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Study on Individual and Combined Relationship of Angiotensin Converting Enzyme, Apolipoprotein E and Angiotensinogen Genes Polymorphism in Patients with Ischemic Cerebrovascular Disease

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The homozygous deletion allele of the angiotensin converting enzyme gene (ACE/DD), homozygous threonine allele of the angiotensinogen gene (AGN/TT), and the 4 allele of the apolipoprotein E gene (apoE/4) are reported to be associated with ischemic heart disease. Ischemic cerebrovascular disease (ICVD) is another atherosclerotic disease, and the effects of these polymorphisms on ICVD have been confusing.

In this study, I investigated whether ACE/DD, AGN/TT, and apoE/4 genotypes are associated with ICVD and whether genetic risk is enhanced by the effect of one upon another. I ascertained these genotypes in patients with ICVD (n=121) diagnosed by brain computed tomography. Control subjects for the ICVD were randomly selected from subjects matched for age, gender, and history of hypertension with patients.

Frequency of ACE/DD genotype was somewhat higher in the patients with ICVD than in the controls (18% vs. 15%). Incidence of ICVD was higher in subjects with the apoE/4/4 genotype than in the other genotypes (50% vs. 27-29%). Incidence of ICVD was much higher in subjects with the AGN/TT genotype than in AGN/MM genotype (36% vs. 17%). Furthermore, the AGN/TT genotype greatly increased the relative risk for ICVD in the subjects with ACE/DD genotype (80.0% vs. 20.0%, P=0.089). Finally, incidence of ICVD was much higher in the subjects with both apoE/2/4 and AGN/TT genotype than in the other genotypes (83.3% vs. 16.7%, P=0.095). These results suggest that AGN/TT enhances the risk for ICVD associated with ACE/DD and apoE/2/4. *(Korean J of Oriental Med 2003;24(4):102-112)*

Key Words: polymorphism, angiotensin converting enzyme, angiotensinogen, apolipoprotein E, ischemic cerebrovascular disease

Introduction

Ischemic cerebrovascular disease (ICVD) 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 (apoE) [8-11)] have been extensively examined. A homozygous deletion allele in intron 16 of the ACE gene (ACE/DD) has been reported to be associated with an increase in the

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incidence of ischemic heart disease and left ventricular hypertrophy[12-14]. 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 [15,16] and myocardial infarction[5-7]. ApoE is a key protein modulating the highly atherogenic apoB-containing lipoproteins[17] and is a candidate gene for the development of coronary artery disease (CAD). The 2/2 genotype was the first to be implicated in premature coronary artery disease[17], which resulted in this polymorphism being extensively studied. These studies have not shown any clear relationship with the apoE polymorphism and risk of CAD, although in some there was a positive association[18,19] yet in others no relationship[20,21].

In general, ICVD and ischemic heart disease have risk factors in common, such as hypertension, hyperlipidemia, and smoking; both types of diseases are pathologically based on atherosclerosis. However, genetic risk factors in ICVD have not been extensively studied as compared with those involved in ischemic heart disease. The genetic polymorphism of ACE/DD and of AGN/TT are suggested to be involved in atherosclerosis via activation of angiotensin generation [1,15,22,23], yet several reports on the effect of ACE/DD and AGN/TT on the incidence of ICVD have shown conflicting results[24-28]. The apoE/4 allele also influences atherogenesis indirectly through an effect on circulating levels of low density lipoprotein cholesterol and apolipoprotein B[9,29]. A recent report, however, showed no association between apoE/4 and ICVD in Caucasian men[30,31]. Therefore, I investigated whether the gene polymorphisms of ACE, AGN, and apoE associated with the incidence of ICVD as well as ischemic heart disease in Koreans. 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.

Materials and Methods

1. Subjects and Measurements

Patients with documented ICVD were identified from clinical records from December 1999 to July 2002 of Wonkwang University Hospital in Iksan, Korea.

Patients aged younger than 30 and older than 80 years were excluded. Final diagnosis of ICVD was confirmed with brain computed tomography or brain magnetic resonance imaging.

I identified 121 patients with ICVD. The control group were randomly recruited and matched with study patients for age and gender. All cases and controls (all Korean) gave informed consent before participating in the research protocol, which was approved by the ethics committee of the hospital.

2. Determination of genotypes

The blood was stored at -20 ℃ until it was ready to be extracted. The genomic DNA was extracted by inorganic procedure[32]. The concentration of DNA was estimated by absorbance at 260 nm.

