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Genotyping via Amplification and HhaI Cleavage of Apolipoprotein E (ApoE) Gene in Patients with Coronary Artery Disease

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= Abstract = **DNA amplification and HhaI cleavage for the apolipoprotein E (apoE) genotyping is identified in 108 patients with coronary artery disease (CAD). A group of randomly selected 100 normal individuals analysed as control samples. There were no significant differences in apoE genotype frequencies between the two groups. Analysis of variances was performed to test for mean differences in plasma lipids, lipoprotein and apolipoprotein B (apoB) levels. Compared to controls, patients with CAD had significantly higher levels of total cholesterol (TC) and low density lipoprotein-cholesterol (LDL-Chol). The ratios of LDL-Chol to apoB were also elevated in the CAD group. The levels of TC and LDL-Chol in the E3/3 genotype of patients were significantly higher than those of the controls. The high density lipoprotein-cholesterol (HDL-Chol) levels in E4/3 type of the patients were significantly higher than those of E3/3 subjects. As phenotype analysis for the apoE polymorphism differs somewhat among populations, so we discussed possible reasons for these discrepancies.**

Key Words: *Apolipoprotein E, Genotyping, Coronary artery disease*

INTRODUCTION

Apolipoprotein E (apoE) is one of the most extensively studied of all the apolipoproteins. It is a structural and functional constituent of chylomicrons, very low density lipoprotein (VLDL), and some subfractions of HDL. Apo E serves as a ligand for LDL (apo B,E) and apo E receptors. And it allows a specific uptake of its carrier lipoprotein particles by the hepatic cells (Mahley *et al.* 1984; Brown and Goldstein. 1986; Beisiegel *et al.* 1988). In addition, apoE is also involved in the conversion of VLDL to LDL (Enholm *et al.* 1984). Thus apoE plays an important role in lipoprotein metabolism and may be involved in atherogenesis.

Three major apoE isoproteins, apoE2, E3, and

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E4 are known. These isoforms are coded by three codominant alleles (ε2, ε3, and ε4) at a single locus of chromosome 19. As a result, six apoE phenotypes are possible (Enholm *et al.* 1986; Eto *et al.* 1986; Utermann. 1987; Sing and Davignon. 1985; Menzel *et al.* 1983; Cimming and Robertson. 1984). E4 differs from E3 by an amino acid substitution (Cys → Arg) at position 112, while E2 differs from E3 due to cystein for arginine replacement at residue 158. Accordingly, apoE4 has one more positive charge than E3, whereas apoE2 has one less (Weisgraber *et al.* 1981; Weisgraber *et al.* 1982). In addition, other rare isoforms (E1, E5 and E7) have been reported (Gregg *et al.* 1983; Weisgraber *et al.* 1984; Yamamura *et al.* 1984; Yamamura *et al.* 1984). Epidemiological studies have demonstrated the association of different apoE phenotypes with lipid disorders. Although some studies have failed to establish such an association, ε2 allele is associated with lower TC and LDL-Chol levels compared to ε3. Most patients with type III hyperlipoproteinemia and hypertriglyceridemic state are homozygous for E2 isoform but some are heterozygous E3/2 or E4/2 patterns (Utermann *et al.* 1975; Utermann *et al.* 1977; Utermann *et al.* 1979; Zannis and Breslow. 1980). The 4 allele is associated with elevations in TC, LDL-Chol, and apoB levels (Robertson and Cumming. 1985; Utermann *et al.* 1984; Assmann *et al.* 1984). It is also more frequent in subjects with angiographically documented CAD, myocardial infarction and hypercholesterolemic state (Cumming and Robertson. 1984; Lenzen *et al.* 1986; Tsuchiya *et al.* 1987). According to recent investigation, the apoE4 allele appears to be a possible risk factor or susceptibility gene in late-onset familiar and sporadic Alzheimer disease (Strittmatter *et al.* 1993).

