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Original Articles

Study on the Relationship between Polymorphism in Apolipoprotein E Gene and Korean Ischemic Cerebrovascular Disease Patients

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The association between apolipoprotein E (*apo* E) gene polymorphism and ischemic cerebrovascular disease (ICVD) has been controversial. These controversies may be due to inaccurate classification of patients and ethnic differences. We investigated the association between *apo* E genotypes and ICVD patients by case-control study in a Korean population. The association between *apo* E polymorphism and ICVD was examined in 121 patients with ICVD and 132 controls without ICVD. The E3/E4 phenotype was more frequent in control subjects (23.8%) than in patients (13.0%) ($p<0.05$). The E2/E3 phenotype was more frequent in patients (14.8%) than in control subjects (10.8%), but the difference was not statistically significant ($p>0.05$). These results suggest that the E4 allele may be a protective factor against early vascular morbidity, and the E2 allele may be a risk factor for cerebrovascular morbidity. *(Korean J of Oriental Med 2003;24(4):113-119)*

Key Words: polymorphism, apolipoprotein E, ischemic cerebrovascular disease

Introduction

Apolipoprotein E (*apo* E) is a 299 amino-acid protein with a central role in cholesterol transport and lipoprotein metabolism. The gene for *apo* E is located on chromosome 19 in linkage with the genes encoding for other apolipoproteins: apo C-I and C-II and the low-density lipoprotein (LDL) receptor gene. It is polymorphic, with three common alleles, E4, E3, E2, which code for three major isoforms in plasma designated *apo* E4, *apo* E3, and *apo* E2 respectively, resulting in six common genotypes[1)]. *Apo* E3 is the predominant isoform. *Apo* E4 differs from E3 by an amino acid substitution at position 112 (cys/arg) and from E2 by a substitution at position 158 (arg/cys). *Apo* E3 acts as a ligand for two receptors: the *apo* E or "remnant" receptor, which is specifically hepatic, and the LDL receptor (apo B/E receptor). The catabolism of TG-rich lipoproteins appears to be modulated by the affinity of *apo* E for *apo* E or *apo* B/E receptors. *Apo* E2 binds defectively to receptors, and this results in an increase in the number of LDL receptors, thereby lowering cholesterol levels. *Apo* E4 is not covalently

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bonded to *apo* A-II, and its transfer from high-density lipoproteins (HDL) to TG-rich lipoproteins is enhanced. This accelerates hepatic remnant captivation by *apo* E receptors and downregulates the number of LDL receptors, thereby enhancing cholesterol levels.

Apo E is a key protein modulating the highly atherogenic *apo* B containing lipoproteins[2] and is a candidate gene for the development of coronary artery disease (CAD). The E2/ E2 genotype was the first to be implicated in premature coronary artery disease[2], which resulted in this polymorphism being extensively studied. These studies have not shown any clear relationship with the *apo* E polymorphism and risk of CAD, as in some there was a positive association[3,4] yet in others no relationship[5,6]. Similarly, the evidence supporting a role for the *apo* E gene polymorphism as a risk factor for stroke is contradictory[7]. These controversies may be due to inaccurate classification of subjects and ethnic differences. Furthermore, genetic risk factors in ischemic cerebrovascular disease (ICVD) have been less studied as compared with those involved in CAD. Therefore, the aim of this study was to compare the prevalence of the three most frequent alleles of *apo* E in a defined group of patients with ischemic cerebrovascular disease with those in a control group.

Materials and Methods

1. Patients

Patients with ICVD (n=121) during acute stage were identified according to well-defined criteria that included computerized tomography scanning (CT), magnetic resonance imaging (MRI), and clinical signs (hemiparesis, hemiplegia, slurred speech, facial palsy, and so forth) at Wonkwang University Hospital in Iksan, Korea. The control group consisted of 132 individuals undergoing routine health screening. None of the controls had a history of ICVD. All cases and controls (all Korean) gave informed consent before participating in the research protocol, which was approved by the ethics committee of each hospital.

2. Determination of *apo* E genotypes

The blood was stored at -20 °C until it was ready to be extracted. The genomic DNA was extracted by inorganic procedure[8]. The concentration of DNA was estimated by absorbance at 260 nm. The *apo* E polymorphism was detected by PCR amplification[9].

