OPEM

Oriental Pharmacy and Experimental Medicine 2004 **4(2)**, 104-111

Gene-gene interaction in cerebral infarction patients: Relationship between apolipopreotein E gene polymorphism and Sasang-constitution

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SUMMARY

Sasang Constitutional Medicine is a major branch of Korean Traditional Oriental Medicine. The differences of disease susceptibility to be shown in Sasang constitution may be due to genetic factors. Therefore, we examined interrelationship among cerebral infarction (CI), apolipoprotein E (apoE) gene polymorphism, and Sasang constitutional classification. ApoE is a key protein modulating the highly atherogenic apoB containing lipoproteins and is a candidate gene for the development of coronary artery disease (CAD). The ε2 and/or ε4 alleles were the first to be implicated in premature CAD, which resulted in this polymorphism being extensively studied. We investigated the association between apoE genotype and CI by case-control study in a Korean population. We also classified CI patients and control group into groups according to Sasang Constitutional Medicine. 196 CI patients and 379 controls without CI were examined. ApoE genotype was determined by 8% polyacrylamide gel separation after DNA amplification. A significant difference in the apoE genotype distribution was observed in the CI patients compared with that in controls (χ^2=14.920, *df*=4, *P*=0.005). Also, the frequency of Taeumin constitution in patients with CI was significantly higher than that in controls (58.0% vs. 36.9%; *P*<0.001). However, the Taeumin constitution did not enhance the relative risk for CI in the subjects with apoE ε2 and/or ε4 alleles. No differences in the apoE genotypes frequencies were observed in the Taeumin compared with that in the other constitutions. In addition, we investigated whether the DD genotype of angiotensin converting enzyme (ACE) gene, a candidate gene for CI, was associated with CI, Taeumin constitution, and apoE polymorphism. As a result, the frequency of Taeumin constitution was significantly higher in CI patients with both apoE ε3/ε4 and ACE ID/DD genotypes than in the remaining Sasang constitutions (14.5% vs. 8.3% and 0%) (χ^2=13.521, *df*=6, *P*=0.035). In summary, we concluded that the apoE polymorphism is a major risk factor for CI in Koreans and the ACE ID/DD genotype enhanced the relative risk for CI in the subjects with apoE ε3/ε4 genotype and Taeumin constitution.

Key words: Apolipoprotein E (apoE); Cerebral infarction (CI); Gene; Polymorphism; Sasang constitution

INTRODUCTION

The Sasang Constitutional Medicine, a major branch of Korean traditional medicine, classifies people's constitutions into four types, according to the strengths and weaknesses in functions of the internal organs. Sasang constitutional philosophy forms the basis of treatment by correcting the imbalance of the internal organs caused by the constitutional properties in each body type. Accordingly, it presents different treatments according to constitution (Lee, 1996). The different constitutions bring about different reactions to the

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same disease. The differences of disease severity to be shown in Sasang constitutional classification may be due to genetic factors.

Cerebral infarction (CI) is a multifactorial disease caused by the interactions of several genetic and environmental factors. Recent advances in genetic epidemiology have revealed that some genetic variants increase the risk for cerebrovascular disease. Apolipoprotein E (apoE) is a 299 amino-acid protein with a central role in cholesterol transport and lipoprotein metabolism. The gene for apoE 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, ε4, ε3, ε2 which code for three major isoforms in plasma designated apo E4, apo E3, and apo E2 respectively, resulting in six common genotypes (Siest *et al.*, 1995). Also, apoE is a key protein modulating the highly atherogenic apoB containing lipoproteins (Davignon *et al.*, 1988) and is a candidate gene for the development of coronary artery disease (CAD), including hypertension. The ε2/ε2 genotype was the first to be implicated in premature coronary artery disease (Davignon *et al.*, 1988), which resulted in this polymorphism being extensively studied.

