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Lack of Association Between Apolipoprotein E Polymorphism and Acute Myocardial Infarction in a Pakistani Population

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Abstract.- The aim of this study was to examine the association of apolipoprotein E(ApoE) polymorphism with acute myocardial infarction (AMI) in a Pakistani population. A cross-sentional study involving 218 AMI patients (166 males and 51 females; age 30-75 years and 171 healthy controls (121 males and 50 females, age 19-76 years) was carried out on a hospital-based Pakistani population. Fasting serum concentrations of total cholesterol, low density lipoprotein (LDL)-cholesterol, high density lipoprotein (HDL)-cholesterol, triglycerides and glucose were determined using kit methods. DNA was extracted and ApoE polymorphism was studied by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP). Apo E3/E3 genotype was the most common genotype in both cases and controls (78% and 74%, respectively), while E2/E3 genotype was the least common one in this cohort (6.5% and 7.97%, respectively). Frequencies of E2, E3 and E4 alleles in AMI patients (0.078, 0.766 and 0.155, respectively) were not significantly different from the frequencies of these alleles in healthy controls (0.11, 0.708, 0.18, respectively; χ^2=1.95, p value 0.37). Moreover, no association was observed between genotypes – E3/E4, E2/E3, E3/E3 and serum lipids in AMI patients. ApoE polymorphism was not found to be associated with AMI in a hospital-based population in Karachi.

Key Words: ApoE polymorphism, ApoE genotypes, acute myocardial infarction, coronary artery disease, Pakistani population, serum lipids.

INTRODUCTION

There is an epidemic of coronary artery disease (CAD) in Pakistan (Jafar *et al.,* 2008). Besides nutritional and environmental factors, genetic architechture of Pakistani population appears to be playing a major role in increasing the risk of CAD (Saleheen *et al.*, 2009). A number of studies have been carried out in the region to investigate the role of apolipoprotein E (ApoE) gene polymorphism towards the risk of myocardial infarction (MI) (Kumar *et al*., 2003; Singh *et al*., 2008; Fallah *et al.*, 2011; Chaudhary *et al.,* 2012). Most of these studies have shown association of ApoE genotypes, especially E3/E4 with coronary heart disease (CHD). No studies have been carried out to investigate the relationship of ApoE polymorphism with CHD in a Pakistani population. The present study was undertaken to investigate whether there is any association of ApoE polymorphism with acute myocardial infarction (AMI) in a hospital-based Pakistani population.

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PATIENTS AND METHODS

In this cross-sectional study, 218 consecutive AMI patients (167 males and 51 females; age range: 30-75 years) admitted to the National Institute of Cardiovascular Diseases (NICVD), Karachi were enrolled with written informed consent following the Guidelines of the Helsinki Declaration for the protection of human subjects. They were the first time AMI. Confirmation of their diagnosis was on the basis of WHO criteria of clinic history suggestive of myocardial ischaemia; ECG indications of myocardial damage and elevation of biochemical markers (creatine kinase and troponin T). Similarly, 171 body mass index (BMI) matched healthy controls (121 males and 50 females; age range 19-76 years) were selected from the personnel of the Aga Khan University and Civil Hospital, Karachi. These control subjects had no evidence of CAD on the basis of clinical history and physical examination and were also not suffering from any other chronic illness such as diabetes mellitus, liver disease, hypertension, uremia and cancer. Those who were pregnant were also not included in the study.

Measurement of biomarkers

Fasting blood was obtained and serum was analyzed for glucose, total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides using kit methods (Roche Diagnostics, USA). The minimum concentrations of detection for serum cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides were 9.7 mg/dl, 3.9 mg/dl, 3.0 mg/dl, 8.9 mg/dl, respectively.

DNA extraction and ApoE genotyping

Genomic DNA was extracted from EDTA-treated blood samples according to published methods (Sambrook, 2001). The ApoE genotype was determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The DNA was amplified by PCR using oligonucleotide primers obtained from MWG, USA. The forward primer was
5' TAAGCTTGGCACGGCTGTCCAAGGA 3'
and reverse primer
5' ACAGAATTCGCCCCGGCCTGGTACAC 3' (Hixson and Vernier, 1990; Lahoz *et al.,* 1996). For the PCR, 100 ng of genomic DNA was added to 100 pmol/µl of each primer. To this, 5% of dimethylsulphoxide and 1.5U of Taq polymerase (Promega Corp. USA) were added to make the final reaction volume to 10 µl with distilled water. The ApoE gene containing region of DNA was amplified in thermal cycler (Mastercycler gradient Eppendroff) with PCR condition as follows: Each reaction mixture was initially denatured for 5 min at 95°C, followed by 30 cycles of melting for 1 min at 95°C, annealing for 1 min at 65°C and extension for 1 min at 72°C, final elongation for 10 min at 72°C and then cooling at 4°C. The amplified product was run on 2% agarose gel. The PCR product of the ApoE gene polymorphic sequence was digested with the restriction enzyme *Hha*I (New England Biolab, USA) at 37°C for 3 hours to ensure full digestion. A polymorphism in ApoE gene causes a shift in *Hha*I restriction site. *Hha*I cleavage sites clearly distinguished the 3 major alleles and the 6 homozygous and heterozygous combinations. The digested products were then resolved on 12% polyacrylamide gel. Bands were observed under UV light after staining with ethidium bromide (0.2 mg/l) for 10 min.

