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Genetic variants on apolipoprotein gene cluster influence triglycerides with a risk of coronary artery disease among Indians

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Abstract Apolipoprotein C3 and apolipoprotien A5 are proteins coded from the APOA1/C3/A4/A5 gene cluster. Sst I polymorphism on apolipoprotein C3 and −1131C polymorphism of apolipoprotien A5 are key variants involved in triglyceride metabolism and cause a significant cardio-metabolic risk. Here, we have evaluated these two variants for their roles in coronary artery disease in patients of the Indian population. The apolipoprotein gene cluster variants were analysed in 416 angiographically determined coronary artery disease patients and matched 416 controls using polymerase chain reaction—restriction fragment length polymorphism. The characteristics of the study subjects were analyzed statistically for their association with the polymorphisms. The alleles were combined as haplotypes and their combined risks were evaluated. The minor allele genotypes of both apolipoprotein C3 (S2) and apolipoprotien A5 (C) had a significant risk for coronary artery disease. The S2 allele genotyped patients had a significantly increased triglyceride level ($P < 0.001$) and increased triglycerides were observed among both patient and control CC genotype carriers. We identified the haplotype S2/C with a significant increased risk ($P < 0.001$) to coronary artery disease with increased levels of circulating triglycerides compared to other haplotypes in patients. We conclude that the variants on apolipoprotein C3 and apolipoprotien A5 modulate serum triglyceride levels and increase the risk of coronary artery disease.

Keywords Coronary artery disease · Apolipoprotein C3 · Apolipoprotein A5 · Triglycerides · Risk

Introduction

Coronary artery disease (CAD) is a multifactorial disease and has complex patho-physiologic characteristics. The interplay of many molecular and biochemical pathways and interactions with environmental factors, contribute towards individual susceptibility [1]. Atherosclerosis, the major event leading to CAD is characterized by the events following accumulation of lipids and fibrous elements in coronary arteries [2]. Several risk factors, genetic and environmental have been identified; most importantly levels of HDL, triglycerides, lipoprotein (a), diabetes mellitus, smoking and hypertension [3]. Among South Asian populations, independent of gender, smoking, high ApoB100/Apo-I ratio, hypertension and diabetes are the most important risk factors for CAD [4, 5]. An individual risk for CAD is wide-ranging among the different ethnic groups in Indians.

The apolipoprotein A5 (APOA5) and apolipoprotein C3 (APOC3) genes are located on chromosome 11q23, on the APOA1/C3/A4/A5 gene cluster. The APOA5 protein is predominantly synthesized in the liver and has roles in

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triglyceride and HDL metabolism. A higher plasma circulating APOA5 would result in lower triglyceride levels [6]. The −1131T→C polymorphism on APOA5 is a key variation claimed for association with circulating triglyceride concentrations [7]. The −T1131C polymorphism has been associated with hyperlipidemia, hypoalphalipoproteinemia, hyperalphalipoproteinemia [8].

ApoC3 is the apolipoprotein constituent of chylomicrons, VLDL, and HDL. The gene expression is high in liver and intestine and is controlled by regulatory elements located in the APOA1/C3/A4/A5 gene cluster [9]. In fasting state, the protein is mainly associated with HDL, whereas in the post-parandial state, the protein redistributes to chylomicron and VLDL. The protein inhibits lipoprotein lipase activity and hepatic uptake of triglycerides [10]. The Sst I polymorphism (C3238G) of the gene has been found to be associated with altered plasma triglyceride concentrations [11].

The current study is an investigation among the south Indian CAD patients for two variations on the APOC3 and APOA5 genes having a significant cardio-metabolic risk. The ethnicity of the Indians and high prevalence of CAD along with diabetes are crucial factors highlighting the need for assessing their genetic components. South Indians have a hallmark of higher triglycerides [12], smoking [13] and hypertension [14]. It is therefore important to elucidate the genetic variants playing roles in the modifying events of atherosclerosis and CAD.

