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ORIGINAL ARTICLE

ApoE gene polymorphism and its relationship with coronary artery disease in ethnic Kashmiri population

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Abstract Apolipoprotein E is a fundamental component of various lipoproteins and plays substantial role in cholesterol/lipid transport among cells of various tissues. The *ApoE* gene is polymorphic with three alleles *ε2*, *ε3*, and *ε4*, coding for isoforms E2, E3, and E4 having different binding inclination for corresponding receptors. This work aimed to investigate the association between *ApoE* gene polymorphism and coronary artery disease (CAD) in Kashmiri population. *APOE* genotyping was done by polymerase chain reaction-restriction fragment length polymorphism. Our study indicated ApoE ε3/ε3 to be the most common genotype in both CAD and control group. The frequency of *ε2, ε3*, and *ε4* alleles of *ApoE* gene in cases was observed to be 0.06, 0.72, and 0.20, while in control subjects it was 0.075, 0.82, and 0.11, respectively. A significant difference was found between cases and controls with respect to TC, LDL, and HDL levels. Our data showed that frequency of ε4/ε4, ε4/ε3 genotype and ε4 allele was significantly higher in cases than in controls ($p = 0.02$, $p = 0.004$, $p < 0.001$ respectively). Moreover, the CAD patients carrying ε4 allele had significantly higher TC and LDL levels (*p* value <0.01). Thus our data showed a significant association of ApoE ε4 allele with the risk of CAD. The data revealed that ApoE ε4 allele is associated with increased risk of CAD and increased levels LDL and TC in Kashmiri population.

Keywords Apolipoprotein *E* · Polymerase chain reaction-restriction fragment length polymorphism · Coronary heart disease · Polymorphism

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Introduction

Coronary artery disease (CAD) is the most important cause of mortality and morbidity from non-communicable diseases with an estimated 17.5 million deaths worldwide. Countries with low-to-middle income contribute the highest proportion of the burden compared with any other region globally [1]. South Asians are among the most vulnerable population with an alarming high incidence [2]. Various genetic and environmental factors influence the development of CAD. Lipoproteins play an extensive part in the advancement of atherosclerotic CAD in humans. The levels of lipoproteins in plasma are dictated by apolipoproteins present on their surface. Apolipoproteins function as ligands for various receptors and determine the metabolic fates of the lipoprotein [3]. Therefore, mutations in the genes coding for any of the apolipoproteins may result in impaired clearance of lipoproteins. The genetic variation could thus be a major determinant of the interindividual variation in susceptibility to CAD [4].

Apolipoprotein E (ApoE) is a structural constituent of chylomicrons and very low-density lipoprotein (LDL) remnants. Apolipoprotein E plays a critical role in CAD by regulating the hepatic uptake of remnant lipoproteins, by facilitating cholesterol efflux from macrophage foam cells

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within atherosclerotic lesions and by modifying inflammatory responses [5]. Thus, ApoE plays a dual role by clearing the plasma of chylomicron remnants and excessive cholesterol, thereby playing a key protective role in atherosclerosis [6].

ApoE is a polymorphic protein which is coded by three alleles at the same locus. The gene coding for ApoE is located on chromosome 19. Three different alleles, ε2, ε3, and ε4, account for ApoE polymorphism and determine the six genotypes which give rise to three homozygous (apo ε2/2, apo ε3/3, apo ε4/4) and three heterozygous genotypes (apo ε2/3, apo ε2/4, apo ε3/4) [7]. The major effect of genetic variation at this locus is its influence on cholesterol levels, one of the major risk factors for CAD. Variation in ApoE binding to ApoE or LDL receptors results in different levels of lipids or lipoproteins and risk among individuals predisposed to CAD [8]. Evidence exists to suggest that the variability of ApoE has differential effects on the atheroprotective potential attributed to different polymorphic variants of ApoE. The ApoE allele ε4 is associated with increased LDL cholesterol levels and decreased ApoE plasma concentrations [9, 10]. Conversely, the ε2 allele is associated with reduced LDL cholesterol levels and higher ApoE plasma concentrations. Among patients with CAD, the ε4 allele has been related to more severe disease and the ε2 allele to less severe disease [11]. The presence of the ε4 allele has been associated with increased death rates in patients with CAD [4]. Allelic frequencies of Apo E differ in different populations [12, 13]. Wide-range population studies have indicated inter-ethnic variations in ApoE polymorphism. Indian population showed the frequency of ε3 allele to be higher than ε2 and ε4 [14]. Our previous study also reported ε3 allele to be the most common allele in Kashmiri Population [15]. This study was carried out to determine the role of ApoE genotypes in the risk of CAD in Kashmiri population.

