RESEARCH ARTICLE

Study of the Association of PCSK9/Eam1104I Gene Polymorphism with Plasma Lipid Concentration and CAD in West Bengal Population

Santanu Maiti[1], Pranamita Biswas[2], Sukanya Banerjee[2], Nandan Kumar Jana[3]*

[1]B.Tech, Department of Biotechnology, Heritage Institute of Technology, Kolkata, West Bengal, India
[2]M.Tech, Department of Biotechnology, Heritage Institute of Technology, Kolkata, West Bengal, India
[3]Assistant Professor, Department of Biotechnology, Heritage Institute of Technology, Kolkata, West Bengal, India

***Address for Correspondence:** Dr. Nandan Kumar Jana, Assistant Professor, Department of Biotechnology, Heritage Institute of Technology, 994, Chowbaga Road, Anandapur, Kolkata-700107, West Bengal, India

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ABSTRACT- Coronary artery disease (CAD) is suspected as a leading cause of mortality in developed countries. Due to cholesterol and fat deposit plaque is forming into the inner walls of the arteries of the heart, which leads to narrowing of blood vessels of the heart and reduce the blood flow rate into heart. Proprotein convertase subtilisin-like kexin type 9 (PCSK9) is one of the candidate gene that regulate lipoprotein retention pathway of CAD development. It is a newly discovered serine protease that plays a key role in LDL-C homeostasis by mediating LDL receptor (LDLR). The LDL receptor is breakdown through a post transcriptional mechanism and induces the production of very low-density lipoprotein in the fasting state. The aim of this study was to investigate the frequency of single nucleotide polymorphism (SNP) of the PCSK9 gene of 155 CAD patients and 102 ages matched healthy controls. Serum lipids, including total cholesterol (TC), triglycerides (TG), HDL, LDL, and VLDL were analyzed. PCR-RFLP analysis was carried out in genotype regions carrying Eam1104I restriction site in the PCSK9. Gene considers significant difference in serum TC, TG, HDL-C, LDL-C and VLDL-C levels ($P<0.001$, <0.0001) of patients and control samples. In CAD patients, the G allele frequency was less than A allele frequency. G allele is responsible for decreasing the LDL: HDL ratio, which had shown that evidence having its protecting effect on the occurrence of CAD in West Bengal Population.

Key-words- CAD, Eam1104I, Polymorphism, PCSK9, SNP, West Bengal population

INTRODUCTION

The human circulatory system or the cardiovascular system circulates blood and other essential nutrients throughout the body with the help of various blood vessels. CAD is one of the most common type of disease that is related to this system [1]. According to a WHO report, 7.3 million people die from coronary heart disease every year, accounting for approximately 13% of global deaths [2]. The scenario in India is amongst the worst making it the "coronary heart disease capital of the world". This has led to the growing demand for additional tools to help clinicians identify the "vulnerable" patient at risk for CAD.

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So we need to develop highly potential diagnostic and therapeutic techniques which will efficiently help to reduce the number of death worldwide. Before doing this we need to know the exact pathology of CAD. The disease is caused by plaque building up along the inner walls of the arteries, making them narrow and rigid due to stenosis, which restricted blood flow to the heart. The heart becomes starved of oxygen and the vital nutrient that is really needed to pump properly [3]. There are several mechanisms in the body that leads to the plaque formation in the arteries. These are (i) Lipoprotein retention, (ii) Endothelial dysfunction, (iii) Immune, and the inflammation response of the artery, (iv) Vascular smooth muscle cell (VSMC) proliferation, (v) Lipid absorption by macrophage and the formation of foam cells and, (vi) Platelet activation, and thrombosis [4,5]. From these mechanisms, it could be said that CAD can be formed of multiple gene-gene and gene-environment interaction [6]. Because of this many research is going on to identify the inherited risk factors for CAD [7]. But the genetic mechanism is still not properly known [8]. So to

know the genetic role behind the development of the disease now a day many experimental strategies are developed. Such as genome wide linkage scan, global microarray gene expression analysis, proteomics etc. These strategies are applied the studies of CAD [9].