Also, the genes of angiotensin converting enzyme (ACE) was examined. ACE is a membrane bound dipeptidyl carboxypeptidase ectoenzyme and it has a key role within the renin angiotensin system by converting angiotensin I into the potent vasoconstrictor angiotensin II and inactivating the vasodilator bradykinin (Wiemer *et al.*, 1991; Ueda *et al.*, 1995). Plasma and tissue levels of ACE are partly under genetic control. Patients with a double deletion of a 287 bp in intron 16 (genotype DD) have higher plasma or tissue levels of ACE than individuals with genotype ID or II (Rigat *et al.*, 1990; Tiret *et al.*, 1992). The ACE genotype is considered to be associated with hypertension, coronary artery disease, left ventricular hypertrophy, myocardial infarction, diabetic nephropathy, CI, brain infarction and ischemic stroke (Sharma *et al.*, 1994; Markus *et al.*, 1995; Kario *et al.*, 1996; Abbud *et al.*, 1998; Ledru *et al.*, 1998; Seino *et al.*, 1998; Vleming *et al.*, 1999).

Therefore, the aim of this study was to compare the prevalence of the three most frequent alleles of apoE in a defined group of CI patients with those in a control group, and to investigate the association between ACE, apoE polymorphisms and CI according to Sasang constitutional classification.

MATERIALS AND METHODS

Subjects

Patients with CI (n=196) during acute stage were chosen according to well-defined criteria that included computerized tomography scanning, magnetic resonance imaging (MRI), and clinical signs (hemiparesis, hemiplegia, slurred speech, facial palsy, and so forth). The control group consisted of 379 individuals undergoing routine health screening. None of the controls had a history of CI.

Discrimination of Sasang constitution of individuals

Individuals were discriminated into four types by QSCC II program; Teaeumin, Taeyangin, Soyangin, and Soeumin. QSCC II is the program for "objective 4-constitutional body types" under PC, which is developed by the Department of Sasang Medical in Kyung Hee University Oriental Medicine Hospital, Seoul, Korea. It has been proved for providing its accuracy and universal logical ground with its standardized diagrams according to diagnostic clinical data.

Determination of apoE genotypes

The blood was stored at -20°C until it was ready to be extracted. The genomic DNA was extracted by inorganic procedure (Miller *et al.*, 1988). The concentration of DNA was estimated by absorbance at 260 nm. The apoE polymorphism was detected by PCR amplification (Hixon, 1991). Briefly a PCR reaction was carried out in a 20 μl volume containing 200 ng of genomic DNA, 10 μM Tris-HCl (pH 8.3), 1.5 μM $MgCl_2$, 200 μM of each dNTP, and 1 U of r*Taq* DNA polymerase (Takara, Japan), with 1 mM of *apo* E F4/F6 (Bioneer, Korea). The primer pairs for each gene were as follows (Fig. 1); F4: 5-ACAGAATTCGCCCCGGCCTGGTACAC-3, F6: 5-TAAGCTTGGCACGGCTG-TCCAAGGA-3 (Emi *et al.*, 1988). Amplification conditions were 5 min preincubation step at 95°C, 40 cycles of

