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ORIGINAL ARTICLE

Effect of Apolipoprotein A1 Genetic Variants at G-75A (Promoter Region) on Lipid Parameters in Acute Myocardial Infarction

[1]Udaya Kumari Ganapathy, [2]Bhuvaneswari Rajendran

ABSTRACT

This study aimed to find an association between G-75A sequence variations in the promoter region of apolipoprotein A1 (APO-A1) gene with respect to myocardial infarction (MI) and serum lipid profile. Fifty-two MI cases and 52 age-, gender-, and risk factor-matched controls were included in this study. Fasting venous blood was collected from each patient and baseline investigations, lipid profile, and polymorphic studies were done. Genotype frequency distribution between cases and controls was compared by chi-square test. Analysis of variance (ANOVA) was done to know the relationship between fasting serum lipid variables with the genotype distribution. Genotypes across the cases and control reveal that "GG" homozygous genotype was higher among cases and "GA" genotype seen more in controls, AA approximately equal among cases and controls. But the difference was statistically insignificant. "G" allele was higher among cases (0.75) than among controls (0.69) and frequency of "A" allele was higher among controls (0.30) than among cases (0.25). In GA genotype, mean high-density lipoprotein (HDL), APO-A1 were high and low APO-B/APO-A1 ratio as compared with GG genotype. But there were no statistically significant differences between G allele and A allele carriers for any lipid variables. There were no statistically significant differences across genotype as well as lipid variables in G-75A (promoter) region of APO-A1 gene for the development of MI.

Keywords: Alleles, APO A1 gene, Genotypes, Lipid variables, Myocardial infarction.

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INTRODUCTION

Myocardial infarction, the most imperative form of ischemic heart disease, is one of the major consequence of atherosclerosis. As on date, coronary artery disease (CAD) is considered to be the largest single contributor to global mortality and it has been denoted as "true pandemic that respects no borders" and is expected to dominate more morbidity and mortality trends in the days ahead. Asian Indians living both in India and abroad are found to have highest rates of CAD in the world. The CAD among Indians is usually more aggressive at the time of presentation compared with any other population. The overall impact is considered to be much higher because the CAD in Asian Indians affects the "younger" working-class population which will affect the dizzying economic boom, for a developing country like India and to sustain this growth, it should have a healthy populace.

Lipoproteins are involved in the pathogenesis of atherosclerosis. Epidemiologic studies have established that there exist an transposed relationship between serum levels of HDL, APO-A1 the main component of HDL, and the occurrence of CAD (antiatherogenesis), whereas low-density lipoprotein has been established as an proatherogenic factor.[1-6]

The current study in genetic cause for MI is being unraveled at an accelerated pace. The future assessment of a person's lifetime risk for developing atherosclerotic vascular disease by genetic analysis, formerly an idea is now evolving into reality. These findings could help in lifestyle modification and the choice and dosage of specific drugs. The best way to fight the burden of CAD is the preventive approach.

There are several investigations that are useful after the disease has set in. Genetic analytic study will be the screening tool before the development of CAD. In future, the genetic analysis may lead to the development of gene therapy mechanism, which could be useful in the treatment of CAD and for the prevention of MI and will provide more insight on the genetic basis of CAD, to the circumstances leading to the infarct. Various studies established relationship between genetic polymorphism in APO-AI-CIII-AIV gene cluster and its association with variation in the levels of triglycerides, HDL, APO-A1 levels, premature atherosclerosis, MI, and CHD in different populations.[7-12] The APO-AI-CIII-AIV gene cluster is approximately 15 kb size is found on chromosome

[1,2]Assistant Professor

[1,2]Department of Biochemistry, Government Kilpauk Medical College, Chennai, Tamil Nadu, India

Corresponding Author: Udaya Kumari Ganapathy, Assistant Professor, Department of Biochemistry, Government Kilpauk Medical College, Chennai, Tamil Nadu, India, Phone: +919176435514, e-mail: udayasaravanan15@gmail.com

11q23.31.[13] This closely placed gene complex which evolved from the same evolutionary sequence is thought to have significant role in lipid and lipoprotein metabolism.[7] Genetic variation in this gene cluster will affect the gene expression in the hepatocyte as well as in the intestinal epithelial cells.

