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APO A1-75 G to A substitution associated with severe forms of CAD, lower levels of HDL and apoA-I among Northern Indians

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Abstract. Apolipoprotein A-I (*APOA1* gene, apoA-I protein) is the major protein for plasma high density lipoprotein (HDL). The relationship of *APOA1*-75G/A polymorphism with lipid profile and coronary artery disease (CAD) is unclear. Out of 370 individuals initially recruited, 164 angiographically proven CAD patients ($\geqslant$ 70% stenosis) and 36 individuals with normal coronaries or insignificant CAD (NCAD, $\leqslant$50% stenosis) from Delhi and adjoining areas were selected for analysis based on the set criteria. Polymorphism was determined by PCR followed by MspI restriction digestion. Lipid profile was estimated by enzymatic kit and apoA-I levels by immunoturbidimetry. A highly significant increasing trend in 'A' allele frequency was observed with the rise in severity of CAD: NCAD (0.097) $<$ SVD (single vessel disease) (0.117) $<$ DVD (double vessel disease) (0.223) $<$ TVD (triple vessel disease) (0.291). In comparison to *GG* individuals, the OR of 'A' allele carriers to develop SVD, DVD, TVD was 1.3, 2.8 and 4.2 respectively ($p_{\mathrm{trend}} = 0.007$). Analysis of intergenotypic variations in the lipid profile revealed significantly lower levels of HDL and apoA-I among 'A' allele carriers as compared to *GG* (patients). Our study, first of its kind from India, suggests that 'A' allele may contribute to severity of CAD and low levels of HDL & apoA-I. However, an in depth study with a larger set of sample is necessary.

Keywords: *APOA1*, apolipoprotein, CAD, polymorphism, India

1. Introduction

Central to pathogenesis of atherosclerosis is deposition of cholesterol in artery wall and within activated macrophages (foam cells). HDL is involved in the transport of cholesterol away from arterial walls to the liver, a process referred to as reverse cholesterol transport (RCT). In addition, HDL has other important atheroprotective functions including anti-inflammatory, anti-oxidant properties of HDL, though their elucidation is as yet unclear [1].

Apolipoprotein A-I (*APOA1* gene, ApoA-I protein) is the major protein of high density lipoprotein (HDL). It is a 243 amino acid long peptide, synthesized mainly in the liver and to some extent in the small intestine. ApoA-I is the *in vivo* activator of Lecithin: cholesterol acyl transferase (LCAT), responsible for the esterification of cholesterol and thus, a major participant in the regulation of RCT [2].

APOA1 gene is present along with *APOC3* and *APOA4* genes on chromosome 11 (11q23.3-qter) [3]. Variations in the *APOA1*-C3-A4 genes have been associated with dyslipidemia and coronary artery disease (CAD) [4]. In this context, a common G to A transition, 75 bp upstream from the transcription start site in *APOA1* gene, (denoted as -75 bp, -76 bp or -78 bp in different studies) has received most of the attention [5]. Many of the studies showed an association of rare 'A'

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allele of *APOA1*-75G/A polymorphism with higher levels of HDL and/or apoA-I [6–14]. However, others did not confirm this association [15–21]. Interestingly, an inverse association has also been reported [22]. A meta-analysis showed that rare 'A' allele might be associated with only marginally higher (by $\sim$0.5 g/l) apoA-I concentration [23].

Studies pertaining to the association of 'A' allele with CAD have also revealed controversial findings. In an Australian CAD patient study, Wang et al. described a positive relationship between 'A' allele and severe forms of CAD [20]. There was another report of higher 'A' allele frequency in myocardial infarction (MI) and angina patients as compared to healthy individuals [24]. However, studies on Solvenian [25] and Taiwanese population [26] did not reveal any preferential distribution of 'A' allele among the CAD population. Recently, in a Japanese study [21], no association was found between the polymorphism and MI.

APOA1-75G/A polymorphism has been studied in different ethnic groups with the notion that 'A' allele may determine an individual's HDL levels and propensity to develop CAD. India is heading towards a CAD epidemic. Low HDL is one of the predominant cardiovascular risk factor among Indians. Despite all these fact, no data has so far been made available from this part of the world. We are reporting here our findings on the *APOA1*-75G/A polymorphism and its association with lipid profile and severity of CAD among Northern Indians.

