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APOLIPOPROTEIN B rs1042031 POLYMORPHISM AND LIPID PROFILE OF CORONARY ARTERY DISEASE IN THE PAKISTANI POPULATION

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Abstract

Apolipoprotein B (*APOB*) is linked to atherosclerosis emergence and Coronary Artery disease (CAD) pathophysiology. Single nucleotide polymorphism (SNP) in the *APOB* gene influences disease susceptibility. This study aimed to determine the association of rs1042031 and serum levels of lipids on the risk of CAD in a population from Pakistan. A cross-sectional study of 200 subjects was conducted at the University of Health Sciences, Lahore. Angiographically diagnosed cases of CAD were recruited from the Punjab Institute of Cardiology, Lahore, during 2021-2022. The lipid profile was done through the calorimetric method. Genotyping was done through Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). The frequency of AA genotype was significantly higher in CAD cases 98 (72.6%) as compared to non-CAD group 4 (6.2%) (p=0.018). The adjusted odds ratio for the AA genotype was 2.09 and 0.937 for the GA genotype. Compared with the lipid profile, a significant increase in cholesterol, serum LDL, and total lipids was observed for the AA genotype in the CAD group (p<0.001).

Conclusion: The AA genotype of the *APOB* rs1042031 gene is a risk factor for CAD in the Pakistani population.

Keywords: *Apolipoprotein B*, genetic polymorphism, lipid profile, RFLP

Introduction

Coronary artery disease (CAD) is a highly prevalent chronic condition, and it is predicted that it will eventually overtake other diseases as a global threat to human health.[1] In those with type 2 diabetes (DM2), atherosclerotic coronary artery disease is now thought to be the leading cause of chronic illness and early death. Anomalies in the metabolism of lipids and lipoproteins could, at least in part,

be responsible for the higher cardiovascular disease burden in people with diabetes type 2.[2] One of the known independent risk factors for CAD and DM2 is hyperlipidemia. The prevalence of dyslipidemia among the Pakistani population is around 96%.[3] Dyslipidemia was linked to several single nucleotide polymorphisms (SNPs), mainly in the apolipoprotein genes.[4] Apolipoprotein B (ApoB) is crucial for the emergence of dyslipidemia.[5] Low-density lipoprotein (LDL) has a structural protein called apolipoprotein B (ApoB). One molecule of ApoB100 is present in each atherogenic lipoprotein particle (LDL, VLDL, IDL, and lipoprotein a). However, LDL particles account for over 90% of the plasma's total ApoB concentration. So, ApoB can influence blood lipid levels by controlling LDL.[6] It is well recognized that having high LDL cholesterol is one of the risk factors for cardiovascular disease.

One type of single nucleotide polymorphism (SNP) that alters the amino acid sequence is found in the *APOB* EcoRI gene. At least 24 polymorphic locations in the *APOB* gene are located on the short arm of chromosome 2.[7,] There have been reports that the polymorphisms EcoRI (rs1042031) affect plasma levels of total and LDL cholesterol.

Benn showed in his population that the AA genotype lowered the LDL levels and the onset of ischaemic stroke.[8] Studies done on the Turkish and Chinese populations showed no association between rs1042031 in cases and control.[9,10] No study to date has explored the role of this gene mutation in the Pakistani population. So, it has been challenging from previous studies to conclude the association of EcoR1 with lipid profile in our population. The present study was designed to establish the association between EcoRI polymorphism of the Apo B gene and changes in serum lipid profiles in CAD patients of the Pakistani population.