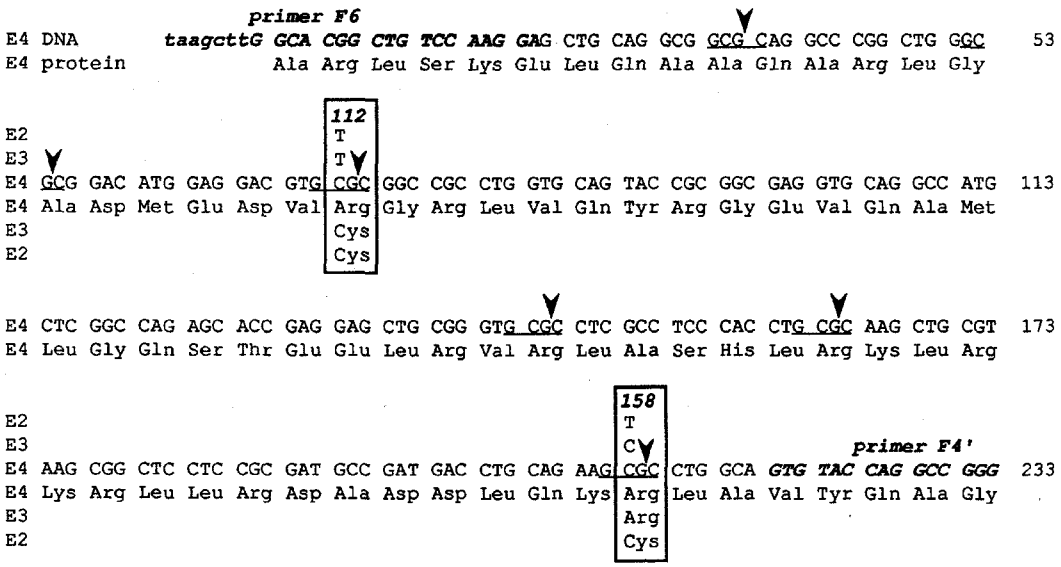

Fig. 1. DNA and protein sequences of amplified regions encoding common apoE isoforms and locations of *Hha*I cleavage sites. The amplified E4 nucleotide sequence (244bp, numbered to the right) is shown above the E4 amino acid sequence. The sequences of amplification primers (F6 and F4, the reverse complement of F4) are also shown (upper case italics are apoE 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.

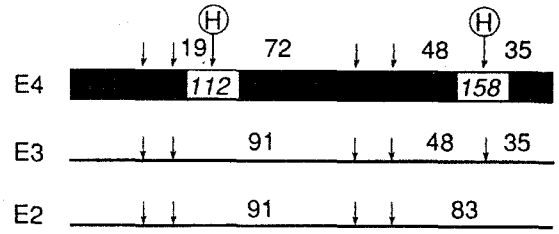

Fig. 2. *Hha*I cleavage maps. *Hha*I cleavage maps (downward arrows show sites) are given for amplified sequences (E4 is shown as a filled box containing codons 112 and 158, E3 and E2 maps are shown below E4). The distances (in bp) between polymorphic *Hha*I sites (circled H) that distinguish isoforms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

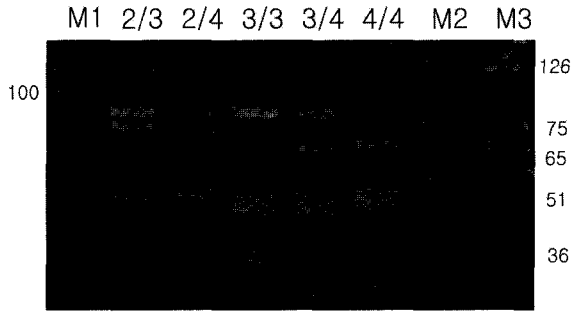

Fig. 3. Electrophoretic separation of *Hha*I fragments after gene amplification of DNA from subjects with known apoE isoforms. A polyacrylamide gel is shown after electrophoresis of *Hha*I fragments from an ε2/ε3 heterozygote (lane marked 2/2), ε2/ε4 heterozygote (lane marked 2/4), ε3/ε3 homoozygote (lane marked 3/3), ε3/ε4 heterozygote (lane marked 3/4), and ε4/ε4 homoozygote (lane marked 4/4). The fragment sizes (in bp) of a DNA standard (100bp ladder, ACE genotypes (86bp and 64bp), and pGEM DNA marker, lane marked M1, M2, and M3, respectively) are shown to the gel.

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 (Eppendorf). The PCR product was digested for 16h at 37°C with 5.5 units *Hha* I in the presence of 2 μg 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. 2 and 3). The following fragments were obtained after restriction enzyme digestion: apoε2: 91, 81, 21, 18, 16, apoε3: 91, 48, 21, 18, 16, apoε4: 72, 48, 33, 21, 19, 18, 16 (Fig. 2 and 3). DNA of a subject with known apo ε2/ε2 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.

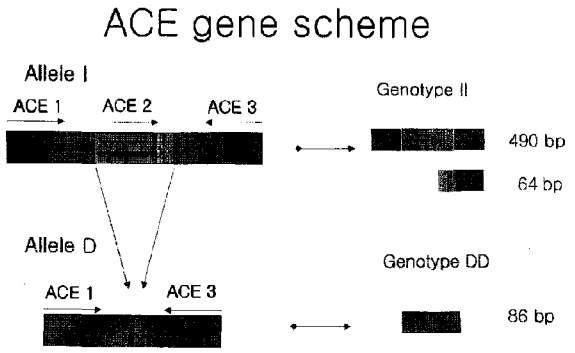

Fig. 4. ACE gene scheme. These primers allow the detection of an 86 bp fragment in the absence of the insertion and of two fragments including 490 bp and 64 bp in the presence of the insertion.