Statistical analysis

All data were analyzed with the help of SPSS® Statistical Package for Social Sciences) software version 19 for windows® (Apache Software Foundation, USA). Quantitative variables were expressed as the mean±SD, while qualitative variables were expressed in percentages. Mean values of quantitative variables between AMI patients and healthy controls were compared using Independent sample t test. Hardy-Weinberg proportions of allele distribution were assessed by chi-square test. Analysis of variance (ANOVA) was undertaken to see the difference of lipid profile across the ApoE genotypes. A p value < 0.05 was considered statistical significant.

RESULTS

Figure 1 shows a typical gel after digestion of PCR product by restriction enzyme *Hha*I. Five different genotypes with their unique banding patterns can be visualized in this Figure. E2/E2 genotype is represented by 3 bands – 91 bp; 83 bp and 38 bp; E2/E3 genotype also has 3 bands – 83 bp, 48 bp and 38 bp; E3/E3 genotype has 4 bands – 91 bp, 48 bp, 38 bp and 35 bp; E3/E4 has 5 bands – 91 bp, 72 bp, 48 bp, 38 bp and 35 bp, while E4/E4 has 4 bands of sizes – 72 bp, 48 bp, 38 bp and 35 bp.

Demographic and clinical characteristics of patients and normal healthy controls show significant differences in the mean serum levels of cholesterols and glucose in AMI patients and healthy controls (Table I). Mean age in the control group was significantly less compared to the patient group (48±10.4 years vs. 53±10 years, respectively; p value < 0.001). This is primarily because of the inclusion of some young controls compared to AMI patients. Serum levels of total cholesterol, LDL-cholesterol and glucose were significantly higher in AMI patients compared to the control group (p value < 0.05), while serum HDL-cholesterol was significantly low in AMI patients compared to controls (p value < 0.05).

Table II shows the distribution of alleles and 3 major genotypes (E3/E3, E3/E4, E2/E4) in AMI patients and control subjects. Frequencies of E2, E3 and E4 alleles in AMI patients (0.078, 0.766 and

