Apolipoprotein E Gene Polymorphism as a Risk Factor for Atherosclerosis in Egyptians

Olfat G. Shaker, Mohamed A. Ay El Deen*
Departments of Medical Biochemistry and Vascular Surgery*
Faculty of Medicine, Cairo University

ABSTRACT

Apolipoprotein E (apo E) plays a role in the regulation of lipid metabolism by mediating cellular uptake of lipoprotein particles via apo E and apo B/E-specific receptors in liver and other tissues. It is classified into 3 major isoforms in human (E_2, E_3 and E_4) according to the differences of amino acids in position 112 and 158. In this study, 100 persons were classified into 3 groups: control group (26), atherosclerosis group (36) and high risk group (diabetic, hypertensive and obese patients) (38). Apo E genotyping was done using the PCR. The frequency of the E_2 allele was relatively high in the control group (30.8%) and in the persons with normal serum cholesterol level (46.6%). Meanwhile, the frequency of the E_4 allele was relatively high in the atherosclerosis group (29.2%) and also in hypercholesterolemics (61.3%). We can conclude that E_2 allele is protective against the development of atherosclerosis, while E4 allele is associated with an increased risk of atherosclerosis.

INTRODUCTION

Atherosclerosis is a major cause of death in the industrialized world. Though, much work on the pathogenesis of atherosclerosis points to low density lipoprotein (LDL) as a key etiological feature in the generation of atherosclerotic plaque (1). The pathological appearance and progression of atherosclerosis is dependent on the presence of injurious agents in the vascular endothelium and variations in different subsets of genes (2). During the last few years, active expression of several genes has been reported in developing atherosclerotic lesions. These genes include scavenger receptors, lipoproteins lipase, tissue factor, apolipoprotein E and various cytokines (3). Abnormalities in apolipoprotein E (apo E) are associated with an increased risk of atherosclerosis and coronary artery disease (CAD) (4). Therefore, apo E genotyping is of increasing importance in clinical practice to identify individuals at risk for atherosclerosis and cardiovascular diseases (5). Apo E is a constituent of intermediate density lipoprotein (IDL), very low density lipoprotein (VLDL) and chylomicrons and is required for receptor mediated endocytosis in the liver. Two hepatic receptors are known to bind apo E containing lipoproteins: LDLR (low- density- lipoprotein receptors) and LRP (lipoprotein receptor related protein). Apolipoprotein E is a single polypeptide chain of 229 amino acids (6). Apolipoprotein (apo) E gene code forms three major isoforms (E_2, E_3, and E_4) with variable effects on lipid metabolism (7). All isoforms are distinguishable on the DNA level by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) (8) Apo E_2 isoform is supposed to play a protective role against the development of atherosclerosis. Also Apo E epsilon2 has a protective effect with regard to the development of ICVD (9). Apo E_2 shows a significantly decrease binding activity to LDL and chylomicron remnant receptors and is therefore, catabolized more slowly than apo E_3. Consequently the accumulation of triglyceride rich VLDL remnants and a low level of LDL cholesterol are observed (10). On the other hand, apo E_4 is associated with an increase risk of atherosclerosis and hyperlipidemia (type III). Apo E_4 is catabolized more rapidly than Apo E3 leading to an increase in LDL- cholesterol, and therefore, the development of atherosclerosis (11). In this study, polymorphism at human apo E locus was typed by using PCR amplification performed on genomic DNA and compared between atherosclerotic patients, patients at high risk of developing atherosclerosis and normal individuals.

SUBJECTS AND METHODS

Blood samples were obtained from (26) normal healthy people who visited Kasr El- Aini Cairo University Hospital for routine check up. They had no family or past history of metabolic diseases and their laboratory data were normal. Also samples were taken from (36) atherosclerotic group and (38) patients of high risk for developing atherosclerosis.

DNA preparation

DNA was isolated from 100μL of anticoagulated peripheral blood according to standard procedures (12). Proteins were salted- out by using a saturated solution of sodium chloride. The supernatant was transferred to another microfuge tube and two volumes of absolute ethanol were added and the DNA was precipitated at -20ºC overnight. The mixture was centrifuged in a microcentrifuge at 14,000 rpm for 20 min. to pellet the DNA. The DNA pellets were washed in 75% ethanol and reprecipitated by centrifugation, air dried and dissolved in 20 μL of nuclease free sterile water.

