Association of Single Nucleotide Polymorphism of Apo A-1 Gene and Lipid Variables in Patients with Myocardial Infarction: A Case-control Study

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ABSTRACT

Introduction: Genetic and environmental factors play a significant role in the development of Cardiovascular Disease (CVD). With advancements in molecular techniques, it is now possible to study genes that may provide evidence of the role of genetic factors in Coronary Artery Disease (CAD). Genetic variants of the Apo A-1 gene might play an important role in regulating lipid profiles and their alteration, leading to the subsequent development of Myocardial Infarction (MI).

Aim: The aim of this study was to evaluate the association between C+83T sequence variations in the first intron of the Apo A-1 gene and lipid variables in patients with MI.

Materials and Methods: A case-control study was conducted at the Department of Biochemistry in association with the Department of Cardiology, Kilpauk Medical College Hospital, Chennai, Tamil Nadu, India, from October 2013 to March 2014. The study included 52 MI cases and 52 age-, gender-, and risk factor-matched controls. Fasting venous blood samples were collected from each patient, and routine investigations, lipid profile assessments, and polymorphic studies were carried out. Allele frequencies between cases and controls were compared using the Chi-square test. Analysis of Variance (ANOVA) was performed to determine the association between fasting serum lipid variables and allele distribution.

Results: Both study groups were matched for age, gender, and risk factors such as alcohol consumption, smoking, Hypertension (HT), and Diabetes Mellitus (DM). The results were compared and found to be statistically insignificant. Genotype analysis between the groups disclosed that the TT homozygous genotype was more prevalent in cases, while the 'CC' genotype was more prevalent in controls, and the CT genotype was approximately equal in both cases and controls. The genotype difference between cases and controls was statistically significant (p-value=0.006). The 'T' allele frequency was higher among cases (0.36) compared to controls (0.13), while the frequency of the 'C' allele was higher among controls (0.86) compared to cases (0.63). In the CC genotype, there was a higher mean value of Apo A-1 and a lower ApoB/Apo A-1 ratio compared to the TT genotype. It was observed that carriers of the C allele and T allele showed no statistical difference in lipid variables except for High-density Lipoprotein (HDL).

Conclusion: This study found a significantly increased T allele frequency in cases compared to controls, suggesting that the presence of the T allele in the C+83T (first intron) of the Apo A-1 gene may increase the risk of developing MI.

INTRODUCTION

Ischaemic heart disease is the number one cause of morbidity and mortality worldwide [1]. Apo A-1 and Apo B serve as important risk predictors for the development of CAD. Risk factors, including dyslipidaemia, smoking, diabetes mellitus, hypertension, obesity, psychosocial stress, poor diet, physical inactivity, and alcohol consumption, contribute to the early etiology of CAD. Novel risk factors like C-Reactive Protein (CRP), adiponectin, homocysteine, Plasminogen Activator Inhibitor-1 (PAI-1), Interleukin-6 (IL-6), and fibrinogen have a multiplicative effect in predicting the progression of the disease but are not completely effective [2,3]. The occurrence of a family history suggests that specific genetic predisposition should be taken into consideration [4]. Holistic approaches are needed to identify the multiple factors, including genetic/epigenetic influences, behind atherosclerosis, which eventually leads to MI [5-8].

A wide variety of candidate genes have been investigated for their role in the evolution and development of CAD. One such candidate gene is the Apo A-1 gene [5-7]. It has been observed that identifying the genomic basis of complex traits like CAD is still in the early phase. From a public health perspective, no other polygenic trait is more vital than atherosclerotic CAD and MI, as it is the number one cause of mortality worldwide [1]. Linkage analysis, Genome Wide Association Studies (GWAS), and specific genetic epidemiologic studies have provided more understanding of the candidate genes underlying this common clinical condition [9,10].