Recently, many genomic regions and many variants in many genes have been implicated as markers of greater susceptibility to develop CAD. Proprotein convertase subtilisin/kexin type 9 or PCSK9 genes, one of the well established genetic biomarker for polymorphisms. PCSK9 gene is a newly discovered serine protease gene. It plays a key role in LDL-C homeostasis by the breakdown of LDL receptor (LDLR) through a post transcriptional mechanism. Human PCSK9 gene consists of 12 exons with the length of 22 kb and it was mapped to chromosome 1p32. This gene produces a3636 bp mRNA which encodes a 692 amino acid glycoprotein. This gene is expressed mostly in the liver, kidney, and small intestine [10-12]. This gene may also regulate lipoprotein production that contains apolipoprotein-B (ApoB) and its help to secrete the ApoB protein. In the fasting state this secretion is promoting the production of very low density lipoprotein [13-16]. Several studies have found that due to missense mutations the activity of PCSK9 gene is increasing (i.e., gain-of-function mutations) that's result the increase of LDL-C levels and CAD [17]. But when nonsense mutations are occurring, then the activity of PCSK9 gene is reduced (i.e., loss-of-function mutations) that's result the lowering of LDL-C levels and reducing the risk of CAD [18]. So this gene is reported to their association with CAD and/or high lipid levels in different populations round the globe, but not a single report found till date in the case of the West Bengal population.

The present study was designed to analyze the association of Eam1104I DNA polymorphism in the PCSK9 gene locus with plasma lipid concentrations and CAD in West Bengal population. We have tried to identify a potential genetic marker that can be used to infer the abnormal lipid levels and predict the occurrence of CAD.

MATERIALS AND METHODS

Blood Sample Collection

Angiographically proven 155 blood samples were collected from the department of Cardiology, R.G Kar Medical College & Hospital and NRS medical collage & Hospital Kolkata as well as a Blood donation camp organized by the department of Biotechnology, Heritage Institute of Technology, Kolkata, India. The 102 controls were subjected to treadmill test to be sure that they were not suffering from any coronary disease. Further, all control subjects with hypertension, diabetes and endocrine or metabolic disorders were excluded from the control group. For this study, we got the approval of the ethical committee from Heritage Institute of Technology, Kolkata, WB, India.

Human Genomic DNA isolation from Blood

Genomic DNA was extracted from a corresponding frozen blood sample using HiPura™ Blood Genomic DNA Miniprep Purification Spin Kit (MB505) of HIMEDIA slightly modified [19] and DNA was visualized by 1% Agarose Gel Electrophoresis (AGE) with Lambda HindIII ruler.

Polymerase Chain Reaction (PCR)

The isolated genomic DNA was used in PCR reaction to amplify a particular sequence using following set primers designed by Aung *et al.* [20]. The sequences of forward and reverse primer were mentioned in Table 1.

Table 1: Primers for PCSK9

Primer	Sequence
Forward primer ($T_{m=}$ 56.8^{0}C)	5'-CACGGTTGTGTCCCAAATGG-3'
Reverse primer ($T_{m=}$ 57.3^{0}C)	5'-GAGAGGGACAAGTCGGAACC-3'

Each amplification reaction was performed in a total volume of 25µl containing 1µl of forward and reverse primer, 2.5µl of 10X PCR buffer, dNTP mix 1.5µl, Double distilled water 16.5µl, Taq DNA Polymerase 0.5µl and Genomic DNA 2µl. After Initial denaturation at 95˚C for 3 min was followed by 30 cycles of denaturation at 95˚C for 30 sec, annealing at 56˚C for 30 sec and extension at 72˚C for 1 min, with a final extension at 72˚C for 7 min.

Then the PCR product was visualized in 2% Agarose Gel Electrophoresis with 100bp DNA ladder under ultraviolet light.

RFLP Analysis

For the RFLP Eam1104I enzyme was used for PCSK9 to digest the respective PCR products at 37˚C overnight, which was then visualized on 2.5% agarose gel electrophoresis (AGE) with 100bp DNA ladder to identify the genotype [20].