Polymerase Chain Reaction (PCR)

All PCR assays were carried out in glass capillaries in an air thermocycler (Idaho Tech., Idaho, and USA). Amplification mixture (50 μL) contained 10 mM Tris (pH 8.3), 1.0 mM $MgCl_2$, 0.2 mM of each of the four deoxynucletide triphosphates, 20 pmole of each primer, 10 ng of genomic DNA, and 2.5 units of Taq DNA polymerase. DNA Amplification cycles were as follows: hard denaturation at 94 C for 50 sec. (1 cycle), followed by 35 cycles of denaturation at 94°C for 10 sec., annealing at 55°C for 10 sec. and extension at 72°C for 25 sec.

Oligonucleotide primers used in each PCR are underlined in figure (1). For genotyping, two different PCR runs were performed on each DNA sample. As illustrated in figure (2), reaction #1 was specific for genotypes E_2 and E_3, while reaction #2 was specific for genotypes E_3 and E_4.

Electrophoresis

PCR products were electrophoresed on 2% agarose gels containing 0.5 μg/mL ethidium bromide in 1 X TAE (40 mM Tris-HCl, pH 7.4, 20 mM sodium acetate, 1 mM Na_2 EDTA) at 5 V/cm. DNA bands were visualized under UV light and data interpreted as shown in figure (2).

RESULTS

This study included 100 subjects presenting to Kasr El-Aini Cairo University Hospital from March 2002 to March 2004. The classification of the subjects and their clinical presentation and investigation findings were illustrated in Table (1).

As regards pattern of lipoprotein E in the three groups; there are 3 isoforms of Apo E (E_2, E_3 and E_4) each is formed of 2 alleles. So we have 100 patients with 200 alleles. Table (2) shows Apo E genotypes in the 3 groups.

Comparison of allele frequencies between group I and group II revealed a significant difference (Chi^2= 9.9, p=0.007). As shown in Table 2, the frequency of E_2 is higher in group I than in group II. On the contrary, the frequency of E_4 is higher in group II than in group I.

CCCGGCTGGGCGCGGACATGGAGGACGTG**1**GCGGCCGCCTGGTGCAGTACCGCGGCGAGG
TGCAGGCCATGCTCGGCCAGAGCACCGAGGAGCTGCGGGTGCGCCTCGCCTCCCACCTGC
GCAAGCTGCGTAAGCGGCTCCTCCGCGATGCCGATGACCTGCAGAAG**2**GCCTGGCAGTGT
ACCAGGCCGGGGCCCGCGAGGGCGCCGAGCGCGGCCTCAGCGCCATCCGCGAGCGCCTGG
GGCCCCTGGTGGAACAGGGCCGCGTGCGGGCCGCCACTGTGGGCTCCCTGGCCGGCCAGC
CGCTACAGGAGCGGGCCCA

Position	1	Position	2
E_2/E_3	T	E_2	T
E_4	C	E_3/E_4	C

Figure 1
Nucleotide sequence of a part of the human apolipoprotein E gene. There are at least six distinct genotypes derived from the apolipoprotein E gene on chromosome 19. This epsilon-4 (E_4) allele had a C nucleotide at position 1 (underlined). On the other hand, the epsilon-2 (E_2) allele had a T nucleotide at position 2 (underlined). Oligonucleotide primers used in PCR- based genotyping are underlined (single and double underlines indicate minus and plus sense primers, respectively).

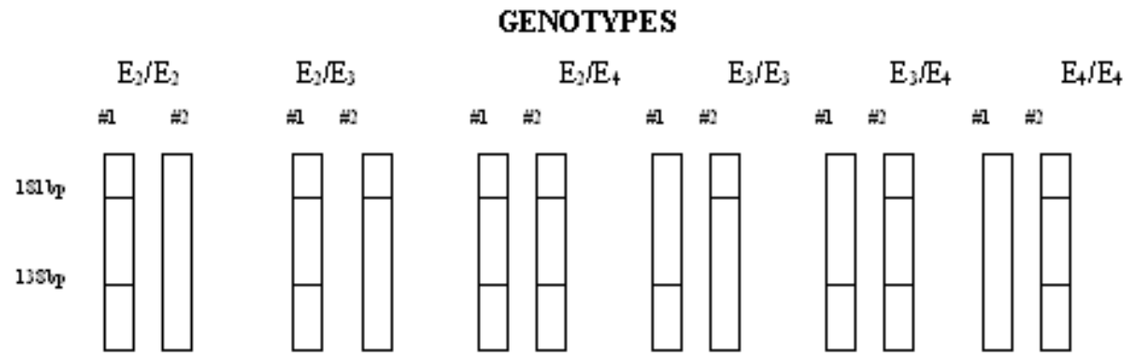

Figure 2
Each of the six genotypes has a characteristic banding pattern after gel electrophoresis. The PCR products (either 181 or 138 bp) expected from each genotype were resolved by agarose gel electrophoresis. The PCR runs, #1 and #2, performed on each DNA sample were specific for E_2/E_3 and E_3/E_4 genes, respectively.

