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Apolipoprotein-E Gene Isoforms Genetic Spectrum in Pakistani Survivors of Myocardial Infarction

Akhtar Ali,[1,*] Masroor Ellahi Babar,[1] Ali Raza Awan,[2] Muhammad Tayyab[2] and Wasim Shehzad[2]

[1]*Faculty of Science and Technology, Virtual University of Pakistan*
[2]*Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore, Pakistan*

ABSTRACT

Apolipoprotein E (APOE) gene have two common polymorphic risk alleles *APOE112* and *APO158* which constitute six isoforms categorized in three genotypes E2 (ε2ε2, ε2ε3, ε2ε4), E3 (ε3ε3) and E4 (ε3ε4, ε4ε4). We aimed to assess *APOE* genotypes association with myocardial infarction in Pakistani cohort. Serum lipid levels were determined of survivors of myocardial infarction (n=100) and control (n=100) and genotyped using high throughput fluorescence based TaqMan and KASPar assays. The *APOE158* risk allele frequency was significantly lower in diseased (*p*-value 0.025). Three alleles E2 (15%), E3 (71%), E4 (14%) and E2 (5%), E3 (76%), E4 (18%) were observed among control and MI patients respectively. The E2 genotype found significantly lower for total cholesterol compared to E3 (*p*-value=0.013) and E4 (*p*-value=0.006). LDL-cholesterol values of E2 genotypes were also significantly lower to E3 (*p*-value=0.002), E4 (*p*-value=0.009) and triglycerides levels (*p*-value=0.039) to E4. The isoform ε3ε3 is more common among Pakistani population and genotype E2 can be considered as lipid lowering and protective. These findings conclude that *APOE* isoforms have genetic contribution with lipid levels and cardiovascular disease in Pakistan patients.

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Authors' Contributions
AA and MEB conceived and designed the study, collected samples and wrote the article. AA, ARA and MT performed experimental work. AA and WS analyzed the data.

Key words
***Apolipoprotein E*, risk allele, isoforms, high-throughput assay.**

INTRODUCTION

Coronary artery disease (CAD) and its complication, myocardial infarction (MI) is the more common cause of death in the world. A number of risk factors have been identified associated with coronary artery disease such as plasma concentration of lipids, inflammation markers, blood pressure, diabetes and smoking. These risk factors are consistent across the race and regions (Berry *et al.*, 2012). MI is presented clinically due to atherosclerosis plaque development (Zdravkovic *et al.*, 2004) and atherosclerosis is major cause of MI in Pakistan (Mushtaq *et al.*, 2006, Wilson *et al.*, 1998). Atherosclerosis is a chronic inflammatory and fibroproliferative disease mainly affecting the coronary arteries (Falk, 1999). Atherosclerosis development largely depends on plasma lipid concentrations and inflammatory response of the vascular tissues, in which cholesterol starts to deposit in peripheral tissues and accelerate the atherogenesis leading to premature cardiovascular events (Alonso *et al.*, 2009). Atherosclerotic lesions block the coronary passage which ultimately leads to heart, brain or extremities ischemia resulting in infarction to ischemia of the heart, brain or extremities, resulting in infarction.

Apolipoprotein-E (ApoE) molecule was firstly recognized as lipoprotein component of triglyceride rich very low density lipoproteins (Shore & Shore, 1973). It has been found an important in all lipoproteins (except LDL1) and has role in cholesterol homeostasis and increases the hepatic uptake by binding the lipoprotein with their receptors. It plays a key protective role in atherosclerosis by efflux of cholesterol from macrophages, preventing platelet aggregation, inhibiting the proliferation of T-lymphocytes and endothelial cells (Duan *et al.*, 1997). It assists in removal of triglyceride-rich lipoprotein remnants by LDLR and proteoglycan-LDL receptor related protein pathways (Linton *et al.*, 1998). ApoE is synthesized mainly in the liver but brain, spleen, kidneys, gonads, adrenals and macrophages can also produce (Mahley, 1988). *APOE* gene spans about 3.6kb, located at chromosome position 19q13.2 and has four exons encoding a polypeptide of 299 amino acids (Scott *et al.*, 1985, Rall *et al.*, 1982). *APOE* gene expresses in three common isoforms E2, E3 and E4 encoded by e2, e3 and e4 co-dominant alleles respectively. These isoforms are classified on the basis of T and C nucleotide substitutions at 112 and 158 amino acid position (Table I). *APOE* e4 genotype is reported as highly associated with severe coronary stenosis patient of Pakistan (Cheema *et al.*, 2015). However, in another survey no significance association observed of *APOE* allele frequencies with MI (Mehboob *et al.*, 2015). This study designed to substantiate the association of *APOE* genotype allele frequencies with myocardial infarction and their relation with lipid levels.