As the Human Genome Project study was completed, this in turn helps us to identify the genetic, physical, and single-nucleotide polymorphism (SNP) maps of the human genome, and this provides the opportunity to map and identify the susceptibility genes for not only single-gene (Mendelian) disorders, but also complex polygenic (non-Mendelian) traits. The evidence is well distinguished for heritability of MI (the consequence of CAD), so it is well established that the inheritance is one of the most important risk factors for this polygenic trait. The saying "complex trait" as such indicates interaction between genes and with environment, along with the complex inheritance which is common and highly probabilistic in phenotypic manifestation as compared with "simple" Mendelian traits. The impact of genetic factors is more important and recent research in this field has led to identifying candidate genes associated with elevated risk of susceptibility for MI and the response to treatment.

The purpose of my study is to explicate the relationship between SNP in the APO-A1, 11q 23.3, namely the G-75A (promoter region) with MI which is the sequel of CAD.

MATERIALS AND METHODS

This study was conducted in the Department of Biochemistry in association with the Department of Cardiology, Kilpauk Medical College Hospital, Chennai. All procedures concerning human subjects or patients were permitted by the institutional ethical committee. Explicit written consent was obtained from the study population.

The study group consisted of 52 documented ST elevation myocardial infarction (STEMI) patients who got admitted in cardiology intensive care unit older than 40 years of either gender. Random selection of patients was done who fulfilled the inclusion criteria of ischemia symptoms, pathological Q waves traced in electrocardiography, new or recognized significant ST-segment–T wave changes, occurrence of new left bundle branch block, and new loss/regional wall motion abnormality of viable myocardium.

The control group consisted of 52 age-, gender-, and risk factor-matched patients with no history or clinical evidence indicative of CAD with exclusion criteria being patients with liver, renal, and thyroid disorders.

For the study, 5 mL of 12 hours fasting venous blood was collected under sterile conditions from the antecubital vein. From the cases, the sample was collected within 24 hours of the episode of STEMI; 3 mL of blood was collected in plain vials and serum was separated after centrifugation at 3000 rpm for 10 minutes and aliquoted, into three Eppendorf and stored at –20°C and were not thawed until the batch was analyzed for extended lipid profile and routine chemistry examinations.

All the biochemical analyses were performed using automated (Robonik) clinical chemistry analyzer. All biochemical analyses were done using Randox calibrator (lot no 2351-562UE) and controls (lot no 768UN level 2) for checking internal quality. The coefficient of variations of all the analytes performed were within the prescribed limits in accordance with Clinical Laboratory Improvement Amendments.

For gene polymorphic studies, fasting blood was collected in 2 mL ethylenediaminetetraacetic acid-coated tubes and deoxyribonucleic acid (DNA) extraction was done using minipreparation kit from Helini biomolecules, Chennai. The DNA quantity was in the range of 5 to 10 ng/µL of fresh blood and purity was assessed by calculating A260/A280 ratio of 1.6 to 1.8 using Eppendorf spectrophotometer ruling out ribonucleic acid contamination. Extracted DNA was identified by 1% agarose gel electrophoresis and comparison with a known molecular weight 1 kb DNA (Lambda DNA) ladder.

The 203 bp fragment of APO-A1 gene was amplified using forward primer 5′-GCAGCTTGCTGTTTG-CCCACTC-3′ and reverse primer 5′-ACGCACCTC-CTTCTCGCAGTCT-3′.

A 2 × polymerase chain reaction (PCR) Master Mix was used in the following composition of Tris HCl: pH 8.5, $(NH_4)_2 SO_4$, $MgCl_2$ 3 mM acts as catalyst and 0.2% Tween 20. Deoxyribonucleotide triphosphates were used in a concentration of 0.4 mM each. Taq polymerase was in a concentration of 0.2 U/µL. Primers were used in a concentration of 10 pmol. The PCR was carried out in a reaction in volume of 20 µL with the following components in the following manner: 10 µL reconstituted PCR Master Mix (contains gel loading dye), 5 µL of reconstituted primers, and 5.0 µL of DNA. Amplification of the extracted DNA was carried out in Cyberlab Smart PCR-PRO thermal cycler with the following cycling conditions: Initial denaturation for 95°C/5 minutes, 34 cycles of denaturation at 94°C for 30 seconds, annealing at 55.9°C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72° C for 10 minutes. Amplicons of 203 bp were identified by 2.5% agarose gel electrophoresis by comparison with a known 100 bp DNA ladder (Fig. 1).