Comparison of allele frequencies between group I and group III didn't show any significant difference (Chi^2= 3.3, p=0.2).

The PCR products (either 181 or 138 bp) expected from each genotype are presented in figure (3). For each DNA sample, two different PCR runs were performed in glass capillaries in an air thermocycler (PCR 1 & 2). Reaction (1) was specific for E_2 and E_3 genes, while reaction (2) was specific for E_3 and E_4 genes.

Comparison of allele frequencies at different serum cholesterol level revealed a significant difference (Chi^2= 91.6, p<0.001). As shown in Table (3), the frequency of E_2 allele at ≤200 mg/dL is higher than the other levels. On the contrary, the frequency of E4 is higher when the total cholesterol> 250 mg/dL than the other 2 levels. Ther is also a significant difference (Chi^2= 66.8, p<0.0001) in comparison of allele frequencies in relation to HDL-Cholesterol (Table 3). The frequency of E_2 allele at HDL cholesterol ≥40 mg/dL group is higher than the other group (<40 mg/dL). On the other hand, the frequency of E4 allele is higher at HDL cholesterol <40 mg/dL than the other group.

Also, Table (3) shows the relation between Apo E alleles and the level of LDL cholesterol. The E_2 allele at LDL cholesterol ≤190 mg/dL is more frequent than its frequency at the LDL- cholesterol >190 mg/dL. Meanwhile, at LDL- cholesterol >190 mg/dL, the E_4 allele had a higher frequency than its frequency at ≤190 mg/dL, and so the test revealed a significant difference (Chi^2= 64.8, p<0.0001).

The frequency of E_2 allele doesn't differ significantly at serum triglycerides >165 mg/dL from that ≤165 mg/dL. On the other hand, the frequency of E_4 allele is higher at level > 165 mg/dL than at level ≤165 mg/dL (Table 3). The frequency of E_3 allele is higher at level ≤165 mg/dL than that > 165 mg/dL, so the test revealed a significant difference (Chi^2= 38.3, p<0.0001).

The same Table (3) demonstrates the comparison of allele frequencies between obese and non-obese subjects, revealed no significant difference (Chi^2= 0.854). The present work shows no statistically significant differences between hypertensive and Normotensive subjects. Also, no significant difference was found by comparing the allele frequencies between diabetic and non-diabetics (Table 3).

Table 1
The clinical presentation and the investigation findings in relation to the 3 groups

Items	Group I n=26		Group II n=36		Group III n=38	
	n	%	n	%	n	%
Sex: Males	19	73,1	30	83,3	25	65,8
: Females	7	26,9	6	16,7	13	34,2
Occupation: Physical work	18	69,2	18	50,0	8	21,1
: Sedentary	8	30,8	18	50,0	30	78,9
Smoking	0	0,0	28	77,8	19	50,0
Family history of D.M.	0	0,0	5	13,9	4	10,5
Family history of hypertension	0	0,0	1	2,8	3	7,9
Overweighs (obese)	0	0,0	5	13,9	16	42,1
Impaired pulses	0	0,0	36	100,0	8	21,1
Hypertension	0	0,0	12	33,3	16	42,1
D.M.	0	0,0	13	36,1	29	76,3
E.C.G: Ischaemic changes	0	0,0	20	55,6	10	26,32
S. Cholesterol>250 mg/dL	0	0,00	18	50,0	13	34,2
S. Cholesterol 201- 250 mg/dL	11	42,3	15	41,7	14	36,8
S. Cholesterol ≤ 200 mg/dL	15	57,7	3	8,3	11	29,0
S. LDL Cholesterol>190 mg/dL	0	0,00	18	50,0	13	34,2
S. LDL Cholesterol ≤190 mg/dL	26	100,0	18	50,0	25	65,8
S. HDL Cholesterol ≥40 mg/dL	26	100,0	17	47,2	25	65,8
S. HDL Cholesterol<40 mg/dL	0	0,0	19	52,8	13	34,2
S. triglycerides>165 mg/dL	0	0,0	17	47,2	17	44,7
S. triglycerides ≤ 165 mg/dL	26	100,0	19	52,8	21	55,3