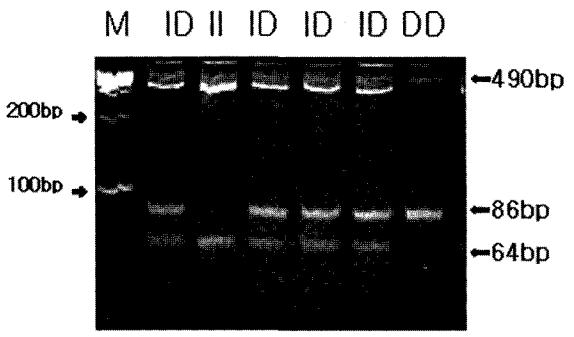

Fig. 5. Electrophoretic separation of ACE genotypes. The amplified alleles were analyzed on 7.5% polyacrylamide gel. The alleles were visualized by etidium bromide staining.

Determination of ACE genotypes

The ACE polymorphism was detected by PCR amplification. The reaction was run with a sense primer; ACE1: 5-CATCCTTTCTCCCATTTCTC-3, an antisense primer; ACE3: 5-TGGGATTACAGGCG-TGATACAG-3 and the primer for inserted region (286 bp); ACE2: 5-ATTTCAGAGCTGGAATAAAATT-3 as described previously (Evans *et al.*, 1994). These primers allow the detection of an 86 bp fragment in the absence of the insertion and of two fragments including 490 bp and 64 bp in the presence of the insertion (Fig. 4). 100 ng of genomic DNA was added to 25 μl of reaction mixture containing each primer (Bioneer, Korea); 1 μM of ACE1 and ACE3, 0.3 μM of ACE2 , 40 μM dNTP, 2.5 μM $MgCl_2$, 10 μM Tris-HCl (pH 8.3), and 1.5 U of *Taq* DNA polynerase (Takara). Amplification conditions were 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. A final extension for 10 min at 72°C was included (MJ Research). The amplified alleles were analyzed on 7.5% polyacrylamide gel. The alleles were visualized by etidium bromide staining (Fig. 5).

Statistical analysis

Comparisons of the allele frequencies of the apoE and ACE polymorphisms between the control and patients were carried out using the two-tailed chi-square test (SPSS 10.0). A *P*-value less than 0.05 were considered statistically significant.

RESULTS

Distribution of apoE genotypes

The genotype distribution in patients and controls did not deviate significantly from Hardy-Weinberg equilibrium. The distribution of apoE genotype in 196 patients with CI were as follows: ε2/ε2, 0 (0%); ε2/ε3, 30 (15.3%); ε2/ε4, 0 (0%); ε3/ε3, 149 (76.0%); ε3/ε4, 16 (8.2%); and ε4/ε4, 1 (0.5%), which was significantly different from the distribution in 379 control subjects: ε2/ε2, 0 (0%); ε2/ε3, 54 (14.2%); ε2/ε4, 16 (4.2%); ε3/ε3, 251 (66.2%); ε3/ε4, 51 (13.5%); and ε4/ε4, 7 (1.8%) (χ=14.920, *df*=4, *P*=0.005) (Table 1).

Association between apoE polymorphism and Sasang constitution

Table 2 shows the association between apoE genotypes and Sasang constitutions in CI patients. The frequency of Taeumin constitution was significantly higher in patients than in the remaining constitutions (59.3%, 30.5%, and 10.2%

Table 1. Distribution of apoE genotype in controls and CI patients

	Genotype					Statistic analysis*
	ε2/ε3	ε2/ε4	ε3/ε3	ε3/ε4	ε4/ε4	
Controls(n=379), n(%)	54(14.2)	16(4.2)	251(66.2)	51(13.5)	7(1.8)	*P*=0.003,
CI patients (n=218), n(%)	32(14.7)	1(0.5)	165(75.7)	19(8.7)	1(0.5)	χ^2=15.923, *df*=4

*Statistical test by χ^2 test (2-sided).