Materials and methods

Patients

A homogeneous well-defined South Indian population diagnosed with CAD ($n = 416$) was studied with distinct age and sex matched controls ($n = 416$) who had come for health checkups were collected from International Center for Cardio Thoracic and Vascular Diseases (ICCTVD), Frontier lifeline, Dr. K. M. Cherian heart foundation at Chennai. The patients and controls groups underwent similar diagnostic protocols prescribed for demonstrating CAD or normal coronary arteries. The cases had all undergone a voluntary coronary angiogram and had atleast one artery with 50% stenosis. The controls chosen were ruled out for CAD after angiogram, CT scan and/or normal cardiac enzymes and treadmill test as evidence of no atherosclerosis.

The clinical investigation of lipid profile, blood sugar and creatinine were performed at the clinical biochemistry department of the hospital using Randox Daytona auto analyser (GMI, Inc., USA). Before administration of medications or surgery, plasma glucose, serum cholesterol, serum triglycerides and HDL cholesterol, LDL cholesterol (Randox Enzymatic Kits, UK) were measured on fasting blood samples. Blood was collected for DNA isolation on confirming diagnosis and before any event of surgery. Informed written consent was obtained from all participants after explaining the objectives and details of the study. According to the Institutional human ethics committee, all the study groups were from the random population and are unrelated.

Genotyping

Genomic DNA was isolated using Lahiri's method [15] and quantified following which the samples were labeled with codes for further analysis. The SNP containing regions were amplified using polymerase chain reaction of 20 µl reaction volumes. The amplified products were subjected to restriction digestion and the genotypes were determined according to the product sizes (Table 1).

Statistical analysis

The genotyping information of each subject (inclusive of controls) was assembled and statistically evaluated with clinical parameters. Consistency of the genotype frequencies with the Hardy–Weinberg equilibrium was tested by chi-square (χ^2) analysis and risks between study groups were performed using SPSS software version 10.5. Multivariate logistic regression analysis and ANOVA were

Table 1 The primers, annealing temperature, PCR product size, identifying restriction enzymes and digested product sizes used in the study

Sl. no.	Gene, variation name	Primer	Product size
1.	Apolipoprotein A5, −1131T→C polymorphism (Talmud et al. 2002)	5′- GTGCCTGTCACCACCGTTTGG -3′	TT-162 bp
		5′- ATGCATTAGCCTC TGCTGTTC -3′	TC-162, 141 and 21 bps
		$T_m = 60$°C, 162 bps, BsuR I (Hae III) digestion	CC-141 and 21 bps
2.	Apolipoprotein C3, Sst I polymorphism, C3238G (Waterworth et al. 1999)	5′- CATGGTTGCCTACAGAGGAGT -3′	S1S1-590 bp
		5′–TGACCTTCCGCACAAAGCTGT -3′	S1S2-590, 365, 225 bps
		$T_m = 55$°C, 590 bps, Sac I digestion	S2S2-365, 225 bps

Primers synthesized from Sigma Genosys, Bangalore. Restriction enzymes and reaction conditions as supplied by MBI Fermentas, USA

performed using an SPSS. The haplotype frequency data and pair wise linkage dis-equilibrium in-betweeen SNP's were obtained using HaploView 4.0 (http://www.broad.mit.edu/mpg/haploview/).

Results

A total of 416 angiographically determined subjects (patients with coronary artery disease) were studied and compared with 416 matched controls. The key identifying characteristics of the studied subjects are described in Table 2. No statistically significant differences were found in the mean age ($P = 0.567$) and sex ($P = 0.515$) in-between the subjects. Body mass index (BMI) showed significant difference among CAD patients and controls ($P < 0.001$). Similarly, cigarette smoking, diabetes and hypertension were highly prevalent among CAD patients ($P < 0.001$).

The allelic and genotypic frequencies of the APOA5 and APOC3 gene polymorphism in patients and control subjects are given in Table 3. Both the polymorphisms were in Hardy Weinberg equilibrium. The allele frequencies of the APOA5 polymorphism showed a high prevalence of C allele among patients (32.1%) compared to controls (23.9%). The odds ratio were computed between the genotypes CC vs. TT, TC vs. TT, CC vs. TC+TT and CC+TC vs. TT (Table 3). All the values are significantly greater than one. The odds ratio is highest for CC vs. TT (2.39, 95% CI 1.378–4.139, $P = 0.001$). We observed that individuals with 'C' allele appear to be at risk (Table 3) compared to those with 'T' allele (OR = 1.50, 95% CI 1.211–1.865, $P < 0.001$). The base line characteristics of the cases and controls were grouped under various genotypes and we observed that there is an increase in circulating triglyceride level for the CC genotype carriers irrespective of the disease condition ($P < 0.01$) (Fig. 1).

