Association between genetic loci linked to HDL-C levels and Indian patients with CAD: a pilot study

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ABSTRACT

Objective To examine the association between loci linked to high-density lipoprotein cholesterol (HDL-C) levels and coronary artery disease (CAD).

Methods A pilot study consisting of age-matched and gender-matched angiographically confirmed CAD cases (n=150) and non-CAD controls (n=150) was performed to test an association. Illumina's Human Cardio-Metabo BeadChip containing 3112 variants associated with HDL-C levels was used for genotyping.

Results A preliminary analysis identified 36 variants from 16 genes that were statistically significant ($p<0.05$) between cases and controls. However, none of the variants remained statistically significant after correction for multiple testing. Besides, variants rs11039159 (*MADD*), rs749067 (*MADD*), rs367070 (*LILRA3*) and rs330921 (*PPP1R3B*) showed modest association with HDL-C levels.

Conclusions None of the HDL-C associated loci included in this study were found to be a significant risk factor for CAD. However, the study could replicate the findings of four variants influencing HDL-C levels.

INTRODUCTION

Coronary artery disease (CAD) is the leading cause of death worldwide, responsible for over 7 million deaths annually. India experiences among the highest number of potentially productive life years lost due to cardiovascular disease, 9.2 million years in 2000 and is expected to double to 17.9 million years by 2030.¹

Dyslipidaemia is a known risk factor of CAD.² Asian Indians are found to have a unique pattern of dyslipidaemia with lower high-density lipoprotein cholesterol (HDL-C), increased triglyceride (TG) levels and higher proportion of small dense low-density lipoprotein cholesterol (LDL-C).³ In a recent study on the prevalence of dyslipidaemia in urban and rural India, it was reported that low HDL-C was the most common lipid abnormality (72.3%) among all the study subjects and it was present as an isolated abnormality in 44.9% of subjects.⁴ HDL-C thus, is an important variable to be considered while evaluating the risk of CAD in an Asian Indian population.

Lipid levels are complex genetic phenotypes, influenced by both environmental and genetic factors.⁵ Genetic variants associated with HDL are found to affect HDL-C levels and HDL functionality.⁶ Genome-wide association studies (GWAS) of plasma lipoprotein fractions have improved our understanding of lipid metabolism and its central role in CAD. GWAS has identified several single nucleotide polymorphisms (SNPs) from newer loci to be associated with HDL-C levels.^{7–11}

Few of the HDL-associated loci have been associated with CAD.¹¹ However, there is still a need to elucidate the role of variants on loci reported to be associated with HDL-C levels, in the pathophysiology of CAD. Moreover, data on these genetic variants in patients of Indian origin with CAD are very limited. The Human Cardio-Metabo BeadChip is a custom Illumina iSelect genotyping array that contains 196 000 SNPs.¹² The array comprises SNPs of different metabolic and atherosclerotic traits such as type 2 diabetes, LDL-C, HDL-C, QT interval, systolic and diastolic pressure, body fat percentage, total cholesterol (TC), height and others. The Cardio-Metabo BeadChip has been used in assessing genetic determinants of CAD in different populations.^{13 14}

The aim of the study was to identify the genetic risk variants of CAD in the loci reported to be associated with HDL-C, in an Indian cohort. The Cardio-Metabo BeadChip is a useful tool for screening as it includes the loci reported to be associated with HDL-C levels.

METHODS

Study participants

The study was a pilot, 1:1 case-control study. Subjects visiting the catheterisation laboratory of a tertiary hospital in Mumbai were included in the study. One hundred and fifty angiographically verified CAD cases ($\geq 50\%$ stenosis in at least one of the three major coronary arteries) were age-matched and gender-matched with 150 angiographically confirmed CAD controls ($\leq 30\%$ stenosis in all the three major coronary arteries).¹⁵ Subjects with abnormal liver or kidney function were excluded from the study.

Informed consent was obtained from all the subjects. Information was collected on demographics, presence of coronary risk factors such as diabetes, hypertension, smoking, and so on, from all the study participants. Current medication, family history and previous coronary intervention, if any, were noted.

DNA extraction

Venous blood sample 5 mL was collected from all the subjects in EDTA tube (for plasma and DNA extraction) and plain tube (for serum). The DNA was extracted by the modified salting out procedure of Miller *et al.*¹⁶ DNA quantitation was done using DU@800 Spectrophotometer (Beckman Coulter). Plasma samples were separated by centrifugation (3000 rpm for 10 min). DNA and plasma aliquots were preserved at -80°C until analysis.

