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Genetic Variants at the Apo-A1 Gene in Association with Coronary Artery Disease

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Abstract

The aim of this study was to investigate whether the genetic variants of ***apolipoprotein A1***: *Msp*I polymorphisms in non translated region at -75bp upstream and +83bp in the first intron, had any impact on the development of CAD. A total of 400 unrelated adult subjects were enrolled in the study (200 CAD patients & 200 CAD-free controls) from the Kashmir region. Plasma levels of lipids were estimated for each sample by using photometric system. DNA extracted from blood samples was amplified by means of polymerase chain reaction, and then subjected to RFLP by using *Msp*I enzyme; resolving the product on 3% agarose gel stained with ethidium bromide, and visualized under UV light. The G →A substitution at –75bp in *apo*-A1 revealed that GG genotype was more prevalent among cases, whereas GA and AA was more prevalent among controls, however the difference was insignificant (p=0.145). Overall 'G' allele was more prevalent than 'A' allele among the study population but the difference was again insignificant (p=0.0106). At +83bp site, there was complete absence of 'T' allele both in cases and controls in *apo*-A1, so that no individual for genotypes C/T and T/T at *apo*-A1 gene was found. Instead the 'C' allele, in homozygous dominant condition (C/C), was present throughout (100%) the study population. From our study, it can be concluded that polymorphism at -75bp (G/A) and +83bp (C/T) polymorphism of Apo-A1 gene appears to have no apparent association with the development of CAD in this study population.

Keywords: Coronary artery disease; Polymerase chain reaction; RFLP; Genetic variants.

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1. Introduction

Coronary artery disease (CAD) is the leading cause of death worldwide. Approximately, 3.8 million men and 3.4 million women die worldwide each year from CAD and the number is on rise; it has become a true pandemic that respects no borders [1].

CAD is a multifactorial disorder and has a complex etiology [2]. It involves both genetic and non-genetic factors; and the interactions among them. Evidence for genetic links comes from factors like positive family history of CAD, altered plasma lipid levels [3,4,5], hypertension, obesity, diabetes, and the metabolic syndrome, all of which have significant heredity element and involves many genes in their susceptibility [6,7].

Lipids whether their intake or the metabolism have always been linked directly or indirectly to the cardiac health. In fact, abnormal lipid metabolism is a major predisposing factor to CAD and the genes involved have been suggested to be an important set of '*candidate genes*' for atherosclerosis [8]. One such set involves the genes coding for apolipoproteins.

Apolipoproteins bind plasma lipids (such as fat and cholesterol) to form lipoproteins and transport them through the lymphatic and circulatory system. Genetic factors have been reported to influence the distribution of lipids and lipoprotein levels [9].The genes coding for apolipoproteins are polymorphic, meaning that different people have different forms, and consequently may handle fats in different ways. **Apolipoprotein A1**- the principal protein component of HDL, which promotes cholesterol efflux from tissues to the liver for excretion, and clear cholesterol from arteries. Apolipoprotein-A1 in humans is encoded by the apo-A1 gene, and consists of three introns and four exons.

Several studies have reported positive associations between *apo*-A1 polymorphisms and CAD. Two such polymorphisms are the nontranslated regions of the *apo*-A1 with guanine to adenine substitution (G→A) at -75bp upstream from the start of transcription and the other with cytosine to thymine substitution (C→T) at +83bp in the first intron of *apo*-A1, which results in the elimination of *Msp*I restriction site. These G-75A and C+83T substitution in nontranslated regions of *apo*-A1 have been shown to be associated with CAD and its severity [10] and have been shown to be associated with plasma HDL-cholesterol levels [11,12,13] and thus may be susceptibility alleles for myocardial infarction.

In light of this, we designed a prospective, case-control study in a homogeneous sample of 400 individuals (200 each of cases and controls). The objective of resolve the question of whether there is i) an association between apo-A1 genetic polymorphism and angiographically documented CAD with sufficient statistical significance ii) to establish the possible effects, if any, of these polymorphisms on circulating levels of plasma lipids and iii) on various CAD risk factors in these patients. Some attempts of carrying out genetic association studies on CAD have been made previously on Indian populations but never in ethnically distinct Kashmiri population, which has high CAD prevalence [14].