Materials and methods

Study subjects

This study comprised of 650 ethnic Kashmiri subjects including 200 CAD patients and 450 control subjects. Patients and controls were recruited from the tertiary care hospital; Sheri-I-Kashmir Institute of Medical Sciences (SKIMS) and belonged to the same geographic area, ethnic background, and approximately similar age group. A total of 200 patients were recruited from a cohort of patients undergoing clinically indicated coronary angiography. Subjects with one or more lesions that significantly reduced the lumen of any coronary artery (≥50 %) were defined as CAD patients. The controls comprised of subjects with no evidence of any personal or family history of CAD, cancer, renal disease, hepatitis, diabetes, etc. Approval for the study was given by the Ethics Committee of SKIMS, and a written informed consent was obtained from each recruited subject. Patient information including demographic data, history, and detailed medical information like hypertension, diabetes mellitus, and smoking status, was extracted from patient files, from investigation reports, and by detailed questionnaire.

General and clinical examination and anthropometric measurements including body weight, body height, BMI, and cardiovascular risk factor assessment including systolic and diastolic pressure and fasting serum lipids levels, were done for every patient. With the subjects wearing light indoor clothes and no shoes, body weight (BW) and body height (BH) were measured. Hypertension was defined as SBP ≥140 mmHg or DBP ≥90 or subjects taking medication for hypertension. Diabetes mellitus was defined as when one has FPG ≥126 mg/dL or subjects under treatment with oral antidiabetic drugs. Dyslipidemic or hyperlipidemic was defined as when one has level of TC >200 mg/dL, TG >150 mg/dL, LDL-C >130 mg/dL, HDL-C <40 mg/dL, TC/HDL-C ratio >4.0, or under medication of lipid lowering drugs. BMI was calculated as per WHO recommended cutoff points for Asians.

Sample collection/storage

Samples were collected from the patients attending OPD and department of cardiology SKIMS. After proper consent from each subject, 5 mL of blood was collected after 12- to 16-h overnight fasting. Each sample was divided in two aliquots, one in EDTA vial and the other in plain vial. The sample with EDTA was used for DNA extraction, while serum was separated from the sample in other vial and used for biochemical analysis. The samples were stored at −20 °C until processed.

Lipid profile

The serum samples of both patients and controls were subjected to lipid analysis. Lipid profiling of the serum samples was done on Autoanalyser (Olympus AU640) using Beckman coulter Kits.

ApoE genotyping

DNA extraction Genomic DNA was isolated using DNA extraction kit (Gene jet genomic DNA Purification Kit) from whole-blood samples of both cases and controls. The quality of the isolated DNA was determined by running each sample on ethidium bromide stained 1 % agarose gel.

PCR amplification The ApoE genotyping was done by a PCR-based restriction fragment length polymorphism (RFLP) analysis. A 218-base-pair (bp) fragment was amplified using the primers: 5′TCCAAGGAGCTGCAGG CGGCGCA 3′(forward) and 5′GCCCCGGCCTGGTACA CTGC CA- 3′ (reverse). The amplification reaction was carried out in 25-μL reaction volume consisting of 50 ng genomic DNA template, 1× PCR buffer (Biotools, B&M Labs, Madrid, Spain) with 2 mmol/L MgCl2, 0.4 mmol/L of each primer (Genscript, Piscataway, NJ), 50 mmol/L dNTPs (Biotools, B&M Labs, Madrid, Spain), 10 % DMSO and 1.5 U Taq polymerase (Biotools, B&M Labs, Madrid, Spain). The PCR thermal cycling profile consisted of an initial denaturation at 95 °C for 7 min followed by 30 cycles of 30 s DNA denaturation at 95 °C, primer annealing for 30 s at 63 °C, 30 s extension at 72 °C, followed by one cycle of a final extension step for 7 min at 72 °C. The amplified products (218 bp) were resolved on 2 % agarose gel containing ethidium bromide.