Methods

The Institutional Review Board of The Institute of Molecular Biology and Biotechnology, The University of Lahore (IMBB/UOL/21/1033) reviewed and approved the study protocol. Informed consent was submitted by all subjects when they were enrolled. Two hundred study subjects were included from November 2021 to April 2022. One hundred and thirty-five were CAD, and 65 were controls. The sample size calculation was done using a 95% confidence interval with 5% absolute precision and 80% power of the study. The patients were recruited from the Punjab Institute of Cardiology, Lahore (PIC). Consultants on Coronary angiography made a diagnosis of CAD. This study has excluded patients in which troponin levels were higher with acute heart syndrome, unstable angina pectoris before resuscitation of cardiopulmonary arrest, cerebrovascular accidents in the previous six months, and constant sepsis. Ten ml venous blood was obtained by sterile venipuncture. Five ml of blood was transferred into vacutainers containing EDTA. Standard protocol was followed to transfer blood samples[11] to the Physiology lab, University of Health Sciences, Lahore. Samples were kept at -20°C till DNA extraction, whereas 5 ml blood was used for serum isolation, which was stored for serum biochemistry. Serum was obtained by centrifugation (3000 rpm for 10 minutes) and stored at -80°C. Plasma lipids, including total cholesterol (TC) and triacylglycerols (TG), were measured utilizing commercially accessible kits in auto-analyzer (Model No. REF 9D93,304985/R03) purchased from Abbott Pvt. Ltd. VLDL and LDL fixations were acquired from estimations of all out cholesterol, TG, and HDL cholesterol utilizing the recipe of Friedewald et al.[12] as displayed underneath:

[LDL] = [total cholesterol] - HDL - [TG]/5, where [TG]/5 relates to an assessment of VLDL cholesterol.

All absorbance estimations were made utilizing an Epoch microplate spectrophotometer (BioTeck U.S., Winooski, VT, USA).

Genotyping

DNA was extracted through Favor Prep Blood Genomic DNA Extraction Kit (Biotech Corp, Taiwan, China). The DNA samples were quantified using nanodrop (ND8000, USA). The polymerase chain reaction was used to determine genotyping (PCR–RFLP). The following primers (Macrogen Inc Korea) were used to amplify rs1042031: (forward primer: 5'-TAGGCAAATTGATGATATCGA-3';

reverse primer: 5'-ACCTGGGACAGTACCGTCCCTA-3'. The reaction included 35 cycles of denaturation at 94 °C for five minutes, followed by 40 cycles of 30 s at 94 °C, 30 sec at 54 °C, 30 s at 70 °C, and one cycle of seven minutes at 72 °C and five minutes at 4 °C. BioRad thermal cycler (iCycler, BioRad USA) was used to perform the PCR reaction. The *APOB* gene's polymorphism site produces an EcoR1 restriction site. Thus, the *APOB* polymorphisms were genotyped using the EcoR1 restriction enzyme (Thermoscientific). The PCR product was checked under the ultraviolet gel documentation system (Bio-Rad USA), and the results were recorded. 10% of the samples were re-genotyped by other laboratory staff to increase the genotyping quality and validation; the results were reproducible, and the genotyping was accurate.

Statistical Analysis

The data was entered using Package of Statistical Software version 25. Shapiro-Wilk's statistics checked data distribution. Mean ± SD was taken for quantitative variables. Student t-test was used to compare normally distributed quantitative variables between two groups. ANOVA was used for comparison in more than two groups. Frequency and percentages were used to express categorical variables. The Chi-square test was used for calculating Genotype frequencies. The correlation coefficient was used to see the association between the variables. All tests were two-sided. A p-value < 0.05 was considered statistically significant.

Results

Table 1 shows the baseline characteristics of the study participants. The mean HDL among CAD was 39.75±3.09mg/dl, and non-CAD was 41.31±3.16mg/dl, which was statistically significant ($p<0.001$). On comparison of genotypes with lipid profile, we found a significant association of AA genotype with dyslipidemia. A significant increase in serum cholesterol, LDL, total lipids, and cholesterol/HDL ratio was observed with AA genotype ($p<0.001$) (Table 2). The frequency of the GG variant of *APOB* ECoR1 in CAD was 12 (8.9%), and in controls, 53 (81.5%), GA was 25 (18.5%), and 8 (12.3%), AA was 98 (72.6%) and 4 (6.2%). The genotype frequency differs ($p=0.018$) significantly between the study groups (Table 3). Both groups observed the Hardy-Weinberg equilibrium ($X2<3.84$; $p<0.05$). The major allele frequency in cases and control was 49(18%) and 114(88%) and of the minor allele was 221(82%) and 16(12%) respectively. No significant association of genotypes was observed in the dominant and recessive models.