2. Method

Study population and blood sampling The study was carried out in the Department of Biochemistry, University of Kashmir, in collaboration with the Department of Medicine, SMHS Hospital, Srinagar, from where patients were recruited, during the period May, 2011 to Dec, 2012. The study was approved by the Ethics Committee of Govt. Medical College, Srinagar. A total of 400 unrelated adult subjects were enrolled in the study, with informed consent, ethnically self identified as Kashmiri, from the Kashmir region of the J&K State. They were selected from the ICU of SMHS Hospital. The choice of each group (case and control) is critical to the correct interpretation of the data. The criteria for selection of the subjects for study are given in Table 1.

Coronary artery disease subjects-The CAD group consisted of 200 patients, 149 men and 51 women, with clear evidence of CAD and not older than 65 years. The criteria for the diagnosis of coronary artery disease were (a) a

history of angina or infarction and previously diagnosed disease (b) an affirmative response to Rose questionnaire, and (c) ECG finding (includes Q wave changes, ST segment depression or elevation and T wave inversions). But the definitive diagnosis of CAD came from coronary angiography. Only those cases with atleast 40% stenosis in one or more coronary vessels were considered as positive. The presence of all these criteria was taken as confirmation of the diagnosis of CAD. Subjects with concomitant liver or renal disease were not included. Also excluded were those who were on lipid lowering diet and medications.

Controls-The other group, the control group, consisted of 200 age-matched unrelated Kashmiri subjects, 131 men and 69 women, recruited from a population of individuals screened for CAD risk factors with no history or clinical evidence suggestive of presence of CAD or any cardiovascular disease. Exclusion criteria were: 1) age below 30 and above 65 years, 2) the presence of type 2 diabetes, and 3) the complete absence of CAD related symptoms and history.

Both cases and controls were subjected to questionnaire to record age, gender, with special reference to cardiovascular disease risk factors like detailed history of hypertension, diabetes, smoking, and sedentary behaviour-assessed mainly by inquiring about work-related and spare time activities.

Fasting blood samples (1-2 ml) were drawn by vacutener into sodium EDTA tubes from the antecubital vein, for biochemical and gene polymorphic studies. Patients coming for various investigation at clinical labs for other ailments, with no evidence/or history of any cardiac disease, were selected as controls (n = 200). Other inclusion criteria were that all subjects recruited for study, be from the Kashmiri population; and that the maximum clinical data and sufficient biological material be available. The recruited CAD patients comprised of 149 males and 51 females.

2.1. Clinical and biochemical assessment

The samples were centrifuged at 2,500g for 10 minutes. Plasma was separated from each sample, and the remaining cell aggregates were stored at –20°C until DNA was extracted from them, for genetic studies. Plasma samples were also stored at –20°C and were not thawed until analyzed for biochemical investigation.

Cholesterol, triglyceride, HDL-C and LDL-C levels were determined from plasma samples stored at –80°C, on photometric system (AT-112 Biochemistry Analyzer) by using commercially available kits (DiaSys, Holzheim, Germany).

2.2. Genetic analyses/Determination of Apo-A1 gene polymorphism

DNA was isolated from frozen EDTA blood by standard Phenol-Chloroform Extraction. The extracted DNA was stored at 4°C, until analysis. Once it was confirmed that the genomic DNA is present, and the concentration and purity is also desirable, genotypic variants at *apo*-A1 were studied through polymerase chain reaction and restriction fragment length polymorphism. The variants analysed were: *apolipoprotein A1*: *Msp*I polymorphisms in non translated region at -75bp upstream and at +83bp in the first intron.

The 25µl PCR reaction mixture were composed of 50-150ng genomic DNA, 0.4pmole/µl of each primer and PCR Master Mix (Fermentas) (2X Hot Start PCR buffer, 400µM each dNTP and 4mM Mg^{2+}). Reaction mixture in PCR tubes was gently mixed and placed in a twenty-five well automated thermal cycler (Imagene) for amplification. Following set of primer [15] was used in the polymerase chain reaction: 5'AGGGACAGAGCTGATCCTTGAA CTCTTAAG3' and 5'TTAGGGGACACCTAGCCCTCAGGAAGAGCA3'. The amplification conditions for *apo*-A1 was- initial denaturation at 94°C for 5min, denaturation at 94°C for 1min, annealing at 60°C for 1.5min, extension at 72°C for 1.5min and final extension at 72°C for 10min. The total number of cycles was 35. Amplification and specificity of amplicons obtained (433bp for *apo*-A1) in the PCR reaction was analyzed by agarose gel electrophoresis on 1.5% gel. After the electrophoresis, the gel was visualized and photographed on a Gel Doc (Pharmacia).