* Corresponding author: drakhtar.ali@hotmail.com
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MATERIALS AND METHODS

Sample selection

Myocardial infarction (MI) study was carried out on samples collected from Mayo Hospital Lahore. Hospitalized MI patients diagnosed on the basis of clinical symptoms, changes in electrocardiogram and biomarkers values (Antman *et al.*, 2000) were registered after their consent. Clinically diagnosed 100 MI patients with mean age 40.7±4.9 (year ± standard deviation) were included in this study. For association study, 100 individuals with average age 39.7±3.5 were included after informed consent. The age difference between both groups was not significant *p*-value 0.07. Each participating individual was briefly explained about this study. This study was approved by the ethical committee of Bioequivalence Study (BeSt) Center, University of Veterinary and Animal Sciences, Lahore.

Lipid profiling

Serum samples were used for estimation of total cholesterol, triglycerides and HDL-cholesterol by using UV-spectrophotometry after following the manufacturer's instructions. LDL-cholesterol was calculated by using the formula (Friedewald *et al.*, 1972).

DNA extraction and quantification

Blood samples were collected using sterile syringe and about 3 mL was collected from each participating individual. Falcon tubes (50 mL) containing 120µL of 0.5M EDTA (Ethylene diamine tetra acetic acid) were prepared before blood collection. DNA was extracted from leukocyte using standard organic DNA extraction method. All the DNA samples were also quantified using NanoDrop (Eppendrof, USA). DNA was quantified on the basis of 260/280 wave length ratio. All the samples were brought at the same concentration at 30ng/µL and working dilutions were prepared at 1.25ng/µL for genotyping.

APOE gene polymorphisms

Two common variants in *APOE* gene 112 and 158 were amplified using forward primer

5′GGAACTGGAGGAACAACTGACC3′ and
5′ACCTGCTCCTTCACCTCGTC3′.

Polymerase chain reaction mixture for each reaction was prepared using template DNA 25-30 ng/µL, Taq. DNA polymerase 0.1µL (5U/ µL, Fermentas, USA), dNTPs mix 2.5µL (2.5 mM Fermentas, USA), $MgCl_2$ (Fermentas, USA) 2.5µL, PCR buffer 2.5 µL (10X, Fermentas, USA), forward Primer 1.0 µL (10 pmol/ µL), reverse primer 1.0 µL (10 pmol/ µL) and deionized H_2O 14.4 µL was added for each reaction mixture of 25 µL volume. Thermocyclic condition were carried out as initial denaturation at 95°C for 5 min and cyclic denaturation for ten cycles at 94°C for 30 sec, annealing temperature at 60°C for 30 sec with decreasing 1C for every cycle, cyclic extension at 72°C for 45 sec. Then twenty five cycles were repeated at 50°C as annealing temperature for 30 sec and final extension at 72°C for 10min was performed to amplify the targeted region. PCR product was cleaned using Qiagen Kit following the manufacturer's instructions.

Genotyping of APOE polymorphisms

High throughput genotyping was done using fluorescence based Taqman and KASPar assays. Both genotyping methods rely on the emission of fluorescence from different fluorophores to denote the presence of a particular allele. Fluorescence was detected using the 7900HT Fast Real-Time PCR System with Sequence Detection System software v2.1. Allele specific primers 5′TGGAGGACGTG3′ and 5′cacgtcctcca3′ were used to locate the allele APOE112 (rs429358) and TaqMan assay was used for amplification. The make-up of the reaction mix depended upon which buffer was available. For 210 samples KAPA buffer 233µL, SNP assay 11.2 µL and water 204 µL. Finally 2.0µL of this mixture was added into each well of the plate. Thermocycler conditions; initial at 50°C for 2 min, 95°C for 10 min, then forty cycles of 95°C for 15 sec and 60°C for 60 sec.