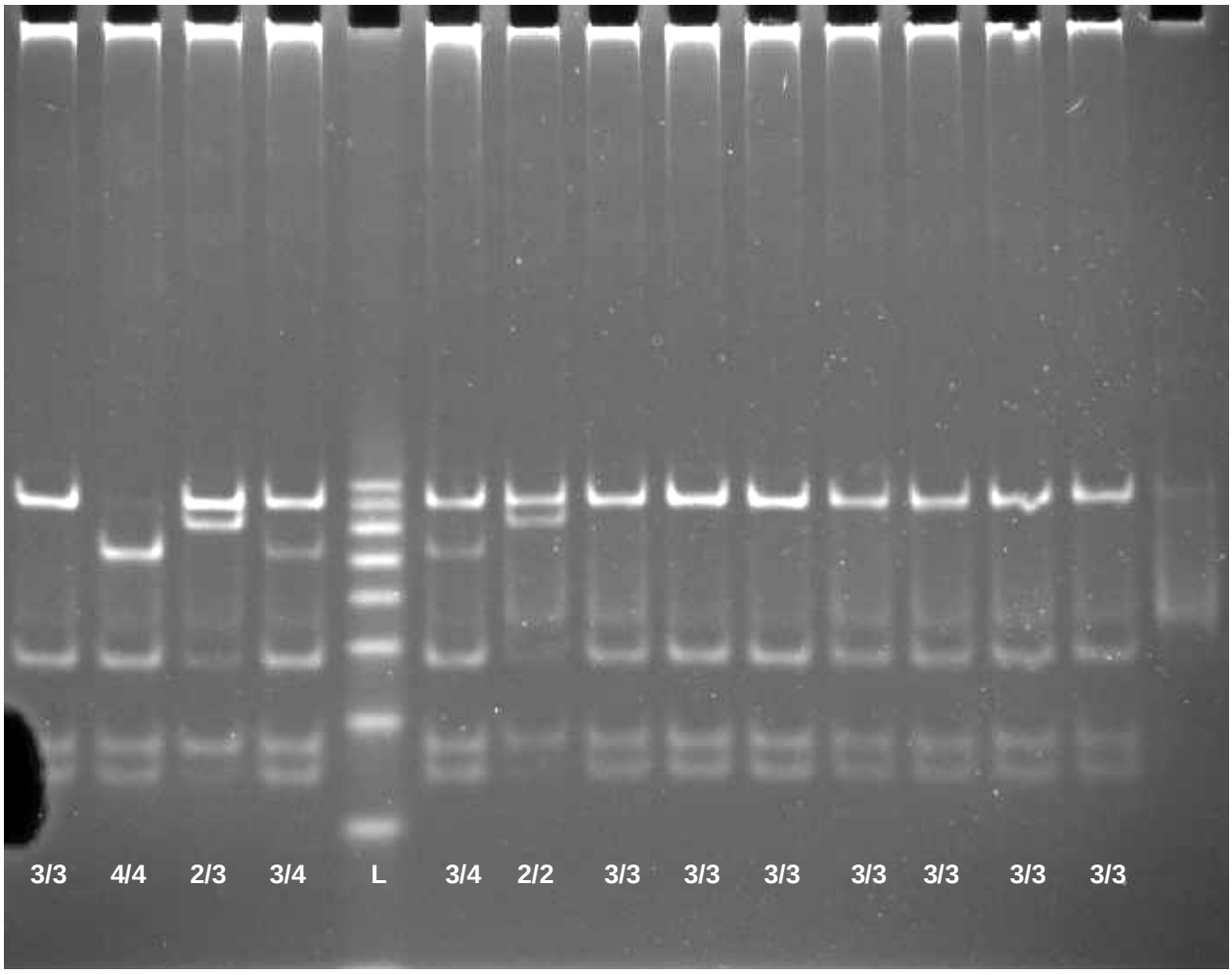

Fig. 1. Visualization of ApoE polymorphism by PCR-restriction endonuclease (*Hha*I). Molecular size marker 100 bp DNA ladder (L) is in fifth column from the left hand side. Genotype 3/3 with 4 bands (91,48,38,35) is in the first column; while genotype 4/4 with 4 bands (72,48,38,35) is in the second column. Genotype 2/3 with 4 bands (91,83,48,38) is shown in the third column, while genotype 3/4 with 5 bands (91,72,48,38,35) is in the fourth column. Genotype 2/2 with 3 bands (91,83,38) is in seventh column from the left. Negative control is shown in the fifteenth column from the left. Genotype 2/4 is not shown in this gel.

0.155, respectively) were not significantly different from the frequencies in the controls (0.11, 0.708 and 0.18, respectively; χ^2=1.95, p value = 0.37). Similarly, the percentages of E3/E3, E3/E4, E2/E3 genotypes were also not significantly different between the patient and control groups (χ^2 = 0.76; p value = 0.68).

Apo E3/E3 was the most common genotype in both cases and controls (78% and 74%, respectively), while E2/E4 genotype was conspicuously absent in this cohort. ApoE genotypes were found to occur in Hardy Weinberg proportions in patient as well as control groups. Relationship of serum lipid levels in AMI group and 3 major genotypes (E3/E3, E2/E3, E3/E4) was examined using one-way ANOVA. No association was found between these genotypes and serum lipid levels in AMI patients (Table III).

Table I.- Demographic and clinical characteristics of AMI patients and healthy controls

Characteristics	AMI patients (n=218)	Healthy controls (n=171)	p value**
Gender*			
Males	166 (76.1)	121 (70.8)	0.179
Female	51(23.4)	50(29.2)	
Age (years)	53 ± 10	48 ± 10.4	<0.0001
BMI (kg/m^2)	24.5 ± 3.5	24.4 ± 5.4	0.82
Total cholesterol (mg/dl)	182 ± 47	172 ± 35	0.02
Triglyceride (mg/dl)	173 ± 111	167 ± 102	0.58
HDL-cholesterol (mg/dl)	33 ± 10.5	37 ± 11	0.0003
LDL-cholesterol (mg/dl)	115 ± 46	101 ± 33	0.001
Glucose (mg/dl)	144 ± 76	102 ± 33.6	<0.001

*Males and females are expressed as n(%)
**p value compares the mean values in two groups using Independent sample t test, while in case of proportions of males and females, chi-square test is used.