The frequency of APOC3 S2 allele was increased in CAD patients (31.4%) as compared to 26.4% in control samples. The distribution of genotypes (S1S1, S1S2, S2S2) of APOC3 polymorphism were observed to be: 52.4, 42.3, 5.3% in controls and 45.4, 46.4, 8.2% in patients, respectively. The prevalence of S2 allele in CAD patients confers an increased risk (OR = 1.29; CI 1.040–1.592; $P = 0.02$). Similarly, the genotypes S2S2+S2S1 vs. S1S1 (Table 3) conferred a greater than one risk (OR = 1.35; CI 1.026–1.77; $P = 0.32$). Regression analysis revealed a significant association of APOC3 S2S2 genotype carriers with serum triglycerides ($P = 0.003$) and fasting glucose levels ($P = 0.007$) (Fig. 2).

Further, the two variants were constructed as haplotypes and their haplotype frequencies are shown in Table 4.

Table 2 Anthropometric, clinical, lipid and other biochemical characteristics in coronary artery disease (CAD) patients and age matched controls

	Cases ($n = 416$)	Controls ($n = 416$)	P
Gender: M/F (n)	322/94	315/101	0.567
Age (year)	53.23 ± 7.80	53.59 ± 8.06	0.515
Body mass index (kg/m^2)	25.77 ± 3.90	24.92 ± 2.77	**<0.001**
Systolic blood pressure (mm/Hg)	133.76 ± 19.77	130.36 ± 16.20	**0.007**
Diastolic blood pressure (mm/Hg)	83.33 ± 10.02	83.29 ± 7.87	0.951
Serum total cholesterol (mg/dl)	173.92 ± 36.90	171.04 ± 30.05	0.218
Serum triglycerides (mg/dl)	159.54 ± 80.91	145.30 ± 74.31	**0.008**
HDL cholesterol (mg/dl)	37.88 ± 5.29	40.12 ± 4.66	**<0.001**
LDL cholesterol (mg/dl)	105.60 ± 28.22	101.74 ± 17.96	**0.019**
Glucose fasting (mg/dl)	140.94 ± 49.74	128.25 ± 27.25	**<0.001**
Glucose post parandial (mg/dl)	198.89 ± 74.02	173.21 ± 50.85	**<0.001**
Urea (mg/dl)	29.85 ± 9.63	29.47 ± 1.95	0.430
Creatinine (mg/dl)	0.90 ± 0.22	0.90 ± 0.21	0.999
Angina (n)	229	NA	NA
Type II diabetes (n)	182	53	**<0.001**
Hypertension (n)	181	93	**<0.001**
Current and ex-smoking (n)	203	24	**<0.001**
Familial CAD (n)	74	50	0.108
Dyslipidemia (n)	48	44	0.856
Myocardial infarction (n)	120	NA	NA
Vessel disease (single/double/triple) (n)	155/102/159	NA	NA

Quantitative variables are expressed in means ± standard deviation

M males, *F* females, *kg/m^2* Kilogram/metre^2, *%* percentage, *mg/dl* milligram/deciliter, *HDL* high density lipoprotein, *LDL* low density lipoprotein, *NA* not applicable

P values <0.05 are shown in bold

Table 3 Genotype, allele frequency distribution with their odds ratios and confidence intervals (95%) for polymorphisms on genes for coronary artery disease patients and controls