Detecting of apoE phenotypes was mainly done by isoelectric focusing (IEF) of delipidated VLDL or by a combination of IEF and immunoblotting of native serum. This method still requires pretreatment of neuraminidase and may lead to misreading of the phenotype as a result of post-translational modifications. Genotypes have also been determined by hybridization with allele specific oligonucleotide probe or automated sequence analysis for amplification products. But there is a problem for use of radiation. The authors were undertaken in order to determine the genotypes and allele frequencies of the apoE using polymerase chain reaction (PCR) in Korean patients with CAD. It was performed to obtain further insights into the question of whether apoE genotypes influence conventional plasma lipid parameters.

MATERIAL AND METHODS

Subjects

One hundred and eight patients (72 males and 36 females) with relatively severe CAD referred to Seoul National University Hospital were determined by angiography to have at least a 50% obstruction of one or more coronary arteries. Patients with secondary hyperlipidemia, liver disease and diabetes were excluded from this group. No patient was under therapy with other lipid-lowering drugs. In addition, a randomly selected normal population of 100 matched subjects (64 males and 36 females) living in Seoul, Korea, was analysed as control samples. Blood samples were obtained in EDTA tubes from patients and controls who had been fasting for 12~16 hours.

DNA amplification and restriction isotyping with HhaI

Genomic DNA was extracted from whole blood by the Trixton X-100 lysis method with slight modifications (Kunkel *et al.* 1979). The oligonucleotides used as primers for PCR were synthesized on an Applied Biosystems 380B DNA synthesizer and purified on OPC cartridges (Applied Biosystem, Foster City, CA) according to the manufacturer's instructions. Cleavage patterns with restriction enzyme HhaI of each type are shown in Figure 1. The sequences of the two primers are as follows; F4: 5′-ACAGAATT-CGCCCCGGCCTGGTACAC-3′, F6: 5′-TAAGCTT-GGCACGGCTGTCCAAGGA-3′. Procedure of PCR using thermostable Taq polymerase (Cetus) was slightly different from the method described by Saiki et al (Saiki *et al.* 1988). Briefly, a total 100ul volumes of the reaction mixture contained 200~

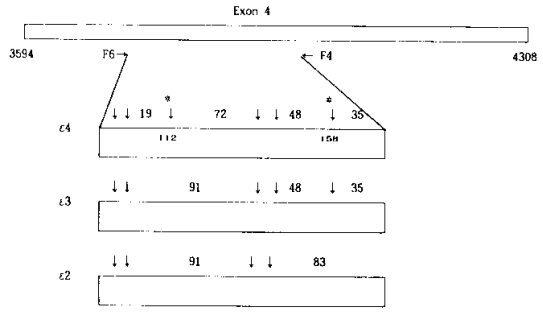

Fig. 1. Cleavage patterns of the three common alleles of the apoE gene containing amino acid positions 112 and 158. Cleavage sites for Hhal are arrowed. Asterisks and numbers indicate the variant sites and base pairs of cleavage sizes, respectively.

400ng of genomic DNA, 200ng of each primer, 200uM of each dNTP, and buffers recommended by the manufacturer. Reaction mixtures were covered with light mineral oil to reduce evaporation, and initial denaturation was at 95℃ for 10 min. Thirty cycles consisting of 1 min denaturation at 95℃, primer annealing at 60℃ for 1 min, and 2 min chain extension at 70℃ were performed. PCRs were carried out using a DNA thermal cycler (Ericome). After amplification, an aliquot (10ul) of each reaction was digested with 10 units of restriction enzyme Hhal for overnight at 37℃. Then the digested DNA samples were electrophoresed on nondenaturing 10% polyacrylamide slab gels at 70V for 16 hrs.

Determinations of the conventional plasma lipid parameters

The concentrations of plasma TC and TG were determined enzymatically using commercial kits (Boehringer-Mannheim, FRG) by Hitachi 736-40 automatic analyzer. HDL-Chol was determined by measuring cholesterol in the supernatant liquid after precipitation of the plasma with $MgCl_2$ and dextran sulfate by Gilford Impact 400E automatic analyzer. Levels of LDL-Chol were then calculated using the formula of Friedewald et al (Friedewald *et al.* 1972). Apo B levels were determined by immunoturbidimetric method with reagent kits (Eiken Chemical Co, Japan).