Table II.- Distribution of ApoE alleles and 3 major genotypes in AMI patients and healthy controls in a hospital-based Karachi population

	AMI group n(%)	Control group n(%)	p value*
Alleles			
E2	17(7.8)	19(11.1)	0.06
E3	167(76.6)	121(70.8)	0.19
E4	34(15.5)	31(18.1)	0.41
Genotypes			
E3/E3	167(78.0)	121(74.2)	0.39
E3/E4	33(15.4)	29(17.8)	0.61
E2/E3	14(6.54)	13(7.97)	0.51

*p value compares percentages in AMI patients and normal healthy subjects (control) using Independent sample t-test.

DISCUSSION

Though ApoE gene is one of the most studied candidate genes in relation to CAD, there is hardly any study carried out on ApoE polymorphism in Pakistani population that has very high prevalence of this disease. A meta-analysis of the studies from 1966 to 2004 indicated that carriers of ApoE4 allele had 42% higher risk of CHD, while E2 allele had no significant relationship with this disease (Song *et al.,* 2004). Studies carried out in Asian countries such as Saudi Arabia and Iran also showed E3 to be the most prevalent isoform that appears have some relationship with CHD (Hallman *et al.,* 1991; Dzimiri *et al.,* 1994; Nabatchian *et al.,* 2008). In the present study, we also found E3 allele to be the most prevalent allele in Pakistani population, however, ApoE polymorphism was not found to be associated with AMI. Our findings conform well to those reported by Al-Bustan *et al.* (2009) and Yilmaz-Aydogan *et al.* (2010) who have found no association between ApoE genotypes and CHD in Kuwaiti and Turkish populations, respectively. However, our results are different from those reported by Baum *et al.* (2006), Kharrazi *et al.* (2006), Chaudhry *et al.* (2012) and Singh *et al.* (2008) who have shown positive association between ApoE genotypes and CHD in Chinese, Iranian, Thai and Indian populations, respectively.

Table III.- Relationship of serum concentrations of lipids with different ApoE genotypes in AMI patients.

Lipids (mg/dl)	Genotype			p value*
	E3/E3 (n=167)	E2/E3 (n=14)	E3/E4 (n=33)	
Total cholesterol	182±45.5	181±54	191±50	0.59
Triglycerides	171±117.5	202±122	161±61.3	0.51
HDL-cholesterol	33±10.2	37±11.7	34±11	0.22
LDL-cholesterol	115±43	100±61	124± 49	0.24

*p value compares mean values in 3 genotypes using one-way ANOVA

The association between ApoE genotypes and CHD appears primarily because of the relationship between ApoE and serum lipids. ApoE is generally considered as a major protein maintaining the metabolism of atherogenic lipoprotein (Lenzen *et al.,* 1986). Lipoprotein concentrations are related to ApoE isoforms in such a manner that individuals with E3/4 and E4/4 genotypes have high serum lipids, whereas individuals with E2/4, E2/3 and E2/2 genotypes have low levels (Yin *et al.,* 2008). Most significant effect of ApoE polymorphism is its

relationship with dyslipidemia which has recently been shown to be one the major risk factors for AMI in men (Madssen *et al.*, 2013).

Most studies showing positive relations of ApoE polymorphism and CHD had deranged cholesterol metabolism (Kharrazi *et al.*, 2006., Singh *et al.*, 2008, Aydogen *et al.*, 2009, Fallah *et al.*, 2011). Since we did not find any association of ApoE genotypes with serum lipid levels in this cohort, this could be one of the reasons for lack of association of ApoE polymorphism and AMI in this Pakistani population.

C-reactive protein (CRP) has been known to be involved in the development of atherosclerosis. A recent study by Grammer *et al.* (2011) has shown association between ApoE genotypes and circulating CRP. This could be a potential confounder in the relationship of ApoE polymorphism and CAD. However, we did not measure CRP in this study. Siddiqui and Cheema (2009) have shown an association of myocardial infarction with apolipoprotein B and apolipoprotein A-1 in Pakistani patients. Perhaps, the variation in these apoproteins rather than ApoE is more unique to Pakistani AMI patients.

Our result must be viewed in the light of certain limitations of this study. Our study questionnaire did not include information about the use of lipid lowering drugs. There is a possibility that some of the AMI patients could have been using statins or fibrates. These drugs could potentially mask any association between ApoE genotypes and serum lipids. Moreover, our sample size was also quite modest. With low frequencies of E2 and E4 alleles (active predictors of CHD) in Pakistani population, the role of sample size becomes even more important. Therefore, it is suggested that studies involving a large sample size comprising of subjects belonging to all the main ethnic groups in Pakistan should be carried out while measuring major biomarkers for CAD including CRP to arrive at a conclusive evidence about the association of ApoE polymorphism and CAD in this population.

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