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Original Articles

Study on the Relationship between Candidate Genes of Cerebral Infarction and Sasang Constitution

Hye-Sun Park[1], Kyung-Yo Kim, Jong-Cheon Joo[2], Jong-Yeol Kim[3]

Department of Sasang Constitutional Medicine, College of Oriental Medicine, Donshin University[1]
Department of Sasang Constitutional Medicine, College of Oriental Medicine, Wonkwang University[2]
Department of Medical Research, Korea Institute of Oriental Medicine[3]

The author investigated whether ACE/DD, AGN/TT, and ApoE/ε4 genotypes are associated with CI and whether genetic risk is enhanced by Sasang constitutional classification. The author ascertained these genotypes in patients with CI (n=211), diagnosed by brain computed tomography. Control subjects for the infarction group were randomly selected from 319 subjects matched for age, gender, and history of hypertension with patients. The ACE/DD genotype was not associated with CI. However, there was significant association between ApoE polymorphism and CI (χ^2=15.089, p<.05). Furthermore, frequency of AGN/TT genotype was higher in the patients with CI than in the controls (χ^2=20.072, p<.05). The frequency of T allele was 0.91 in patients and 0.82 in controls (χ^2=17.237, p<.05). However, the Sasang constitutional classification did not increase the relative risk for CI in the subjects with ApoE/ε4 or AGN/T allele. These results suggest that ApoE and AGN polymorphism predict CI, but Sasang constitutional classification does not enhance the risk for CI associated with ApoE/ε4 or AGN/TT in a Korean population.

Key Words: Cerebral infarction, ACE polymorphism, Apo E polymorphism, AGN polymorphism, Sasang constitution, Korean population

Introduction

Cerebral infarction (CI) is a multifactorial disease caused by the interactions of several genetic and environmental factors, as with ischemic heart disease. Recent advances in genetic epidemiology have revealed that some genetic variants increase the risk for myocardial infarction. The genes of angiotensin converting enzyme (ACE)[1-4], angiotensinogen (AGN)[5-7] and apolipoprotein E (Apo E)[8-11] have been extensively examined. Numerous studies have attempted to relate the ACE/DD polymorphism with myocardial infarction and/or coronary artery disease (CAD), leading to conflicting results. However, a recent meta-analysis conducted on 15 studies published before 1995 (3394 cases of myocardial infarction and 5479 control subjects) demonstrated a mean odds ratio for myocardial infarction for the DD vs. the ID/II genotypes of 1.26. The relative risk appeared to be

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Correspondent to:Jong-Cheon Joo.
Department of Sasang Constitutional Medicine, Wonkwang University Oriental Medical Hospital in Suncheon, 544, Joryedong, Suncheon, Jeonnam, Korea. Tel : 82-61-720-7522, Fax : 82-61-720-7550, E-mail: jcjoo@wonkwang.ac.kr

variable according to ethnicity, and higher in the Japanese population[3,12]. Um et al.[13] reported that ACE polymorphism is not a risk factor for the development of cerebral infarction in a Korean population. A homozygous molecular variant of the AGN gene, with threonine instead of methionine at position 235 (AGN/TT), is known to be one of the inherited predisposing factors for essential hypertension[14,15] and myocardial infarction[5-7]. However, several reports on the effect of AGN/TT on the incidence of myocardial infarction have shown conflicting results[16,17]. The ε2/ε2 genotype of Apo E was the first to be implicated in premature CAD[18], 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, although in some there was a positive association[19,20] yet in others no relationship[21,22]. Also, the ApoE/ε4 allele also influences atherogenesis indirectly by an effect on circulating levels of low density lipoprotein cholesterol and apolipoprotein B[23]. A recent report, however, showed no association between ApoE/ε4 and cerebrovascular disease in whites[24,25].

In general, CI and ischemic heart disease have risk factors in common, such as hypertension, hyperlipidemia, and smoking; and both types of diseases are pathologically based on atherosclerosis. However, genetic risk factors in CI have not been extensively studied as compared with those involved in ischemic heart disease. Therefore, the author investigated whether the gene polymorphisms of ACE, AGN, and Apo E associated with the incidence of CI in a Korean population. Ethnic difference is an important factor in evaluating genetic risk. Furthermore, analysis of three genes in one population would be informative in optimizing our understanding of interaction among genetic effects of three genes.