The ApoAI-CIII-AIV gene cluster is approximately 15 kb in size and is located on chromosome 11q23.3 [11-13]. Genetic variation in this gene cluster affects gene expression in hepatocytes and intestinal epithelial cells. The DNA region between nucleotides -256 and -41 upstream from the transcription start site of the human Apo A-1 gene is essential and adequate for maximal expression [14]. The Apo A-1 gene promoter contains a TATA-like motif close to the transcriptional start site (25-30 bp upstream). Additionally, the Apo A-1 gene is regulated by promoter/enhancer sequences containing closely spaced cis-acting elements. These elements assemble different combinations of hepatocyte-enriched and ubiquitous factors in a positive or negative manner in response to changes in hormonal or metabolic status, diet, and environmental factors in the liver and intestine. In hepatocytes, Apo AI gene transcription is maintained by three cis-acting elements bound by members of the nuclear receptor superfamily, hepatocyte-enriched factors, and CCAAT/Enhancer Binding Proteins (C/EBP) [13].

These factors synergistically stimulate Apo AI enhancer activity by recruiting an uncharacterised transcriptional co-activator(s). Apo A-1 gene transcription is also induced by Peroxisome Proliferator Activated Receptors (PPARα), which interacts with the positive Peroxisome Proliferator Responsive Element (PPRE) located in the A site of the Apo A-1 gene promoter liver-specific enhancer region [13].

The hypothesis in the current study was to establish the relationship between the C+83T polymorphism (First Intron) in the Apo AI gene and lipid profile, as well as the occurrence of MI. The Apo A-1 gene is involved in HDL metabolism and is considered a candidate gene for MI. The association between reduced HDL cholesterol levels and increased risk of heart disease is well-established, independent of Triglyceride (TGL) levels and other lipid parameters [12]. Apo A-1, Apo B, and the Apo B/Apo A-1 ratio are effective predictors and markers of MI. Polymorphisms in the Apo A-1 gene and alterations in lipid variables might further increase the risk of MI. The C+83T polymorphism in the Apo AI gene results in a single nucleotide transition, leading to the loss of *Moraxella* species 1 (MspI) restriction sites. This polymorphic site was detected using PCR and MspI digestion in a single run. This nucleotide change occurs at regulatory hotspots [13]. The single nucleotide base change at +83 bp (in the first intron) has been found to alter either transcription or translation, but the underlying mechanism has not yet been established.

Hence, the present study was conducted to evaluate the association between C+83T sequence variations in the first intron of the Apo A-1 gene and lipid variables in patients with MI.

MATERIALS AND METHODS

This case-control study was conducted in the Department of Biochemistry in association with the Department of Cardiology, Govt Kilpauk Medical College Hospital, Chennai, Tamil Nadu, India, over a period of six months, from October 2013 to March 2014. All procedures performed in this study involving human participants were in accordance with the ethical standards of the institution. Permission for the study was obtained from the Institutional Ethical Committee (IEC) (Ref No: 3393/ME-1/Ethics/2013 Dt: 27.09.2013). Explicit written consent was obtained from the study population.

Inclusion criteria: In the present study, 52 documented acute ST Elevation Myocardial Infarction (STEMI) patients aged >40 years, with cardiac troponin (cTn) values above the 99th percentile Upper Reference Limit (URL) and one of the following ischaemia symptoms were included: pathological Q waves traced in electrocardiography, new or recognised significant ST-segment-T wave (ST-T) changes, occurrence of new Left Bundle Branch Block (LBBB), and new loss/regional wall motion abnormality of viable myocardium [13]. These patients were admitted to the cardiology Intensive Care Unit (ICU). A total of 52 age-, gender-, and risk factor-matched healthy controls (with no history or clinical evidence indicative of CAD) were included in the study.

Exclusion criteria: Subjects with liver, renal, and thyroid disorders were excluded from the study.

Sample size: A total of 52 subjects with MI who presented in the department within the study duration and 52 healthy controls were enrolled in the study using purposive sampling.

Data collection: For the study, 5 mL of fasting venous blood was collected under sterile conditions from the antecubital vein after 12 hours of fasting. In the case of STEMI patients, the sample was collected within 24 hours of the episode. A 3 mL blood sample was collected in plain vials, and serum was separated after centrifugation at 3000 rpm for 10 minutes. The serum was aliquoted into three eppendorfs and stored at -20°C without thawing until the batch was analysed for extended lipid profile and routine chemistry examinations. All biochemical analyses were performed using an automated (ROBONIK) clinical chemistry analyser.