Table 2
Apo E genotype and allele frequency in 100 subjects (200 alleles)

	Genotype frequency %						Allele frequency %		
	E_2/E_2	E_2/E_3	E_2/E_4	E_3/E_3	E_3/E_4	E_4/E_4	E_2	E_3	E_4
Group I	19,2	23,1	-	38,5	7,7	11,5	30,8	53,9	15,4
GroupII	-	11,1	8,3	38,9	33,3	8,3	9,7	61,1	29,2
Group III	10,5	15,8	5,3	34,2	18,4	15,8	15,8	51,3	27,6

DISCUSSION

Apo E genotypes polymorphism is divided into 3 isoforms of E_2, E_3 and E_4 depending on the net charge of amino acids on isoelectric focusing. The three isoforms are classified by changes of

their amino acids components of cysteine and arginine (13). In isoform E_2, arginine is substituted by cysteine at position 112 (14). The isoform E4 produces by changes the codon at position 158 results in change of cysteine to arginine. Apo E plays an important role in the regulation of human plasma lipoprotein concentration (15).

In this study, apo E gene polymorphism was evaluated as a risk factor for atherosclerosis among Egyptians by using PCR for genotyping of apo E. The percentages of the 3 alleles were (E_2 =19.5%, E_3 =55.5% and E_4=25%) i.e. E_3 is the most common allele, E_4 is the next and E_2 is the least common one. These data run parallel with the results of (16). They stated that E_3 allele is the most common allele, while E_2 is the lowest one. The relative allele percentages in Japanese were (E_2: 3.8%, E_3: 84.3% and E_4: 11.2%) and in Finnish were (E_2: 4.1%, E_3: 73.3% and E_4: 22.7%). Also in healthy Thais apo E genotype frequencies were 5.5 % for E_2/E_2, 12.4 % for E_2/E_3, 81.1 % for E_3/E_3 and 0.9 % for E_4/E_4 (17). These data suggested that racial differences were existed in apo E polymorphism (8). The $E_{2/2}$ genotype was 19.23% at group I, absent at group II, and 10.53% at group III. So, this genotype was relatively high in the normal group while it was completely absent at atherosclerotic patients i.e. it has a protective role against the development of atherosclerosis. These data correspond to Sing et al (11) who stated that there is a growing body of evidence for a protective, anti atherogenic role of E_2 allele in the absence of any environmental or genetic challenge. Also our results are in accordance to Lin et al (9) who stated that Apo E epsilon 2 has a protective effect with regard to the development of ICVD for Taiwan Chinese below the age of 65. Utermann (18) found that there was no $E_{2/2}$ genotype among the myocardial infraction group (n= 239). They sated that this supports the hypothesis that the presence of the E2 allele protects against atherosclerosis.

The $E_{3/3}$ genotype was 38.46% in group I, 38.89% in group II, and 34.21% in group III, i.e. it didn't show much variation between the 3 groups and it is the most common genotype in each group. Black et al (19) stated that the apo $E_{3/3}$ homozygote was the most frequent genotype in all the populations. At the same time, our results are in agreement with Chanprasertyothin et al (17) who stated that E3/E3 was 81.1 per cent in his study.

Meanwhile, $E_{4/4}$ genotype was 11.54% in group I, 8.33% in group II, and 15.79% in group III. Although it was relatively low at group II in comparison to its percentage at group I, but by the addition of the result of the heterozygote $E_{3/4}$ genotype, this will explain the prevalence of the E_4 allele in the atherosclerotic group.

By studying the alleles in relation to the groups, the E_2 allele was relatively high in the normal group: (30.77%), while it was relatively low in the atherosclerotic group 9.72%, (Chi^2= 9.9 and p=0.007). The E_2 allele is supposed to play a protective role against the development of atherosclerosis. It shows a significantly decreased binding activity to LDL and chylomicron remnant receptors (20).

The E_4 allele was relatively low in normal group: 15.38%, while it was relatively high in atherosclerotic group: 29.17 % (Chi^2= 9.9 and p=0.007). This significant difference revealed that the apo E4 is associated with atherosclerosis, i.e., in subjects with E_4 allele (especially $E_{4/4}$ or $E_{3/4}$ genotypes), there will be an increased incidence to develop atherosclerosis. These results are in accordance with Yang et al (21) who stated that apo E_4 has a very close relation to CHD, suggesting that apo E_4 is an independent genetic factor of the early onset of CHD. Also, Yan et al (22) detected the genetic polymorphism of the apo E gene and stated that apo E gene might contribute to the determination of serum lipid profile and the development of CHD.