2. Materials and methods

2.1. Study subjects

A total of 370 individuals coming for angiography at the department of cardiology, AIIMS, New Delhi were recruited in the study. Of these, only 200 individuals (36 controls, 164 patients) were selected for the investigation based on the criteria that all of these were 1) residents of Delhi or adjoining areas, sharing fairly similar socioeconomic, cultural pattern and dietary habits, 2) voluntary participants in the study and 3) with no cardiomyopathy or valvular disease. Individuals angiographically proven to have normal coronaries ($N = 27$) or $\leqslant$ 50% stenosis ($N = 9$) in one of the major coronary artery, formed the NCAD group (control group). The criteria for the control group were taken from the pioneer work of Wang et al. [20]. Subjects having $\geqslant$ 70% stenosis in one, two and three major coronary arteries were classified as SVD (single vessel disease, $N = 43$), DVD (double vessel disease, $N = 54$) and TVD (triple vessel disease, $N = 67$) patients respectively. A statistician was consulted for the study design. The study was approved by the ethical committee of All India Institute of Medical Sciences, New Delhi and its guidelines were observed.

2.2. Lipids, lipoproteins and apolipoprotein A-I

Venous blood sample was collected from each individual after at least 12 hours of fasting. Lipid profile was monitored using enzymatic kits (Randox laboratories limited, UK). All the chemicals used in the study were procured from Sigma Chemical Co., USA until or unless the source is specified.

ApoA-I levels were estimated in plasma by immunoturbidimetry using anti-human apoA-I antibody (from rabbit, DAKO) and modifications were made to the method reported by Mount et al. [27].

2.3. APOA1-75G/A polymorphism

DNA was extracted from blood by salting out method [28]. 100–500 ng of DNA was amplified in a thermocycler (PTC-100, MJ Research Inc., USA) using 1 unit of Taq DNA Polymerase (Life Technologies Inc., USA) in a 25 ul reaction mixture containing 10 picomole forward primer: 5'-AGG GAC AGA GCT GAT CCT TGA ACT CTT AAG-3' and reverse primer: 5'-TTA GGG GAC ACC TAG CCC TCA GGA AGA GCA-3' (MWG Biotech GmbH, Germany) [8]. DNA was initially denatured for 5 minutes at 95 °C, annealed at 57°C for 2 minutes and heated at 70 °C for 1 minutes. The cycling conditions were set to heat the samples at 95°C for 30 seconds, at 57°C for 45 seconds and at 72°C for 1 minute. The cycle was repeated 40 times followed by final extension at 72 °C for 10 minutes.

About 9 ul of the PCR product was digested at 37 °C overnight with 10 units of MspI restriction enzyme (New England Biolabs Inc., USA) in the presence of 1 ul of 10 X buffer provided with the restriction enzyme. The digested PCR product was resolved on an 8% polyacrylamide gel using 1XTBE buffer (89 mM Tris Borate, 2 mM EDTA, pH8.3) at 250 V for 2 hours and visualized by silver staining. Substitution from G to A at -75 bp results in the loss of the MspI site. The presence of 183 bp represents the 'A' allele. The genotypes were referred to as *GG*, *GA* and *AA*.

Table 1
Clinical and biochemical characteristics of patients and NCAD individuals

Parameters	CAD patients ($N = 164$)	NCAD ($N = 36$)
Age (yrs)	53.07 ± 10.37	51.14 ± 9.70
Sex (M,F)	145 M, 19F	29 M, 7F
Diabetes (%)	32.3	22.2
Hypertension (%)	50.6	55.6
Smoking (%)	28	22.2
TC (mg/dl)	196.01 ± 52.03	185.61 ± 46.27
LDL-C (mg/dl)	123.5 ± 44.07	115.32 ± 36.87
HDL-C (mg/dl)	38.23 ± 9.78	39.17 ± 9.86
LDL-C/HDL-C ratio	3.37 ± 1.41	3.07 ± 1.15
TG (mg/dl)	168.20 ± 74.53	152.81 ± 67.29
ApoA-I (g/l)	0.91 ± 0.14	1.05 ± 0.24

2.4. Statistical analysis

Allelic frequencies were estimated by gene-counting method. The sample-size dependent standard error of alleles was calculated in terms of 95% confidence interval (CI) of the estimates. Chi-square goodness-of-fit was used to verify the agreement of the observed genotype frequencies with those expected ones (*Hardy-Weinberg equilibrium*). Chi-square test was applied to compare genotypic frequencies between two or more groups. Contingency table approach (Fisher's Row X Column test) was used to determine if there are significant differences in allele frequencies among the groups. Pooling of relevant genotypes was done to have sizeable number of samples in each category. Linear regression was used to study the effect of genotypes on lipids and lipoproteins, after adjusting for age and sex. Polytomous logistic analysis was performed taking NCAD as the referent category. We computed the Odds ratio (OR) with 95% CI for the 'A' allele carriers (*GA/AA*) to develop SVD, DVD and TVD as compared to *GG* individuals, after age and sex adjustment. All the statistical analysis was preformed using SPSS (Statistical Package for Social Sciences) for windows (version 9.0.0, SPSS Inc., Chicago) with the consultation of a statistician. Statistical significance was set at $p < 0.05$.