In logistic regression analysis, GA has an odds ratio of 0.397, while AA has an odds ratio of 2.090 (Table 3). Figure 1 shows the analysis of *APOB* EcoR1 G> A gene polymorphism found using the PCR-RFLP technique; three genotypes are at this locus. The Lane (GG) wild-type homozygote's result showed that it had been digested by a restriction enzyme into 330 base pairs. The restriction enzyme could not digest the (AA) mutant type and the homozygote 181bp and 149bp bands. Lane (G/A) heterozygote's 330 bp, 181 bp, and 149 bp bands were produced after restriction enzyme digestion, as shown in (Figure 1).

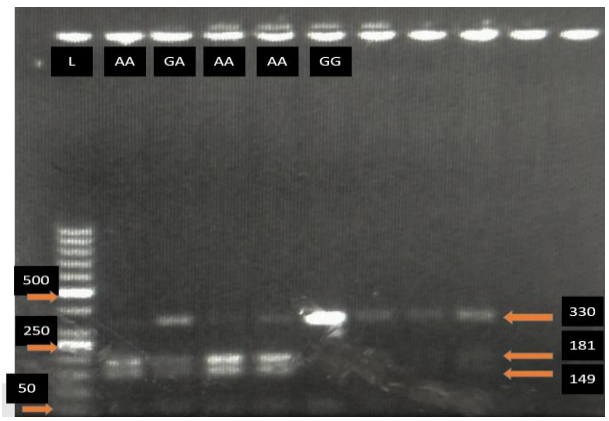

Figure 1: Agarose gel electrophoresis, RFLP-PCR product analysis of *APOB* EcoR1 G>A Gene polymorphism, by EcoRI restriction enzyme.

L: ladder (50-10000bp). Lane (AA) mutant type homozygote, the product undigested by restriction enzyme and still 181bp and 149bp bands. Lane (G/A) heterozygote, the product digested by restriction enzyme into 330bp, 181bp and 149bp bands. Lane (GG) wild type homozygote that showed digested by restriction enzyme into 330 bp band: Electrophoresis condition: 2% agarose gel, Voltage: 110AM.and Time: 45 min.

TABLE 1: CHARACTERISTICS OF THE POPULATION

Parameters	Cases	Control	p-value
Age(years)	50.53±11.70	44.43±13.57	0.018
Systolic BP (mm Hg)	132.79±13.72	123.85±13.07	0.000*
Diastolic BP (mm Hg)	85.77±12.10	89.69±1.21	0.010*
Heart Rate	78.82±4.22	85.00±0.00	0.002*
Serum cholesterol (mg/dl)	179.58±32.64	171.49±30.17	0.085
Serum LDL (mg/dl)	108.98±25.96	102.38±25.58	0.090
Serum HDL (mg/dl)	39.75±3.09	41.31±3.16	0.001*
Serum VLDL (mg/dl)	33.09±5.62	31.94±7.04	0.263
Triglycerides (mg/dl)	163.88±27.81	156.60±36.09	0.164
Cholesterol/HDL ratio	4.48±0.78	4.30±0.81	0.164