1) 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' -TGGGATTACA GGCG TGATACAG-3' and the primer for inserted region (287 bp), ACE2: 5' -ATTTCAGAGCTGGAATAAA ATT-3' as described previously[33]. 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 L of reaction mixture containing each primer (Bioneer, Korea): 1 M of ACE1

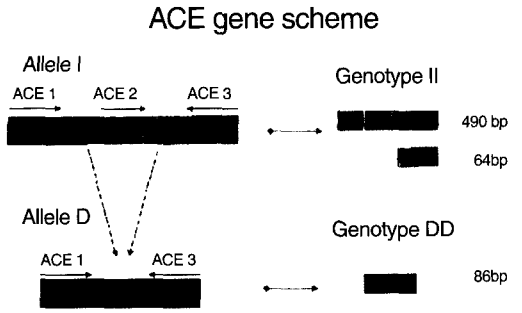

Fig. 1. Scheme of ACE gene polymorphism and polymerase chain reaction.

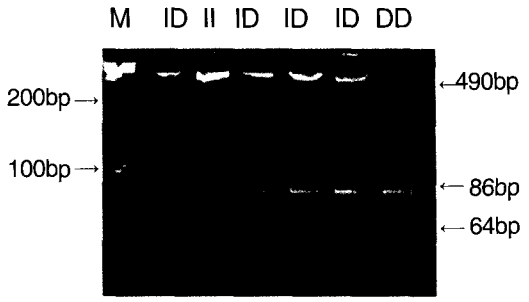

Fig. 2. Genotyping of ACE gene.
Agarose gel electrophoresis with ethidium bromide staining showing the three genotypes of the ACE polymorphism in DNA obtained from whole blood samples, using primers ACE^1 (forward), ACE^1 (reverse), and ACE^2 (insert). M represents molecular size marker.

and ACE3, 0.3 M of ACE2, 40 M dNTP, 2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), and 1.5 U of Taq DNA polymerase (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 (Eppendorf). The amplified alleles were analyzed on 7.5% polyacrylamide gel. The alleles were visualized by ethidium bromide staining(Fig. 2).

2) Determination of apoE genotype

The apoE polymorphism was detected by PCR amplification[34]. 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 rTaq DNA polymerase (Takara, Japan), with 1 M of apoE F4/F6 primers (Bioneer, Korea). The primer pairs for each gene were as follows: F4: 5' -ACAGAATTCGCCCCGGCCTGGTACAC-3', F6: 5' -TAAGCTTGGCACGGCTGTCCAAGGA-3' (Fig. 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 16 h at 37 ℃ with 5.5 units HhaI 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. The following fragments were obtained after restriction enzyme digestion: apoE2: 91, 81, 21, 18, 16, apoE3: 91, 48, 21, 18, 16, apoE4: 72, 48, 33, 21, 19, 18, 16(Fig. 4, 5). DNA of a subject with known apo 4/4 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.

3) Determination of AGN genotype

The AGN polymorphism was detected by PCR amplification[35]. Briefly, a PCR reaction was carried out in a 20 L volume containing 200 ng of genomic DNA, 10 mM Tris-HCl (pH8.3), 1.5 mM $MgCl_2$, 200 M of each dNTP, and 1 U of rTaq DNA polymerase (Takara, Japan), with 1 M of AGN upstream/downstream primers (Bioneer, Korea). The primer pairs for each gene were as follows:

downstream: 5' -CAGGGTGCTGTCCACACTGGAC CCC-3',

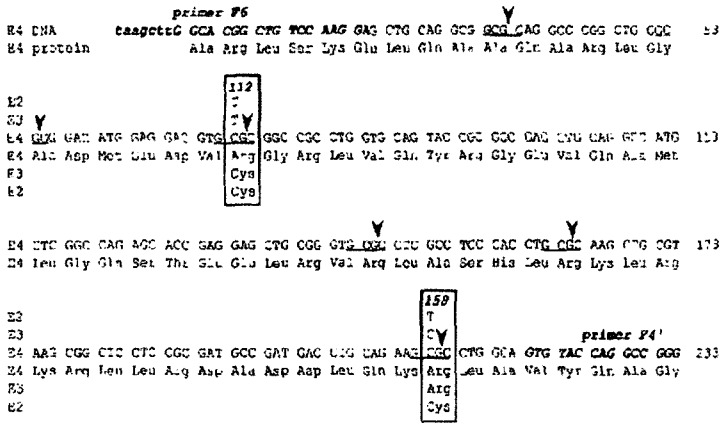

Fig. 3. DNA and protein sequences of amplified regions encoding common apoE isoforms and locations of HhaI cleavage sites.
The amplified 4 nucleotide sequence (244 bp, numbered to the right) is shown above the 4 amino acid sequence. The sequences of amplification primers (F6 and F4, the reverse complement of F4) are also shown (upper case italics are apoE sequences, lower case italics are synthetic cleavage sites). Nucleotide substitutions that distinguish 2 and 3 isoforms are shown above the 4 nucleotide sequences, and amino acid substitutions are shown below the 4 amino acid sequence (substitution sites at codons 112 and 158 are boxed). The sites for HhaI cleavage in the 4 nucleotide sequences are underlined and marked by arrows.

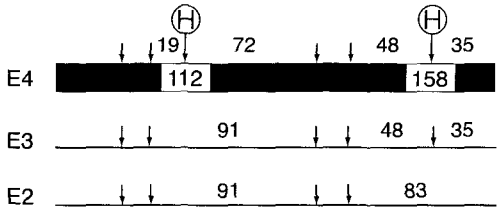

Fig. 4. HhaI cleavage maps.
HhaI cleavage maps (downward arrows show sites) are given for amplified sequences (4 is shown as a filled box containing codons 112 and 158, 3 and 2 maps are shown below 4). The distances (in bp) between polymorphic HhaI sites (circled H) that distinguish isoforms are shown for each cleavage map.

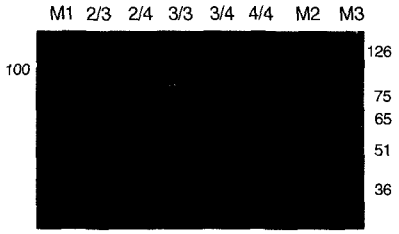

Fig. 5. Genotyping of apoE gene.
Electrophoretic separation of HhaI fragments after gene amplification of DNA from subjects with known apoE isoforms. A polyacrylamide gel is shown after electrophoresis of HhaI fragments from an 2 3 heterozygote (lane marked 2/3), 2 4 heterozygote (lane marked 2/4), 3 3 homoozygote (lane marked 3/3), 3 4 heterozygote (lane marked 3/4), and 4 4 homoozygote (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.

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 L of PCR product were diluted to 15 L in the recommended restriction buffer containing 5 units of TthIII 1 and digested for at least 2 hours. Fig. 7 shows 10 consecutive samples from our screening program resolved on 8% polyacrylamide gel.

3. Statistical analysis

All numerical values were tested by Student's *t*-test or one-way ANOVA.

Comparisons of the frequencies of all 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

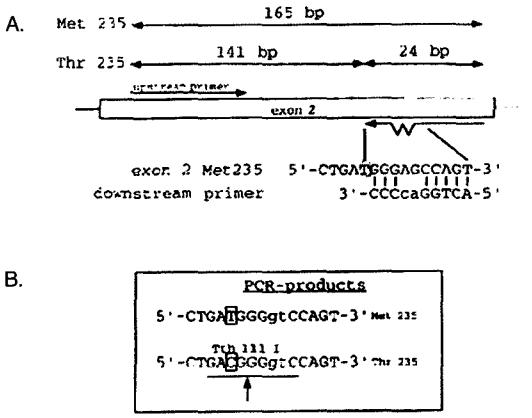

Fig. 6. Scheme of AGN gene polymorphism and polymerase chain reaction-restricted fragment length polymorphism.

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

Characteristics	
Age (year)	66.1±11.2*
Male, %	44.2
Total cholesterol (mg/dL)	191.1±47.3
HDL cholesterol (mg/dL)	46.7±11.8
LDL cholesterol (mg/dL)	121.2±35.9
Triglyceride (mg/dL)	139.2±78.9
Embolism, %	19.1
Diabetes, %	28.6
Obesity, %	16.9
Ischemic heart disease, %	28.9

* Mean ± S.D.

was considered statistically significant.

Results

1. 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.

1) ACE genotyping by PCR amplification

The 490 bp, 64 bp, and 86 bp fragments yielded by PCR amplification were identified as II (490 bp and 64 bp) and DD (86 bp) homozygous genotype, respectively (Fig. 2).