DNA extraction kit procured from Helini biomolecules was used to isolate DNA from the 2 mL fasting venous samples collected in EDTA-coated test tubes. DNA quantity and purity were analysed using a UV spectrophotometer, with values within the range of 5-10 ng/μL for quantity and an A260/280 ratio between 1.6-1.8 for purity [Table/Fig-1].
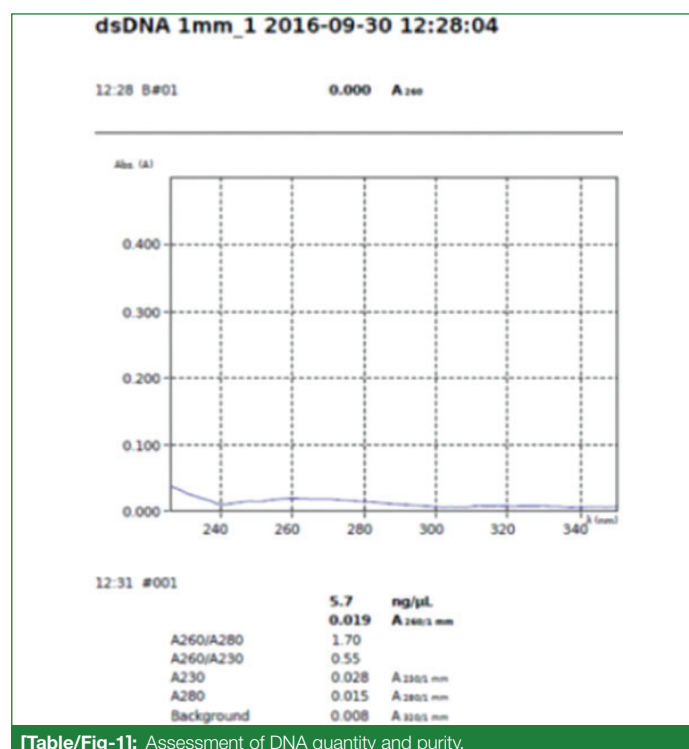
[Table/Fig-1]: Assessment of DNA quantity and purity.

The extracted DNA was identified by 1% agarose gel electrophoresis and compared to a known molecular weight 1kb DNA (Lambda DNA) ladder. A 353bp fragment of the Apo A-1 gene was amplified using the forward primer 5'GGCCACGGGGGATTTAGGGAGAA-3' and reverse primer 5'AGCTGGCTGCTTAGAGACTGCGA-3'. The 2×PCR Master mix was used [Table/Fig-2] [15].
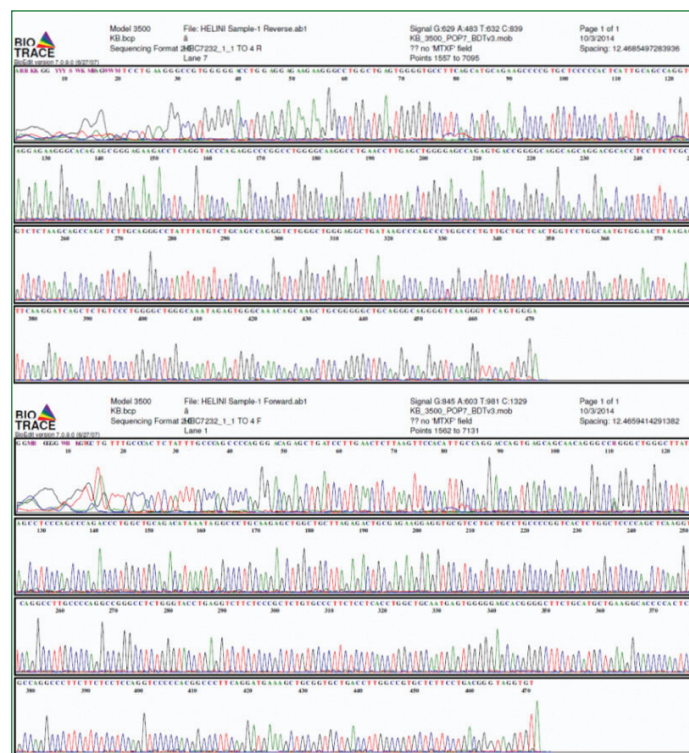
[Table/Fig-2]: Gene confirmation by sequencing using forward and reverse primer.

