL cholesterol levels were calculated from the formula given by Friedwald *et al.* [17]. Serum apoB levels were estimated using an immunoturbidimetric kit (Diasys, Holzheim, Germany). The LDL was isolated from serum. Briefly, the precipitation buffer consisted of 0.064 M trisodium citrate adjusted to pH 5.05 and contained 50,000 IU/L heparin. The insoluble lipoproteins were sedimented by centrifugation at 1,000g for 10 min. The pellet was resuspended in 1 mL of 0.1 M sodium phosphate buffer at pH 7.4 containing 0.9% normal saline [18]. The LDL protein oxidation as apoB carbonyl content was assessed. Briefly, 0.2 mL of dinitrophenylhydrazine (10 mM DNPH in 2 M HCl) was added to the isolated LDL solution in a centrifuge tube. The contents were incubated for 1 hour at room temperature. Then 0.6 mL of denaturing buffer (0.15 M sodium phosphate buffer, pH 6.8, containing 3% sodium dodecyl sulphate) was added and the tubes were allowed to vortex for 1min. After mixing, an equal volume of ethanol and heptane mixture was added and the contents were mixed again. The tubes were centrifuged at 1,000 g for 5 min. The LDL was recovered from the interface and washed three times with ethanol ethyl acetate (1:1 v/v) mixture. Each DNPH sample was dissolved in denaturing buffer and was scanned from 320 to 410 nm. The peak absorbance was used to calculate protein carbonyls with extinction coefficient 22,000 M⁻¹ cm⁻¹ [19]. Results were expressed as nmoles carbonyl/mg LDL protein. The LDL lipid peroxidation as malondialdehyde (MDA) levels was estimated by the method of Beuge and Aust [20]. The protein content of the LDL sample was estimated according to the protocol of Lowry *et al.* [21].

DNA was isolated from the blood samples by the phenol chloroform method [22] and the quality was

checked on 0.8% agarose gel electrophoresis. DNA amplification kits and primers were procured from Genei, Bangalore, India. A 480 bp sequence of *APOB* gene containing the *EcoR*I restriction site was amplified by polymerase chain reaction (PCR) on a thermal cycler (Bio-Rad India Pvt Ltd., Gurgaon, India). The following primers were used to amplify the desired sequence in *APOB* gene: forward primer (F) 5'-CTG AGA GAA GTG TCT TCG AAG-3' and reverse primer (R) 5'-CTC GAA AGG AAG TGT AAT CAC-3'. The reaction mixture was prepared to a final volume of 50 μ L; 2 μ L of DNA (1 μ g/ μ L) was added to the assay mixture. The amplified sequence was isolated after 0.8% agarose gel electrophoresis and digested overnight at 37°C with 20 U of *EcoR*I. The digested products were resolved on 2.5% agarose gel electrophoresis in 1X Tris-acetate ethylenediaminetetraacetic (TAE) buffer, pH 8.0, at a constant voltage of 50 V. A 480 bp sequence containing the *EcoR*I restriction site was digested into fragments of 253 and 227 bp, respectively. In the absence of the *EcoR*I restriction site, the 480 bp sequence produced only a single band on the agarose gel. The respective alleles were designated as R+ and R-.

Statistical Analysis. Results were expressed as mean \pm standard deviation (SD). Chi-Square was applied to determine the significance in allele frequency. The Student's *t* test was applied to assess the significance in other parameters. Statistical significance was determined at $p < 0.05$.

RESULTS

The subjects under study had either the R+R+ genotype or R+R- genotype. No individual with homozygous mutant alleles, *i.e.*, R-R-, was identified (Figure 1). The frequency of R+ and R- alleles was calculated (with an allele counting method) in control subjects as well as in CAD patients. The frequency of the R- allele was significantly higher ($p < 0.05$) in CAD patients when compared to controls.

In order to study the association of the R- allele with CAD, variations in serum lipids, lipoprotein cholesterol and apoB levels of CAD patients and control subjects with different apoB genotypes were investigated (Table 1). Total cholesterol, triglycerides, very low-density lipoprotein-cholesterol (VLDL-C), low-density lipoprotein-cholesterol (LDL-C) and high-density lipoprotein-cholesterol (HDL-C) levels did not differ significantly in the subjects, whether they



Figure 1. Electrophotogram of *EcoR*I digested *APOB* gene after PCR amplification.

were CAD patients or controls carrying the R+R+ or R+R- *APOB* genotype. The data were analyzed after segregating subjects on the basis of age and gender. However, CAD patients with the R+R- genotype had significantly raised ($p < 0.05$) serum apoB levels as compared to those having the R+R+ genotype.