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Biochemical assay

Levels of HDL-C, TC and TG were estimated from serum samples as per lab protocol on Unicel DXC800 Automated Analyser (Beckman Coulter). For quality control, three levels (low/medium/high) Beckman Synchron comprehensive chemistry controls for HDL-C, TC and TG were run daily. LDL-C levels were calculated using the Friedewald formula.¹⁷

DNA microarray

The samples for DNA microarray were processed through the Human Cardio-Metabo BeadChip array using the Illumina Hi-Scan technology (Infinium II Assay). The steps include sample normalisation to uniform concentration, denaturation of the DNA and isothermal whole-genome amplification, DNA fragmentation and hybridisation to the beadchip. Further, single base extension of the hybridised sample and chip staining and quality control (QC) were performed,¹⁸ followed by scanning of the beadchip and generation of the output data using the Genome Studio software (V2011.1). Of the 196 000 SNPs present on the chip, we included 3112 SNPs located in the intronic, exonic and untranslated region (UTR) regions of the reported HDL-associated genes in the study (see online supplementary table S1).

Statistical analysis

Statistical analysis was done by PLINK V1.07 and SPSS V20. PLINK was used to calculate the minor allele frequencies, test for Hardy-Weinberg equilibrium (HWE), perform linkage disequilibrium (LD) pruning, model association testing and correction for multiple testing. SPSS was used to test for normality of the phenotypical variables and compare with the genotypical variables. HDL-C, LDL-C and TG were expressed as median; TC was expressed as mean±SD. Smoking, alcohol, diabetes, hypertension and family history were expressed in percentage (%). Student's t-test was used to compare the phenotypical variables between cases and controls, and Kruskal-Willis test/analysis of variance was used to compare HDL-C levels between genotypes. A p value <0.05 was considered statistically significant.

RESULTS

DNA microarray QC

DNA microarray QC test was done using Illumina software Genome Studio (V2011.1) for sample-dependant QC and PLINK V1.07 for sample-independent parameters. Of the two women that were found to be related to each other (based on 'identity by state' in PLINK), one was excluded from further analysis. None of the samples were removed for low genotyping. The total genotyping rate in the remaining individuals (n=299) was 99.1%. For effective association testing, SNPs failing the HWE ($p < 0.001$), having an SNP failure rate of >5% or a minor allele frequency <0.01 were excluded.¹⁹

We had included 3112 reported HDL-associated SNPs present on the Cardio-Metabo BeadChip, for analysis. Of these, 31 markers failed the HWE test in controls, 53 SNPs failed the missingness test and 212 SNPs failed the frequency test. These SNPs were excluded from further analysis. SNPs that were in LD ($r^2 > 0.8$), were pruned out from subsequent analysis.

Patient demographics

The distribution of phenotypical variables among the study participants is as given in table 1.

Ninety-four male and 56 female cases with age-matched and gender-matched controls were included in the study. One female

Table 1 Phenotypical variables

Variable	Cases (n=149)	Controls (n=150)	p Value
Age (mean±SD)	52.71±8.29	52.84±8.92	0.89
Diabetes (%)	54 (36)	43 (28.67)	0.16
Hypertension (%)	81 (54.67)	76 (50.67)	0.52
Smoking (%)	23 (15.33)	20 (13.33)	0.61
Alcohol (%)	18 (12.0)	22 (14.67)	0.51
Family history (%)	60 (40.0)	35 (24.0)	0.002*
TC (mean±SD, mg/dL)	168.22±43.93	164.60±38.39	0.45
TG (median, mg/dL)	131.0	119.0	0.028*
HDL-C (median, mg/dL)	35.0	38.0	0.006*
LDL-C (median, mg/dL)	103.0	98.0	0.277
Non-HDL-C (median, mg/dL)	128	126.5	0.228
TC/HDL-C (median)	4.85	4.35	0.0004*

HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides.