2) ApoE restriction isotyping by PCR amplification and cleavage with HhaI

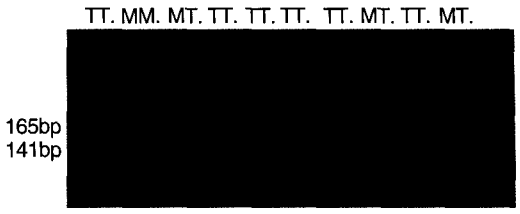

Fig. 7. Genotyping of AGN gene.
Electrophoretic separation of TthIII 1 fragments after gene amplification of DNA from AGN gene. A polyacrylamide gel is shown after electrophoresis of TthIII 1 fragments from an MT heterozygote (lane marked MT), TT homozygote (lane marked TT), and MM homozygote (lane marked MM).

Determination of apoE genotypes relies on cleavage at polymorphic HhaI sites to distinguish 2, 3, and 4 sequences. Fig. 3 shows the sequence (244 bp) encoding the 4 isoforms after amplification by PCR with F4 and F6 primers and shows the six HhaI cleavage sites (GCGC) in the amplified 4 sequence, including HhaI sites at codons for arginine residues (GCGC) at positions 112 and 158. The 3 sequence encodes a cysteine residue at position 112 (GTGC), which abolishes the HhaI cleavage site in the 4 sequence, resulting in a total of five HhaI cleavage sites. The 2 sequence encodes cysteine at positions 112 (GTGC) and 158 (GTGC) that abolish two cleavage sites relative to the 4 sequence, resulting in a total of four HhaI cleavage sites(Fig. 4).

Fig. 5 shows gel-separated products of apoE amplification and HhaI digestion. Namely, with the exception of a shared 38 bp fragment, each genotype possessed unique combinations of HhaI fragment sizes. The 2/ 2 sample contained 91 and 83 bp HhaI fragments reflecting the absence of sites at 112 cys and 158 cys. The 3/3 sample also contained the 91 bp fragment (112 cys), as well as 48 and 35 bp fragments from cleavage at the HhaI site at 158 arg. The 4/ 4 sample also contained these 48 and 35 bp fragments (158 arg), as well as a unique 72 bp fragment from cleavage at 112 arg.

Table 2. Characteristics of ICVD Patients (n=121) According to ACE Genotypes

Characteristics	II	genotype ID	DD
Total cholesterol (mg/dL)	202.4±41.0*	181.9±42.0	186.6±67.6
HDL cholesterol (mg/dL)	47.4± 9.6	44.8±12.9	49.4±11.2
LDL cholesterol (mg/dL)	129.2±33.8	110.7±32.8	133.4±43.7†
Triglyceride (mg/dL)	144.4±88.6	143.4±80.4	123.2±53.1
Embolism, %	26.7	60.0	13.3
Diabetes, %	29.6	51.9	18.5
Obesity, %	38.5	35.8	23.1
Ischemic heart disease, %	37.0	51.9	11.1

* Mean ± S.D.
† $p<0.05$; Statistical tests by one-way ANOVA

Table 3. Characteristics of ICVD Patients (n=121) According to apoE Genotypes

	Genotype				
Characteristics	ε2/ε3	ε2/ε4	ε3/ε3	ε3/ε4	ε4/ε4
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	46.8±12.6	43.6±6.9	43.0
LDL cholesterol (mg/dL)	108.3±34.2	135.8±40.5	122.4±38.1	124.7±24.8	135
Triglyceride (mg/dL)	126.2±47.6	106.6±20.7	136.1±79.8	184.1±107.7	146.0
Embolism, %	12.5	6.3	68.8	12.5	0
Diabetes, %	11.5	7.7	53.8	23.1	3.8
Obesity, %	23.1	7.7	53.8	15.4	0
Ischemic heart disease, %	18.5	3.7	70.4	7.4	0

* Mean ± S.D.

Table 4. Characteristics of ICVD Patients (n=121) According to AGN Genotypes

	Genotype	
Characteristics	TT	MT
Total cholesterol (mg/dL)	193.6±47.8*	181.8±45.6
HDL cholesterol (mg/dL)	46.9±10.3	46.1±14.4
LDL cholesterol (mg/dL)	124.7±35.1	114.1±37.9
Triglyceride (mg/dL)	138.1±77.8	139.9±81.4
Embolism, %	81.3	18.8
Diabetes, %	63.0	37.0
Obesity, %	76.9	23.1
Ischemic heart disease, %	81.5	18.5

* Mean ± S.D.