Statistical analysis

Data analysis was performed with the Statistical Analysis System (SAS). Differences of allele frequencies and conventional lipid parameters were determined by the Student t-test. Two groups or more were compared by the Mann-Whitney U test and the Kruskal-Wallis 1 way ANOVA. We compared the observed genotype frequencies with the expected ones calculated by the Hardy-Weinberg genetic hypothesis equation.

RESUITS

The products of PCR amplification and Hhal cleavage in apoE sequences containing amino acid positions 112 and 158 from genomic DNA were separated by nondenaturing 10% polyacrylamide gel electrophoresis (PAGE). Six distinguishable genotypes were observed among 208 Korean subjects (108 patients with CAD and 100 controls, Figure 2). This research appears to show that apoE gene are encoded by three common alleles ($\varepsilon 2$, $\varepsilon 3$ and $\varepsilon 4$) as in other populations. However, five of the six potential apoE patterns in the patient samples were identified. That is, the patient group had not homozygous type for the 2 allele. Comparison of the genotypes and allele frequencies between the two groups are shown in Table 1. E3/3 showed almost the same frequency in both groups as the most prevalent genotype and is considered to be the normal form. The next frequent genotype was E4/3. The other genotypes showed frequencies of less than 10 percent. E4/4 and E4/2 genotypes showed more frequencies in the patients than those of controls. On the other hand, E3/2 genotype was more frequent in the control group. Apo E genotype frequencies were not significantly different between the control and patient groups (Student t test, $P = 0.480$). Two groups were no significant deviation of observed gene frequencies from the expected ones calculated by the Hardy-Weiberg genetic hypothesis equation ($\chi^2 = 1.6486$,

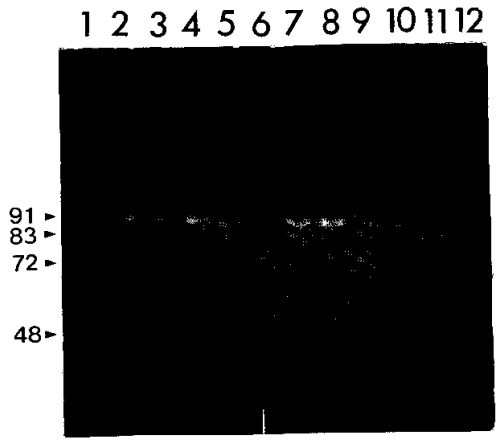

Fig. 2. Products of PCR amplification and HhaI cleavage for apoE gene by nondenaturing 10% polyacrylamide gel electrophoresis. Each lane is represented the genotype of samples studies: E2/2 (1 and 7 lanes), E3/3 (2 and 8 lanes), E4/2 (3 and 9 lanes), E4/3 (4 and 10 lanes), E3/2 (5 and 11 lanes), and E4/4 (6 and 12 lanes). Although 48 bp of E3/2 type is weak in intensity, it is well recognized on gel.

d.f = 3, in control group; 2 = 1.6157, d.f = 3, in patient group).

Age, sex, and lipid profiles between the two groups are compared in Table 2. Mean age of the patients was significantly higher than that of controls ($P<0.01$). Compared to controls, patients had significantly higher levels of LDL-Chol ($P<0.01$), TC and LDL-Chol/apoB ($P<0.001$). Also these mean parameters of patients in both sexes were all higher than those of controls. Distribution in each category of LDL-Chol and apoB levels between the two groups are compared in Figure 3 and Figure 4, respectively. In the control group, the skewness of the apoB distribution showed more increase than that of the LDL-Chol. Considering that most of the controls were normotriglyceridemics, distributions of LDL-Chol and apoB may be resulted from significant variation of the composition of LDL particles in each individual. Although some exceptions showed in the categories of high level, there are increased patterns for apoB and LDL-Chol in the patient group. Levels of LDL-Chol and apoB were also analysed for each age category within both groups (Table 3 and 4). In 40~64 age, the normal