The APO-A1 gene polymorphism at the site G-75A (promoter region) was spotted by digestion of the amplified PCR product with the MspI restriction enzyme using 3 µL of Tango buffer (1×) for 100% MspI digestion 2.0 µL

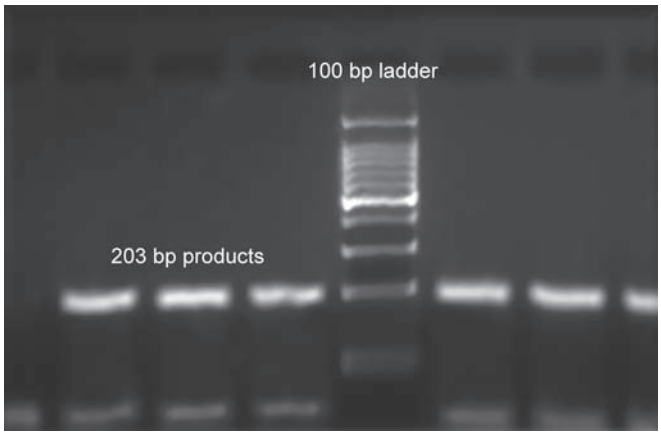

Fig. 1: The PCR product of G-75A (promoter region)

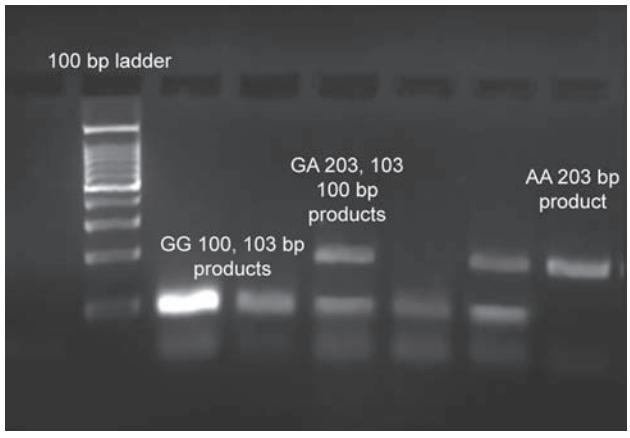

Fig. 2: Restriction digestion fragments of G-75A (promoter region)