The allele APOE158 was genotyped using allele specific primers 5′AGATTTTATC3′ and 5′gataaaatct3′. This polymorphism was genotyped using KASPar assay. KASPar genotyping is based upon Kompetitive Allele Specific PCR (KASP) technology (Cuppen, 2007). The reaction mixture for 210 sample; KASPar mixture 409 µL, SNP assay 11.25 µL, water 409 µL. Finally 3.8 µL of this mixture was added into each well of the plate. Thermocyclic conditions; initial at 94°C for 15 min, 10 cycles at 94°C for 20 sec and 94°C for 60 sec. Then 26 cycles starting at 65°C with 0.8°C per cycle decrease for 20 sec and 94°C for 60 sec.

Data analysis

Conformance of allele frequency and level of significance for Hardy-Weinberg Equilibrium was tested by Chi-square test. Fischer exact test (http://www.socscistatistics.com) used for association of genetic polymorphism with MI. Lipid levels and their relation with genotypes was analyzed through online t-test calculator (http://www.quantitativeskills.com/sisa/statistics/t-test.htm).

RESULTS

Lipid values of control and MI groups were measured for this study (Table I). Total cholesterol, tri-glyceride and low density lipoprotein cholesterol (LDL-C) values in disease group were significantly higher while HDL-C values were lower. No significant difference observed between ages of both participating groups.

Table I.- Lipid levels and age of control and disease samples MI, myocardial infarction; TC, total cholesterol; Tri-gly, Tri-glycerides; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; St. Dev. standard deviation.

Parameters	Control Group (n=100)	MI Group (n=100)	P value
Age (years)	39.7±3.5	40.7±4.9	0.07
TC (mmol/L)	4.4±0.6	4.8±1.0	< 0.05
Tri-gly (mmol/L)	2.5±0.9	3.2±1.1	< 0.05
HDL-C (mmol/L)	1.4±0.2	1.1±0.2	< 0.05
LDL-C (mmol/L)	2.6±0.6	3.1±0.9	< 0.05

Apolipoprotein-E (APOE) gene was sequenced for two common variant 112 and 158 in the selected samples of patients and controls. Amplified fragments were sequenced and one polymorphic change identified c.526C>T resulting in Arginine to Cysteine amino acid change at p.R130C position (18 amino acid are cleaved as peptide signal hence literature reported as 112 position). Other single nucleotide change found at c.664T>C coding for Arginine to Cysteine amino acid change at p.C176R position (18 amino acid are cleaved as peptide signal hence literature reported as 158 position).

Genotyping of risk alleles

After amplification, genotyping results of two common variants APOE112 and APOE158 were detected through Sequence Detection System (SDS) software (Fig.1). The detectable alleles in MI and control groups for APOE112 were 83 and 89, and for APOE158 were 86 and 92, respectively. The undetermined alleles were not considered for analysis and all the alleles followed the Hardy-Weinberg equilibrium (Table II).

Genotyping of both alleles were analyzed by applying chi-square test using online Fisher exact calculator (http://www.socscistatistics.com), we found no significant difference (p value=0.850) between MI and control samples for APOE112 allele. APOE158 risk allele frequency was significantly higher (p-value 0.025) in controls than patient (Table III).

Six isoforms of *APOE* gene are encoded by three e2, e3 and e4 co-dominant alleles. Isoforms are categorized into three subtype E2 (ε2ε2, ε2ε3, ε2ε4), E3 (ε3ε3) and E4 (ε3ε4, ε4ε4). These isoforms are classified on the basis of T and C nucleotide substitutions at 112 and 158 amino acid position (Table IV). Three alleles E2 (15%), E3 (71%), E4 (14%) and E2 (5%), E3 (76%), E4 (18%) were observed among control and MI patients respectively. The isoform ε3ε3 observed more than 70% in both groups which is more common and is considered as wild type.