The PCR reaction mixture consisted of Tris HCl-pH 8.5, (NH4)2SO4, MgCl2 (3 mM), acting as a catalyst, and 0.2% Tween 20. dNTPs were used at a concentration of 0.4 mM each, and Taq polymerase

was used at a concentration of 0.2 U/μL. The primers were used at a concentration of 10 pmol. The PCR reaction was carried out in a volume of 20 μL with the following components: 10 μL reconstituted PCR master mix (containing gel loading dye), 5 μL of reconstituted primers, and 5.0 μL of DNA. The amplification of the extracted DNA was performed in a CYBERLAB SMART PCR-PRO thermal cycler, with the following cycling conditions: initial denaturation at 95°C for 5 minutes, 34 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 10 minutes. The amplicons of 353 bp were identified by 2.5% agarose gel electrophoresis, compared to a known 100 bp DNA ladder [Table/Fig-3] [15].
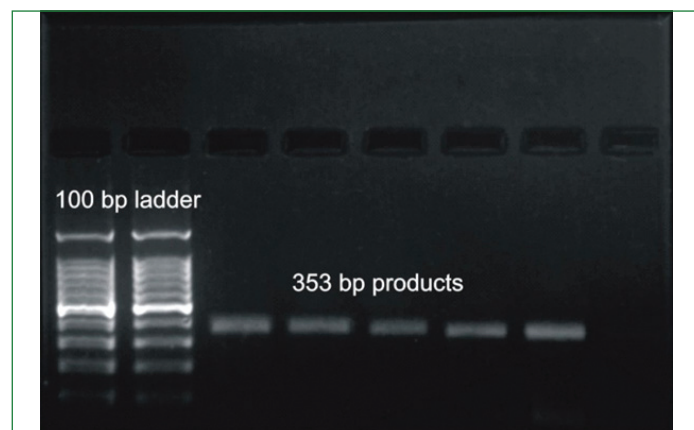
[Table/Fig-3]: PCR product of C+ 83T (first intron).

The Apo A-1 gene polymorphism at the C+83T site (First Intron) was detected by digesting the amplified PCR product with the MspI restriction enzyme. The reaction mixture contained 3 μL of Tango buffer (1x), 2.0 μL of MspI (10 U/μL), 10.0 μL of PCR product, and 15.0 μL of distilled water. The entire procedure was carried out on ice. The contents were mixed thoroughly and then placed in a 37°C water bath for one hour. The restriction-digested product was subjected to 2.5% agarose gel electrophoresis for genotyping [15,16].

There were two restriction sites for MspI within the 353 bp product. The analysis was performed using a 100 bp and 50 bp ladder. The C allele had a restriction site between 96-97 bp (polymorphic site) as well as a normal restriction site at 50-51 bp [Table/Fig-4,5]. The T allele destroyed its site for restriction at position 96-97 bp. CC (homozygous individuals) would yield 257 bp, 46 bp, and 50 bp. CT (heterozygous individuals) would yield 257 bp, 46 bp, 50 bp, 303 bp, and 50 bp. TT (homozygous individuals) would yield 303 bp and 50 bp [15,16].
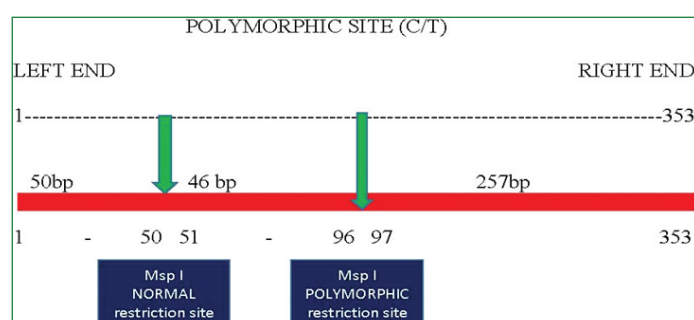

[Table/Fig-4]: Msp1 restriction site on Apo A-1 gene.