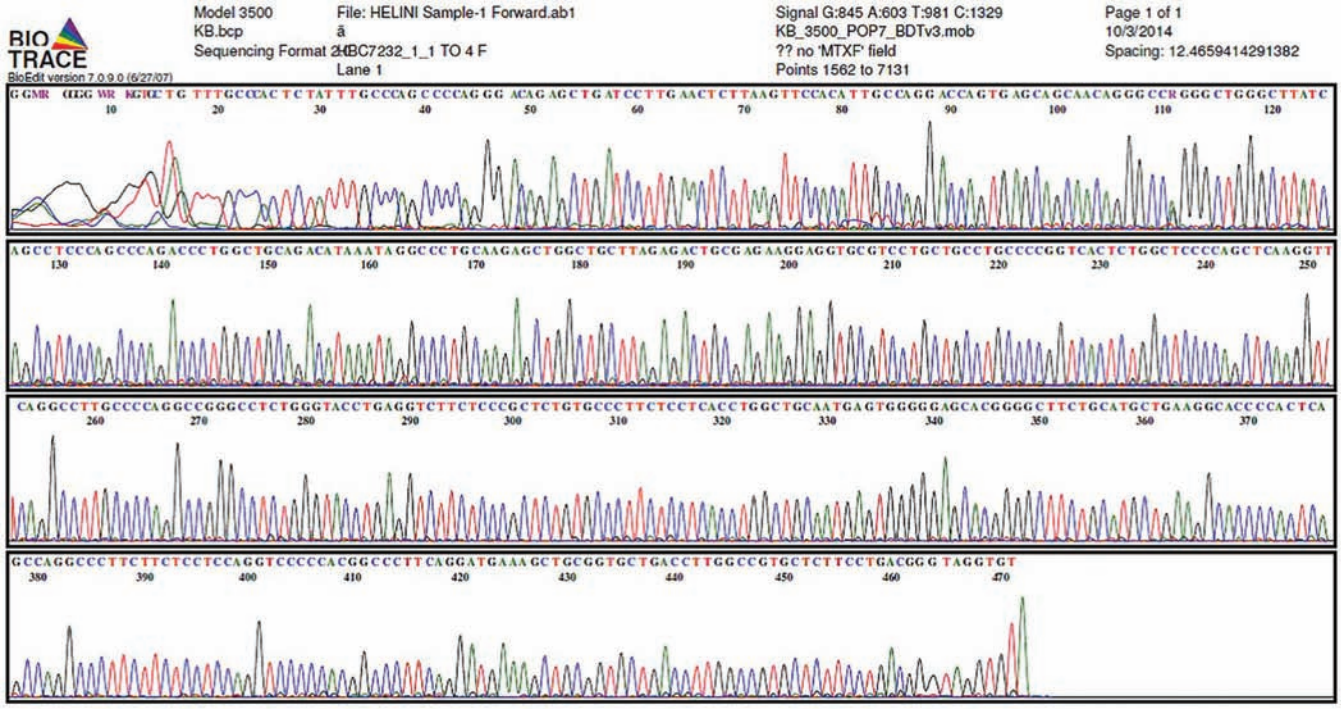

Fig. 3: Gene confirmation by sequencing using forward primer

MspI (10 U/µL), 10.0 µL of PCR product, and 15.0 µL of distilled water. The entire procedure was carried out in ice. The contents were mixed thoroughly. The Eppendorf was then placed in a 37°C water bath for 1 hour. Restriction-digested product was subjected to 2.5% agarose gel electrophoresis for genotyping.

There was one restriction site for MspI within 203 bp. G allele had restriction site between 103 and 104 bp (polymorphic site), yielding 103 and 100 bp A allele destroying its site for restriction at position 103 to 104 bp, so A (individuals) would yield only 203 bp (Fig. 2). Gene confirmation was done using with forward and reverse primer (Figs 3 and 4).

Statistical Analysis

Statistical Package for the Social Sciences, version 19, was used for statistical analysis. The groups were compared using unpaired Student's t-test and p-value of <0.05 was considered significant. Genotype frequency with Hardy–Weinberg equilibrium distribution between cases and controls was compared with the χ^2 test for 2 × 2 contingency table. Analysis of variance was used to know the relationship between fasting serum HDL, APO-B, APO-A1, and ratio of APO-B/APO-A1 with the genotype distribution.

RESULTS

The controls and the acute MI study population were matched for their age, gender and evaluated statistically. There was no statistical difference in history of smoking, alcohol, diabetes mellitus (DM), and hypertension (HT) except in the occurrence of CAD, which was higher in study groups when matched with controls (Tables 1 to 4).