Raised serum apoB levels indicate the predominance of small dense LDL particles that are more prone to oxidation. The MDA-LDL levels and apoB carbonyl content of LDL did not differ significantly in CAD patients with different *APOB* genotypes (Table 2). A similar trend was observed in Log triglyceride/high-density lipoprotein-cholesterol (HDL-C) value which was taken as an index of LDL particle size. Overall, CAD patients had increased predominance of small dense LDL particles resulting in their increased oxidation. However, this observation was independent of *APOB* genotypes.

DISCUSSION

In the present study, the role of the *APOB* gene polymorphism (c.12669G>A,p.Gln4154Lys) was

Table 1. Variations in lipid, lipoprotein cholesterol and apolipoprotein B levels among different *APOB* genotypes in coronary artery disease patients and normal subjects.

Subjects	Sex	Ages (years)	TC (mg/dL)		Triglyceride (mg/dL)		VLDL-C (mg/dL)		LDL-C (mg/dL)		HDL-C (mg/dL)		ApoB (mg/dL)	
			R+R+	R+R-	R+R+	R+R-	R+R+	R+R-	R+R+	R+R-	R+R+	R+R-	R+R+	R+R-
Control Subjects	Males (n=44)	35-44	184±12.6 (n=10)	193±12.8 (n=6)	136±11.2	138±11.2	27±8.2	27±8.0	113±10.0	121±10.6	44±9.5	44±9.0	78±12.3	86±12.6
		45-54	191±12.0 (n=8)	205±12.6 (n=5)	142±10.9	146±10.6	28±8.1	29±8.4	121±10.5	135±10.6	42±9.8	41±9.6	85±12.4	99±12.5
		55-64	220±11.8 (n=15)	-	155±10.4	-	31±8.4	-	147±10.8	-	41±9.5	-	102±12.2	-
	Females (n=30)	35-44	189±12.4 (n=7)	198±12.5 (n=3)	138±11.1	142±11.6	27±8.4	28±8.6	117±11.2	126±11.6	45±9.6	44±9.5	72±12.5	80±12.9
		45-54	195±12.6 (n=6)	208±12.8 (n=4)	148±11.4	150±11.7	29±8.2	30±8.5	123±11.4	136±11.4	43±9.5	42±9.6	80±12.8	85±12.9
		55-64	225±12.8 (n=10)	-	160±11.6	-	32±8.5	-	151±11.6	-	42±9.4	-	95±12.8	-
CAD Patients	Males (n=40)	35-44	230±12.9 (n=10)	236±12.6 (n=3)	236±10.8	238±11.2	47±8.2	47±8.0	145±10.0	149±10.6	38±9.5	40±9.0	141±12.3	154±11.5 ^a
		45-54	248±13.0 (n=12)	245±12.6 (n=1)	242±10.9	246±10.9	48±8.3	49±8.4	161±10.5	155±10.6	39±9.8	41±9.6	149±12.4	163±11.0 ^a
		55-64	259±12.8 (n=13)	265±12.8 (n=1)	255±10.5	265±10.8	51±8.6	53±8.5	169±10.8	173±10.6	40±9.4	39±9.5	155±12.2	160±11.4
	Females (n=47)	35-44	232±12.4 (n=9)	239±12.7 (n=5)	238±11.1	242±11.6	47±8.4	48±8.6	145±11.2	151±11.6	40±9.5	40±9.5	130±12.5	142±11.2 ^a
		45-54	245±12.7 (n=10)	248±12.7 (n=5)	249±11.4	250±11.7	49±8.2	50±8.4	156±11.6	158±11.4	40±9.4	38±9.6	140±11.8	156±12.6 ^a
		55-64	255±12.8 (n=15)	260±12.9 (n=3)	253±11.8	255±11.8	51±8.6	51±8.6	165±11.6	179±11.8	39±9.6	38±9.6	142±12.8	159±11.5 ^a

TC: total cholesterol; VLDL-C: very low-density lipoprotein cholesterol; LDL-C: low-density lipoprotein-cholesterol; HDL-C: high-density lipoprotein cholesterol; ApoB: apolipoprotein B.

*p<0.05 (significant difference in serum apoB levels among the different *APOB* genotypes).