Table 2. Distribution of apoE genotype according to Sasang constitution in CI patients

Genotype	Sasang constitution, n(%)			Statistic analysis*
	Taeumin	Soyangin	Soeumin	
ε2/ε3	16(15.5)	9(15.8)	1(4.8)	P=0.139, χ^2=12.282, df=8
ε2/ε4	0(0)	1(1.8)	0(0)	
ε3/ε3	77(74.8)	41(71.9)	18(85.7)	
ε3/ε4	10(9.7)	6(10.5)	1(4.8)	
ε4/ε4	0(0)	0(0)	1(4.8)	

*Statistical test by χ^2 test (2-sided).

Table 3. Distribution of apoE allele according to Sasang constitution in CI patients

Genotype	Sasang constitution, n(%)			Statistic analysis*
	Taeumin	Soyangin	Soeumin	
ε2	16(7.8)	10(8.8)	1(2.4)	P=0.681, χ^2=2.296, df=4
ε3	180(87.4)	97(85.1)	38(90.5)	
ε4	10(4.9)	7(6.1)	3(7.1)	

*Statistical test by χ^2 test (2-sided).

for Taeumin, Soyangin, and Soeumin respectively) (data not shown). However, no association of apoE polymorphism was observed with Sasang constitution for genotype or allele in CI patients (Table 2 and 3). In addition, patients were grouped with Taeumin vs. other constitutions, since the frequency of Taeumin was significantly higher in patients than in the remaining constitutions. However, we did not find an association between apoE polymorphism and Sasang constitution (Table 4).

Gene-gene interaction: association among apoE, ACE polymorphisms and Sasang constitution in CI patients

We also analyzed the genotypes of apoE and ACE in combination to evaluate whether combination of these genotypes is associated with Sasang constitution in CI patients. Taeumin constitution was significantly higher in CI patients with both apoE ε3/ε4 and ACE ID/DD genotypes than in the remaining Sasang constitutions (14.5% vs. 8.3% and 0%.) (χ=13.521, df=6, P=0.035) (Table 5). These

Table 4. Distribution of apoE allele according to Sasang constitution in CI patients

Genotype	Sasang constitution, n(%)		Statistic analysis*
	Taeumin	Soyangin + Soeumin	
ε2	16(7.8)	11(7.1)	P=0.796, χ^2=0.457, df=2
ε3	180(87.4)	135(86.5)	
ε4	10(4.9)	10(6.4)	

*Statistical test by χ^2 test (2-sided).

Table 5. Distribution of apoE genotype according to Sasang constitution in CI patients with ACE ID/DD genotypes

Genotype	Sasang constitution, n(%)			Statistic analysis*
	Taeumin	Soyangin	Soeumin	
ACE ID/DD + apoE ε2/ε3	10(16.1)	8(22.2)	1(11.1)	P=0.035, χ^2=13.571, df=6
ACE ID/DD + apoE ε2/ε4	0(0)	0(0)	0(0)	
ACE ID/DD + apoE ε3/ε3	43(69.4)	25(69.4)	7(77.8)	
ACE ID/DD + apoE ε3/ε4	9(14.5)	3(8.3)	0(0)	
ACE ID/DD + apoE ε4/ε4	0(0)	0(0)	1(11.1)	

*Statistical test by χ^2 test (2-sided).

Table 6. Distribution of apoE allele according to Sasang constitution in CI patients with ACE ID/DD genotypes

Genotype	Sasang constitution, n(%)			Statistic Analysis*
	Taeumin	Soyangin	Soeumin	
ACE ID/DD + apoE ε2	10(8.1)	8(11.1)	1(5.6)	P=0.733, χ^2=2.017, df=4
ACE ID/DD + apoE ε3	105(84.7)	61(84.7)	15(83.3)	
ACE ID/DD + apoE ε4	9(7.3)	3(4.2)	2(11.1)	

*Statistical test by χ^2 test (2-sided).

Table 7. Distribution of apoE allele according to Sasang constitution in CI patients with ACE ID/DD genotypes

Genotype	Sasang constitution, n(%)		Statistic analysis*
	Taeumin	Soyangin + Soeumin	
ACE ID/DD + apoE ε2	10(8.1)	9(10.0)	P=0.798, χ^2=0.451, df=2
ACE ID/DD + apoE ε3	105(84.7)	76(84.4)	
ACE ID/DD + apoE ε4	9(7.3)	5(5.6)	

*Statistical test by χ^2 test (2-sided).

results suppose that Taeumin with both apoE ε3/ε4 and ACE ID/DD genotypes have higher risk on CI than either Soyangin or Soeumin. However, no significant association was observed in the combined genotypes of ACE ID/DD genotype with apoE ε4 allele, even though in Taeumin vs. other constitutions (Table 6 and 7).