In addition, 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[26]. The different constitutions bring about different reactions to the same disease. The differences of disease severity to be shown in Sasang constitutional classification may be due to genetic factors.

Therefore, the author investigated whether ACE/DD, AGN/TT, and ApoE/ε4 genotypes are associated with CI and whether genetic risk is enhanced by Sasang constitutional classification.

Materials and Methods

1. Subjects

Patients with CI (n=211) during the 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 319 individuals undergoing routine health screening. None of the controls had a history of CI.

2. Discrimination of Sasang constitution of individuals

Individuals were discriminated into four types by Questionnaire for the Sasang Constitution Classification (QSCC)II program and clinically important characteristics such as physical frame, facial features, personalities, emotions, and reactions to herbal medicines Teaeumin, Taeyangin, Soyangin, and Soeumin. QSCC II is the program for "objective 4-

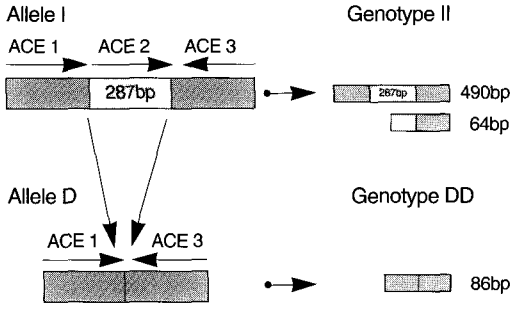

Fig 1. ACE gene scheme. These primers allow the detection of an 86 bp fragment in the absence of the insertion and of two fragments including 490 bp and 64 bp in the presence of the insertion

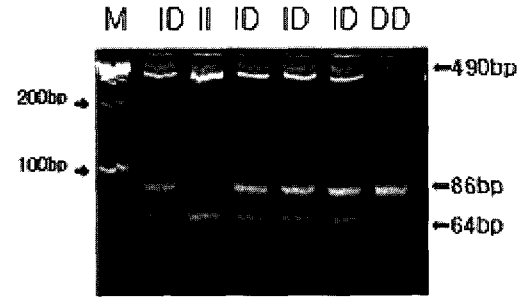

Fig 2. Electrophoretic separation of ACE genotypes. The amplified alleles were analyzed on 7.5% polyacrylamide gel. The alleles were visualized by ethidium bromide staining.

constitutional body types" under PC, which is developed by The Society of Sasang Constitutional Medicine. It has been proved for providing its accuracy and universal logical ground with its standardized diagrams according to diagnostic clinical data. The clinical characteristics were based on the Donguisusebowon-Longevity and Life Preservation in Oriental Medicine which is basic book that explains how to identify each constitution.

This program is checked by the consciousness.

3. Determination of genotypes

The blood was stored at -20℃ until it was ready to be extracted. The genomic DNA was extracted by inorganic procedure[27]. The concentration of DNA was estimated by absorbance at 260 nm.

4. Determination of ACE genotype

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'-TGGGATTACAG GCGTGATACAG-3' and the primer for inserted region (287 bp); ACE2: 5'-ATTTCAGAGCTGGAATAAA ATT-3' as described previously[28]. 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. 1). 100 ng of genomic DNA was added to 25 ㎕ of reaction mixture containing each primer (Bioneer, Korea); 1 ㎛ of ACE1 and ACE3, 0.3 ㎛ of ACE2, 40 ㎛ dNTP, 2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), and 1.5 U of Taq DNA polynerase (Takara). Amplification conditions were 35 cycles of denaturation at 94℃ for 1 min, annealing at 55℃ for 1 min, and extension at 72℃ for 1 min. A final extension for 10 min at 72℃ was included (MJ Research). The amplified alleles were analyzed on 7.5% polyacrylamide gel. The alleles were visualized by etidium bromide staining (Fig. 2).