Lipid-profile analysis

Serum lipids, including total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL) were determined by enzymatic methods with commercially available kits ERBA Mannheim Cholesterol Liquid Stable Reagent CHOD-PAP End Point, Triglycerides estimation kit (ENZOPAK) and ERBA Mannheim HDL Direct Liquid Stable Reagent respectively. The VLDL cholesterol and LDL cholesterol were calculated using the William Friedewald's formulae [21].

STATISTICAL ANALYSES

The statistical calculations in this study were performed using Graphpad Quickcals (http://www.graphpad.com/quickcals/ttest1.cfm) and one way ANOVA software (http://vassarstats.net/anova1u.html). Two types of analysis were done using this software's. Unpaired Student t Test (two tailed) and One Way ANOVA. Epidemiological data were recorded on a pre-designed form and managed with Excel software. All statistical analyses were done with the

Graphpad Prism 5.0. For each variable, the values were expressed as mean±SD. Data was evaluated by student's t test and one-way Analysis of Variance (ANOVA) followed by Tukey's multiple comparison tests. Allele and genotypic frequencies were calculated with the gene counting method. "p" value<0.05 was considered significant.

RESULTS AND DISCUSSION

Genomic DNA isolation

After the isolation of genomic DNA, according to the procedure previously described by Aung *et al.* [19], 1% Agarose Gel Electrophoresis (AGE) was run to check the presence of DNA in the eluted solution. The genomic DNA was seen in 1% AGE (Fig. 1). The genomic DNA band is visible above Lambda Hind III.

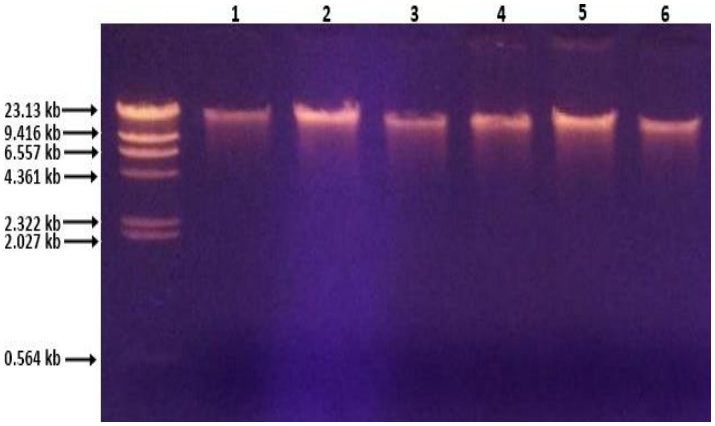
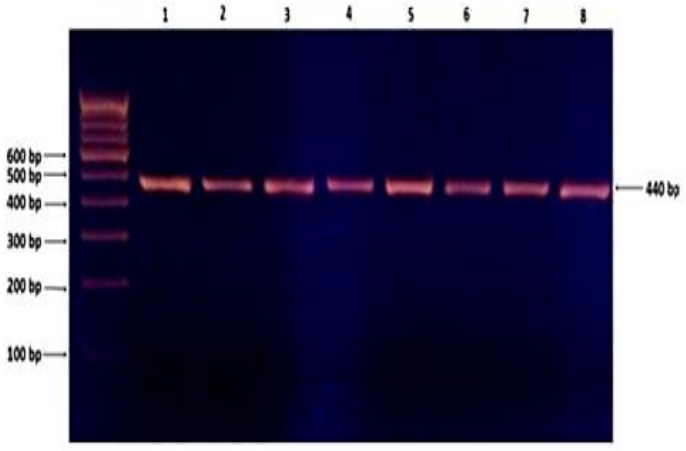