Table 1. Comparison of apoE genotypes and gene frequencies in Korean population

	Controls		CAD	
	No. observed	(%)	No. observed	(%)
E4 / 4	2	(2.0)	8	(7.4)
E3 / 3	66	(66.0)	72	(66.6)
E2 / 2	2	(2.0)		
E4 / 3	20	(20.0)	18	(16.7)
E4 / 2	4	(4.0)	8	(7.4)
E3 / 2	6	(6.0)	2	(1.9)
Total	100	(100.0)	108	(100.0)
Gene frequencies				
$\varepsilon 4$	0.140 ± 0.0244		0.195 ± 0.0283	
$\varepsilon 3$	0.790 ± 0.0435		0.760 ± 0.0412	
$\varepsilon 2$	0.070 ± 0.0387		0.046 ± 0.0283	

The genotype frequencies between the two groups were not statistically significant by Student t-test ($P = 0.480$). There are no significant deviation of observed gene frequencies from the expected ones calculated by the Hardy-Weinberg genetic hypothesis equation ($\chi^2 = 1.6486$, df = 3, in control group; $\chi^2 = 1.6157$, df = 3, in CAD group). CAD: coronary artery disease

Table 2. Lipid parameters in the study subjects

	Patients (n = 108)		Controls (n = 100)	
	Males (n = 72)	Females (n = 35)	Males (n = 64)	Females (n = 36)
Age (yr)[a]	57.0 ± 10.2	54.5 ± 11.2	53.4 ± 6.7	45.0 ± 6.2
Cholesterol[b]	214.1 ± 40.5	221.8 ± 53.4	194.1 ± 22.4	186.5 ± 26.4
Triglycerides	197.6 ± 120.9	172.0 ± 122.6	189.3 ± 81.0	133.4 ± 73.9
HDL-cholesterol	40.2 ± 10.2	46.1 ± 11.0	39.5 ± 8.1	45.6 ± 10.2
LDL-cholesterol[a]	134.1 ± 35.2	141.3 ± 54.2	116.4 ± 20.3	115.0 ± 20.5
ApoB	85.8 ± 26.0	78.0 ± 31.1	83.7 ± 14.2	82.3 ± 28.4
LDL-Chol / apoB[b]	1.6 ± 0.4	1.9 ± 0.7	1.3 ± 1.4	1.4 ± 0.7

Values are means ± SD, (mg/dl).
[a]$P<0.01$; [b]$P<0.001$: Significance of mean difference between the two groups.

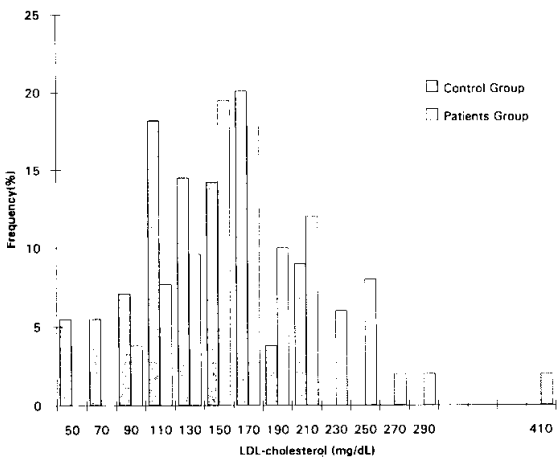

Fig. 3. Comparison of frequency distribution of plasma LDL-cholesterol levels between controls and patients with CAD in Koreans.

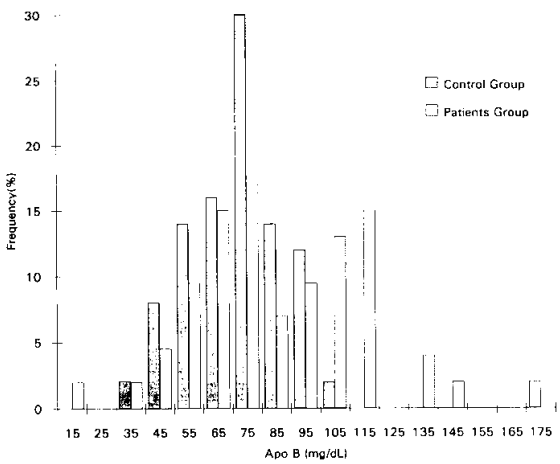

Fig. 4. Comparison of frequency distribution of plasma apo B levels between controls and patients with CAD in Koreans.

group generally showed a gradually increase in the levels of LDL-Chol and apo B with age. These patterns were not adjusted to LDL-Chol of the patient group, but the levels of apoB increased with age. Based on the genotypes of the apoE, the overall mean ± SD of each lipid variable are listed in Table 5.