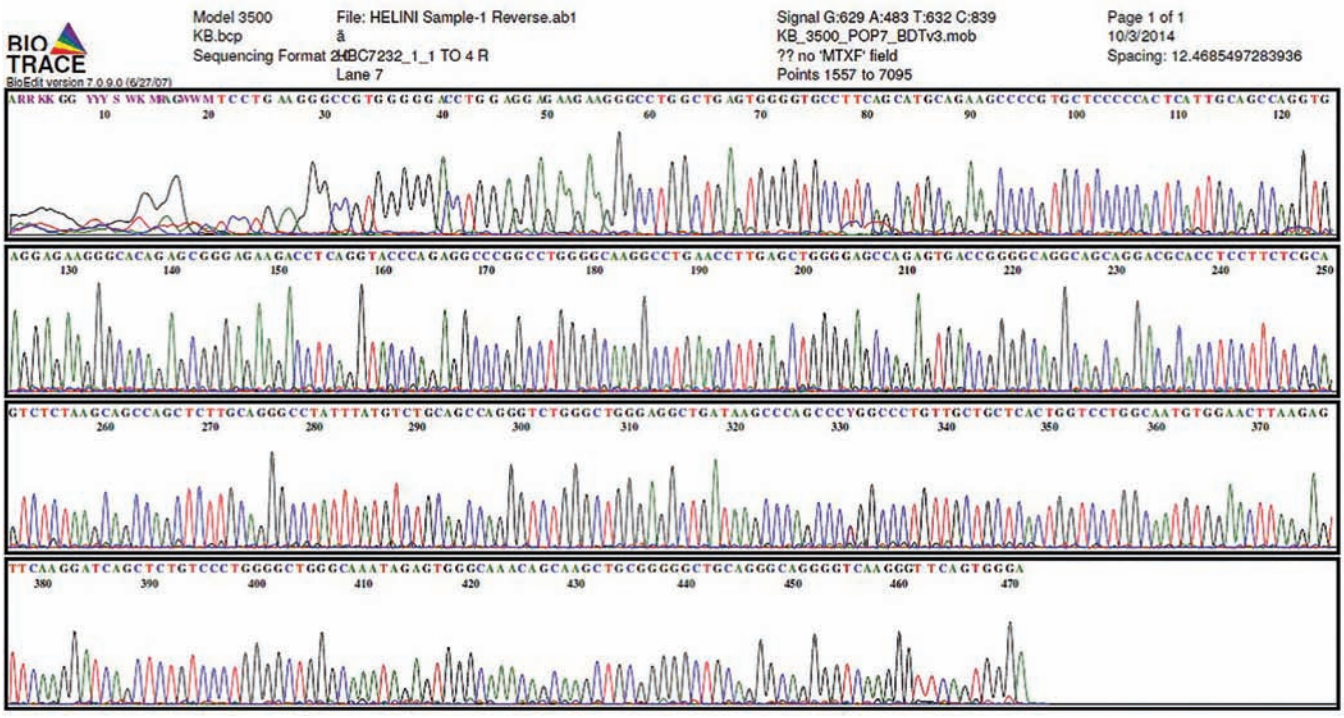

Fig. 4: Gene confirmation by sequencing using reverse primer

Table 1: Baseline characteristics of the two groups

Variables	*Distribution*	*Study group (n = 52)*	*Control group (n = 52)*	*p-value*	*Significance*
Age		53.77 ± 8.34	53.6 ± 10.04	0.92	NS
Gender distribution	Male	45	44	1	NS
	Female	7	8		
Personal history	Smoking	16	10	0.257	NS
	Alcohol	17	14	0.669	NS
	DM	24	21	0.692	NS
	HT	13	11	0.816	NS
	CAD	8	0	0.003	S

NS: Nonsignificant; S: Significant

Table 2: Comparison of serum HDL, APO-A1, APO-B, APO-B/APO-A ratio between cases and controls

Lipid variables	*Group*	*Mean ± SD*	*p-value*	*Significance*
HDL (mg/dL)	Cases (n = 52)	48.54 ± 12.74	0.000	S
	Controls (n = 52)	63.08 ± 13.80		
APO-A1 (mg/dL)	Cases (n = 52)	81.46 ± 22.30	0.000	S
	Controls (n = 52)	114.02 ± 18.14		
APO-B (mg/dL)	Cases (n = 52)	98.79 ± 25.04	0.004	S
	Controls (n = 52)	84.48 ± 23.84		
APO-B/APO-A1 (mg/dL)	Cases (n = 52)	1.29 ± 0.52	0.000	S
	Controls (n = 52)	0.74 ± 0.21		

SD: Standard deviation; S: Significant. The mean HDL value of cases was 48.54 ± 12.74 mg/dL and for controls, 63.08 ± 13.80 mg/dL with p-value of 0.000 (significant). The mean APO-A1 value of cases was 81.46 ± 22.30 mg/dL, and for controls, 114.02 ± 18.14 mg/dL, p 0.000 (significant). The APO-B value of cases was 98.79 ± 25.04 mg/dL, and for controls, 84.48 ± 23.84 mg/dL, p-value of 0.004 (significant). The mean APO-B /APO-A1 ratio value of cases was 1.29 ± 0.52 mg/dL, and for controls, 0.74 ± 0.21 mg/dL, p 0.000 (significant).