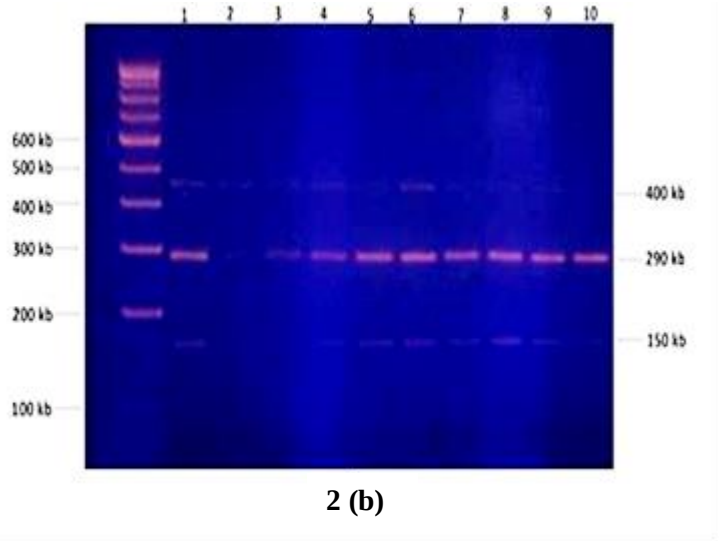
Fig. 1: Genomic DNA for blood for (sample 1-6) run on 1% gel with lambda/HindIII ruler as control

PCR amplification and RFLP analysis

After the PCR reaction was done in the presence of the amplified DNA were checked by running 2% AGE. The size of the amplified specific segment for PCSK9 gene was 440 bp in size Fig. 2 (a). The gene specific PCR products were then digested by specific restriction endonuclease and analyses of the restriction fragment length polymorphism were done by running 2.5% AGE Fig. 2 (b).



2 (a)



2 (b)

Fig. 2 (a): Amplified DNA product of PCSK9 gene for (sample 1-8) on 2% AGE with 100 bp ladder as control **(b):** PCR-RFLP: Eam1104I Digested PCR DNA product of PCSK9 gene (for sample 1-10) on 2.5% AGE with 100 bp ladder as control

For PCSK9 gene, genotyping was done based on the absence of Eam1104I cut site (G) and presence of cut site (A). When the cut site of Eam1104I is present in both alleles (AA), it produced two bands of size 150 bp and 290 bp. When there is no cut site of Eam1104I gave only one band (GG) of the size 440 bp. When the cut site is present in one allele (AG) it produced three bands of size 150 bp, 290 bp and 440bp. In Fig. 3, two bands (AA) were observed for sample number 8, 9, and 10, three bands (AG) were observed for sample number 1, 3-6 and one band (GG) was observed for sample number 2. The total numbers of all three types of genotypes were counted by analyzing all the subjects 155 patients and 102 controls for PCSK9 gene.

Genotyping on the basis of RFLP analysis

For PCSK9 gene, in Table 2 A allele frequency was 0.838 and G allele frequency was 0.162 in the control group, whereas in the patient group the values were 0.642 and 0.358 respectively.

Table 2: PCSK9/Eam1104I genotype and allele frequencies in patient and control groups

Genotype *	Patient's n (%) n= 155	Control n (%) n= 102
AA	48	72
AG	103	27
GG	4	3
Allele Frequency *		
A	0.6415	0.8375
G	0.3585	0.1625

***P<0.0001 between the Two groups**

The distribution of PCSK9/ Eam1104I genotypes/alleles frequencies among healthy subjects and hyperlipidaemia patients showed that in the control group, 70 percent (72 of 102) were homozygous for A and 2.9 percent (3 of 102) for G allele, and also the patient group the values were 30 percent (48 of 155) and 2.5 percent (4 of 155) respectively. There was a significant difference in the frequency distribution between the two groups (P<0.0001) (Table 2). The distribution of genotypes observed in both the groups was in Hardy-Weinberg equilibrium.

Lipid Profile Analysis

Table 3 were shows that there is a highly significant difference in TG, TC and LDL (all p value is 0.001) and significant difference in HDL and VLDL (p value= 0.0455, p value=0.0142) when compared between control and the patient. TC, TG, HDL-C, LDL-C and VLDL-C levels were significantly (P<0.001, <0.0001) higher in patients compared with control. Age and sex were similar in two groups (Table 3).