DISCUSSION

In Western societies, atherosclerosis related to ischemic heart disease is the most common cause of deaths. Although the incidence of coronary artery disease (CAD) in Koreans is not high as in Caucasians, it has also been gradually increasing. This may be due to environmental variations such as changes in diet or life-style. But differences in the genetic background may be a more important factor. The present study focussed on the determination of the genotype and allele frequencies of the apoE gene between patients with CAD and control groups.

Table 3. Levels of plasma LDL-cholesterol for each age group

Age group (yr)	Patients	Sample size	Controls	Sample size
30~34			91.4 ± 4.9	4
35~39	146.9 ± 27.4	6		
40~44	132.5 ± 50.1	16	113.9 ± 15.8	22
45~49	125.5 ± 44.3	10	115.4 ± 19.3	24
50~54	161.9 ± 43.9	10	116.4 ± 36.1	10
55~59	130.8 ± 40.2	22	117.5 ± 17.3	28
60~64	149.9 ± 40.7	16	123.9 ± 23.9	12
65~69	129.3 ± 46.9	22		
> 70	123.6 ± 33.7	6		

Values are means ± SD, (mg/dl)

Table 4. Levels of plasma apoB for each age group

Age group (yr)	Patients	Sample size	Controls	Sample size
30~34			59.5 ± 7.1	4
35~39	86.7 ± 12.7	6		
40~44	68.9 ± 29.9	16	84.0 ± 34.1	22
45~49	71.4 ± 37.4	10	85.7 ± 14.5	24
50~54	95.6 ± 27.3	10	86.2 ± 25.5	10
55~59	89.4 ± 30.1	22	80.8 ± 10.8	28
60~64	90.0 ± 28.5	16	88.5 ± 10.3	12
65~69	77.2 ± 23.3	22		
> 70	98.7 ± 16.5	6		

Values are means ± SD, (mg/dl)

Previously a number of studies have reported that 6 genotypes of apoE have been distinguished by isoelectric focussing (IEF) and polymerase chain reaction (PCR) techniques in various ethnic group. In the present study we used the method of a combination of PCR amplification and HhaI cleavage with slight modification followed by non-denaturing polyacrylamide gel electrophoresis (PAGE) (Hixson and Vernier, 1990; Wenham *et al.* 1991).

As in the case of other populations, this research appears to show that genotypes of apoE gene were determined to 6 different genotypes coded by three common alleles ($\varepsilon 2$, $\varepsilon 3$ and $\varepsilon 4$). There were no significant differences in apoE genotype frequencies between patients with CAD and control groups in Koreans ($p = 0.480$, Student t-test). Also the frequency of the apoE alleles in the samples studied here were similar to those observed in the other ethnic groups studied.

To evaluate whether the genotype variations at the apoE locus significantly affect the lipid parameters, relationships of its were analysed in this study. Because the number of individuals with E2/2, E3/2, E4/4 and E4/2 genotypes was small, the two common genotypes (E3/3, E4/3) were compared. Significant mean differences between the two groups were observed for TC and LDL-Chol in the E3/3 type. The levels of TC and LDL-