Table 3: Genotype distribution of APO-A1 G-75A (promoter) region in acute MI cases and control

Genotype frequency	*GG (%)*	*GA (%)*	*AA (%)*
Cases (n = 52)	30 (58)	18 (34)	4 (8)
Control (n = 52)	23 (44)	26 (50)	3 (6)
Total (n = 104)	53 (50.59)	44 (42.43)	7 (6.73)

The genotype frequency was found to be: Cases: GG 58%, GA 34%, and AA 8%, and in controls: GG 44%, GA 50%, and AA 6%. This was found to be in Hardy–Weinberg equilibrium; G allele frequency in cases and controls was 0.75 and 0.69 respectively; while A allele frequency in cases and controls was 0.25 and 0.30 respectively. χ^2 value 2.522, p-value was 0.283

Table 4: Comparison of HDL, APO-A1, APO-B, and APO-B/APO-A1 levels in different genotypes at G-75 A base pairs individually

Lipid variables across different genotypes	*GG (mean ± SD)*	*GA (mean ± SD)*	*AA (mean ± SD)*	*p-value*	*Significance*
HDL (mg/dL)	53.40 ± 11.404	59.95 ± 18.406	48.00 ± 10.583	0.592	NS
APO-A1 (mg/dL)	95.42 ± 23.891	100.91 ± 29.479	95.43 ± 17.803	0.263	NS
APO-B (mg/dL)	97.00 ± 22.374	85.66 ± 28.498	88.57 ± 19.121	0.535	NS
APO-B/APO-A1 (mg/dL)	1.11 ± 0.542	0.93 ± 0.419	0.95 ± 0.247	0.390	NS

SD: Standard deviation; NS: Nonsignificant. The mean HDL value across genotype GG was 53.40 ± 11.404 mg/dL, GA 59.95 ± 18.406, and AA 48.00 ± 10.583 with p-value of 0.592 (nonsignificant). The mean APO-A1 value across genotypes, GG was 95.42 ± 23.891 mg/dL, GA 100.91 ± 29.479, and AA 95.43 ± 17.803 with p-value of 0.263 (nonsignificant). The mean APO-B value across genotypes, GG is 97.00 ± 22.374 mg/dL, GA 85.66 ± 28.498, and AA 88.57 ± 19.121 with p-value of 0.535 (nonsignificant). The mean APO-B/APO-A1 ratio value across genotypes, GG 1.11 ± 0.542, GA 0.93 ± 0.419, and AA 0.95 ± 0.247 with p-value of 0.39 (nonsignificant)

DISCUSSION

The comparison of genotypes across the cases and control reveals that "GG" homozygous genotype was higher among cases and "GA" genotype was seen more in controls, AA more or less equal among cases and controls. But the difference was statistically insignificant. As far as frequency, "G" allele was higher among cases (0.75) as compared with controls (0.69); and the frequency of "A" allele was higher among controls (0.30) as compared with cases (0.25). Such findings are in concordance with studies conducted in Indian populations and from other subjects where the "A" allele was found to be higher in frequency among control groups.[14] The allele frequency, however, was also insignificant in those studies.[15,16] This study is in discordance with some previously reported data, particularly studies carried out in Kashmiris, Australian populations, and by Reguero et al[17] with high frequency of the A allele in subjects younger than 50 years with a diagnosed MI.

In GA genotype mean HDL, APO-A1 was high and low APO-B/APO-A1 ratio, as compared with GG genotype. But no statistically significant differences were observed between G allele and A allele carriers for any lipid variables. The MspI-75(G/A) promoter polymorphism was first recorded by Pagani et al.[19]A potential description for the effect of the A allele on APO-A1 levels suggests that the presence of the A allele at-75 bp from transcription start site of the gene increases the transcriptional efficiency of the promoter.[19]