5. Determination of Apo E genotype

The Apo E polymorphism was detected by PCR amplification[29]. Briefly a PCR reaction was carried out in a 20㎕ volume containing 200ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 1.5mM MgCl2, 200㎛ of each dNTP, and 1 U of rTaq DNA polymerase (Takara,

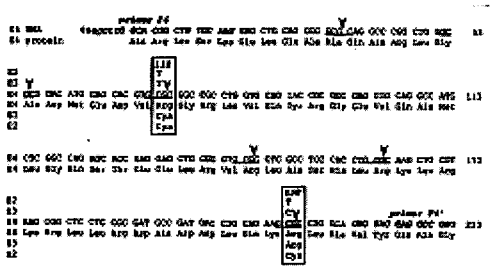

Fig. 3. DNA and protein sequences of amplified regions encoding common ApoE isoforms and locations of *Hhal* cleavage sites. The amplified E4 nucleotide sequence (244bp, numbered to the right) is shown above the E4 amino acid sequence. The sequences of amplification primers (F6 and F4, the reverse complement 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 *Hhal* cleavage in the E4 nucleotide sequence are underlined and marked by arrows.

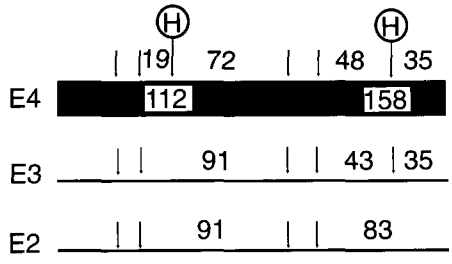

Fig. 4. *Hhal* cleavage maps. *Hhal* cleavage maps (downward arrows show sites) are given for amplified sequences (E4 is shown as a filled box containing codons 112 and 158, E3 and E2 maps are shown below E4). The distances (in bp) between polymorphic *Hhal* sites (circled H) that distinguish isoforms are shown for each cleavage map.

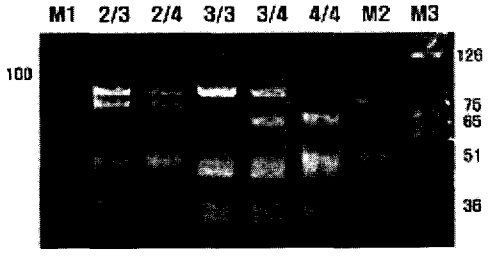

Fig. 5. Electrophoretic separation of Hhal fragments after gene amplification of DNA from subjects with known ApoE isoforms. A polyacrylamide gel is shown after electrophoresis of Hhal fragments from an ε2/ε3 heterozygote (lane marked 2/2), ε2/ε4 heterozygote (lane marked 2/4), ε3/ε3 homoozygote (lane marked 3/3), ε3/ε4 hetero-zygote (lane marked 3/4), and ε4/ε4 homoo-zygote (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

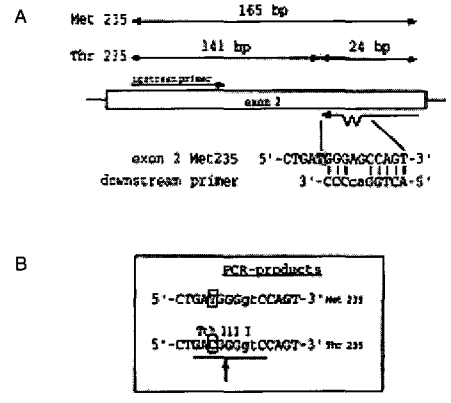

Fig. 6. Scheme of AGN gene polymorphism and polymerase chain reaction-restricted fragment length polymorphism.

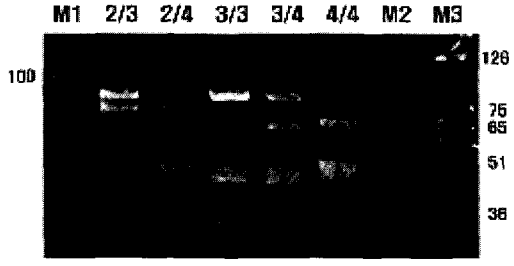

Fig. 7. Genotyping of AGN gene. Electrophoretic separation of TthIII I fragments after gene amplification of DNA from AGN gene. A polyacrylamide gel is shown after electrophoresis of TthIII I fragments from an MT heterozygote (lane marked MT), TT homoozygote (lane marked TT), and MM homozygote (lane marked MM).

