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Research Article

Expression of e4 Mutant *APOE* Gene in a select South Indian Population Indicates Relation to Coronary Artery Disease

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Abstract

The polymorphic human Apolipoprotein E (*APOE*) gene encodes 3 common epsilon (ε) alleles (ε2, ε3, and ε4), reported to influence the risk of Alzheimer's disease (AD) and cardiovascular diseases. Studies indicated that individuals with the e4 version of APOE gene is associated with higher cholesterol levels. The genetic architecture of coronary artery disease (CAD) is largely determined by the effects of genetic variants, yet genetic detection is not usually followed in CAD. There have been no prospective studies so far in India. The present study attempted to evaluate the correlation between the expressions of *APOE* gene in a selected south Indian sample. Genetic detection among the CAD patients to screen for associations between *APOE* and CAD by polymerase chain reaction, (PCR) followed by evaluation of the mRNA expression of the e4 variant of the gene by the reverse transcriptase-polymerase chain reaction (RT-PCR). A significant association of *APOE4* gene variant to CAD was observed in the studied subjects. Twelve out of fourteen cases and one out of thirteen controls carried the *APOE e4* variant. Confirmation of *APOE4* mRNA expression in the CAD patients by RT-PCR implied that all the CAD cases transcribed mRNA of the gene in study, suggesting a potential candidature of the *APOE e4* allele in CAD pathogenesis. To conclude, expression of *APOE4* variant is a significant predictor of CAD in a sample of south Indian people. The findings reveal a possibility of using the expression of the *APOE4* variant as prognosis in suspected CAD patients when confirmed by studies on larger cohorts or populations.

Keywords: Cardiovascular; CAD; Allele Variant; Epsilon; RT-PCR

Introduction

Coronary artery disease, abbreviated as CAD, also known as coronary heart disease [CHD] characterized by cholesterol build up on the inner walls of arteries called as atherosclerosis [1], is a complex disease with multifactorial etiologies. It is well established that development of CAD is influenced by either genetic mutations or environmental factors or by gene-environment interactions through biological pathways or networks [2] and has rapidly evolved as a leading cause of mortality and morbidity worldwide [3]. Factors including age, arterial hypertension, dyslipidemia, diabetes mellitus, smoking, and poor diet have been associated with high risk for the disease [4,5] eventually leading to clinical events such as myocardial infarction (MI), ischemic heart failure, and cardiac death [6,7]. Along with these factors, studies reported obesity, low-density lipoprotein (LDL) cholesterol, increased plasma triglycerides (TG), and decreased plasma high-density lipoprotein (HDL) cholesterol are significant independent risk factors for CAD [8].

Epidemiological and family studies reported that host genetics accounts for 40% to 60% of the risk [9]. Studies indicated the lipid-associated genes and its variants have effects on lipoprotein metabolism, atherosclerosis and CAD, holding links between circulat-

ing levels of plasma lipids [LDL-C, HDL-C, and TG] and the disease [10,11], which can also be heritable risk factors for cardiovascular disease [10,12]. As genetic studies can provide novel insights into the disease pathogenesis, a focus of keen investigation on *APOE* [apolipoprotein-E] gene, involved in lipoprotein metabolism, has arisen.

APOE, located on chromosome 19 at position q13.2, near the genes for Apo C-I and C-II, codes for a major apoprotein of the chylomicron [13]. The human *APOE* gene is polymorphic, encoding one of three common epsilon [ε] alleles [ε2, ε3, and ε4] and the three isoforms viz, *APOE2*, *APOE3*, and *APOE4* which give rise to six different genotypes [14] and each isoform differs in their binding affinities to low density lipoprotein receptors, and lipoprotein particles [15]. As *APOE4* allele is associated with hyperlipoproteinemia, higher total and low-density lipoprotein cholesterol levels, it imparts cholesterol-raising effects in atherosclerosis and premature cardiovascular diseases (CVD) [13,15]. Studies have reported the *APOE* polymorphisms in cardiovascular and cerebrovascular disease with inconsistent results [16].

Evident from the earlier candidate gene studies that the functional effects of *APOE* polymorphism on the hepatic binding and triglyceride rich lipid binding and its metabolism plays a central role in the lipoprotein transportation and many processes on the arterial wall [17]. It becomes very obvious that the risk for atherosclerosis and other heart diseases increases with age and can be marked by presence of altered lipid metabolism which can be linked to *APOE* polymorphism. The *APOE4* allele [Arg-112 and Arg-158] with *e4/e4* genotype has been linked to atherosclerosis [13,18]. Therefore, a need to study the relationship between *APOE4* variant and the risk of CAD has become very important.