Independent sample t-test
$p<0.05$ significant

Table 2: GENOTYPING AND ASSOCIATION WITH LIPID PROFILE IN CASES AND CONTROL

***APOB* rs1042031**	**CAD**				**Non-CAD**			
	GG	**GA**	**AA**	**p-value**	**GG**	**GA**	**AA**	**p-value**
Serum Cholesterol (mg/dl)	169.00 ±1.90	166.50 ±19.78	182.36 ±34.87	0.000*	176.75 ±42.29	176.88 ±31.54	169.47 ±28.16	0.152
Serum LDL (mg/dl)	95.00 ±0.70	89.00 ±16.04	113.06 ±26.49	0.000*	101.33 ±25.66	106.68 ±26.97	101.41 ±25.36	0.396
Serum HDL (mg/dl)	45.00 ±0.00	41.23 ±3.30	40.00 ±1.07	0.000*	38.67 ±4.21	40.40 ±2.74	39.72 ±3.01	0.041*
Serum VLDL (mg/dl)	29.00 ±0.00	37.50 ±4.81	32.71 ±5.61	0.000*	32.33 ±12.37	32.28 ±6.15	31.82 ±6.46	0.737
Total Lipids	684.00 ±0.00	720.00 ±63.61	738.21 ±87.70	0.000*	710.38 ±153.52	725.33 ±96.67	691.83 ±110.29	0.109
Triglycerides (mg/dl)	146 ±0.00	187.50 ±24.05	161.49 ±27.51	0.000*	158 ±63.17	159.40 ±28.34	155.72 ±33.84	0.611
Cholesterol/HDL ratio	3.7 ±0.00	4.15 ±0.59	4.60 ±0.80	0.000*	4.33 ±1.14	4.47 ±0.91	4.25 ±0.75	0.078

***p-value <0.05 significant, ANOVA**

Table 3: GENOTYPING AND ASSOCIATION WITH CAD

Genotypes	CAD	Controls	p-value	Odds ratio(CI)
Co-dominant model				
GG	12 (8.9%)	53 (81.5%)	0.371	1.00
GA	25 (18.5%)	8 (12.3%)	0.348	0.397(0.058-2.737)
AA	98 (72.6%)	4 (6.2%)	0.018	2.090(0.402-10.809)
Allelic frequency				
G	49(18%)	114(88%)		
A	221(82%)	16(12%)		
Dominant model				
AA	98 (72.6%)	12 (18.5%)	0.16	1.00
GA+GG	37 (27.4%)	53 (81.5%)		0.60 (0.29-1.25)
Recessive model				
AA+GA	123 (91.1%)	4 (6.2%)	0.5	1.00
GG	12 (8.9%)	61 (93.8%)		0.67 (0.21-2.17)
Over Dominant model				
AA+GG	110 (81.5%)	57 (87.7%)	0.26	1.00
GA	25 (18.5%)	8 (12.3%)		0.62 (0.26-1.46)

A “Chi-Square” test was utilized to calculate the p-value and “Confidence Interval” (CI).

DISCUSSION

In this study, we aimed to determine the effect of rs1042031 and serum levels of lipids on the risk of CAD in a Pakistani population. Our study showed a significant association of *APOB* rs1042031 with dyslipidemia among CAD patients. The frequency of AA genotype was significantly higher in CAD cases 98 (72.6%) compared to non-CAD group 4 (6.2%). We observed a strong correlation of the AA genotype of *APOB* rs1042031 in CAD cases (p=0.018) with an adjusted odds ratio of 2.09, indicating a 2.09 times greater chance of having CAD with this genotype. A meta-analysis by Chiodini *et al.* showed a greater risk of CAD with EcoR1 polymorphism.[7] Chen *et al*. 2016 concluded that the E-(A) allele might be susceptible to the development of CAD.[13] Hegele *et al*. showed an increase in the frequency of EcoR1 polymorphism in cases. However, no significant association was found between plasma LDL levels.[14] The amino acid glutamic acid is changed to lysine by the *APOB* rs1042031, found in coding region 4181. In multiple meta-analysis reports, Xiao *et al*. 2015, and Zhang *et al*., 2015 concluded that *APOB* rs1042031 mutation may alter the binding affinity between *APOB* and the LDL receptor, causing a buildup of LDL in the circulation and ultimately culminating in coronary disease.[15,16] Belgin S. Duman conducted a study on the Turkish population and found no association in the ECoR1 genotype frequencies between patients and the control group.[9] Ju-Pin Pan conducted a study on the Chinese population and found no association.[10] The influence of the study design, sample size, and ethnicity can be used to account for the discrepancy in results.