Gene/polymorphism	Genotype/ allele	Controls ($n = 416$) (%)	Cases ($n = 416$) (%)	P	Genotype/ allele	OR	95% CI	P
1. Apolipoprotein A5, −1131T→C polymorphism	TT	57.5	45.9	**<0.001**	C vs. T	1.5	1.211–1.865	**<0.001**
	TC	37.2	44		CC vs. TT	2.39	1.378–4.139	**0.001**
	CC	5.3	10.1		TC vs. TT	1.48	1.109–1.967	**0.007**
	χ^2_{HWE}	0.235	0.036		CC vs. TC+TT	2.01	1.178–3.433	**0.009**
	T	76.1	67.9		CC+TC vs. TT	1.66	1.260–2.186	**<0.001**
	C	23.9	32.1					
2. Apolipoprotein C3, Sst I polymorphism, C3238G	S1S1	52.4	45.4	**0.015**	S2 vs. S1	1.29	1.040–1.592	**0.02**
	S1S2	42.3	46.4		S2S2 vs. S1S1	1.8	1.016–3.182	**0.042**
	S2S2	5.3	8.2		S2S2+S1S2 vs. S1S1	1.35	1.026–1.770	**0.032**
	χ^2_{HWE}	0.061	0.068					
	S1	73.6	68.6					
	S2	26.4	31.4					

n number/frequency, % percentage, *C* cytosine, *T* thymine, *S1* presence of Sst I restriction site, *S2* absence of Sst I restriction site, χ^2_{HWE} Chi-square with one-degree of freedom for departure from Hardy Weinberg equilibrium, *P* probability for chi-square with one degree of freedom

P values <0.05 are shown in bold

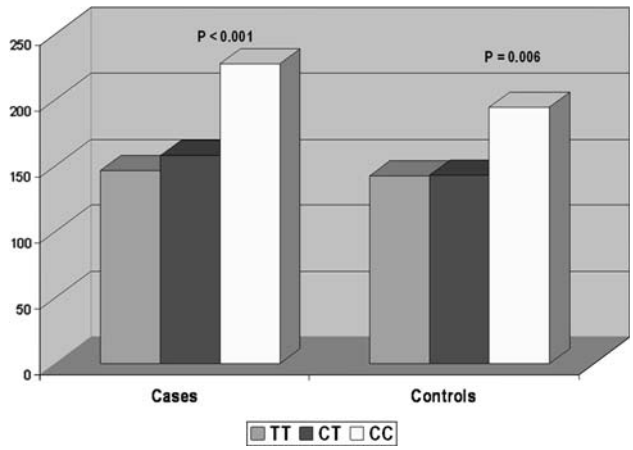

Fig. 1 Comparison of serum triglyceride levels among APOA5 −1131T→C genotype carriers

The cases and controls showed significant differences ($P < 0.001$) among the distribution of haplotypes. The risk alleles viz., S2 of APOC3 polymorphism and C of APOA5 polymorphism have shown two times increased risks in the cases and controls than other haplotypes (OR = 2.88, 95%CI 1.596–5.205). This was further emphasized by the observation of a reduced risk for the non risk allele haplotype 'S1/T' (OR = 0.76, CI 0.575–0.993, $P = 0.044$). The haplotypes (S1/T, S1/C, S2/T, S2/C) among the cases were grouped and their clinical characteristics were compared. The 'S2/C' haplotype was seen with increased circulating triglycerides ($P = 0.003$) compared to all the other possible haplotypes observed (Table 5).

Discussion

The increasing rate of coronary artery disease among the Indian population is a cause of concern. Comprehensive studies to date have identified risk factors (both genetic and non-genetic) for CAD prognosis including myocardial infarction, or angina pectoris and stroke (1, 3). However, these factors are moderately conclusive for the Western population alone. This study on risk alleles and their effects is an effort to identify the difference in risks among the Indian population. The present study reports 416 CAD patients and 416 matched controls from the Indian population and the effect of variants on apolipoprotein gene cluster to CAD. Among the studied variants apolipoprotein C3, Sst I polymorphism had similar frequencies as reported previously for the Indian population [16]. Apolipoprotein A5 −1131T→C polymorphism had a slightly higher frequency (24%) than the previous study (20%) published from a Pune population [17].