* $p < 0.05$: statistically significant

case, after microarray QC check was removed from subsequent analysis. The mean age of the subjects was 52 years. The percentage of subjects smoking ($p=0.61$) and consuming alcohol ($p=0.51$) was similar between the cases and the controls. The percentage of patients with diabetes ($p=0.16$) and hypertension ($p=0.52$) was higher in cases over controls, but not statistically significant. Lipid profiles of all the subjects were taken. The Shapiro-Wilk test was used to check for normal distribution of data. In the study population, TC values were normally distributed, while those of LDL-C, HDL-C and TG did not follow normal distribution. The mean TC and median LDL-C levels were similar between both groups. Median HDL-C levels were lower in cases (35 mg/dL) than controls (38 mg/dL) ($p=0.006$). The median TG value was significantly higher in cases (131 mg/dL) than controls (119 mg/dL) ($p=0.028$). Of the subjects, 114 patients (76.5%) and 70 controls (46.6%) were on lipid-lowering drugs. Family history was found to be statistically significant between the groups ($p=0.002$) with 40% cases and 24% controls having a positive family history. On performing regression analysis of all the phenotypical variables, HDL-C levels and family history were found to be independent risk factors for CAD.

Association testing

Using PLINK, a total of 755 variants were subject to model association testing between cases and controls. In the preliminary analysis, with a threshold of $p < 0.05$, 36 variants from 16 genes were found to be statistically significant. The allele frequencies and the p values of the variants are given in table 2. Twenty-two variants had a higher allele frequency in cases than controls, while 14 variants had a higher allele frequency in controls than cases.

Thirty allelic SNP variants of genes *APOA1*, *GALNT2*, *COBL1*, *SLC39A8*, *TRPS1*, *ABCA1*, *MADD*, *UBASH3B*, *MVK*, *SCARB1*, *VDR*, *LACTB*, *LILRA3* and *HNF4A* were found to be statistically significant ($p < 0.05$) between cases and controls on performing allelic association testing. On performing dominant model association testing, 11 variants were found to be statistically significant ($p < 0.05$). They include the variants of *APOA2*, *GALNT2*, *PPP1R3B*, *TRPS1*, *MADD*, *NR1H3*, *LILRA3* and *HNF4A* genes. On performing additive model association testing of the above variants, five variants were found to be statistically significant ($p < 0.05$). They include *GALNT2* variant rs11620, *APOA2* variant rs6413453, *PPP1R3B* variant rs330921 and *MADD* variant rs8027027.

Table 2 Allele frequencies and association testing

Chr.	rs no.	Gene	A1 (minor allele)	MAF in cases	MAF in controls	A2 (major allele)	Model	p Value
1	rs6413453	<i>ApoA2</i>	A	0.228	0.147	G	Allelic	0.01062
							Dominant	0.01698
							Additive	0.04895
1	rs11620	<i>GALNT2</i>	A	0.510	0.420	G	Allelic	0.02725
							Dominant	0.00837
							Additive	0.03091
1	rs3088075	<i>GALNT2</i>	G	0.128	0.063	A	Allelic	0.00753
1	rs3213495	<i>GALNT2</i>	A	0.154	0.087	G	Allelic	0.01098
1	rs4846903	<i>GALNT2</i>	A	0.030	0.007	G	Allelic	0.03314
1	rs4846904	<i>GALNT2</i>	G	0.175	0.243	A	Allelic	0.03846
1	rs4846911	<i>GALNT2</i>	C	0.030	0.003	G	Allelic	0.01041
1	rs6682741	<i>GALNT2</i>	A	0.175	0.243	G	Allelic	0.03846
1	rs7547269	<i>GALNT2</i>	G	0.285	0.370	A	Allelic	0.02725
1	rs78988790	<i>GALNT2</i>	A	0.030	0.000	G	Allelic	0.002422
2	rs116305649	<i>COBLL1</i>	A	0.020	0.000	G	Allelic	0.01351
4	rs10489124	<i>SLC39A8</i>	A	0.003	0.027	G	Allelic	0.01923
8	rs330921	<i>PPP1R3B</i>	A	0.329	0.403	G	Dominant	0.008218
							Additive	0.01988
8	rs2737259	<i>TRPS1</i>	A	0.000	0.020	G	Allelic	0.01414
8	rs3808409	<i>TRPS1</i>	G	0.463	0.390	A	Dominant	0.02759
8	rs7006935	<i>TRPS1</i>	A	0.003	0.023	C	Allelic	0.03349
9	rs72735008	<i>ABCA1</i>	A	0.044	0.013	G	Allelic	0.02585
11	rs10838687	<i>MADD</i>	C	0.306	0.372	A	Dominant	0.04301
11	rs11039159	<i>MADD</i>	A	0.285	0.210	C	Allelic	0.03304
11	rs11039182	<i>MADD</i>	G	0.235	0.163	A	Allelic	0.0284
11	rs3816725	<i>MADD</i>	C	0.453	0.537	A	Allelic	0.04054
							Dominant	0.008399
							Additive	0.03
11	rs7124955	<i>MADD</i>	T	0.453	0.524	A	Dominant	0.01863
11	rs749067	<i>MADD</i>	G	0.292	0.210	A	Allelic	0.02081
11	rs7944419	<i>MADD</i>	A	0.238	0.160	G	Allelic	0.01655
11	rs80270279	<i>MADD</i>	G	0.318	0.375	A	Dominant	0.01693
							Additive	0.0184
11	rs7120118	<i>NR1H3</i>	G	0.456	0.524	A	Dominant	0.02567
11	rs10750225	<i>UBASH3B</i>	A	0.440	0.356	G	Allelic	0.0364
12	rs72648010	<i>MVK</i>	A	0.024	0.003	C	Allelic	0.0327
12	rs10773105	<i>SCARB1</i>	G	0.450	0.370	A	Allelic	0.04763
12	rs12581963	<i>SCARB1</i>	A	0.154	0.093	G	Allelic	0.02345
12	rs989892	<i>SCARB1</i>	C	0.440	0.520	A	Allelic	0.0491
12	rs2239186	<i>VDR</i>	G	0.148	0.277	A	Allelic	0.000115
15	rs77125097	<i>LACTB</i>	A	0.070	0.033	G	Allelic	0.04055
19	rs367070	<i>LILRA3</i>	G	0.232	0.163	A	Allelic	0.03612
							Dominant	0.02649
20	rs188921652	<i>HNF4A</i>	C	0.037	0.003	A	Allelic	0.003413
20	rs6065725	<i>HNF4A</i>	A	0.201	0.140	G	Allelic	0.04615
							Dominant	0.02964