Table 3: Comparative analysis of Lipid Profile data between control and patient for PCSK9 polymorphism

	Patients (n=155)	Control (n=102)	Significance level (p-value*)
TC (mg/dl)	189.32±51.88 *	165.16±43.36	0.0001
TG (mg/dl)	152.49±56.9 *	123.93±42.26	0.0001
HDL-C (mg/dl)	49.06±6.14 **	45.235±9.726	0.0455
LDL-C (mg/dl)	110.42±49.23 *	89.43±31.56	0.0001
VLDL-C (mg/dl)	29.83±10.088 *	26.32±10.27	0.0142

***p value≤ 0.05 are considered to be statistically significant**

The serum lipid levels in normalipidaemic and hyperlipidaemic subjects according to PCSK9/ Eam1104I genotype are shown in Tables 4 and 5 respectively. In both groups, the plasma HDL-C was higher in the GG genotype than in the AA and AG genotypes, whereas the plasma LDL-C was higher in the AA genotype than in the AA and AG genotype. Other parameters such as TC, TG and VLDL-C were not significantly different between various genotypes in normal individuals. In the patients group, TC level was lower in GG genotype than in the AA and AG genotypes. Other parameters such as TG, VLDL-C was not significantly different between various genotypes in patient.

Table 4: Lipid levels in control group according to PCSK9/ Eam1104I genotype

	AA	AG	GG
N	**72**	**27**	**3**
TC (mg/dl)	171.26±45.97	150.19±34.34	153.33±2.5166
TG (mg/dl)	129.68±44.32	110.63±35.46	105.667±4.509
HDL-C (mg/dl)	45.33±10.035	44.148±8.254	79±14.74
LDL-C (mg/dl)	92.48±31.245	82.89±33.34	75±3.6
VLDL-C (mg/dl)	28.24±10.87	22.04±7.154	19±3

Values are Mean ± SD

Table 5: Lipid levels in patients group according to PCSK9/ Eam1104I genotype

	AA	AG	GG
N	**48**	**103**	**4**
TC (mg/dl)	189.39 ± 50.016	191.56 ± 52.49	130.75 ± 23.77
TG (mg/dl)	146.46 ± 68.35	157.37 ± 50.56	99.25 ± 30.24
HDL-C (mg/dl)	50.31 ± 7.69	48.38 ± 5.3	51.75 ± 2.06
LDL-C (mg/dl)	121.6 ± 47.76	111.82 ± 50.106	60.25 ± 18.025
VLDL-C (mg/dl)	27.48 ± 9.22	31.36 ± 10.15	18.75 ± 7.37

Values are Mean ± SD

In One way ANOVA, Table 6 only TC values were shown statistically significant in all three genotypes in male patients. In Table 7 only HDL-C values show statistically significant in all three Genotypes in female patients.

Table 6: Comparative analysis of different genotypes of Male patients

	AA	AG	GG	p-value*
TG	145.95±71.28	156.67±48.53	99.25±30.23	0.105
TC	188.47±46.43	191.11±50.32	130.75±23.76	0.05
HDL	50.26±8.03	49.046±5.37	51.57±2.06	0.45
LDL	110.76±44.98	110.77±47.92	60.25±18.02	0.105
VLDL	27.47±9.44	31.29±9.91	18.75±7.36	0.009

***p value≤ 0.05 are considered to be statistically significant**

Table 7: Comparative analysis of different genotypes of Female patients

	AA	AG	p-value*
TG	150 ±47.29	161.125± 67.11	0.69
TC	195.83±76	194±64.89	1.0
HDL	50.67±5.08	44.75±2.93	0.002
LDL	117.5±67.31	117.56±62.13	1.00
VLDL	27.5±8.16	31.68±11.74	0.43

***p value≤ 0.05 are considered to be statistically significant**

In Fig. 3 (a) comparative analysis was done in the patient group. Here also seen that HDL-C level was higher in GG genotype than AA and AG genotype. Whereas in LDL-C level was much higher in AA genotype than AA and AG genotype. In Fig. 3 (b) also comparative analyses was done in control group. Here also seen that HDL-C level was much higher in GG genotype than AA and AG genotype. Whereas in LDL-C level was much higher in AA genotype than AA and AG genotype.