The allele A possibly would be in linkage disequilibrium with a different polymorphic site in the nearby gene,[20] which in turn might impact the HDL levels. The polymorphic site might be in a sequence with the APO-A1 gene promoter that regulates its expression in response to hormonal stimulation and by various metabolic signaling pathways.[18,20-21] The probable mechanism is that it may be due to decreased stability of a DNA–protein complex that inhibits transcription. The nucleotide region between 199 and –40 bp is needed for repression of transcription by the binding factor (MWt 90 kDa) and is present in all cells that do not express the APO-A1 gene other than liver and intestine in which the gene is expressed.[22] The nucleotide at the –75 position of the APO-A1 promoter lies in a GC-rich sequence (5′GCC[A/G]GGG-3′). The transcription of these promoters has been shown to be negatively regulated by this GC box. A GC binding factor (90-kDa factor) could mediate the repression of transcription observed in the G allele. The G to A transition decreases its binding affinity to –75 bp position and alleviates the repression of APO-A1 gene transcription. Previous studies have reported the effect of genetic and environmental factors on APO-AI and HDL-cholesterol levels; however, the gene–environment interactions are still incompletely known.

CONCLUSION

This study revealed that GG genotype was more in cases and GA genotype more in controls, AA genotype distribution is more or less equal between cases and control. No statistically significant difference across genotypes was obtained. In GA genotype, mean value of HDL, APO-A1 were high and low APO-B/APO-A1 ratio as compared with GG genotype. G allele frequency is more in cases and A allele more in controls. No statistically significant differences were observed between G allele and A allele carriers for any lipid variables. In this study, no statistically significant differences were obtained across genotypes as well as lipid variables in G-75A (promoter) region of APO-A1 gene for the development of MI.

Limitations of This Study

- Small sample size.
- Probable occurrence of linkage disequilibrium of APO-AI gene with the near by polymorphic site and the gene complex APO-CIII and APO-AIV.
- Gender, hormonal, and various metabolic signaling pathways as in DM, diet, and environmental factors, such as smoking could have modulated the genotype effect on circulating APO-A1 and HDL for the inheritance of such a complex trait CAD which progresses to dreadful complication in MI.

- Low frequency of the presence of rare alleles.
- It is critically important to consider the level of confidence to place the accuracy of result.

Future Scope of the Study

- Genotypic differences across different ethnic groups for this two polymorphic site have to be established.
- More studies with larger samples are needed to confirm the genotypic risk associated with both the polymorphic sites in the development of MI.
- Further research may focus upon the gene–gene interactions and gene–environment interactions and its relationship with the genotypic variation of APO-A1 gene in patients with MI, so as to provide efficient preventive measures to genetically susceptible population in future.
- Probable occurrence of linkage disequilibrium of APO-AI gene with the near by polymorphic site and the gene complex APO-CIII and APO-AIV has to be established.
- Whole genome sequence analysis is needed to reveal extensive level of variation and heterogeneity between individuals and populations, and genome-wide association studies have to be done, as these analyses eliminate biases in the selection of the candidate genes.