In our study, serum HDL was significantly decreased in the AA genotype of *APOB* (p<0.001). A local study by Habib *et al*. found a positive association of *APOB* with lipid profile.[17] Hu *et al*. conducted a study on the Chinese population and reported the A allele carriers to have more dyslipidemic profile than G.[18] In contrast to our findings, a study done by Abaj *et al*., 2022 on 648 Iranian diabetic patients also showed a significant connection between the EcoRI and serum HDL-C. Serum HDL in the GG genotype was 52.96 ± 11.64mg/dl, while in GA, AA genotype was 55.56 ± 15.73 mg/dl (p=0.03). So, the plasma HDL levels in A allele carriers were greater than in G carriers.[19] Protein function and, consequently, lipid levels may be significantly impacted by this polymorphism-induced amino acid change. However, Saha *et al*. found no significant association between plasma lipid levels and ECoR1.[20] The EcoR1 SNP is associated with the substitution of lysine for glutamic acid, which changes the formation and recognition of the LDL receptor binding site and may result in hypercholesterolemia.[21]

Serum LDL was significantly raised with the AA genotype (p<0.001). Our results are in contrast with a prospective study by Benn *et al*. They hypothesized that the AA genotype of the ECoR1 could lower LDL levels and the risk of ischemic stroke.[8] According to Turner *et al*., this gene mutation altered the protein structure of ApoB, making it less able to attach to LDL and LDL receptors and slowing down the pace at which LDL is broken down.[22] This failure to remove ApoB eventually resulted in a buildup of LDL in the plasma. As a result, atherosclerosis would develop more quickly as a result of this dyslipidemia. So, the results of this study could be interpreted by these biological mechanisms. Hence, it could be inferred from the present analysis that the AA genotype (A allele of rs1042031) could confer a risk factor for CAD by altering the lipid profile. Precise molecular pathways and CAD pathophysiology warrant future research with different ethnicities and broad sample sizes.

Limitations

Despite the significant efforts made to test for a potential link between EcoRI polymorphisms and CAD risk, this study has several limitations that should be acknowledged. This study examined one locus SNP (rs1042031) in the *APOB* gene, and it is yet unknown whether other genetic variations affect the risk of CAD. Additionally, it was thought that the combined impacts of several genotypes would serve as risk factors more effectively than a single locus gene. However, the study's strength is the lack of similar data from Pakistan in the literature. A larger sample size and multicentric study should focus on more genetic analysis via whole genome sequencing, as ours is a pilot study providing the foundation for future studies.

Conclusions

This study demonstrated a close relationship between *APOB* rs1042031 polymorphism and CAD in the Pakistani population. Having the A allele causes 2.09 times the genetic risk of CAD by altering lipid profile. Personalized medicine may be designed to meet the patients' physiological conditions, depending on their genotype, which can help manage the disease safely and efficiently.

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Author Contributions:

Hira Sohail: Sample collection, Experimental laboratory work, Data entry, and manuscript writing.

Madeeha Shahzad Lodhi: Supervised research, Statistical analysis and interpretation of data, Review and final approval of the manuscript.

Saba Khaliq: Review the statistical analysis, supervised research, and Experimental laboratory work.

Farhat Ijaz: Study design, Review, and final approval of the manuscript.

Qurban Ali: Formal analysis, Methodology, Resources, Writing – review & editing

Muhammad Arshad Javed: Formal analysis, Methodology

Peng Jin-liang: Investigation, Writing – review & editing

Qiu Chong-Rong: Project administration

All the authors are responsible and accountable for the accuracy or integrity of the work.

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