Japan), with 1 ㎛ of apo E F4/F6 (Bioneer, Korea). The primer pairs for each gene were as follows (Fig. 3); F4: 5'-ACAGAATTCGCCCCGGCCTGG-TACAC-3', F6: 5'-TAAGCTTGGCACGGCTGTCCAAGGA-3'. Amplification conditions were 5 min preincubation step at 95℃, 40 cycles of denaturation at 94℃ for 40 sec, annealing at 67℃ for 40 sec, and extension at 72℃ for 40 sec. A final extension for 10 min at 72℃ was included (Eppendorf). The PCR product was digested for 16h at 37℃ with 5.5 units *Hha* I in the presence of 2 ㎍ 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. 5). 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. 4 and 5). DNA of a subject with known ApoE 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.

6. Determination of AGN genotype

The AGN polymorphism was detected by PCR amplification[30]. Briefly a PCR reaction was carried out in a 20 ㎕ volume containing 200 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 200 ㎛ of each dNTP, and 1 U of rTaq DNA polymerase (Takara, Japan), with 1 ㎛ of AGN upstream/downstream primers (Bioneer, Korea). The primer pairs for each gene were as follows; downstream: 5'-CAGGGTG-CTGTCCACACTGGACCCC-3', upstream: 5'-CCGTTTGTGCAGGGCCTGGCTCT-CT-3' (Fig. 6).Cycling conditions are: initial denaturation at 90℃ 3 min., 10 cycles 94℃ 1 min., 68℃ 1 min., 72℃ 1 min., followed by 30 cycles 90℃ 30 sec., 68℃ 1 min., 72℃ 30 sec., final extension 72℃ 10 min. 5㎕ of PCR product are diluted to 15㎕ in the recommended restriction buffer containing 5units of Tth III 1and digested for at least 2 hours. Fig. 7 shows 10 consecutive samples from our screening program resolved on 8% polyacrylamide gel.

7. Statistical analysis

Comparisons of the frequencies of all genotypes between the control and CI 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 was considered statistically significant.

Results

1. Clinical characteristics of CI patients

The characteristics of the patients with CI and those of control subjects are summarized in Table 1.

Table 1. Clinical Characteristics of Cerebral Infarction (CI) Patients

N	211
Age (year)	57.8±15.4
Male, %	52.3
Total cholesterol (mg/dl)	193.4±46.2
Triglyceride (mg/dl)	150.3±116.5
Diabetes, %	12.5
Hypertension, %	45.4

2. Association between genotypes and CI

The genotype distribution of each gene in patients and control subjects did not deviate significantly from Hardy-Weinberg equilibrium.

The distribution of ACE genotypes was not different between CI patients and control subjects (Table 2). This result for the ACE gene is in consistence with those previously published[13].

The distribution of Apo E genotype in 211 patients with CI were as follows; 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 319 control subjects: ε2/ε3, 45 (14.1%); ε2/ε4, 14 (4.4%) ε3/ε3, 211 (66.1%); ε3/ε4, 43 (13.5%); and ε4/ε4, 6 (1.9%) (χ2=15.089; p<.05) (Table 3). In addition, the allele frequencies of patients with CI were as follows; ε2, 30 (7.7%); ε3, 344 (87.8%); and ε4, 18 (4.6%), which was significantly different from the distribution in control subjects: ε2, 59 (9.2%); ε3, 511 (80.1%); and ε4, 68 (10.7%) (χ2=13.134; p<.05) (Table 4).