Briefly, a PCR reaction was carried out in a 20 L volume containing 200 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 M of each dNTP, and 1 U of r*Taq* DNA polymerase (Takara, Japan), with 1 M of *apo* E F4/F6 primers (Bioneer, Korea). The primer pairs for each gene were as follows: F4: 5' -ACAGAATTCGCCCCGGCCTG GTACAC-3' , F6: 5' -TAAGCTTGGCACGGCTGTCCAAGGA-3' .

Amplification conditions were 5 min preincubation step at 95 °C, 40 cycles of denaturation at 94 °C for 40 sec, annealing at 67 °C for 40 sec, and extension at 72 °C for 40 sec. A final extension for 10 min at 72 °C was included (MJ Research). The PCR product was digested for 16 h at 37 °C with 5.5 units *Hha*I in the presence of 2 g bovine serum albumin. PCR products were then separated electrophoretically through 8% polyacrylamide gel with a pGEM DNA marker (Promega, U.S.A.) and the products visualized by ethidium bromide staining (Fig. 3). The following fragments were obtained after restriction enzyme digestion: *apo* E2: 91, 81, 21, 18, 16; *apo* E3: 91, 48, 21, 18, 16; *apo* E4: 72, 48, 33, 21, 19, 18, 16. DNA of a subject with known *apo* E4/E4 genotype was included with each batch as a control to prevent inaccurate typing resulting from an incomplete digest. Genotypes were determined without reference to case or control status.

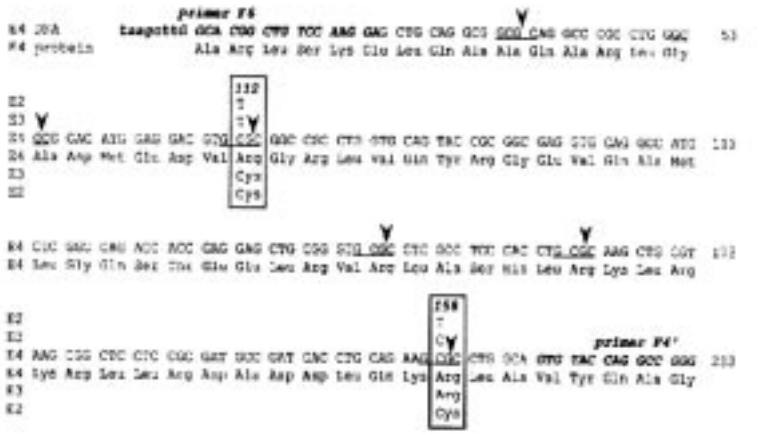

Fig. 1. DNA and protein sequences of amplified regions encoding common apo E isoforms and locations of *Hha*I cleavage sites.
The amplified E4 nucleotide sequence (244 bp, numbered to the right) is shown above the E4 amino acid sequence. The sequences of amplification primers (F6 and F4, the reverse complement of F4) are also shown (upper case italics are *apo* E sequences, lower case italics are synthetic cleavage sites). Nucleotide substitutions that distinguish E2 and E3 isoforms are shown above the E4 nucleotide sequences, and amino acid substitutions are shown below the E4 amino acid sequence (substitution sites at codons 112 and 158 are boxed). The sites for *Hha*I cleavage in the E4 nucleotide sequence are underlined and marked by arrows.

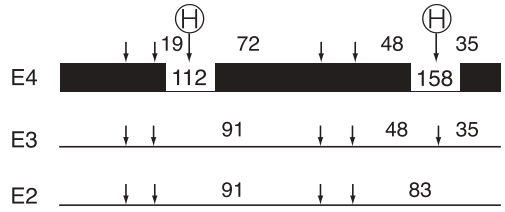

Fig. 2. *Hha*I cleavage maps.
*Hha*I cleavage maps (downward arrows show sites) are given for amplified sequences (E4 is shown as a filled box containing codons 112 and 158, E3 and E2 maps are shown below E4). The distances (in bp) between polymorphic *Hha*I sites (circled H) that distinguish isoforms are shown for each cleavage map.