The E_4 allele is distributed differently from apo E_3 between VLDL and HDL is degraded more rapidly than apo E_3 and may enhance the catabolism of E_4 bearing particles, leading to

Table 3
Apo E allele frequency in all alleles (n = 200), grouped according to different biochemical and clinical characteristics

Characteristics	n.	Apo E allele frequency %			Chi^2	p
		E_2	E_3	E_4		
Cholesterol 200 mg/dL	58	46,5	50	3,5		
Cholesterol 201-250 mg/dL	80	11,3	76,2	12,5	91,6	<0,001
Cholesterol >250 mg/dL	62	4,8	33,9	61,3		
HDL-Cholesterol <40 mg/dL	64	4,7	34,9	60,9	66,8	<0,001
HDL-Cholesterol 40 mg/dL	136	26,5	65,4	8,1		
LDL-Cholesterol 190 mg/dL	138	26,1	65,2	8,7	64,8	<0,0001
LDL-Cholesterol >190 mg/dL	62	4,8	339, 61,3			
Triglyceride 165 mg/dL	132	20,5	67,4	12,1	38,3	<0,0001
Triglyceride > 165 mg/dL	68	17,7	30,9	51,5		
Obese	42	21,4	59,3	19,1	0,85	0,65 (n.s.)
Non-obese	158	19,6	54,4	26		
Hypertensive	56	14,3	50,0	35,7	4,62	0,10 (n.s.)
Normotensive	144	21,5	57,0	21,5		
Diabetics	84	21,4	54,8	23,8	0,44	0,80 (n.s.)
Non-diabetics	116	18,1	55,2	26,7		

other alternations in lipoprotein metabolism which result in elevated levels of LDL (16). Also, in previous study done by Kim et al (8), who stated that apoE_4 is associated with an increase risk of atherosclerosis as it is catabolized more rapidly than apo E_3, leading to an increase in LD cholesterol, and therefore the development of atherosclerosis.

By studying the relation of alleles with obesity, diabetes mellitus and hypertension, it revealed that there is no significant difference for each item. But the relation of the alleles with the lipid profile has a great difference. By studying the relation of alleles and the total serum cholesterol (T.S. cholesterol), we found that at T.S. cholesterol> 250 mg/dL, the E_4 allele is the most prevalent allele (61.3%), while the E_3 allele is the next one (33.9%) and the E_2 allele is the least common one (4.8%) (Chi^2= 91.6 and p<0.01). At T.S. cholesterol 201- 250 mg/dL, E_4 (12.5%), E_3 (76.2%) and E_2 (11.3%). On the contrary, at T.S. cholesterol<200 mg/dL, E_4 (3.5%), E_3 (50%) and E_2 (46.5%). So, the E_2 allele is associated with low total serum cholesterol, while E_4 allele is related to high total serum cholesterol level.

These results agreed with the results of previous studies. Kim et al (8), found that at T.S. cholesterol <200 mg/dL, E_2: 3.8%, E_3: 94.2%, and E_4: 2%, while at T.S. cholesterol>240 mg/dL, E_2: 0%, E_3: 80%, and E_4: 20%. At the same time, Chanprasertyothin (17) stated that subjects having the E_2 allele had lower TC ($r = -0.27$, $P < 0.05$) and LDL-C. ($r = -0.25$, $P < 0.05$).

The E_4 allele is frequently observed with high plasma cholesterol level (23). In a previous study, Davignon et al (16) reported that in seven studies done on different populations for evaluation of the association between apo E genotypes and the average cholesterol level, individuals with $E_{2/2}$ genotype had the lowest, while individuals carrying the $E_{3/4}$ or $E_{4/4}$ genotypes had the highest average cholesterol level.

In view of the significant opposite impacts of the E_4 and the E_2 alleles on plasma LDL concentrations, it is evident that determination of apo E phenotypes will become useful adjunct to the assessment of the risk of atherosclerosis and hypercholesterolemia of an individual. The E_4 allele is associated with higher levels of LDL cholesterol and so could favor the development of atherosclerosis (16).

We can conclude that, apo E gene polymorphism is closely associated with the development of atherosclerosis, detection of apo E genotypes early in life can diagnose persons at high risk for developing atherosclerosis and this helps in early prophylaxis.

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