Studies on role of *APOE* gene in cardiovascular diseases are very limited especially in Indian population. A meta-analysis revealed a strong association of *APOE4* mutation with the increased risk of CHD, indicating a prevalence of approximately 30 - 56% in Indian population [19]. A study on 195 Indian cases of acute myocardial infarction exhibited *APOE3/E4* genotype that found affecting LDL and HDL cholesterol levels, both of which contribute to premature atherosclerosisis [20]. The imperative role of this genetic variant was later supported, when 193 cases of diagnosed coronary angiographed Punjabi (North West India) population exhibited significant association of *E3/E4* genotype with CHD [21] followed by another study on 200 coronary angiographed CAD patients of Kashmiri origin which again, revealed that *APOE* [*ε4*] allele is associated with increased risk of disease along with high LDL and total cholesterol [22]. These studies indicated that *APOE* (ε4) allele polymorphism play a vital role in cardiovascular disease pathogenesis. Thus, a necessity has been emerging for in-depth evaluation on *APOE* gene wherein, a genetic revelation can lead to early detection for CAD in person with family history or other risk factors, especially in Indian population.

Research questions raised on how to explain the contribution of *APOE4* to coronary heart disease risk, as *APOE* is known as a major regulator of blood lipid levels in humans [23] and the underlying mechanism of *APOE4* on CAD pathogenesis is not clearly established yet. Studies, so far, hold important findings on genotype/allele distributions, effects of *APOE* genotypes on clinical features and metabolic parameters such as plasma lipid and lipoprotein concentration in patients. It should be considered, here, that solitary presence or absence of the allele/genotype alone cannot add to CAD pathogenesis. The expression of this dynamic gene variant is equally essential and required.

Aim of the Study

The present study investigated the mRNA expression of *APOE4* variant in the patients, aiming to signify the candidature of the gene polymorphism (*e4* variant) towards the risk of CAD, which further may lead to AD and other severities.

Materials and Methods

Study population

The study enrolled a total of 27 subjects (14 were CAD patients and 13 were normal participants) of age between 38 - 65 years, on obtaining an informed consent, attending OPD at Heart Care Clinic, Vishakhapatnam, Andhra Pradesh. These patients had different states of CAD with high levels of blood cholesterol (above 200 mg/dL). Individuals with normal cholesterol levels (less than 190 mg/dL) were considered as the control group in the present study.

Sample collection

The participants were recruited from the inpatient service of the Heart Care Clinic, Vishakhapatnam, from July 2020 to September 2020. Blood samples were drawn from ante cubital vein and transferred to EDTA (5 mg/ml) tubes, from all patients on admission to the coronary care unit. The samples were transported to the lab by maintaining cold chain. The EDTA samples kept at 4°C,

was used within 24 hours for DNA extraction and subsequent PCR analysis.

DNA extraction

The human genomic DNA from whole blood was isolated within 24 hours of collection using salting method by Miller., *et al*. 1988 [24]. RBC Lysis buffer with proteinase K was used followed by purification using phenol chloroform isoamyl alcohol. Finally, DNA was precipitated using ice cold 95% ethanol, centrifuged and then resuspended in Tris EDTA Buffer. DNA isolation Then the quality of the isolated genomic DNA was determined by running each sample on ethidium bromide-stained 1.0% agarose gel. DNA purity were checked by determining ratio of absorbance at 260nm to 280nm.

PCR amplification of the *APOE* gene

The presence of *e4* variant of *APOE* gene in the isolated genomic DNA was detected by PCR amplification of 3553 bp amplicon of the 3598 bp gene using thermocycler (Bio-Rad). Primers were self-designed using primer quest tool from integrated DNA technologies. The primers used in the PCR are: Forward primer: 5'-CGGTGAGAAGCGCAGTC-3' and the reverse primer: 5'-TTTCTAAGTGGTTCAAAGTGCG-3'.

Each PCR reaction mixture contained 25 µl of 2X PCR master mix, 2 µl of the prepared DNA template, 2 µl each forward primer and reverse primer, 19µl PCR grade water (nuclease free) to make a total volume of 50 µl. Negative control [with water instead of the DNA template] was included in each reaction. The microfuge tubes were then placed in a thermal cycler for PCR amplification. The reaction conditions for initial denaturation at 95°C was for 2 minutes with 35 cycles of denaturation at 94°C (30 seconds), annealing at 57°C (30 seconds) and extension at 72°C (3 minutes). The final elongation was at 72°C for 10 minutes. The 3553 bp amplicons were stored at 4°C until analysis. The products were analysed by electrophoresis on 1% ethidium bromide-stained agarose gel. The size of the amplicon was estimated by comparing it with a DNA molecular size marker of 100 bp ladder (Merck Biosciences).