Table 5. Plasma lipid levels in the two groups according to apoE genotypes

	E2 / 2	E3 / 2	E4 / 2	E3 / 3	E4 / 3	E4 / 4
Cholesterol						
CAD (N)		147.0 ± 37.9 (2)	239.5 ± 44.2 (8)	212.5 ± 44.2 (72)[a]	220.7 ± 50.4 (18)	239.8 ± 41.1 (8)
Control (N)	226.0 ± 17.3 (2)	193.3 ± 15.3 (6)	185.5 ± 13.4 (4)	192.2 ± 25.4 (66)[a]	185.6 ± 24.1 (20)	196.0 ± 17.2 (2)
Triglycerides						
CAD (N)		126.0 ± 68.5 (2)	202.8 ± 85.7 (8)	177.3 ± 97.9 (72)	186.6 ± 156.2 (18)	305.0 ± 229.5 (8)
Control (N)	174.0 ± 22.3 (2)	157.0 ± 33.2 (6)	105.0 ± 5.7 (4)	187.2 ± 86.4 (66)	131.2 ± 66.5 (20)	107.0 ± 32.5 (2)
HDL-cholesterol						
CAD (N)		33.0 ± 10.2 (2)	46.3 ± 11.4 (8)	39.5 ± 10.0 (72)[b]	49.6 ± 10.5 (18)[b]	47.5 ± 9.6 (8)
Control (N)	42.0 ± 4.5 (2)	39.7 ± 14.2 (6)	52.5 ± 3.5 (4)	39.9 ± 8.3 (66)	41.7 ± 9.0 (20)	57.0 ± 9.8 (2)
LDL-chlesterol						
CAD (N)		88.9 ± 37.4 (2)	152.7 ± 34.5 (8)	137.3 ± 41.2 (72)[b]	133.8 ± 55.2 (18)	131.3 ± 31.2 (8)
Control (N)	149.2 ± 12.3 (2)	122.3 ± 17.2 (6)	112.0 ± 8.8 (4)	114.0 ± 22.3 (66)[b]	117.6 ± 15.2 (20)	117.6 ± 17.8 (2)
ApoB						
CAD (N)		89.3 ± 19.5 (2)	84.5 ± 15.2 (8)	82.5 ± 25.9 (72)	77.3 ± 34.3 (18)	87.0 ± 33.8 (8)
Control (N)	107.0 ± 11.7 (2)	84.3 ± 6.8 (6)	121.5 ± 77.1 (4)	81.9 ± 14.9 (66)	78.4 ± 18.5 (20)	74.0 ± 14.2 (2)

Values are means ± SD, (mg/dl).

As numbers of E2/2, E3/2, E4/3 and E4/4 types are small, so the two common types were compared.

Significance of differences between CAD and controls of the E3/3 type; [a]$P<0.05$, [b]$P<0.01$.

Significance of differences between E3/3 and E4/3 types in the HDL-cholesterol levels: [c]$P<0.05$

Chol in the E3/3 genotype of the patients were significantly higher than those of the controls ($P<0.05$, $P<0.01$).

These differences may be due to a more frequent incidence of CAD in patients with hypercholesterolemia (Tsuchiya *et al.* 1987). However, there were no significant difference in the E4/3 type of the two groups. In the patient group, HDL-Chol levels of E4/3 type had significantly higher than those of E3/3 type ($P<0.05$). Differences between E3/3 and E4/3 types of the patient group might be partially attributable to increase in TC and LDL-Chol levels of the $\varepsilon 4$ allele.

Several case-control studies showed higher frequencies of the 4 allele in subjects with angiographically documented coronary artery disease compared with normal subjects (Cumming and Robertson. 1984;Tsuchiya *et al.* 1987). In this study we also observed an association of the 4 allele with elevated TC in the patients group with CAD.

The association of the $\varepsilon 2$ allele with type III hyperlipoproteinemia has been well explained (Sniderman *et al.* 1980); however, we detected $\varepsilon 2$ homozygote having no signs of type III hyperlipoproteinemia in the control group. Another interesting association of the E2 allele with serum lipids is the low levels of serum TC in E3/2 subjects (Menzel *et al.* 1983). In the present study subjects withe E3/2 phenotype also tended to only have a lower concentration of LDL-Chol compared to the most common E3/3 genotype.

There is a little problems on the apoE phenotype assay among populations. It may be due to the methods used to type the apoE polymorphism. The patterns of IEF can lead to erroneous results even after pretreatment with neuraminidase. So, genotyping by PCR analysis can be the method to offset such a defects.

Until now, although some contrdictions have been raised, a number of studies have reported on association between the apoE genotypes and lipid profiles. But the exact control mechanism of the apoE gene on diseases resulting from disorders of lipid metabolism is almost unknown. Accordingly, further study is needed on these aspects of gene levels.

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