Table 2. Low-density lipoprotein oxidation in coronary artery disease patients and control subjects with the R+R+ and R+R- *APOB* genotypes.

Subjects	Control Subjects (n = 74)		CAD Patients (n =87)	
	R+R+	R+R-	R+R+	R+R-
ApoB genotypes				
ApoB carbonyl content (nmol carbonyl/mgLDL protein)	1.53 ± 0.36	1.62 ± 0.39 ^a	2.62 ± 0.44	2.80 ± 0.42 ^a
MDA-LDL (nmol/mgLDL protein)	0.16 ± 0.05	0.17 ± 0.07 ^a	0.38 ± 0.07	0.41 ± 0.09 ^a
Log (triglyceride/HDL-C) ratio ^b	0.23 ± 0.06	0.26 ± 0.07 ^a	0.65 ± 0.15	0.68 ± 0.20 ^a

ApoB: apolipoprotein B; MDA-LDL: malondialdehyde-low-density lipoprotein.

^a p >0.05.

^b This ratio was taken as an index of LDL particle size.

studied in native Indian Punjabi subjects suffering from CAD. Three *APOB* genotypes, *i.e.*, R+R+, R+R- and R-R- are possible. However, only two genotypes R+R+ and R+R- were observed in both control sub-

jects and CAD patients in this study. The R-R- genotype was not observed. The occurrence of this genotype in other populations has been reported to vary from infrequent to nil [23]. Frequency of the R- allele

was observed to be high in CAD patients when compared to control subjects in the population under study. In other populations, increased frequency of the R- allele has also been reported in CAD patients as compared to normal subjects [8,9]. On the other hand, no significant difference in the frequency of the R- allele has been reported in South Asian CAD patients and normal subjects living in Britain [10]. Similar results have been reported in the Brazilian population [11]. Thus, the R- allele is not associated with CAD in all the populations. In the Indian Punjabi population, the R+R- genotype was more frequent in CAD patients in comparison to control subjects and accordingly, the frequency of the R- allele was higher in CAD patients. This clearly indicates that the R- allele is associated with increased susceptibility toward CAD development in our population. In a study on the South Indian Tamil population, the frequency of the R- allele has been reported to be 0.054 [13], which is close to that observed in our population (0.04). It may be noticed that this comparison was done in a healthy population only and it may be possible that frequency of the R- allele would be more in the Tamil population suffering from CAD. However, they have not reported this data. No significant variations in lipid and lipoprotein cholesterol levels in CAD patients having either the R+R+ or R+R- genotype were observed. Similar results have been reported in South Indian [13] and in Brazilian populations [11]. Hegele *et al.* [8] also observed no difference in total or LDL cholesterol levels in CAD patients and normal subjects with different apoB genotypes in the American population. However, positive association of *APOB* gene *EcoR1* polymorphism with elevated lipid levels has been reported in the Russian population [12]. In the present study, we observed raised serum apoB levels in CAD patients with the R+R- genotype as compared to those having the R+R+ genotype. On the contrary, insignificant difference in apoB levels of CAD patients with different apoB genotypes has been reported in the American population. In the Indian population from the city of Lucknow, Uttar Pradesh, India, higher levels of apoB and LDL cholesterol were reported in CAD patients with the R+R+ apoB genotype [24]. Thus, the relationship between the R- allele of apoB and raised serum apoB levels is not consistent in all populations and thus need to be verified in different ethnic groups.

Raised serum apoB levels were associated with the R- allele in our population. It seemed interesting

to investigate if this *APOB* gene polymorphism could affect the heterogeneity of LDL particles and its oxidation. However, no significant difference was observed in MDA-LDL levels and apoB carbonyl content in CAD patients having the R+R+ and R+R- genotypes, thereby indicating that this *APOB* gene polymorphism does not affect the LDL oxidation status in the Indian Punjabi population. Independent of the genotype, both MDA-LDL levels and apoB carbonyl content were raised in CAD patients in comparison to control subjects. Similar results were observed when Log triglyceride/HDL-C values were compared in CAD patients having the R+R+ or R+R- *APOB* genotypes. An increased susceptibility of LDL to oxidation has been reported in CAD patients by other investigators [25,26]. Overall, CAD patients had increased predominance of small dense LDL particles that are more prone to oxidation. However, this observation was independent of *APOB* genotype. This clearly indicates that some other factors such as environment or nutrition might be more potent in bringing structural and functional changes in apoB of LDL.

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