3. Results

Age and sex distribution of NCAD and angiographically proven CAD individuals were statistically comparable (Table 1). There were no significant differences in the frequencies of diabetics, hypertensive individuals and smokers between NCAD and CAD group. Lipid profile was comparable in NCAD and CAD group.

The genotype distribution was in *Hardy-Weinberg equilibrium* in all the study groups. Prevalence of 'A' allele (p trend $= 0.0001$): NCAD (0.097) $<$ SVD (0.117) $<$ DVD (0.223) $<$ TVD (0.291) increased significantly with the rise in severity of CAD (Table 2). After adjusting for age and sex, the OR of 'A' allele carriers to develop SVD, DVD and TVD was 1.3, 2.8 and 4.2 respectively as compared to GG individuals ($p = 0.007$, Table 3). 'A' allele carriers were almost two times more prevalent in CAD group (37.8%) as compared to NCAD group (19.4%, $p = 0.036$, Table 2). Higher prevalence of 'A' allele was observed in CAD patients (0.223) as compared to NCAD group (0.097, $p = 0.012$).

Age and sex adjusted intergenotypic variations in lipid, lipoprotein and apolipoprotein A-I levels with respect to *APOA1*-75G/A polymorphism has been summarized in Table 4. Rare 'A' allele carriers were associated with lower levels of HDL in patients (9.7% lower, $p = 0.014$) and NCAD (10.8% lower, $p =$ NS). Likewise, 'A' allele carriers had 5% lower levels of apoA-I as compared to *GG* individuals in the patients group ($p = 0.0280$). Similar trend was observed in the SVD, DVD and TVD group. No gradient was observed in the HDL and apoA-I levels with the rise in severity of CAD(data not shown).

4. Discussion

Our study, first of its kind from India, brings forth the relationship of *APOA1*-75G/A polymorphism with lipid profile and severity of CAD. People residing in different regions of India may express variable genotype-phenotype relationship and hence, susceptibility to CAD. The study, therefore, was confined to the residents of close geographical premise of Delhi and surrounding areas visiting the department of Cardiology, AIIMS, New Delhi for angiography. The study subjects might be of heterogeneous ethnicity, but shared fairly common socio-economic cultural background and dietary habits. Wang et al., in a study pertaining to the association of 'A' allele with severity of CAD, used NCAD category as the referent [20]. We followed the same study design and criteria for NCAD in this study. The criteria for NCAD was no or $\leqslant$ 50% stenosis in one of the major coronary artery disease and for patients was $>$ 50% stenosis [20]. Our patients, however, were those who were angiographically proven to have $\geqslant$ 70% stenosis in one of the major coronary artery. Of 370 individuals who were initially enrolled, only 200

Table 2
Distribution of genotypes & alleles of *APOA1*-75G/A polymorphism and severity of coronary artery disease

Groups	Genotype frequency (%)[a]			Allele frequency[b] (95% CI)		
	GG	*GA*	*AA*	*GA/AA*	*G*	*A*
NCAD	29	7	0	7	0.903	0.097
($N = 36$)	(80.6%)	(19.4%)		(19.4%)	(0.81–0.96)	(0.04–0.19)
SVD	33	10	0	10	0.883	0.117
($N = 43$)	(76.7%)	(23.3%)		(23.3%)	(0.79–0.94)	(0.06–0.21)
DVD	33	18	3	21	0.777	0.223
($N = 54$)	(61.1%)	(33.3%)	(5.6%)	(38.9%)	(0.68–0.75)	(0.15–0.31)
TVD	36	23	8	31	0.709	0.291
($N = 67$)	(53.7%)	(34.4%)	(11.9%)	(46.3%)	(0.62–0.78)	(0.22–0.38)
CAD Patients (SVD +	102	51	11	62	0.777	0.223
DVD + TVD, $N = 164$)	(62.2%)	(31.1%)	(6.7%)	(37.8%)	(0.73–0.82)	(0.18–0.27)

[a]All genotypes compared: $\chi^2 = 15.678$, df $= 6$, $p = 0.016$; *GG* Vs. *GA/AA*: $\chi^2 = 10.584$, df $= 3$, $p = 0.014$; χ^2 trend $= 10.13$, df $= 1$, p trend $= 0.0015$.
[b]Alleles: $p = 0.0000$, S.E.: 0.0000; $\chi^2_{\text{trend}} = 15.07$, df $= 1$, p trend $= 0.0001$.

individuals fulfilled the selection criteria and could be included for the analysis. This limited the sample size in different groups.