For RFLP, PCR products were digested with 5U of *Afl III* and *Hae II* restriction enzyme (New England Biolabs) at 37 °C overnight. Simultaneous digestion of the 218-bp amplified product yielded E3/E3 (145-, 50- and 23-bp), E4/E4 (195- and 23-bp) and E2/E2 (168- and 50-bp) fragments. Heterozygous genotypes were also resolved based on their respective sizes. For quality control, in each PCR reaction distilled water was used instead of DNA as a negative control, and more than 10 % of the samples were analyzed twice. DNA fragments were subjected to electrophoresis on a 3 % agarose gel for resolution.

Statistical analysis

Statistical comparisons were performed using Fisher's exact/Chi-square test. A probability value (p value) of <0.05 was taken statistically significant. All the statistical calculations were done using GraphPad Prism 5 statistical software. Hardy–Weinberg equilibrium for the distribution of the genotype was performed by Chi-square test.

Results

Lipid profile in study subjects

In the current study, a significant difference was found in the TC, LDL, and HDL levels between cases and controls ($p < 0.05$). However, no significant association was observed between TG levels and CAD ($p > 0.05$) Table 1.

Genotype analysis of ApoE gene variants

The ApoE genotype was successfully determined for all the subjects. Apo E allele frequencies showed moderate deviation from Hardy–Weinberg equilibrium. In CAD group 55 % of the subjects had ε3/ε3 genotype, 26.5 % had ε3/ε4 genotype, 6.5 % had ε4/ε4 genotype, 9 % had ε3/ε2, 2 % had ε2/ε4 genotype, and 1 % had ε2/ε2 genotype Table 2. In control group, the predominant genotype was ε3/ε3 (70 %), followed by ε4/ε3 (14.6 %), ε3/ε2 (7.99 %), ε2/ε4 (2.6 %), ε2/ε2 (2 %) and ε4/ε4 (2.6 %) Table 2. The ApoE allele frequencies in the control subjects were 0.075 for the ε2 allele, 0.82 for the ε3 allele, and 0.11 for the ε4 allele. The frequencies in the CAD group were 0.065 for the ε2 allele, 0.72 for the ε3 allele, and 0.20 for the ε4 allele Table 2. Our results indicated a significant association of ε4/ε3, ε4/ε4 genotype and ε4 allele with the risk of CAD ($p = 0.004$, $p = 0.02$ and $p < 0.001$, respectively) Table 2. Furthermore in this study, we observed that CAD patients carrying ε4 allele (ε3/ε4 & ε4/ε4) had significantly higher levels of LDL and TC ($p < 0.01$) Table 3. No other anthropometric measurement showed an association with ApoE genotype.

Discussion

In this study, we investigated the frequency of distribution of various Apo E genotypes in CAD patients in comparison with the control subjects of Kashmiri population so as to evaluate its association with the risk of CAD. This is for the first time that study of ApoE gene polymorphism in CAD patients has been done in ethnically discrete Kashmiri population. Different populations exhibit different frequencies in the distribution of apo E isoforms, and so far, the most frequent allele in all populations examined including our population is ε3 which codes for the isoform apo ε3 [13, 15, 16]. In our investigation, ApoE allelic frequencies in CAD group were 0.72, 0.20, and 0.065 for ε3, ε4, and ε2, respectively, while in controls the frequency was 0.82 for ε3, 0.11 for ε4, and 0.075 for ε2. In the current study, we observed a much higher frequency of ε4/ε4, ε3/ε4 genotypes and ε4 allele in the CAD group than that of the control group indicating the significance of ε4 in the pathogenesis of CAD. The association of ApoE gene with CAD has been described in many studies and meta-analyses. Compared to the most abundant ε3/ε3 genotype, whose effects are considered neutral the ancestral ε4 allele has been found as a risk factor for CAD [13, 17–20]. However, no association of ε2 allele with the risk of CAD was found in this study. Moreover, the present study