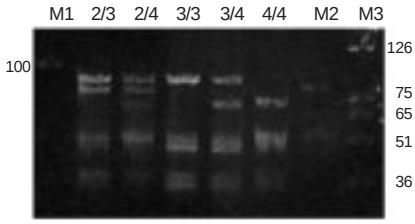

Fig. 3. Electrophoretic separation of *Hha*I fragments after gene amplification of DNA from subjects with known *apo* E isoforms.
A polyacrylamide gel is shown after electrophoresis of *Hha*I fragments from an E2E3 heterozygote (lane marked 2/2), E2E4 heterozygote (lane marked 2/4), E3E3 homozygote (lane marked 3/3), E3E4 heterozygote (lane marked 3/4), and E4E4 homozygote (lane marked 4/4). The fragment sizes (in bp) of a DNA standard (100 bp ladder, ACE genotypes (86 bp and 64 bp), and pGEM DNA marker, lane marked M1, M2, and M3, respectively) are shown to the gel.

3. Statistical analysis

The mean levels of all numerical values were tested by Student's *t*-test.

Comparisons of the allele frequencies of the *apo* E genotypes between the control and ICVD patients were carried out using the Pearson chi-square test. All statistical analyses were performed using SPSS v9.00 (SPSS Inc.) statistical analysis software. A *p*-value less than 0.05 were considered statistically significant.

Results

1. *Apo* E restriction isotyping by PCR amplification and cleavage with *Hha*I

Determination of *apo* E genotypes relies on cleavage at polymorphic *Hha*I sites to distinguishE2, E3, and E4 sequences. Fig. 1 shows the sequence (244 bp)

encoding the E4 isoform after amplification by PCR with F4 and F6 primers (10) and shows the six *Hha*I cleavage sites (GCGC) in the amplified E4 sequence, including *Hha*I sites at codons for arginine residues (GCGC) at positions 112 and 158. The E3 sequence encodes a cysteine residue at position 112 (GTGC), which abolishes the *Hha*I cleavage site in the E4 sequence, resulting in a total of five *Hha*I cleavage sites. The E2 sequence encodes cysteine at positions 112 (GTGC) and 158 (GTGC) that abolish two cleavage sites relative to the E4 sequence, resulting in a total of four *Hha*I cleavage sites (Fig. 2).

Fig. 3 shows gel-separated products of *apo* E amplification and *Hha*I digestion. Namely, with the exception of a shared 38 bp fragment, each genotype possessed unique combinations of *Hha*I fragment sizes. The E2/E2 sample contained 91 and 83 bp *Hha*I fragments reflecting the absence of sites at 112 cys and 158 cys. The E3/E3 sample also contained the 91 bp fragment (112 cys), as well as 48 and 35 bp fragments from cleavage at the *Hha*I site at 158 arg. The E4/E4 sample also contained these 48 and 35 bp fragments (158 arg), as well as a unique 72 bp fragment from cleavage at 112 arg.

2. Clinical characteristics of patients with ICVD

Table 1 shows the clinical characteristics of the present subjects. A total of 121 patients were included in the analysis.

3. Clinical characteristics according to apo E genotypes in patients with ICVD

Table 2 shows the clinical characteristics according to *apo* E genotypes of the present subjects. The levels of total cholesterol were lower in E2/E3 and E3/E3 genotypes than in the rest of the genotypes. The levels of triglyceride had the highest value in the E3/E4 genotype. The frequency of diabetes was higher in the E2/E4, E3/E4, and E4/E4 genotypes than in the E2/E3 and E3/E3. The rest of the variables (i.e., percentage of obesity and smoking) showed no significant differences among genotypes.

4. Association between the frequencies of apo E genotypes and ICVD

The frequencies of *apo* E alleles with ICVD were as follows: E2, 22 (9.6%); E3, 186 (80.9%); and E4, 22 (9.6%). This was significantly different from the distribution in control subjects: E2, 18 (6.9%); E3, 201

Table 1. Clinical Characteristics of ICVD Patients (n=121)

Characteristics	Mean S.D.
Age (year)	48.1 ±22.2
Sex (m:f, %)	44:56
Total cholesterol (mg/dl)	187.5 ±47.0
HDL cholesterol (mg/dl)	46.8 ±12.6
Triglyceride (mg/dl)	134.9 ±83.8
Diabetes, n (%)	25(26.3)
Obesity, n (%)	13(16.9)
Ischemic heart disease, n (%)	27(28.4)

Table 2. Characteristics of ICVD Patients (n=121) According to the *apo* E Genotypes