The E2 subtype showed significantly lower serum lipid contents with respect to other two subtypes (Table V). The E2 was found significantly lower for total cholesterol levels compared to E3 (p-value=0.013), E4 (p-value=0.006), LDL-cholesterol to E3 (p-value=0.002), E4 (p-value= 0.009) and triglycerides levels p-value=0.039) to E4. We found no significant difference among E3 and E4 for total cholesterol, LDL-cholesterol and triglyceride.

DISCUSSION

In this investigation, we examined the genotypic effects of *APOE* genotypes on MI and lipid levels in Pakistani cohort. Exchange of arginine and cytosine at 112 and 158 positions in *APOE* gene have been extensively studied in relation with the cholesterol metabolism and cardiovascular risks. Our genotyping findings of both alleles showed no deviation from Hardy-Weinberg equilibrium. The prevalence of APOE158 risk allele was significantly higher (p-value=0.025) in controls than patients. The six isoforms of *APOE* gene were observed (Table IV), and the most common ε3ε3 was more than 70% in both groups and is considered as wild type (Lahoz *et al.*, 2001). The ε2ε2 genotype was found in control samples only. Frequency of ε3ε4 genotype was higher in MI while ε2ε3 in controls. The frequency of E4 allele in heterozygous form was higher in coronary disease patients which is already reported as associated with premature coronary myocardial infarction (Schmitz *et al.*, 2007). *APOE* e4 genotype is reported as highly associated with severe coronary stenosis patient of Pakistan (Cheema *et al.*, 2015). In contrast to this, homozygous E4 isoform was found in controls group which is supporting to another surveyed data from Karachi-Pakistan (Mehboob *et al*., 2015). Controversially to the largely reported data on *APOE* genotypes, no significant association of E4 have also been reported in American population (Liu *et al*., 2003, Morgan *et al.*, 2007). Similarly in Achen study (Schmitz *et al*., 2007), a pronounced effect of E4 was found in younger patients

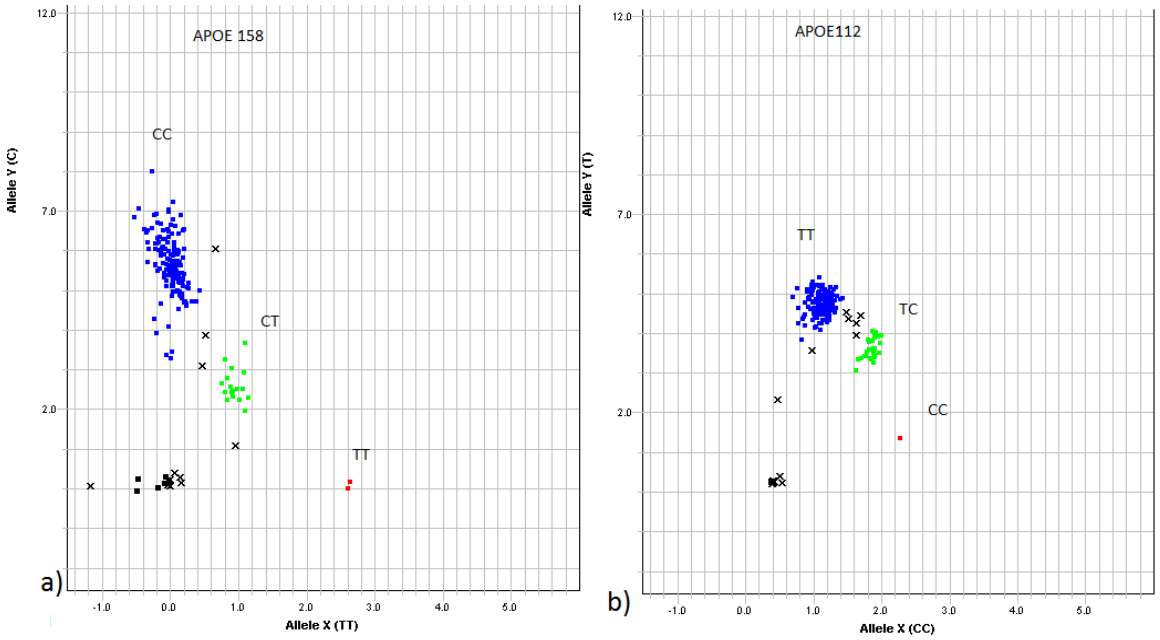

Fig. 1. Genotyping result of APOE112 (a) and APOE158 (b) risk alleles in MI and control samples.