Isolation and Purification of mRNA

RNA isolation was done by TRIzol™ reagent method as suggested by Piotr Chomczynski and Nicoletta Sacchi [25] with some changes. Leucocytes were separated from whole blood by repeated treatment with Erythrocyte Lysis Buffer. The pellets were obtained after centrifugation followed by Trizol extraction of RNA. The matured mRNA was purified using Polythymidyl columns (Oligotex mRNA mini kit, *Qiagen)*. The mRNA was absorbed because of presence of poly-A tail, separating them from other RNA content like t-RNA or rRNA.

Reverse Transcriptase PCR: The RT-PCR was performed for detection of 711bp

mRNA of *APOE4* variant using following primers. The forward-Primer (5'-3'): ACGAGACCATGAAGGAGTTGA and the reverse primer (5'-3'): ACGGGTCGCTGTTAGTGA. The First strand synthesis was accomplished as the reaction components (5 µl of RNA, 3 µl of Primer-R and 28 µl of DEPC water) were mixed and incubated at 65°C for 10 min in a fresh 1.5 ml vial, followed by ice storage.

Reverse transcriptase reaction

The reverse transcription reaction was carried out by incubating the reaction components (5 µl of 10X-RT buffer, 1 µl of RNase inhibitor, 2 µl of dNTPs, 5 µl of 100 mM DTT, 1 µl of Reverse transcriptase) at 37°C for 1 hour followed by boiling for 5 min at 94°C. For the negative control, the reaction components were same except reverse transcriptase was not added. The synthesized first stand was stored in ice.

For the cDNA synthesis, 50 µl PCR reaction mix was prepared that included 2µl of cDNA, 2 µl of each forward and reverse primer, 2 µl dNTPs, 5 µl of 10X Taq Assay buffer, 1 µl of Taq DNA polymerase, 36 µl of PCR grade water. The reaction conditions for initial denaturation were at 94°C for 2 minutes. After 30 cycles of denaturation at 94°C (30 seconds), annealing at 57°C (30 seconds) and extension at 72°C (30 seconds), the final elongation was at 72°C for 2 minutes. The amplified product (705 bp cDNA) was analysed by 2% agarose gel electrophoresis under UV light.

Results and Discussion

The outcomes of the genetic detection of *APOE4* in the studied cohort from Andhra Pradesh by PCR followed by the mRNA expression of the e4 variant of the gene by RT-PCR are summarized in table 1. The study sample consisted of a total 27 participants (fourteen CAD patients and thirteen controls), which showed significant expression of the gene variant in CAD group compared to the control participants.

Sample ID	Study Group	Presence of APOE4 gene (PCR)	APOE4 mRNA Expression (RT-PCR)
1	Diseased	+	+
2	Diseased	+	+
3	Diseased	+	+
4	Diseased	+	+
5	Diseased	+	+
6	Control	-	Not Performed
7	Diseased	-	N/A
8	Diseased	-	N/A
9	Control	-	Not Performed
10	Control	-	Not Performed
11	Control	-	Not Performed
12	Control	-	Not Performed
13	Diseased	+	+
14	Control	-	Not Performed
15	Control	-	Not Performed
16	Control	-	Not Performed
17	Diseased	+	+
18	Diseased	+	+
19	Control	-	Not Performed
20	Diseased	+	+
21	Control	-	Not Performed
22	Control	-	Not Performed
23	Control	-	Not Performed
24	Diseased	+	+
25	Diseased	+	+
26	Diseased	+	+
27	Control	+	-
Summary:			
APOE e4 mRNA		**CAD Group (N)**	**Control Group (N)**
Expression		12	1 (sample:27)
No Expression		2 (Sample:7 and 8)	12
Total Subjects		14	13

Table: 1: *APOE (e4)* gene expression among the cases and the control groups.

"+" Indicates presence of *APOE4* gene and mRNA expression of the gene variant;

"-" Indicates absence of the *APOE4* gene and no expression of the gene variant. N indicates the number of cases.

Presence of *APOE gene* e4 variant

The presence of *APOE e4* gene was confirmed by successful amplification of the gene length of size 3553 bp. Among the diseased samples, twelve subjects out of fourteen, showed expression of *APOE e4* gene. Two diseased samples (sample ID: 7 and 8) showed no presence of *APOE e4* variant, indicating the disease condition might be due to other related genetic factors such as *LDLR, APOA1* gene, or other physiological variables or it could be due to some environmental factors or interaction of both genetic and environment, given that it is a multifactorial disorder. On the contrary, in the control group, only one sample (sample ID: 27) out of total thirteen samples were found carrying the *APOE e4* variant gene. The present study reported the presence of e4 variant form of *APOE* gene among the diseased subjects whereas none of the subjects in the control group showed its presence.