3) AGN restriction isotyping by PCR amplification and cleavage with TthIII 1

The amplification yields a product of 165 bp. In presence of C at position 704 cleavage by TthIII 1 generates a fragment of 141 bp (Fig. 6, 7).

3. Association between clinical characteristics and genotypes

Table 2 shows the clinical characteristics according to ACE genotypes of the present subjects. The levels of LDL cholesterol had the highest value in DD genotype ($P<0.05$). The remaining variables had no significant differences in genotypes. The characteristics of patients were not different among the genotypes of AGN and apoE (Table 3, 4).

4. Association between the frequency of each genotype and ICVD

The genotype distribution of each gene in patients and control subjects did not deviate significantly from Hardy-Weinberg equilibrium. The frequency of subjects with ACE/DD was higher in the ICVD group than in the control group (17.5% vs. 15.2%), although the statistics power was very weak (Fig. 8, Table 5). The distribution of apoE genotype in 121 patients with ICVD were as follows: 2/3, 14.0%; 2/4, 4.4%; 3/3, 63.2%; 3/4, 13.2%; and 4/4, 5.1%, which was little different from the distribution in 357 control subjects: 2/3, 14.6%; 2/4, 4.2%; 3/3, 65.8%; 3/4, 13.4%; and 4/4, 2.0%, the main difference being that the frequency of

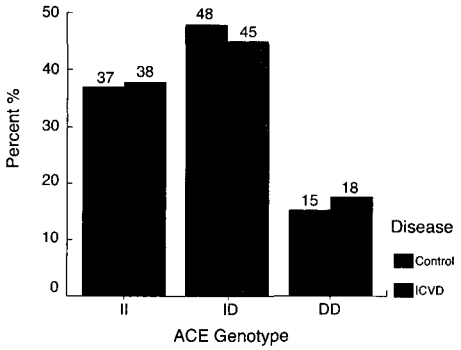

Fig. 8. Distribution of ACE genotypes in ICVD patients and controls.

Table 5. Distribution of ACE Genotypes in ICVD Patients (n=121) and Control Subjects (n=613)

Subjects	II	ID	DD
Patients, n(%)	43(37.7)	51(44.7)	20(17.5)
Controls, n(%)	226(36.9)	294(48.0)	93(15.2)

Statistical tests by χ^2 test (2-tailed)
In ICVD patients, 114 cases of 121 cases were valid and the remaining 7 cases were omitted

Table 6. Distribution of apoE Genotypes in ICVD Patients (n=121) and Control Subjects (n=357)

	Genotype				
Subjects	ε2/ε3	ε2/ε4	ε3/ε3	ε3/ε4	ε4/ε4
Patients, n(%)	17(14.0)	5(4.4)	77(63.2)	15(13.2)	1(5.1)
Controls, n(%)	52(14.6)	15(4.2)	235(65.8)	48(13.4)	7(2.0)

Statistical tests by χ^2 test or Fisher' s exact test (2-tailed)
In ICVD patients, 115 cases of 121 cases were valid and the remaining 6 cases were omitted

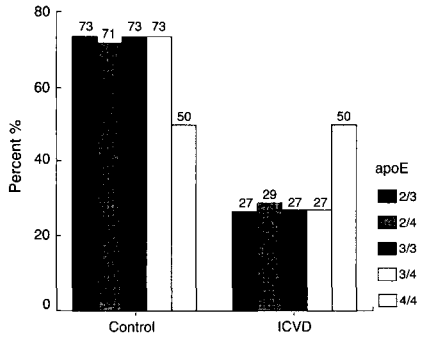

Fig. 9. Distribution of apoE genotypes in ICVD patients and controls.

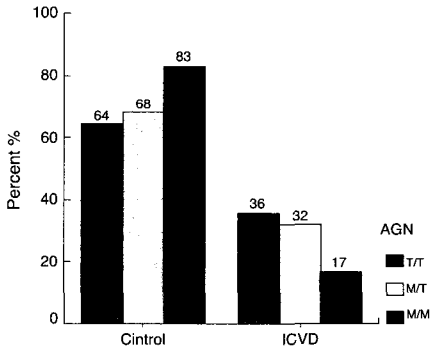

Fig. 10. Distribution of AGN genotypes in ICVD patients and controls.