Table II.- Genotyping of APOE112 and APOE158 MI, myocardial infarction; hom. allele, homozygous allele, het. allele, heterozygous allele; RAF, risk allele frequency; sig. level, significance level.

Genotype/ SNP	Assay	Sample Type	Hom. Allele	Het. Allele	Hom. Allele	Risk Allele	RAF	p-value
		MI	(TT) 68	(TC) 15	(CC) 0		0.09	0.37
APOE112	TaqMan	Control	76	13	1	**C**	0.08	0.60
		All	144	28	1		0.09	0.77
		MI	(CC) 81	(CT) 5	(TT) 0		0.03	0.78
APOE 158	KASPar	Control	79	12	2	**T**	0.09	0.08
		All	160	17	2		0.06	0.06

Table III.- Fisher exact test *p*-value calculated for APOE risk alleles. MI, myocardial infarction.

Genotype/ SNP	Risk Allele	Allele Type	Subject		P value
			MI	Control	
		T	151	165	0.850
APOE112	C	C	15	15	
		C	167	170	0.025
APOE158	T	T	5	16	

but these results are far less robust. Lipid level values suggest higher concentration of cholesterol contents in the diseased group which is considered as risk factor for coronary artery disease. The APOE subtype E2 showed significantly lower serum lipid contents with respect to other two subtypes (Table V) for total cholesterol levels compared to E3 and E4. Genotype E2 is extensively studied and is considered as protective factor against higher lipid levels and cardiovascular disease risk (Luptákova *et al.*, 2013). We found no significant

Table IV.- Genotyping results of six isoforms of *APOE* gene categorized into three subtypes.

	Allele/ (SNP)	E2		E3		E4	
		ε2 ε2	ε2 ε3	ε2 ε4	ε3 ε3	ε3 ε4	ε4 ε4
Genotype	APOE112	TT	TT	TC	TT	TC	CC
	APOE158	TT	CT	CT	CC	CC	CC
Control			11	1	65	12	1
MI		0	4	1	65	15	0

Table V.- Lipid contents and APOE genotype association HDL, high density lipoprotein; LDL, low density lipoprotein; St.Dev, Standard Deviation.

Parameters	APOE2 (n=19)	APOE3 (n=130)	*p*-value	APOE3 (n=130)	APOE4 (n=28)	*p*-value	APOE2 (n=19)	APOE4 (n=28)	*p*-value
TC (Mean±SD)	4.3±0.5	4.6±0.7	0.013	4.6±0.7	4.8±0.8	0.116	4.3±0.5	4.8±0.8	0.006
Tri-gly (Mean±SD)	2.6±0.5	2.8±1.1	0.094	2.8±1.1	3.0±1.0	0.177	2.6±0.5	3.0±1.0	0.039
HDL-C (Mean±SD)	1.3±0.2	1.2±0.2	0.974	1.3±0.2	1.2±0.1	0.990	1.3±0.2	1.3±0.1	1.000
LDL-C (Mean±SD)	2.4±0.5	2.8±0.8	0.002	2.8±0.8	2.9±0.9	0.297	2.4±0.5	2.9±0.9	0.009

difference among E3 and E4 for total cholesterol, LDL-cholesterol, triglyceride and HDL-cholesterol.

Genotypes were not significantly different among both groups and this lack of significance for reported SNP imprints some possible reasons. The first reason for unexpected results could be the random collection of hospitalized patients without family history of coronary heart disease. Randomized collection restricted with family history might give a different picture of the allele, because genetic association of myocardial infarction risk is assessed 40-50% in the family history based studies (Yang *et al.*, 2011). The second could be small number of sample size. International Consortium for Blood Pressure Genome-Wide Association Studies reported small sample number could not give any association of lead SNPs with coronary artery disease (http://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs0005\85.v1.p1), because it reduces the strength of generated data. This study can be replicated on large scale by considering the sample selection criterion, increased sample size and adding other identified risk alleles reported on south Asian ancestry.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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