REFERENCES

1. Packard CJ. Apolipoproteins: the new prognostic indicator? Eur Heart J Suppl 2003 Jun;5(Suppl D):D9-D16.
2. Walldius G, Jungner I. Apolipoprotein B and apolipoprotein A-1: risk indicators of coronary heart disease and targets for lipid modifying therapy. J Intern Med 2004 Feb;255(2):188-205.
3. Gordon DJ, Probstfield JL, Garrison RJ, Neaton JD, Castelli WP, Knoke JD, Jacobs DR Jr, Bangdiwala S, Tyroler HA. High density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. Circulation 1989 Jan;79(1):8-15.
4. Wilson PW, Abbott RD, Castelli WP. High density lipoprotein cholesterol and mortality. The Framingham Heart Study. Arteriosclerosis 1988 Nov-Dec;8(6):737-741.
5. Ballantyne CM, Hoogeven RC. Role of lipid and lipoprotein profiles in risk assessment and therapy. Am Heart J 2003 Aug;146(2):227-233.
6. Miller GJ, Miller NE. Plasma high density lipoprotein concentration and development of ischaemic heart disease. Lancet 1975 Jan;1(7897):16-19.
7. Groenendijk M, Cantor RM, de Bruin TW, Dallinga-Thie GM. The ApoAI-CIII-AIV gene cluster. Atherosclerosis 2001 Jul;157(1):1-11.
8. Ordovas JM, Civeira F, Genest J Jr, Craig S, Robbins AH, Meade T, Pocovi M, Frossard PM, Masharani U, Wilson PW, et al. Restriction fragment length polymorphisms of the apolipoprotein A-I, C-III, A-IV gene locus. Relationships with lipids, apolipoproteins, and premature coronary artery disease. Atherosclerosis 1991 Mar;87(1):75-86.
9. Tybjaerg-Hansen A, Nordestgaard BG, Gerdes LU, Faergeman O, Humphries SE. Genetic markers in the apo AI-CIII-AIV gene cluster for combined hyperlipidemia, hypertriglyceridemia, and predisposition to atherosclerosis. Atherosclerosis 1993 May;100(2):157-169.
10. Paul-Hayase H, Rosseneu M, Robinson D, Van Bervliet JP, Deslypere JP, Humphries SE. Polymorphisms in the apolipoprotein (apo) AI-CIII-AIV gene cluster: detection of genetic variation determining plasma apo AI, apo CIII and apo AIV concentrations. Hum Genet 1992 Feb;88(4):439-446.
11. Kee F, Amouyel P, Fumeron F, Arveiler D, Cambou JP, Evans A, Cambien F, Fruchart JC, Ducimetière P, Dallongeville J. Lack of association between genetic variations of apo A-I-C-III-A-IV gene cluster and myocardial infarction in a sample of European male: ECTIM study. Atherosclerosis 1999 Jul;145(1):187-195.
12. Vavatsi NA, Kouidou SA, Geleris PN, Tachmatzidis C, Gikas T, Tsifodimos DK, Trakatellis AC. Increased frequency of the rare PstI allele (P2) in a population of CAD patients in northern Greece. Clin Genet 1995 Jan;47(1):22-26.
13. Karathanasis SK. Apolipoprotein multigene family: tandem organization of human apolipoprotein AI, CIII, and AIV genes. Proc Natl Acad Sci U S A 1985 Oct;82(19):6374-6378.
14. Liao B, Cheng K, Dong S, Liu H, Xu Z. Effect of apolipoprotein A1 genetic polymorphisms on lipid profiles and the risk of coronary artery disease. Diagn Pathol 2015 Jul;10:102.
15. Padmaja N, Kumar MR, Adithan C. Association of polymorphisms in apolipoprotein A1 and apolipoprotein B genes with lipid profile in Tamilian population. Indian Heart J 2009 Jan-Feb;61(1):51-54.
16. Chhabra S, Narang R, Lakshmy R, Das N. APOA1-75 G to A substitution associated with severe forms of CAD, lower levels of HDL and apo-A1 among northern Indians. Dis Markers 2005;21(4):169-174.
17. Reguero JR, Cubero GI, Batalla A, Alvarez V, Hevia S, Cortina A, Coto E. Apolipoprotein A1 gene polymorphism and risk of early coronary disease. Cardiology 1998 Dec;90(3):231-235.
18. Toptas B, Gormus U, Ergen A, Gurkan H, Kelesoglu F, Darendeliler F, Bas F, Dalan AB, Izbirak G, Isbir T. Comparison of lipid profiles with APOA1 MspI polymorphism in obese children with hyperlipidemia. In Vivo 2011 May-Jun;25(3): 425-430.
19. Pagani F, Sidoli A, Giudici GA, Barenghi L, Vergani C, Baralle FE. Human apolipoprotein A-I gene promoter polymorphism: association with hyperalphalipoproteinemia. J Lipid Res 1990 Aug;31(8):1371-1377.
20. Hargrove GM, Junco A, Wong NC. Hormonal regulation of apolipoprotein AI. J Mol Endocrinol 1999 Apr;22(2): 103-111.
21. Harnish DC, Malik S, Kilbourne E, Costa R, Karathanasis SK. Control of apolipoprotein AI gene expression through synergistic interactions between hepatocyte nuclear factor 3 and 4. J Biol Chem 1996 Jun;271(23):13621-13628.
22. Dawar R, Gurtoo A, Singh R. Apolipoprotein A1 gene polymorphism (G–75A and C+83T) in patients with myocardial infarction: a pilot study in a North Indian population. Am J Clin Pathol 2010 Aug;134(2):249-255.