Table 1 Clinical parameters of CAD cases and controls

Parameter	Cases (n = 200)	Controls (n = 450)	OR (95 % CI)	p value
Age				
<50	47	135	1.39 (0.93–2.09)	0.11
≥50	153	315		
Gender				
M	130	260	1.35 (0.94–1.94)	0.18
F	70	190		
BMI				
<25	92	230	0.82 (0.57–1.15)	0.24
≥25	108	220		
Diabetes				
Yes	79	0	–	–
No	121	450		
Smoker				
Yes	111	248	1.01 (0.72–1.44)	0.93
No	89	202		
SBP				
≤120	89	236	0.73 (0.51–1.03)	0.07
>120	111	214		
DBP				
≤80	101	240	0.89 (0.63–1.26)	0.89
>80	99	210		
LDL				
≥130	130	210	2.12 (1.48–3.04)	<0.001
<130	70	240		
TC				
≥200	82	140	1.5 (1.07–2.21)	0.02
<200	118	310		
HDL				
≥40	87	248	1.5 (1.12–2.26)	0.007
<40	113	202		
TG				
<150	98	254	0.74 (0.52–1.05)	0.07
≥150	102	196		

Table 2 Apo E genotype and allele frequency in cases and controls

Genotype	Controls (n = 450)	Cases (n = 200)	OR (95 % CI)	p value
ε3/ε3	315	110	1.27 (0.95–1.7)	0.08
ε2/ε3	36	18	1.12 (0.59–2.10)	0.76
ε4/ε3	66	53	1.81 (1.19–2.74)	0.004
ε2/ε2	9	2	0.5 (0.07–2.5)	0.52
ε4/ε4	12	13	2.43 (1.02–5.8)	0.02
ε2/ε4	12	4	0.75 (0.20–2.54)	0.78
Allele	Controls	Cases	OR (95 % CI)	p value
ε3	732 (0.82)	291 (0.72)	Reference	
ε2	66 (0.075)	26 (0.065)	0.99 (0.60–1.65)	0.98
ε4	102 (0.11)	83 (0.20)	2.04 (1.46–2.85)	<0.001

Table 3 Association of Apo E polymorphism with the clinical characteristics of CAD patients

Parameter	ε3/ε3 (n = 110)	ε3/ε4 + ε4/ε4 (n = 66)	p value	OR (95 % CI)
Age				
<50	20	11	0.84	0.11 (0.46–2.69)
≥50	90	55		
BMI				
<25	49	27	0.75	1.16 (0.59–2.25)
≥25	61	39		
Diabetes				
Yes	43	30	0.43	0.77 (0.39–1.49)
No	67	36		
Smoker				
Yes	69	38	0.53	1.24 (0.63–2.42)
No	41	28		
SBP				
<140	51	32	0.87	0.92 (0.47–1.77)
≥140	59	34		
DBP				
<90	49	27	0.75	1.16 (0.59–2.26)
≥90	61	39		
LDL				
≥130	60	52	0.001	3.09 (1.46–6.63)
<130	50	14		
TC				
≥200	57	47	0.008	2.3 (1.14–4.65)
<200	53	19		
HDL				
≥40	56	25	0.10	1.70 (0.87–3.32)
<40	54	41		
TG				
<150	42	31	0.27	0.69 (0.36–1.36)
≥150	68	35		

revealed that the carriers with ε4 allele have higher LDL and TC levels compared to ε3/ε3 carriers. ε4 is considered to be a thrifty allele [21], and it has been associated with elevated levels of total cholesterol and low-density lipoproteins [22]. The association between ε4 and cholesterol levels could be due to the better binding attraction of the ε4 allele for the receptor, leading to an increase in plasma cholesterol levels. Another reason could be modification of effects of Apo E isoforms on plasma cholesterol by changes in dietary fat and cholesterol intake [17].

CAD risk is also influenced by several well-established risk factors, such as body mass index (BMI), an indicator of overweight and obesity, blood lipids, and blood pressure. In this study, statistically significant differences between LDL, TC, and HDL Levels were observed between cases and controls ($p < 0.001$, $p \leq 0.03$ and $p = 0.04$, respectively). However, we found no significant difference between BMI, blood pressure, or TG levels in CAD patients and controls.

Conclusion

Thus, our current data favor the concept that there is a significant impact of ApoE polymorphism on the risk of CAD. The data revealed that ApoE ε4 allele is associated with increased risk of CAD and increased levels LDL and TC in Kashmiri population.

Compliance with ethical standards

Conflict of interest None.

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