APOA1-75G/A polymorphism in the promoter region of the gene results in the loss of MspI site (Rare allele: 'A'). The genotype distribution was found to be in *Hardy-Weinberg equilibrium* in all the study groups. The frequencies of various genotypes decreased in the order of *GG* > *GA* > *AA*.

A highly significant increasing trend in the 'A' allele frequency with the rise in severity is the key finding of our study. Similar trend was obvious in the OR of 'A' allele carriers to develop SVD, DVD and TVD ($1.3 < 2.8 < 4.2$, $p = 0.007$). Thus, it may be hypothesized that atypical 'A' allele confers a risk of developing severe forms of CAD, though a study in a larger sample is essential. Wang et al. [20] also showed an increase in the 'A' allele frequency with respect to severity to CAD in Australian male CAD patients. A significant association of 'A' allele with myocardial infarction and angina was shown in a Spanish case-control study [24]. However, no relationship could be established in Solvenian [25] and Taiwanese population [26]. Recently in a Japanese study, no association was observed between 'A' allele and MI [21].

Our data revealed that 'A' allele carriers were associated with lower HDL levels as compared to GG homozygous in patients (9.7% lower, $p = 0.014$) and NCAD (10.8% lower, $p =$ NS). Also, apoA-I levels were found to be lower in *GA* and *AA* individuals as compared to *GG* in the patients (5% lower, $p = 0.0280$). Similar trend was observed in the intergenotypic variations in SVD, DVD and TVD groups (data not shown). The levels of HDL were not different between CAD and NCAD, and also did not show any relationship with the severity of the disease. Similar kinds of observations have been reported in two other Northern Indian studies [29,30]. However, a larger study sample is essential to explain these observations.

Table 3
Odds ratio of 'A' allele carriers to develop single, double and triple vessel disease as compared to *GG*

Groups	Crude OR (95%CI)	p value	Adjusted OR[a] (95%CI)	p value
NCAD	1 (Referent)		1 (Referent)	
SVD	1.25 (0.4–3.7)	$p = 0.012$	1.3 (0.41–4.0)	$p = 0.007$
DVD	2.6 (1.0–7.6)		2.8 (1.0–7.6)	
TVD	3.6 (1.4–9.3)		4.2 (1.6–11.0)	

[a]Adjusted for age, sex.

In conclusion, base change at -75 bp of *APOA1* gene was found to be associated with severe forms of CAD and lower levels of HDL-C & apoA-I in this representative group of Northern Indians. Thus, our findings suggest that individuals harboring -75 base change might be at a higher risk of developing severe forms of CAD and having lower levels of HDL and apo A-I. However, the major limitation of the study has been small sample size, and an exhaustive investigation, with a large sample size, is warranted.

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Table 4
Age and sex adjusted intergenotypic variations in lipid, lipoprotein and apolipoprotein A-I levels with respect to *APOA1*-75G/A polymorphism

Parameter	Groups	*GG*	*GA/AA*	p Value
TC	CAD ($N = 164$)	198.77 ± 52.55 (102)	191.33 ± 52.58 (62)	0.383
	NCAD ($N = 36$)	192.02 ± 46.85 (29)	159.07 ± 49.37 (7)	0.108
LDL	CAD	124.63 ± 44.64	120.63 ± 44.67	0.580
	NCAD	119.02 ± 38.12	99.91 ± 40.18	0.247
HDL	CAD	39.69 ± 9.58	35.83 ± 9.62	0.014
	NCAD	40.01 ± 10.25	35.69 ± 10.80	0.329
LDL/HDL	CAD	3.33 ± 1.43	3.44 ± 1.44	0.636
	NCAD	3.13 ± 1.21	2.85 ± 1.28	0.582
TG	CAD	166.92 ± 74.49	170.57 ± 74.72	0.762
	NCAD	162.10 ± 61.92	114.31 ± 65.25	0.078
ApoA-I (g/l)	CAD	0.93 ± 0.14	0.88 ± 0.14	0.028
	NCAD	0.92 ± 0.14	0.86 ± 0.14	0.3675

All the parameters expressed in mg/dl (except apoA-I).

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