The baseline characteristics, routine basic chemistry analysis including Glucose, urea, creatinine and lipid profile, Apo A genotype distribution, and comparison of HDL, Apo A-1, Apo B, and Apo B/Apo A-1 levels in different genotypes at C+83T base pairs in MI cases and controls were statistically evaluated.

STATISTICAL ANALYSIS
The data were expressed as Mean±SD. The groups were compared using Student's t-test. A p-value of <0.05 was considered significant.
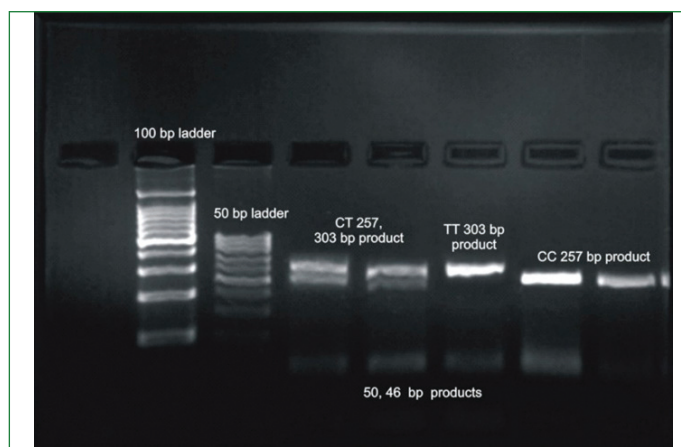
[Table/Fig-5]: Restriction digestion product of C+ 83T (First Intron).

The genotype frequency with Hardy-Weinberg equilibrium distribution between cases and controls was compared using the chi-square test for 2×2 contingency tables. Analysis of Variance (ANOVA) was used to determine the relationship between fasting serum HDL, Apo B, Apo A-1, and the ratio of Apo B/Apo A-1 with the genotype distribution. All statistical analyses were performed using SPSS Software version 19.

RESULTS
In the present study, the mean age in the case group was 53.77±8.34, while in the control group it was 53.6±10.04. The control and acute MI study groups were matched for age and gender, and no significant difference was found. There was also no statistical difference found for risk factors such as smoking, alcohol, history of Diabetes Mellitus (DM), and Hypertension (HT) between the study and control groups [Table/Fig-6].

Variables	Distribution	Study group (n=52)	Control group (n=52)	p-value
Age (years)		53.77±8.34	53.6±10.04	0.92
Gender distribution	Male	45	44	1
	Female	7	8	
Personal history	Smoking	16	10	0.257
	Alcohol	17	14	0.669
	DM	24	21	0.692
	HT	13	11	0.816
	CAD	8	0	-

[Table/Fig-6]: Baseline characteristics between cases and controls.
Student's t-test; Level of significant p-value <0.05

The serum HDL and Apo A-1 levels were significantly lower in the cases than in the control group, while the serum Apo B level and Apo B/Apo A-1 ratio were significantly higher in the cases compared to the control group. However, urea, Total Cholesterol (TC), Triglycerides (TG), Low-density Lipoprotein (LDL), and creatinine were significantly higher in the cases compared to the control group [Table/Fig-7].

Analytes compared	Group	Mean±SD	p-value
Glucose (mg/dL)	Cases (n-52)	136.23±76.63	0.43
	Controls (n-52)	124.94±70.58	
Urea (mg/dL)	Cases (n-52)	30.17±26.20	0.05
	Controls (n-52)	22.79±7.30	
Creatinine (mg/dL)	Cases (n-52)	1.30±1.05	0.01
	Controls (n-52)	0.93±0.15	
Total cholesterol (mg/dL)	Cases (n-52)	197.42±57.05	0.005
	Controls (n-52)	169.33±42.40	
Triglyceride (mg/dL)	Cases (n-52)	164.13±60.85	0.001
	Controls (n-52)	128.90±44.52	
LDL (mg/dL)	Cases (n-52)	118.52±43.50	0.03
	Controls (n-52)	101.44±39.06	