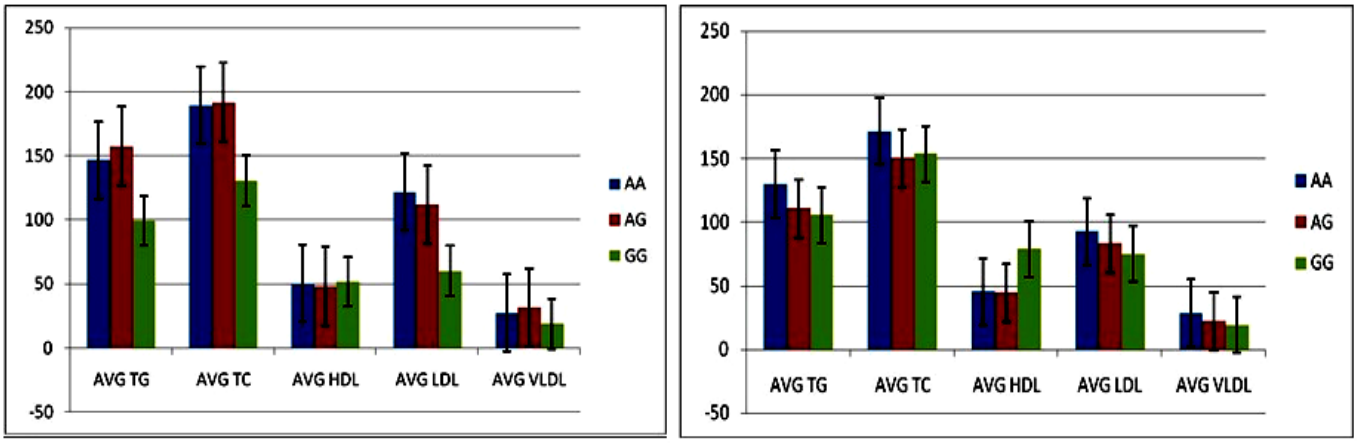


Fig. 3: (a): Comparative analysis of different genotype of patient, **(b):** Control

The purpose of this investigation was to study the Eam1104I polymorphism of PCSK9 gene in West Bengal subjects with and without primary combined hyperlipidaemia. Lipids and lipoproteins metabolism in humans may be controlled by many genes. PCSK9 gene plays very important role among them. The PCSK9 gene polymorphism can result in the lipid metabolism disorder and thus cause the hyperlipidemia which is closely related to the occurrence and development of CAD [22]. We were found that the heterozygous AG genotype being the most common in the patient group of selected population. Allele frequency was significantly higher in both patient and control subjects and the G allele frequency also higher in patients than control subjects. There are no such studies has done previously about this PCSK9 gene on coronary artiery disease. The study of Zhang *et al*. [23] conducted in the Sichuan region showed that G allele frequency of patients in CAD group is higher than that in non-CAD group and another study was done previously by Aung *et al*. [20] in Chinese population based on Drinker and Non Drinker that means they drink alcohol or not. We know from their study that the genotypic and allelic frequency of PCSK9 gene was same between non-drinkers and drinkers (P>0.05 for each). In the case of AA genotype non-drinkers were higher serum LDL-C levels than the AG genotype, whereas in drinkers TC levels was higher in AG genotype than the AA genotypes (P <0.05 for each). The effects of alcohol consumption on TC and LDL-C levels are depended upon genotypes. In their study AA genotype had lower serum TC and LDL-C levels in drinkers than in non-drinkers [19]. In our study similar type of result found that HDL-C level was much higher than LDL-C level in GG genotype than AA and AG genotype in control subject, whereas in patient group HDL-C level was higher than LDL-C level in GG genotype than AA, AG genotype. So it's clear that whenever A allele to G allele conversion is occur, then HDL-C level was higher than LDL-C level. We were validating of our result by one way ANOVA also and in female patients HDL-C values shown statistically significant in all three genotypes. So from this it's proved that in female patients its effect is protective.

CONCLUSIONS

The PCSK9 gene mutation may lead to a change in its normal function and thereby affecting the expressions of a series of metabolite related to it. HDL-C level is higher than LDL-C level in both patient and control subjects in GG genotype than AA and AG genotype that means this genotype may prevent atherosclerosis disease. Also this G allele is responsible for decreasing the LDL: HDL ratio, which shows evidence of having its protecting effect on the occurrence of CAD in West Bengal Population. Due to protective nature of PCSK9 gene this PCSK9/Eam 1104I polymorphism is not a better biomarker for West Bengal Population.

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