The distribution of AGN genotypes in 211 patients with CI was as follows; TT, 174 (82.5%); MT, 35 (16.6%); and MM, 2 (0.9%). It was significantly different from the distribution in 319 control subjects: TT, 206 (64.6%); MT, 108 (33.9%); and MM, 5 (1.6%). The frequency of subjects with AGN/TT was higher than in the CI group than in the control group (χ2=20.072, p<.05). In addition, the allele frequencies of patients with CI were as follows; T, 383 (90.8%); and M, 39 (9.2%), which was significantly different from the distribution in control subjects: T, 520 (81.5%); and M, 118 (18.5%) (χ2=17.237; p<.05). Table 5 and 6 present the genotype and allele frequencies of the subjects.

3. Association among genotype, CI, and Sasang constitution

The distribution of Sasang constitution in 211 patients with CI was as follows; Taeumin, 58.2%; Soyangin, 30.6%; and Soeumin, 11.2%.

Of interest, the frequency of Apo E/ε2 in Taeumin and Soyangin was higher than those in Soeumin, but Apo E/ε4 frequency was lower than those in Soeumin. However, the difference was not statistically significant (p>0.05) (Table 7). The author also did not find the association between AGN polymorphism and Sasang constitution in CI patients (Table 8). In addition, the

Table 2. Distribution of ACE Genotypes in Cerebral Infarction (CI) Patients and Control Subjects

	Genotype			Statistics
	II	ID	DD	
Patients, n (%) n=211	68(32.2)	98(46.4)	45(21.3)	NS
Controls, n (%) n=319	116(36.3)	154(48.3)	49(15.4)	

Statistical tests by χ^2-test; NS, not significant.

Table 3. Distribution of Apo E Genotypes in Cerebral Infarction (CI) Patients and Control Subjects

	Genotype					
Subjects	ε2/ε3	ε2/ε4	ε3/ε3	ε3/ε4	ε4/ε4	Statistics
Patients,n (%)n=211	30(15.3)	0(0)	149(76.0)	16(8.2)	1(0.5)	x^2=15.089
Controls, n(%) n=319	45(14.1)	14(4.4)	211(66.1)	Σ43(13.5)	6(1.9)	p<.05

Statistical tests by x^2-test; NS, not significant.
p=0.002

Table 4. Distribution of Apo E Allele Frequencies in Cerebral Infarction (CI) Patients and Control Subjects

	Allele			Statistics
Subjects	ε2	ε3	ε4	
Patients, n(%) n=211	30(7.7)	344(87.8)	18(4.6)	x^2=13.134
Patients, n(%) n=319	59(9.2)	511(80.1)	68(10.7)	p<.05

Statistical tests x^2test.
In CI patients, 196 cases of 211 cases were valid and the remaining 15 cases were omitted.
p=0.005

Table 5. Distribution of AGN Genotypes in Cerebral Infarction (CI) Patients and Control Subjects

	Genotype			Statistics
Subjects	TT	MT	MM	
Patients, n(%) n=211	174(82.5)	35(16.6)	2(0.9)	x^2=20.072
Patients, n(%) n=319	206(64.6)	108(33.9)	5(1.6)	p<.05

Statistical tests x^2-test.
p=0.001

Table 6. Distribution of AGN Allele Frequencies in Cerebral Infarction (CI) Patients and Control Subjects

	Allele		Statistics
Subjects	T	M	
Patients, n(%) n=211	383(90.8)	39(9.2)	x^2=17.23
Patients, n(%) n=319	520(81.5)	118(18.5)	p<.05

Statistical tests by x^2-test.;OR, odds ratio.

Table 7. Distribution of Apo E Allele according to Sasang Constitution in Cerebral Infarction (CI) Patients

Allele	Sasang constitution,n(%)			Statistics
	Taeumin	Soyangin	Soeumin	
ε2	16(7.8)	10(8.8)	1(2.4)	
ε3	180(87.4)	97(85.1)	38(90.5)	NS
ε4	10(4.9)	7(6.1)	3(7.1)	

Statistical x^2-test(2-sided); NS, not significant.

Table 8. Distribution of AGN Allele according to Sasang Constitution in Cerebral Infarction (CI) Patients

Allele	Sasang constitution,n(%)			Statistics
	Taeumin	Soyangin	Soeumin	
T	204(89.5)	112(93.3)	39(88.6)	NS
M	24(10.5)	8(6.7)	5(11.4)	

Statistical x^2-test(2-sided); NS, not significant.