HDL (mg/dL)	Cases (n-52)	48.54±12.74	<0.001
	Controls (n- 52)	63.08±13.80	
Apo A-1 (mg/dL)	Cases (n-52)	81.46±22.30	<0.001
	Controls (n-52)	114.02±18.14	
Apo B (mg/dL)	Cases (n-52)	98.79±25.04	0.004
	Controls (n-52)	84.48±23.84	
Apo B/Apo A-1 (mg/dL)	Cases (n-52)	1.29±0.52	<0.001
	Controls (n-52)	0.74±0.21	

[Table/Fig-7]: Comparison of serum glucose, urea and creatinine levels and lipid profile between acute MI cases and controls.
Student's t-test and p-value of <0.05

The comparison of genotypes across the acute MI cases and controls revealed that the 'TT' homozygous genotype was higher among cases, while the 'CC' genotype was more common in controls. The genotype difference between cases and controls was statistically significant (p=0.006) [Table/Fig-8]. In terms of allele frequency, the 'T' allele was higher among cases (0.36) compared to controls (0.13), and the frequency of the 'C' allele was higher among controls (0.86) compared to cases (0.63) [Table/Fig-9].

Genotype frequency	CC	CT	TT
Cases (n-52)	31	4	17
Control (n-52)	42	6	4
Total (n-104)	73	10	21

[Table/Fig-8]: Allele distribution of Apo A-1 C+83 T (First Intron) region in acute MI cases and controls.

Alleles	Cases	Controls	p-value
C allele	0.63	0.86	0.006
T allele	0.36	0.13	

[Table/Fig-9]: Allele distribution of Apo A-1 C+83 T (First Intron) region in acute MI cases and controls.

Genotype distribution of Apo A-1 C+83 T (First Intron) region in acute MI cases and controls.

In CC genotype mean value of Apo A-1 were high and low HDL and Apo B/Apo A-1 ratio as compared to TT genotype. Difference in the mean for lipid variables were obtained between C allele subjects and T allele carriers but it was not statistically significant except HDL [Table/Fig-10].

Lipid variables compared	CC (Mean±SD)	CT (Mean±SD)	TT (Mean±SD)	p-value
HDL (mg/dL)	56.05±13.59	51.3±9.522	57.10±21.375	0.036
Apo A-1 (mg/dL)	100.08±26.70	97.80±15.843	89.52±26.726	0.572
Apo B (mg/dL)	93.08±23.703	83.70±24.363	90.38±31.385	0.080
Apo B/Apo A-1 (mg/dL)	1.02±0.480	0.86±0.267	1.12±0.564	0.167

[Table/Fig-10]: Comparison of HDL, Apo A-1, APO B, APO B/Apo A-1 levels in different genopes at C+83T base pairs in cases and controls.

The genotype distribution of the Apo A-1 C+83 T (First Intron) region in acute MI cases and controls was analysed.

DISCUSSION

The study group consisted of 52 patients with documented MI, and the control group included 52 age- and gender-matched individuals. Fasting serum levels of Total Cholesterol (TC), Triglycerides (TGL), LDL, HDL, Apo A-1, Apo B, and the ratio of Apo B/Apo A-1 were measured. TC, TGL, and LDL levels were significantly higher in cases compared to controls. Serum HDL and Apo A-1 levels were significantly lower in cases than in the control group, and the serum Apo B level and Apo B/Apo A-1 ratio were significantly higher in cases than in controls, consistent with the findings of various other studies [17]. High TC, LDL cholesterol, TGL, and low HDL cholesterol play important roles in the development of MI, and the

oxidised fraction of LDL is considered a risk factor. This is due to the receptor-mediated uptake of LDL cholesterol by cells, leading to the incorporation of cholesterol inside the cells [18].

HDL particles are responsible for removing cholesterol from cells through reverse cholesterol transport and carrying it to the liver. The association between reduced HDL cholesterol levels and increased risk of heart disease is well-established, independent of TGL levels and other risk factors. HDL also provides protection against atherosclerosis through its anti-inflammatory and antioxidant properties, inhibiting LDL cholesterol oxidation and the expression of cellular adhesion molecules and monocyte recruitment. HDL may also reduce the risk of thrombosis by inhibiting platelet activation and aggregation. The STAT3 (signal transducer and activator of transcription 3) protein plays a key role in HDL-induced cardioprotection [19].