MAF, minor allele frequency.

Table 3 Correlation with HDL-C levels

SNP	rs11039159 (<i>MADD</i>)	rs749067 (<i>MADD</i>)	rs367070 (<i>LILRA3</i>)	rs330921 (<i>PPP1R3B</i>)	
Mean HDL-C levels (WT) (mg/dL)	38.72	38.65	36.32	36.51	
Mean HDL-C levels (HTZ) (mg/dL)	35.12	35.31	38.80	36.76	
Mean HDL-C levels (MT) (mg/dL)	36.70	36.70	42.33	41.85	
Additive model (p)	0.028*	0.046*	0.014*	0.019*	
Post hoc (p)	0.022* (WT-HTZ)	0.036* (WT-HTZ)	NS	0.020* (WT-MT)	0.025* (HTZ-MT)

HDL-C, high-density lipoprotein cholesterol; HTZ, heterozygous; MT, mutant; SNP, single nucleotide polymorphism; WT, wild type.

*p<0.05: statistically significant

However, after applying Bonferroni correction for multiple testing, none of the variants was found to be statistically significant.

Correlation with HDL-C levels

Genetic variants found to be statistically significant at $p < 0.05$ on preliminary association testing were compared with HDL-C levels, using the additive model testing. The results are given in table 3.

For the *MADD* gene, HDL-C levels were found to be significantly different between the three genotypes for rs11039159 ($p = 0.028$) and rs749067 ($p = 0.046$). Mean HDL-C levels were found to be higher for the wild type 'AA' genotype as compared with the heterozygous 'AG' genotype of rs749067. On post hoc analysis, this difference was found to be statistically significant ($p = 0.036$). A similar result were observed for HDL-C levels between 'CC' and 'CA' genotypes of the variant rs11039159 ($p = 0.022$).

For the *PPP1R3B* gene variant rs330921, HDL-C levels were found to be significantly different between wild type (GG) and mutant (AA) ($p = 0.020$), and between heterozygous (GA) and mutant (AA) genotypes ($p = 0.025$) on post hoc analysis. HDL-C levels were significantly higher in the mutant as compared with the other genotypes.

For the *LILRA3* gene variant rs367070, a statistically significant difference was seen between the HDL-C levels on initial testing ($p = 0.014$). However, after post hoc analysis, the difference was not found to be statistically significant. For the other genetic variants, no significant difference in HDL-C levels was observed between the genotypes.

DISCUSSION

There is little information on the association of the newer HDL-associated loci with CAD in the Indian population. This pilot study was performed on 300 Indian subjects to screen for genetic variants in the loci reported to be associated with HDL-C levels and associate the variants with CAD.