2.3. Restriction digestion

The purified amplification products were subjected to restriction analysis and for which the standard protocol was used. 5µl of the PCR products were digested separately with 10 units (1µl) of *Msp*I (for *apo*-A1). The reaction mixture also included 1µl of 10Xbuffer and 8µl of nuclease-free water. The reaction mixture was mixed gently and spinned down for few seconds. Finally the mixture was incubated at 37°C for 3h. The digestion products were then resolved on agarose gel (2.5% in case of *apo*-A1) stained with ethidium bromide, and visualized under UV light. DNA molecular weight marker of 50bp was used to assess the size of the PCR–RFLP products.

The *Msp*I restriction endonuclease recognizes CCGG sequence, causing cleavage of the *apo*-A1 into four (209, 113, 66, 45) fragments.

2.4. Sequencing of Apo-A1 gene region

Sequencing of the amplified regions of the apo-A1 gene was carried out, in order to confirm that only the desired region has been amplified. The automated sequencing also helps to check sequence variants.

3. Statistical analysis

All statistical analyses were performed by using the GraphPad Prism 6 software program; and Quickcal, an online statistical software. Results were expressed as means ± Standard deviation. Values of $p<0.05$ were considered to indicate statistical significance. CAD patients and CAD-free controls were compared with regard to various risk factors for disease (like hypertension, smoking), medical history, lipid profile, and frequency of alleles. The independent samples were tested with the unpaired t test to analyze differences between controls and CAD for various variables. The genotypic frequencies were estimated from the observed genotypic counts; the statistical significance of difference in genotypic counts between cases and controls was compared by χ^2-square test. Allele frequency was calculated by using the equation p + q =1 where p and q are the frequencies of each allele at the particular locus.

4. Results

4.1. Characteristics of study population

The distribution of demographic and biochemical characteristics; and various cardiovascular risk factors of the population (control vs cases) studied are summarized in Table 1. The mean ages and the number of male and female patients differed insignificantly between the two groups.

Table 1: Characteristics of study population

Variables	CAD-free (n=200)	CAD patients (n=200)	*p*- value
	n (%)	n (%)	
Age (mean)	53.16±12.62	54.45±10.34	p >0.264
Male	131 (65.5)	149 (74.5)	
Female	69 (34.5)	51 (25.5)	p>0.05
Positive family history	-	16 (8.0)	
Current smoking	73 (36.5)	119 (59.5)	P <0.0001
Blood Pressure	51 (25.5)	97 (48.5)	P <0.0001
Systolic(mmHg) Diastolic(mmHg)	120.5±5.75 80.05±3.12	124.1±24.51 79.52±14.77	P=0.0438 P=0.6198

Lipid profile-			
1. Total cholesterol (mg/dl)	162.770±19.60	169.431±17.24	P=0.0003
2. HDL cholesterol (mg/dl)	45.88±4.23	33.66±4.53	P=0.0001
3. Triglycerides (mg/dl)	143.154±31.20	174.66±27.81	P=0.0001
4. LDL cholesterol (mg/dl)	85.61±21.25	124.65±36.21	P=0.0001

Values represent mean ± SE

4.2. Analysis of Apo-A1 gene

Apo-A1 gene region was amplified and then resolved on 1.5% agarose gel forms a 433bp PCR product (Fig. 1). The PCR product has 3 restriction sites for *Msp*I at –75, +37, and +83bp; thus 4 fragments are produced that are 45, 66, 113, and 209bp (Fig. 2). When there is G→A transition at –75bp, the *Msp*I restriction site is lost and it produces a fragment of 179bp, instead of 113 and 66bp (Lane 6). Similarly when a C→T transition takes place, the +83bp restriction site is lost and a fragment of 254bp is produced instead of 209bp and 45bp. The gel picture is interpreted using this information. This was done for each subject in study population, and interpretation (with respect to –75bp and +83bp site polymorphism) was documented as an allelic profile of each subject.