Expression of *APOE e4* variant mRNA in diseased samples

Amplified reverse transcriptase PCR product (705bp) separated on agarose gel, shows mRNA expression of the *APOE4* gene. In the study, twelve subjects exhibited *APOE e4* gene expression, which indicated that the twelve diseased cases were transcribing *APOE e4* variant gene, however, one control sample that carried the inactive form of *APOE e4* gene did not express the gene; thus, confirming active role of *APOE e4* variant gene in coronary artery disease.

CAD is a chronic progressive disorder with high incidences of disability and mortality around the world as a result of interaction of genetic and environmental factors [26]. The *APOE e4* allele has also been linked to increased plasma LDL-C concentrations in normolipidemic subjects [27] earlier, which justifies the present finding that one participant from the control group exhibited the *APOE4* amplification. Studies indicated the *APOE* variants affect the plasma lipid concentration, thereby, increasing the risk of developing CAD through enhanced vascular inflammation [16,28,29], which owes another explanation for the findings in the present study.

Notably different population around the world exhibit different frequencies of *APOE* isoforms distribution [19,27,30], correlating to CAD and lipidemias. Ranjith., *et al*. 2004 examined the association of lipoprotein (a) (Lp (a)) and *APOE* polymorphisms and reported *APOE e3/e4* genotype is strongly associated with the incidence of myocardial infarction in South African Indian patients [20]. Singh., *et al.* 2008 investigated *APOE* polymorphism in the

northwest Punjab population by polymerase chain reaction [PCR] and observed *e3/e4* genotype with CHD [21] to which Graner., *et al*. 2008 indicated that *APOE4* isoform had an increased and more severe CAD status than that of patients with the *APOE3* isoform [31]. Afroze., *et al*. 2015 studied a strong association of the ancestral *APOE4* allele with increased risk of CAD and increased levels LDL and total cholesterol in Kashmiri population [22]. Study by Hou., *et al*. 2020 reported prevalence of 11.72% *APOE allele e4* among CAD patients and 8.46% among control participants from south china population, suggesting *APOE* is a potential susceptibility locus for CAD [16]. The *e3* allele of the gene is usually considered as the normal one with a high frequency but in contrast, the *e4* allele could be an important indication for evaluating the cardiovascular disease risk [32]. Association of *e4* allele with CAD further gets strengthen with conditions like type 2 diabetes, smoking, obesity and high oxidative pressure as *e4* allele has influence on lipid profiles [27,33]. The significant mRNA expression of *APOE4* in CAD patients from the present study supports the findings from the previous work on Indian population, with regards to *APOE* gene polymorphisms and coronary heart disease (CHD) risk as summarized in the table 2 below. The studies highlighted the significance of *APOE4* in the pathogenesis of cardiovascular disease and other heart diseases. Polymorphism in the *APOE* gene strongly affects the level of gene product and the present study is in conformity with this as majority of the CAD cases carried *APOE4* gene variant that showed notable expression in the patients.

Study Group	Distribution of *APOE4* gene polymorphism in CHD/CAD cases			Reference
	Genotype	Cases (%)	Population /Ethnicity	
Ranjith N., *et al*. 2004	APOE (E3/E4) genotype	39.4	South African Indians/Caucasian	[20]
Singh P., *et al*. 2008	APOE (E3/E4) genotype	56.3	Punjabi/Caucasian	[21]
Afroze D., *et al*. 2015	ApoE (E4/E4) Genotype with the risk of CAD	30. 8	Kashmiri/Caucasian	[22]
Present-study	APOE4 mRNA expression in CAD	Twelve out of fourteen cases in South Indian (CAD patients)		

Table 2: Comparison of different studies in Indian population on *APOE* gene polymorphism in relation to coronary heart disease.

It can be suggested that although *APOE4* is not alone a responsible genetic factor, yet plays a major independent role as it is significantly associated with CAD. The molecular basis for expression of human *APOE4* related to elevated level of pro-atherogenic plasma lipoprotein cholesterol distribution has now become a prioritized concern and a research focus for effective therapies to reduce CAD, in future. The study is therefore an initiative towards a decent foundation to start screening of enormous populaces from different ethnicities and also before studies on proteomics and pharmacogenomics of *APOE* gene and other candidate genes.