The −1131T→C polymorphism showed a significant risk for studied CAD patients but increased triglycerides for C allele carriers were observed among both CAD patients and controls. The apolipoprotein A5 −1131T→C polymorphism has been associated with increased triglycerides in human studies. Palmen et al. [18] reasoned increased triglycerides were due to increased APOA5 expression and not circulating plasma apoAV levels. Also, the −1131T→C is located upstream to the proximal promoter region and there is no transcription factor binding sites identified in this location. Therefore, it is justified that

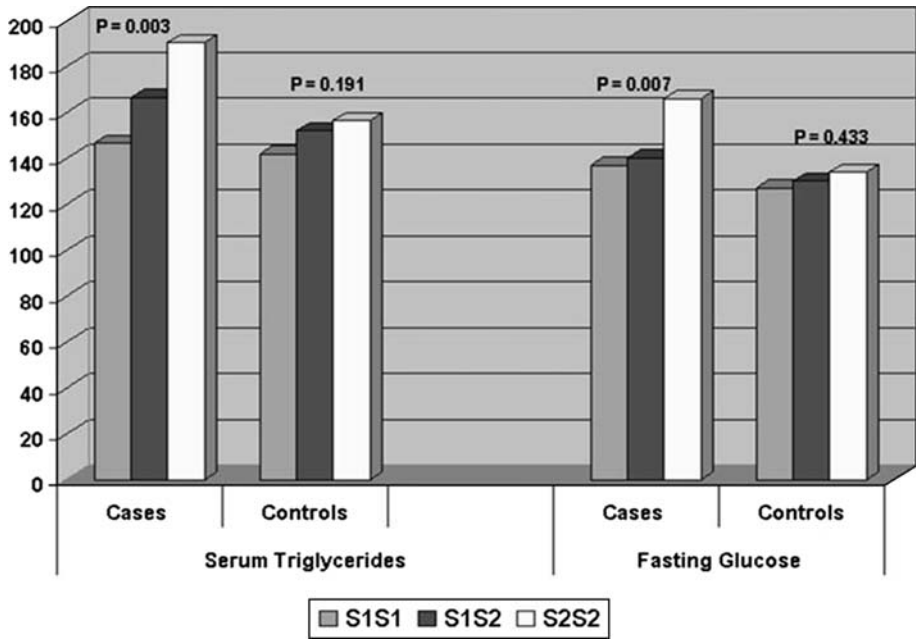

Fig. 2 Comparison of serum triglyceride and fasting glucose levels among APOC3 Sst I (C3238G) genotype carriers

Table 4 Haplotypes of the polymorphisms APOC3 Sst I (C3238G) and APOA5 −1131T→C, with their frequencies and risks

Haplotype	Cases	Controls	OR	95% CI	*P*
S1/T	0.468	0.537	0.87	0.684–1.096	0.231
S1/C	0.218	0.224	0.98	0.711–1.347	0.8933
S2/T	0.211	0.201	1.06	0.763–1.474	0.728
S2/C	0.103	0.038	2.69	1.490–4.847	**<0.001**
	***P* < 0.001**				
S1/T vs. S1/C+S2/T+S2/C			0.76	0.575–0.993	**0.044**
S2/C vs. S1/C+S1/T+S2/T			2.88	1.596–5.205	**<0.001**

OR odds ratio, *CI* confidence interval, *C* cytosine, *T* thymine, *S1* presence of Sst I restriction site, *S2* absence of Sst I restriction site, *P* probability for chi-square with one degree of freedom

the −1131T→C variant may not be functional, but could be in linkage with other functional sites [7]. Although the exact nature of interaction of this promoter variant causing an increase risk for CAD or allied factors is not known, the current study has replicated the observation in an Asian population [19]. Also, among an ethinic Chinese population of Taiwan, the association of APOA5 −1131T→C polymorphism with its effect on triglyceride metabolism was shown to contribute to an increased susceptibility to metabolic syndrome [20].

The response to fenofibrate treatment of patients with metabolic syndrome and elevated plasma triglyceride levels has been shown to vary according to the APOA5 −1131T→C polymorphism status [21]. Finally, it has been postulated that additional genetic factors are necessary for expression of the triglyceride-raising effect of APOA5 variants [22]. But, given the known functions of the APOC3 and APOA5 genes and their known effects on lipid metabolism, the finding of an association between APOA5 and CAD is to be interpreted as noteworthy in the Indian population. The variant thus can be considered as an entity that influences triglycerides among Indian CAD patients.