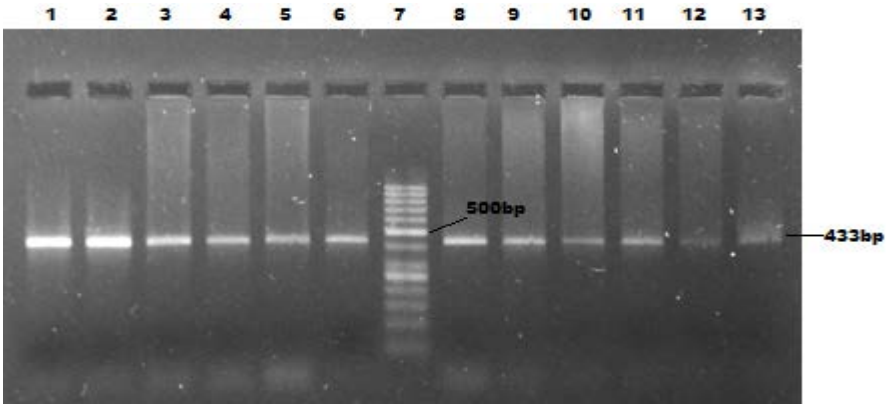

Fig 1. Agarose gel electrophoresis of amplified apo-A1 gene region. All lanes contain a 433bp amplified product, except lane 7 which contains 50bp DNA ladder.

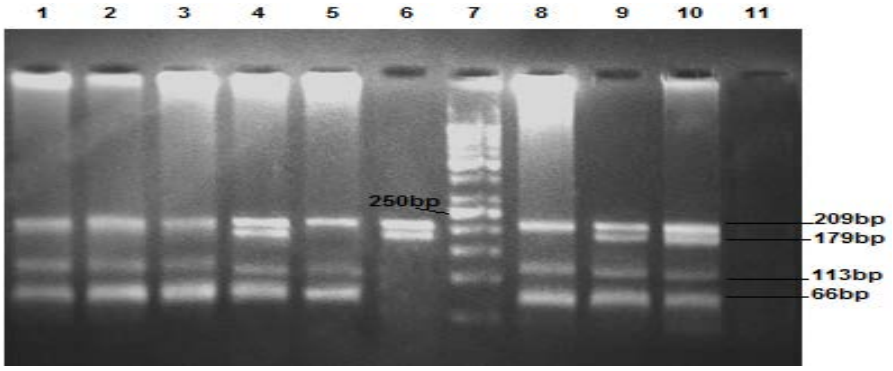

Fig 2. Agarose gel electrophoresis of amplified apo-A1 gene region digested with MspI. Lane 1-5 contains amplicons from CAD patients, whereas Lane 8-10 contains amplicons from CAD-free controls; Lane 7 contains 50bp DNA ladder and Lane 11 contains negative control. Lane 1, 2, 3,5 and 8 contains three bands of 209bp, 113bp and 66bp (homozygous wild-type G/G at -75 and C/C at +83bp); Lane 4, 9 and 10 contains four bands of 209bp, 179bp, 113bp and 66bp (heterozygous G/A at -75 and homozygous C/C at +83bp); and Lane 6 contains two bands of 209bp and 179bp homozygous wild-type G/G at -75 and C/C at +83bp).

4.3. Allele and genotypic frequencies in patients and control subjects

The genotypic frequency of the *apo*-A1 polymorphic variants for 200 patients with angiographically-proven CAD and an equal number of control subjects are described in Table 2. As there were no sex specific differences in frequencies, the comparison of genotypic frequencies between patients and controls subjects was carried out for both sexes combined.

Table 2: Comparison of apo-A1 genotypes among the CAD patients and CAD free controls

Genotype apo-A1		Controls n=200	%	Cases n=200	%	Chi-Square	*p*-value
G-75A	GG	115	57.5	134	67	3.86	0.145
	GA	21	10.5	17	8.5		
	AA	64	32	49	24.5		
C+83T	CC	200	100	200	100	0	1
	CT	Nil		Nil			
	TT	Nil		Nil			

Chi-square analysis was carried out separately for genotypes at two different loci (-75bp and +83bp) in Apo-A1 gene. The distribution of genotypes differ insignificantly both in case of -75bp as well as at +83bp. The numbers of CAD cases carrying GG, GA and AA genotypes were 134, 17 and 49; and 115, 21 and 64 in controls respectively. The difference was however insignificant (p=0.145).

Table 3: Comparison of apo-A1 at G-75A allelotypes among the CAD patients and CAD free controls

Allelotype for apo-A1 at G-75A	Controls n=200	(%)	Cases n=200	(%)	Chi-Square	*p*-value
G	251	62.75	285	71.25	6.54	0.0106
A	149	37.25	115	28.75		

For allelotyping in case of -75bp, frequency for G allele was found to be less in controls as compared to cases; whereas reverse was seen for the A allele which showed high frequency in cases as compared to that in controls. But distribution in frequency was found to be statistically insignificant (p=0.0106). In fact, +83bp at *apo*-A1 there is complete uniformity of C and T alleles between the two groups of cases and controls.