Strengths and limitations of the study

The study investigated the potential association between *APOE* gene polymorphism and the presence of CAD in a sample of south Indian population. The strength of the study is that *APOE4* expression in the CAD subjects is indicative of its association to disease development. This study may be treated as a good pilot study in a South Indian population for the expression of the *APOE4* gene. However, some potential limitations of this study can be the selection of the enrolled control participants who came from a population attending hospital. Second, the sample size of this study is very small, which might have under-powered the study. Owing to the current COVID-19 pandemic, the number of cases visiting the hospital has significantly reduced. Moreover, this study was conducted within restrictions in the matter of time, place and corona pandemic situation. Impacts of *APOE* genotypes and alleles in the etiopathogenesis of coronary artery ailment should be uncovered in more detailed examinations on their effects in lipoprotein mechanism as well as other systemic pathways. Thus, further studies with larger samples are warranted to confirm these findings.

Conclusion

CAD has been linked with several genes such as *LDL* receptor gene, Apolipoprotein (*APO*). Detection for specific genetic mutation is not usually performed in CAD as it is still not clear about the underlying mechanism and correlation of such genetic analysis with cardiac risk due to small number of scientific studies reported.

The findings from the present study confirmed the mRNA expression of *APOE4* gene in the CAD patients which suggest that *APOE4* can be a candidate and a potential gene in studying for risk for CAD development, although the observations also indicates that this is not the only factor. It ought to be viewed as presence or absence of a gene variant continued with expression analysis can be the primer for a reliable basis to understand the correlation of the *APOE* variant to CAD. The study is an indicative step in the direction that active *APOE4* gene expression may help in early prediction of potential risk of CAD and may be treated as a good pilot study in a South Indian population for the expression of the respective gene.

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Informed Consent

Written informed consent was obtained from patients who participated in this study.

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Conflict of Interest

The authors declare no conflict of interests.

Ethical Approval

The study was approved by the Institutional Ethics Committee of the Hospital.

Supplementary

Supplementary provided.

PCR result

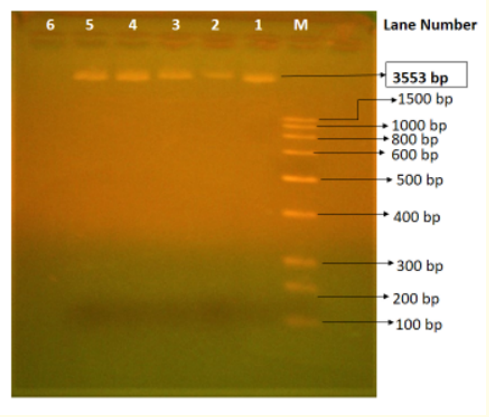

Figure 1: Amplified PCR product separated on agarose gel. Lane 1 - 5: amplified APOE gene from the diseased sample; Lane 6: Sample from control group; Lane M: 100 bp Marker.**: Significant differences ($p < 0.01$),
***: Significant differences ($p < 0.001$).

RT-PCR result

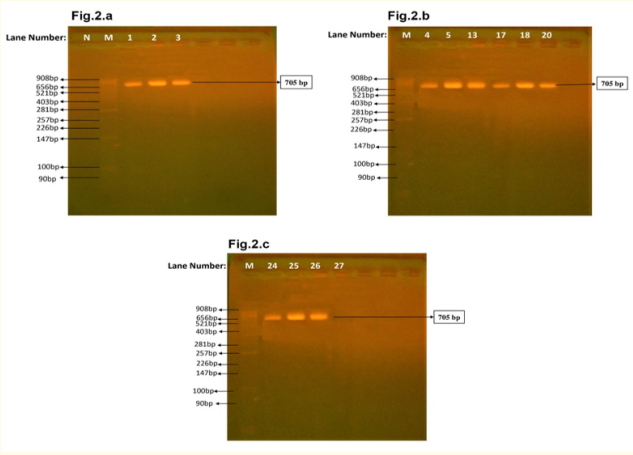

Figure 2: Amplified Reverse Transcriptase PCR product separated on agarose gel. 2.a: Lane M: pBR322 DNA/ AluI Marker, Lane-N: Negative control; Lane: 1-3: Expressed mRNA of APOE4 variant from RT-PCR analysis of diseased cases. 2.b: Lane: 4, 5, 13, 17, 18, 20 are the expressed mRNA samples from RT-PCR analysis. 2.c: Lane: 24-26: Expressed mRNA from RT-PCR analysis from the diseased cases; Lane: 27: control sample that carried the APOE4 gene but did not expressed.

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