Characteristics	E2/ E3	E2/ E4	E3/ E3	E3/ E4	E4/ E4
Age (year)	53.3 ±22.4*	53.3 ±17.4	51.4 ±21.7	40.6 ±20.7	38.7 ±24.8
Sex (m:f)	6:11	0:5	38:38	7:8	0:1
Total cholesterol (mg/dl)	171.0 ±50.8	207.8 ±43.8	190.0 ±49.4	205.6 ±28.4	207.0
HDL cholesterol (mg/dl)	50.1 ±10.5	50.2 ± 7.9	46.5 ±12.6	43.6 ±6.9	43.0
Triglyceride (mg/dl)	126.2 ±47.6	106.6 ±20.7	136.1 ±79.8	184.1 ±107.7	146.0
Diabetes, n (%)	3(21.4)	2(50.0)	14(23.0)	6(50.0)	1(100)
Obesity, n (%)	3(25.0)	1(25.0)	7(14.6)	2(28.6)	0(0)
Smoking, n (%)	3(21.4)	1(25.0)	22(37.9)	3(25.0)	0(0)

* Mean ± S.D.

(77.3%); and E4, 41 (15.8%). In addition, the distribution of *apo* E genotype in 121 patients with ICVD were as follows: E2/E3, 17 (14.8%); E2/E4, 5 (4.3%) E3/E3, 77 (67.0%); E3/E4, 15 (13.0%); and E4/E4, 1 (0.9%), which was a little different from the distribution in 132 control subjects: E2/E3, 14 (10.8%); E2/E4, 4 (3.1%) E3/E3, 78 (60.0%); E3/E4, 31 (23.8%); and E4/E4, 3 (2.3%).

The frequencies of E2/E3 and E3/E3 were higher in ICVD patients than in control groups (controls vs. patients: E2/E3, 10.8% vs. 14.8%; E3/E3, 60.0% vs. 67.0%), but the difference was not statistically significant ($p>0.05$). Especially, the frequencies of E3/E4 were lower in ICVD patients than those of in control groups (controls vs. patients: 23.8% vs. 13.0%). This difference was statistically significant ($p<0.05$) (Table 3). These results indicate that the E2 allele may be implicated as a risk factor for ICVD, whereas the E4 allele may be a protective factor against ICVD.

Discussion

ICVD is a multifactorial disease caused by the interactions of several genetic and environmental factors, including such recognized risk factors as high blood pressure, smoking, diabetes, obesity and advanced age. I examined the relationship between polymorphic genetic factor and ICVD. Apolipoprotein E is a polymorphic glycoprotein that plays a critical role in cholesterol transport. *Apo* E polymorphisms have been extensively examined as a risk factor of vascular disease, including coronary artery disease (CAD)[11-16]. However, studies concerning the relationship between gene polymorphisms potentially implicated and vascular diseases are leading to conflicting findings, due in part, to the difference in ethnic backgrounds between populations.

These led me to evaluate the impact of polymorphisms in the *apo* E gene on ICVD in individuals from Korea.

As a result, the frequencies of E2/E3 and E3/E3 genotypes were higher in ICVD patients than in the control group. In contrast, the frequencies of E3/E4 were lower in ICVD patients than in the control group (controls vs. patients: 23.8% vs. 13.0%) and the difference was statistically significant ($p<0.05$). These data are consistent with the report showing that the frequency of E2/E3 genotype was higher in ICVD patients than those of in control group (10.1% for patients, 1.4% for controls)[7]. On the other hand, my data is inconsistent with the study reporting the protective effect of E3[7]. To date, the *apo* E2 allele has been reported to be associated with ICVD, whereas the *apo* E4 allele was associated not only with ICVD[11,17,18] but also with large-vessel ICVD[19]. Conversely, *apo* E was shown to be unrelated to cerebral infarction in Western populations[14,20,21] and to cerebral infarction in the Japanese population[22].

In conclusion, I examined the distribution of *apo*E genotypes in the Korean population and the association of *apo* E polymorphisms with ICVD, and I suggest that *apo* E2 is a risk factor for ICVD, whereas E4 is a protective factor.

Table 3. Frequency of *apo* E Genotype in Patients (n=115) and Control Subjects (n=130)

Genotypes	Controls n=130(%)	ICVD n=115(%)	*p* value
E2/ E3	14(10.8)	17(14.8)	.346
E3/ E4	4(3.1)	5(4.3)	.598
E3/ E3	78(60.0)	77(67.0)	.260
E3/ E4*	31(23.8)	15(13.0)	.031
E4/ E4	3(2.3)	1(0.9)	.375

Statistical tests by X^2-test or Fisher' s exact test (2-tailed).
* $p<0.05$

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