4.4. Sequencing of amplicons

Sequencing of the amplified *apo*-A1 gene regions (Fig. 3) revealed that -75bp site at *apo*-A1 exist in heterozygous form for two nucleotide substitution (G/A) in the nontranslated region, as have been previously reported, which results in the abolition of *Msp*I site. However, the other variant of *apo*-A1 at +83bp site in the first intron contains a single common polymorphic ‘C’ allele in homozygous form (C/C).

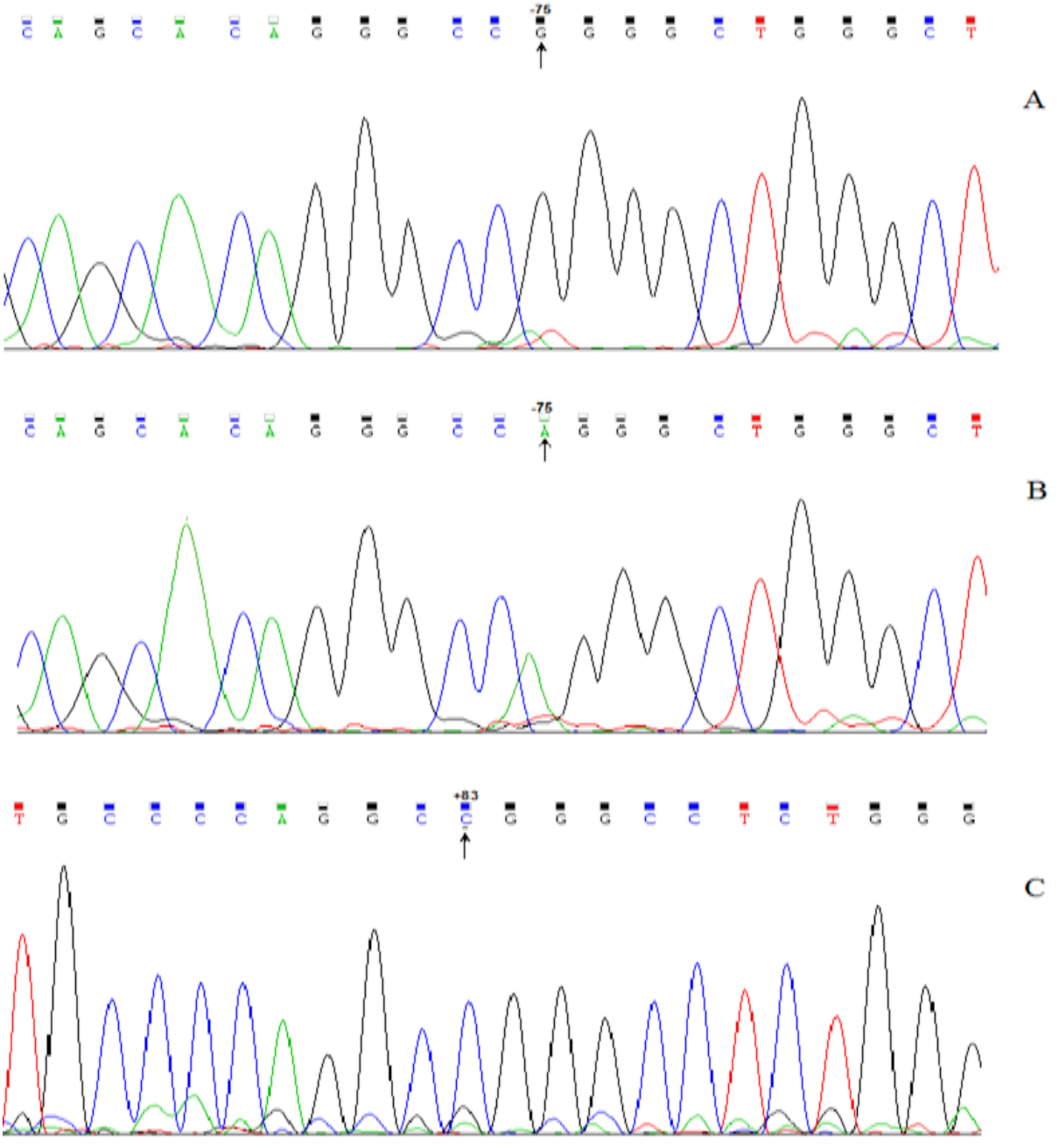

Fig 3. Partial nucleotide sequence of apo-A1 genes. apo-A1 amplicon shows heterozygosity for G→A transition at -75bp site, among some patients as well as controls (panel A & B); apo-A1 (+83) reveals total homozygosity (panel C) among both CAD patients and CAD-free controls (C/C). The arrow indicates the nucleotide involved in the substitution.