In individuals with the CC genotype, the mean values of HDL and Apo A-1 were high, and the Apo B/Apo A-1 ratio was low compared to those with the TT genotype. The T allele frequency was higher in cases, while the C allele frequency was higher in controls. Differences in the means of lipid variables were obtained between individuals carrying the C allele and those carrying the T allele, but these differences were not statistically significant except for HDL.

The comparison of genotypes across the acute MI cases and controls revealed that the 'TT' homozygous genotype was more common among cases, while the 'CC' genotype was more common among controls. The CT genotype was approximately equal among cases and controls. The genotype difference between cases and controls was statistically significant (p-value=0.006). In terms of allele frequency, the 'T' allele was higher among cases (0.36) compared to controls (0.13), and the 'C' allele frequency was higher among controls (0.86) compared to cases (0.63). These findings were consistent with studies conducted in Indian populations [20,21] and in other subjects [22,23], where the 'T' allele was found to be more frequent among CAD populations. However, this study was in disagreement with some previously reported data, particularly studies carried out in the Kashmiri population [16]. In the CC genotype, there was a high mean value of Apo A-1 and a low Apo B/Apo A-1 ratio compared to the TT genotype. However, no statistically significant differences were observed between C allele and T allele carriers for any lipid variables other than HDL, which is similar to a study conducted in Brazilian children [24].

In this study group of acute MI, the single base change at C+83T bp of the TT genotype was more common in acute MI cases compared to controls. The mechanism behind the association between the single base substitution C to T at +83 in the first intron of the Apo A-1 gene and an elevated HDL cholesterol level is established [10]. It is suggested that the methylation pattern of the 5' region of the Apo A-1 gene reveals the extent of its expression.

In tissues expressing the Apo A-1 gene, the 5' region of the gene is hypomethylated, such as in the liver, but in non-expressing tissues, it is heavily methylated. The MspI restriction site at +83 bp is found to contain a CpG dinucleotide. CpG dinucleotide is known to be methylated in non-expressing cells but undermethylated in cells expressing Apo A-1. It is possible that the T substitution at this site leads to further demethylation, resulting in Apo A-1 gene expression [15,25]. Another explanation is that the T substitution in the 5' end leader region for Apo A-1 mRNA influences the translation of Apo A-1 messenger RNA (mRNA), and this could be important for the initiation of translation of mRNA [15].

The discrepancy in this study is due to differences in the genetic susceptibility between different ethnic groups. Additionally, the Apo A-1 gene locus lies in a cluster with CIII and AIV loci, which could be in linkage disequilibrium with the Apo A-1 alleles in some populations but not in others.

Limitation(s)

One of the major limitations of the present study was the small sample size. Additionally, the occurrence of linkage disequilibrium with nearby polymorphic sites was not analysed.

CONCLUSION(S)

In this study, the T allele was significantly increased in acute MI cases compared to controls, suggesting that the presence of the T allele in the C+83T (first intron) of the Apo A-1 gene may contribute to the development of MI. However, no significant differences were found across the lipid variables, except for HDL, indicating that various factors can influence the expression of the Apo A-1 gene. It is important to establish genotypic differences for this polymorphic site across different ethnic groups. Larger studies are needed to confirm the genotypic risk associated with this polymorphic site in the development of acute MI. Future research could focus on gene-gene interactions, gene-environment interactions, and their relationship with the genotypic variation of the Apo A-1 gene in patients with acute MI, in order to provide effective preventive measures for genetically susceptible populations. Factors such as gender, hormones, metabolic signaling pathways, diabetes mellitus, diet, and environmental factors like smoking may have modulated the genotype effect on circulating Apo A-1 and HDL levels in the inheritance of this complex trait, coronary artery disease, which can progress to the dreadful complication of MI.

Authors contribution: The authors' contributions to the study were as follows: Dr. G Udaya Kumari contributed to the study design and PCR analysis; Dr. J Vinodha and Dr. K Rekha contributed to sample collection and data preparation; Dr. A Preethi contributed to DNA extraction; Dr. Prashanth Talikoti contributed to manuscript writing; Dr. A Balasubramanian contributed to critical revising and final approval of the manuscript as the corresponding author; Dr. C Archana Devi and Dr. M Vijayalakshmi contributed to analyte estimation and statistics.

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