Table 9. Distribution of Apo E and AGN Alleles according to Sasang Constitution in Cerebral Infarction (CI) Patients

Genotype	Sasang constitution,n(%)		Statistics
	Taeumin	Soyangin + Soeumin	
AGN T/apoE ε2	16(7.8)	11(7.1)	
AGN T/apoE ε3	154(75.5)	121(78.6)	
AGN T/apoE ε4	10(4.9)	9(5.8)	
AGN M/apoE ε2	0(0)	0(0)	NS
AGN M/apoE ε3	24(11.8)	12(7.8)	
AGN M/apoE ε4	0(0)	1(0.6)	

Statistical x^2-test(2-sided); NS, not significant.

combined analysis of the AGN and Apo E genotypes did not enhance the predictability of CI according to Sasang constitutional classification (Table 9).

Discussion

The present study demonstrated that the renin-angiotensin system related genes were associated with the incidence of CI. Most cerebrovascular disease is related to atherosclerosis of the cerebral arteries. Furthermore, the common and major pathological changes in ischemic heart disease and CI, are atherosclerosis and thrombogenesis in the artery. These findings suggest that the association of the ApoE and AGN/TT genotypes with the incidence of both CI and ischemic heart disease may be related to vascular atherogenesis and thrombogenesis.

Of interest, the AGN/TT genotypes affected the predictability of CI. The genotype is reported to be involved in an increase of angiotensin II generation, not only in the circulation[14], but also in local tissues[31, 32]. Several investigations have revealed that angiotensin II contributes to atherosclerotic changes and plaque

rupture via several mechanisms such as vasoconstriction, vascular smooth muscle cell growth, thrombogenesis, and antifibrinolysis. These findings further support the theory that the AGN/TT contribute to vascular atherogenesis and thrombogenesis via activation of angiotensin II production.

Another gene analyzed, the ApoE/ε4 allele, had an association with CI. The gene is reported to be associated with atherosclerotic disease of the heart, such as myocardial infarction, silent myocardialischemia and restenosis after coronary angioplasty, and carotid artery atherosclerosis. However, the role of Apo E polymorphism in ischemic stroke is still controversial[24.25.33-36]. Also, the Apo E gene polymorphism was shown to be unrelated to cerebral infarction in Western populations[37,38] and to cerebral infarction in Japanese population[39]. This controversy might be due to in part, to the difference in ethnic background between populations. The frequency of the Apo E allele in our study did not deviate from that of Japanese, although the frequencies of ε2 and ε3 were a little higher than those in Japanese. The frequencies of ε 2, ε3, and ε4 alleles in Korean controls were 0.09, 0.80, and 0.11, respectively, while in Japanese controls, these were 0.06, 0.85, and 0.09, respectively[39].

The frequency of ε3/ε3 was higher in CI patients than those of in control groups(76% vs. 66.1%).

In addition, the author investigated the AGN, Apo E and ACE genotypes in CI patients classified by Sasang constitution. As a result, any difference in distributions of the genotypes was not observed in the CI patients.

Also the frequency of Apo E/ε2 in Taeumin and Soyangin was higher than those in Soeumin, but Apo E/ ε3 and Apo E/ε4 frequency were lower than those in Soeumin. However, the difference was not statistically significant(p>0.05).

And Soyangin constitution was higher in cerebral Infarction patients with AGN T allele than in the remaining sasang constitutions. and Soeumin constitution was higher in cerebral Infarction patients with AGN M allele than in the remaining sasang constitutions. However, the difference was not statistically significant.

In summary, the author concluded that the Apo E and AGN polymorphisms are major risk factors for CI in Koreans and Sasang constitutional classification did not enhance the risk for CI associated with ApoE/ε4 or AGN/TT in a Korean population. These results suggest the apparent relationship between gene polymorphism and CI, as well as the novel possibility of molecular genetic understanding of Sasang constitution medicine.

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