5. Discussion

Indians, as a community, are prone to develop CAD at a much younger age [16,17]. By 2020, about 2.6 million Indians are predicted to die due to coronary artery disease [18]. Kashmiri population, although being ethnically distinct, also have a high predisposition to premature CAD; this population is poorly investigated for the genetic factors conferring the high susceptibility. An epidemiological study conducted in Kashmir region shows that the overall prevalence of CAD was 7.54% (rural -6.7%, urban-8.37%, males-7.88%, females-6.63%) [14].

The critical assumption underlying this proposition was that the DNA polymorphism in apolipoprotein genes can be used to detect individuals with a high risk of developing CAD. Our study focused on common apolipoprotein-A1 DNA polymorphisms of the two major SNP's.

Our study reveals that G→A substitutions at -75bp of *apo*-A1 was statistically insignificant (p=0.145). The comparison between different genotypes among the cases and controls reveals that 'GG' homozygous genotype was higher among cases and 'AA' genotype was higher in controls. The 'GA' genotype was seen more or less equal among cases and controls. But the difference was overall insignificant. As far as frequency, 'G' allele was higher among cases (71.25%) as compared to controls (62.75%); and frequency of 'A' allele was higher among controls (37.25%) as compared to cases (28.75%). Such findings were in consistence with studies carried out in Chinese [19,20] and Australian populations [21]; but were in contrast to some previously reported data [10], particularly from Indian populations [22,23,24]where the 'A' allele was found to be higher in frequency among CAD population. The allelotype frequency however was also insignificant.

Our findings show that 'A' allele has higher frequency among controls, and thus may not be involved in the promotion of CAD or may not have any role in the development of CAD among the study population.

As far as C→T substitution at +83bp in *apo*-A1 was concerned, there was complete absence of 'T' allele among the study population whether in cases or controls. No individual for genotypes C+83T and T+83T at *apo*-A1 gene was found. Instead the 'C' allele, in homozygous dominant condition (C/C), was present throughout (100%) the study population, confirming to no apparent role of it in the CAD development, but the study on larger sample size is still required. 'T' allele, in some studies has been associated with increase in susceptibility to CAD or MI [22,23,24], and in some studies has been found insignificant [10].

A study on various CAD risk factors like family history, smoking, hypertension and biochemical parameter like plasma lipid estimation was also carried out. A small proportion (8%) of CAD cases, falling in the age group of 30-45yr had positive familial history of coronary artery disease, confirming to the genetic element of this disease, as was found in previous studies [25]. A significant revelation was that all of them were males having positive maternal history of disease.

Smoking was also significantly ($p< 0.005$) associated with the development of CAD. Of total, 70% were current chain smokers and the rest were former smokers.

About 48.5% CAD patients were hypertensive (with men twice as compared to women) and was significantly associated with CAD ($p< 0.005$). The systolic and diastolic blood pressure of cases and controls varied insignificantly.

Each lipid parameter was an independent risk factor for CAD and comparison between cases and controls revealed significant ($p<0.005$) association with CAD. Abnormal levels of plasma lipids characterize the patients with CAD, with all four lipid components varying significantly among the two groups of cases and controls, representing an additional risk to these patients.

6. CONCLUSION

An important feature of this study is that despite analyzing polymorphisms among very important genes, we failed to find any association of them with CAD disease (except for one SNP).

From our study, it can be concluded that Apo-A1 gene polymorphism at -75bp (G/A) and +83bp (C/T) appears to have no apparent association with CAD in this study population but study on larger sample size is required. Our study also reveals that CAD patients had significantly higher levels of conventional cardiovascular risk factors; with male gender, hypertension, smoking, and favorable lipid profile- characterized by increase in total cholesterol, triglycerides and LDL, but decrease in HDL.

To our knowledge, this study is the first report from Kashmir valley dealing with association of alleles with CAD. A limitation to this preliminary study is a limited sample number; hence, further analysis on larger samples is required.

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