4/4 was higher in ICVD patients than those of in control groups (5.1% vs. 2.0%) (Table 6). Also, incidence of ICVD was higher in subjects with the apoE/4/4 genotype than in the other genotypes (50% vs. 27-29%) (Fig. 9), but the difference was not statistically significant (P>0.05). Incidence of ICVD was higher in subjects with the AGN/TT genotype than in AGN/MM genotype (36% vs. 17%) (Fig. 10, Table 7).

Of interest, AGN/TT genotype appeared to increase the relative risk for ICVD in the subjects with ACE/DD (80.0% vs. 20.0%, P=0.089) (Table 8). Furthermore, incidence of ICVD was higher in the subjects with both apoE/2/4 and AGN/TT genotype than in the other genotypes (83.3% vs. 16.7%, P=0.095) (Table 9). These results suggest that AGN/TT enhances the risk for ICVD associated with ACE/DD and apoE/2/4.

Discussion

The present study demonstrates that the renin-angiotensin system related genes are associated with the incidence of ICVD. Most cerebrovascular disease is related to atherosclerosis of the cerebral arteries. Furthermore, the common and major pathological changes in ischemic heart disease and ICVD are atherosclerosis and thrombogenesis in the artery. These findings suggest that the association of the ACE/DD genotype with the incidence of both ICVD and ischemic heart disease may be related to vascular atherogenesis and thrombogenesis.

Of interest, the combined analysis of the AGN/TT and ACE/DD genotypes further enhanced the predictability of ICVD. Furthermore, both genotypes are reported to be involved in an increase of angiotensin II generation, not only in the circulation[15], but also in local tissues[22,23]. 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 and ACE/DD genotypes contribute to vascular atherogenesis and thrombogenesis via activation of angiotensin II production.

Another gene analyzed, the apoE/4 allele, increased the relative risk for ICVD in the subjects with and AGN/TT genotype. This gene is reported to be associated with atherosclerotic disease of the heart, such as myocardial infarction, silent myocardial ischemia and restenosis after coronary angioplasty, and carotid artery atherosclerosis.

However, the role of apoE polymorphism in ischemic stroke is still controversial[30].

To date, apo2 allele has been reported to be associated with ICVD, whereas apo4 allele was associated not only with ICVD[36,38] but also with large-vessel ICVD[39].

Conversely, apoE was shown to be unrelated to cerebral infarction in Western populations[40-42] and to cerebral infarction in Japanese population[43]. This controversy might be due to in part, the difference in ethnic background between populations.

It is not even known whether the apoE/2/4, ACE/DD

Table 7. Distribution of AGN Genotypes in ICVD Patients (n=121) and Control Subjects (n=296)

	Genotype		
Subjects	TT	MT	MM
Patients, n(%)	84(71.8)	33(28.2)	0(0)
Controls, n(%)	190(64.2)	101(34.1)	5(1.7)

Statistical tests by χ^2 test or Fisher's exact test (2-tailed)
In ICVD patients, 117 cases of 121 cases were valid and the remaining 4 cases were omitted

Table 8. Combined Analysis of ACE Genotypes and AGN Genotypes in ICVD Patients and Controls

Subjects	Genotype		
	DD and T/T	DD and Other Genotypes	*p* value
Patients, n(%)	16(80.0)	4(20.0)	0.089
Controls, n(%)	31(59.6)	21(40.3)	

Statistical tests by Linear-by-Linear χ^2 test (2-tailed)

Table 9. Combined Analysis of apoE Genotypes and AGN Genotypes in ICVD Patients and Controls

Subjects	Genotype		
	ε2/ε4 and TT	ε2/ε4 and Other Genotypes	*p* value
Patients, n(%)	5(83.3)	1(16.7)	0.095
Controls, n(%)	6(42.9)	8(57.1)	

Statistical tests by Pearson χ^2 test (2-tailed)

and AGN/TT polymorphisms are causative variants or just markers of another functional variant.

Further studies are necessary to determine the genetic locus responsible for ICVD, and whether the apoE, AGN and ACE genes themselves, and not other genes beside them, confer susceptibility to cerebrovascular events. Although there is no direct evidence showing that the apoE/2/4, ACE/DD and AGN/TT genotypes could influence ICVD, it may be useful to introduce genetic pharmacology for evaluation of the effects of ACE inhibitors on the prevention of ICVD as well as on myocardial infarction.

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