DISSCUSSION

The Sasang constitutional medicine we examined was established by Je-Ma Lee of Korea in 1894. Since then it has been developed as a major branch of Korean traditional Oriental medicine, which occupies an important position along with Western medicine in Korea. It classifies people's constitutions into four types, such as Taeyangin, Taeumin, Soyangin and Soumin, according to the functions of the internal organs, behavioral characteristics, the symptoms of a disease, and quantitative variations including body size etc. This classification was determined by QSCC II program as well as clinical measurements (weight, height, blood pressure etc.). Sasang constitution philosophy was supported by the report demonstrating that continuous characters governed by a large number of independent mendelian factors (polygenic characters) would display precisely the quantitative variation and family correlations described by the biometrics (Fisher, 1918). Also, Falconer extended the polygenic theory to discontinuous nonmendelian characters by postulating an underlying continuously variable susceptibility (Falconer, 1981). We suggested that Sasang constitutional medicine was similar to the approach to complex disease through the polygenic factor and nonmendelian characters by Fisher and Falconer. So, Sasang constitutional medicine could be applicable to whole world people as well as Korean and Asian. Also, Taeumin, who resembled the typical abdominal type of obesity in Western populations, is thought to have a higher rate of stroke, hypertension and hyperlipidemia than the other types because he or she has a large liver and small lungs. Here my data showed a consistent result with the viewpoint of Sasang Constitutional Medicine.

In this study, we investigated apoE and ACE genotypes of the CI patients classified by Sasang constitution. As a result, the frequency of Taeumin constitution in patients with CI was significantly higher than that in controls. Also, a significant difference in the apoE genotype distribution was observed in the CI patients compared with that in controls. In addition, the frequency of Taeumin constitution was significantly higher in CI patients with both apoE å3/å4 and ACE ID/DD genotypes than in the remaining Sasang constitutions. These results are consistent with the reports that apoE ε4 allele was associated with the occurrence of myocardial infarction and coronary atherosclerosis (Hixon, 1991; Kosunen *et al.*, 1995; Wang *et al.*, 1995).

However, these have produced mainly contradictory results (Pedro-Botet *et al.*, 1992; Couderc *et al.*, 1994; Kuusisto *et al.*, 1995; Nakata *et*

al., 1997; McCarron et al., 1999; Catto et al., 2000; Frikke-Schmidt et al., 2001; MacLeod et al., 2001). Different ethnic groups can also affect the results of these studies (Odawara et al., 1996). The apoE ε2 allelic frequency of Korean controls in this study was similar to that in Japanese controls (0.05 vs. 0.05) (Zaman et al., 1997; Kokubo et al., 2000) and Europeans (0.05 vs. 0.06) (Brega et al., 1998; Kowalska et al., 1998; Kumar et al., 2002), but lower than that in Taiwanese (0.05 vs. 0.08) (Wu et al., 2002). In addition, the apoE ε4 allelic frequency of our controls was lower than that in Japanese controls (0.03 vs. 0.11) (Zaman et al., 1997; Kokubo et al., 2000), and higher than in Taiwanese (0.03 vs. 0.08) (Wu et al., 2002). Even among Europeans there are geographic differences, with an ε4 frequency as high as 0.20 in Norway (Kumar et al., 2002) and as low as 0.07 in Turkey (Brega et al., 1998). Theses indicate that ethnic difference should be carefully considered in the studies on the association between apoE genotype and disease aetiology.

In summary, we concluded that the apoE polymorphism is a major risk factor for CI in Koreans and the ACE ID/DD genotype enhanced the relative risk for CI in the subjects with both apoE ε3/ε4 genotype and Taeumin constitution. These results suggest the apparent relationship between apoE and ACE polymorphisms and Sasang constitutions, as well as the novel possibility of molecular genetic understanding of Sasang constitution medicine.

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