In a previous study done by the hospital on 9000 urban subjects, HDL-C levels were found to be abnormally low in 64.2% of the men and 33.8% of the women.²⁰ In the current study, a similar trend of low HDL-C levels was observed among all the subjects. A majority was managed on lipid medications. Further, the angiographically confirmed cases had significantly lower ($p < 0.05$) HDL-C levels than the controls. Additionally, family history, a known risk factor of CAD² was found to be a statistically significant ($p < 0.05$) variable between cases and controls.

GWAS have reported newer gene loci to be involved in the regulation of HDL-C levels.^{9 10 11} Their exact function in the metabolism of HDL is yet to be determined. In a study on 200 unrelated probands with extremely low (≤ 10 th centile) or high (≥ 90 th centile) HDL-C levels, *LILRA3* gene was found to be associated with elevated HDL-C levels, using next-generation sequencing techniques.²¹ The association of the *PPP1R3B* gene locus with HDL-C levels, from animal studies, have been reported.¹¹ Similarly, SNPs in the *MADD-FOLH1* gene locus have been reported to affect lipids and lipoprotein levels, including HDL-C and also associated with the risk of coronary heart disease and ischaemic stroke in the Chinese Han population.^{22 23}

In our study, variants of *MADD*, *PPP1R3B* and *LILRA3* genes were found to be correlated with HDL-C levels. Although, these SNPs were found to influence HDL-C levels as has been previously reported, it must be noted that the data are not completely reflective of baseline HDL-C levels as 70% of the cases

and 46% of the controls were on statins. Since statins are found to modestly affect HDL-C levels (2–10%), their effect on HDL-C levels cannot be ruled out at this time.²⁴

There are limited studies exploring the relationship of newer HDL-associated genes with CAD.¹⁰ The most significant results have been reported for the *GALNT2* variant. In a study on promoter DNA methylation status, it was seen that the promoter DNA hypermethylation of the *GALNT2* gene was associated with an increased risk of CAD.²⁵ In a recent study to identify the genetic determinants of 10-year progression of intima media thickness (IMT) using the genome-wide association approach in a Chinese cohort, it was seen that rs12040273 of the *GALNT2* gene was suggestive of a significant association ($p < 10^{-5}$) with carotid IMT.²⁶ In our study, during the preliminary genotypical association testing, *GALNT2* variant rs11620, *APOA2* variant rs6413453, *PPP1R3B* variant rs330921 and *MADD* variant rs8027027 were found to be statistically significant with CAD cases. However, the results were not found to be statistically significant after correction for multiple testing.

There are certain limitations to this study. As pointed out earlier, most of the patients and control subjects included in the study were on lipid-modifying drug(s). Besides, the study cohort consists of an ethnically heterogeneous population (from a cosmopolitan city like Mumbai) and hence may skew the study results. Furthermore, the potential effects of confounding factors (eg, lifestyle, etc) on the genetic variants could not be estimated due to a lack of sufficient data.

To summarise, this study confirms the low HDL-C pattern seen among Indians. This pilot study presents, for the first time, data on reported HDL-associated genetic loci and CAD in Indian subjects. Some of the literature-reported variants demonstrate a possible relation with CAD. Further studies would be needed to establish their association. The functional role of the HDL-associated variants and their clinical significance needs to be evaluated.

Identifying the HDL-associated genetic risk markers of CAD is particularly important in the Indian context due to the rising trend of dyslipidaemia and incidence of CAD seen among young Indians.²⁷ A genetic risk score for the Indian population, if developed, can be used as a tool along with the traditional risk markers for early and improved prediction of CAD risk in subjects.

Key messages

What is already known about this subject?

Genome-wide association studies have helped elucidate the role of lipid metabolism in coronary artery disease (CAD). Newer genetic loci have been reported to be associated with high-density lipoprotein cholesterol (HDL-C) levels.

What does this study add?

Large-scale genetic data on Indian subjects are limited. The study generates pilot genetic data from angiographically confirmed Indian CAD subjects. The study examines the genetic polymorphisms on loci reported to be associated with HDL-C levels and their association with CAD.

How might this impact on clinical practice?

The results of the study were not statistically significant. Further studies would be needed to identify genetic risk loci, which can be used in clinical practice as a risk marker for CAD.

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Contributors Study conception and design: TFA, AS, CKP, RMR; Data Collection: AS, CKP, RMR; Experimental work and data analysis: AS; Manuscript writing: AS, TFA; Approval of final version: TFA, AS, CKP, RMR.

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