The apolipoprotein C3 Sst I variation has also been shown to be associated with CAD and its genotypes with fasting glucose and triglycerides in the present study. The Sst I polymorphism has been previously shown to be a risk for CAD or MI in combination with other variation on the cluster in a Indian [23] and other populations [24]. In the present study we observed that the S2S2 and S1S2 genotypes had increased levels of fasting glucose and triglycerides among the CAD patients. Along with the influence on characteristics modifying risk of CAD, the S2 allele showed a significant risk for CAD.

The human APOC3 gene expression is controlled by elements that are spread through out the APOA1-C3-A4-A5 gene cluster on the long arm of chromosome 11. Also,

Table 5 Comparison of APOC3 Sst I and APOA5 −1131T→C Haplotypes with clinical characteristics among CAD patients

	S1/T+S1/C+S2/T	S2/C	*P*
Age (year)	53.33 ± 7.79	52.71 ± 8.42	0.418
Body mass index (kg/m^2)	25.77 ± 3.58	25.84 ± 2.76	0.830
Systolic blood pressure (mm/Hg)	133.72 ± 19.15	135.81 ± 19.25	0.262
Diastolic blood pressure (mm/Hg)	83.31 ± 9.93	84.45 ± 9.85	0.239
Serum total cholesterol (mg/dl)	173.67 ± 38.00	177.65 ± 42.70	0.291
Serum triglycerides (mg/dl)	157.63 ± 75.56	181.17 ± 109.28	**0.003**
HDL cholesterol (mg/dl)	37.94 ± 5.52	37.51 ± 5.29	0.423
LDL cholesterol (mg/dl)	105.90 ± 29.15	105.37 ± 32.57	0.854
Glucose fasting (mg/dl)	138.94 ± 45.26	145.82 ± 58.26	0.135
Glucose post parandial (mg/dl)	197.41 ± 71.87	204.27 ± 92.72	0.347
Urea (mg/dl)	29.81 ± 9.63	30.50 ± 10.38	0.465
Creatinine (mg/dl)	0.90 ± 0.23	0.92 ± 0.28	0.344

Quantitative variables are expressed in means ± standard deviation

C cytosine, *T* thymine, *S1* presence of Sst I restriction site, *S2* absence of Sst I restriction site, *M* males, *F* females, *kg/m^2* kilogram/metre^2, *%* percentage, *mg/dl* milligram/deciliter, *HDL* high density lipoprotein, *LDL* low density lipoprotein

P values <0.05 are shown in bold

plasma concentrations of apoC3 in human populations correlate well with TG levels. Although, the Sst I polymorphism has been previously established with association for CAD and triglycerides, the influence of this variant to APOC3 function is unclear. The Sst I polymorphism is located in the 3′ untranslated region of *APOC3* gene. Therefore, it is more likely that S2 allele is in linkage disequilibrium with other causative mutations/polymorphisms hitherto unknown in APOC3 or nearby gene involved in determining the TG levels [25].

The haplotypes analysis performed for APOA5 −1131T→C and APOC3 Sst I polymorphisms indicated significant differences ($P < 0.001$) in distribution. Most significantly, a risk haplotype S2/C was obtained ($P < 0.001$) and was associated with increased triglycerides ($P = 0.003$). Previously, haplotype analysis performed by Talmud et al. [26], suggest that APOC3 −482C>T interacts with Sst I variant, but does not rule out functional changes elsewhere in the gene cluster. The current study has indicated that APOC3 *Sst* I variant is influenced by the APOA5/4 −1131T→C promoter polymorphism.

The genes governing lipid transport and metabolism have always been considered as classical risk factors for coronary artery disease. Although, the above variants have been identified with discrete evidence on influence on triglycerides and coronary artery disease, the roles of other variants in the apolipoprotein gene cluster cannot be ruled out. A comprehensive study on the APOA1/C3/A4/A5 gene cluster could yield interesting results.

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