Genotype distribution and gene frequency of angiotensin I-converting enzyme in Korean population

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The angiotensin converting enzyme (ACE) is a key component of the renin-angiotensin system thought to be important in the pathogenesis of hypertension and cadiovascular diseases. Deletion polymorphism in the ACE gene may be a risk factor for myocardial infarction. The insertion/deletion (I/D) polymorphism of the ACE detected by PCR analysis appears to be associated with hypertension in Koreans and its nucleotide was subcloned into T-vector and its nucleotide sequences were determined. We also examined an association between hypertension and genetic variance of ACE. We identified the angiotensin I-converting enzyme genotype in 127 hypertensive and 189 normotensive Korean subjects. The distribution of ACE genotype II, ID, DD were 39.2%, 40.2%, 20.6% respectively and the frequency for ACE alleles I and D were 0.593 and 0.407, respectively in all subjects. The frequency of D allele in Korean males is higher than that of Korean females (male; 0.438 : female; 0.267), and the frequency of I allele in Korean females is higher than that of Korean males (female; 0.733 : male; 0.562). Genotype distributions of angiotensin I-converting enzyme genes in Korean normal adult population were different from that of Caucasians ($P<0.001$). There were no significant differences in genotype frequency between the hypertensive control group (n=127) and the normotensive group (n=189). We observed significant differences of ACE genotype distribution between the male group and the female group in total ($P=0.001$) and in hypertensive Korean subjects ($P=0.013$).

Keywords: Angiotensin I-converting enzyme, ACE genotype, Gene frequency, Genetic polymorphism

INTRODUCTION

Essential hypertension is a common human disease believed to result from the interplay of multiple genetic and environmental determinants. The functions of the dicarboxypeptidase angiotensin converting enzyme (ACE) include the metabolism of bradykinin and the conversion of angiotensin I to angiotensin II. Angiotensin II is an octapeptide that has vasoactive and sodium-retaining activities along with a capacity to stimulate proliferation of vasculature (Zee *et al.*, 1992). The ACE gene insertion/deletion (I/D) polymorphism may have important clinical relevancer. A number of associations of the ACE gene I/D polymorphism with cardiovascular diseases now have been recognized. This may be related to the high levels of ACE that accompany the presence of the D allele (Cambien *et al.*, 1992; Tiret *et al.*, 1993).

The present study describes an association study of the polymerase chain reaction (PCR) technique to determine 287bp insertion/deletion polymorphisms in groups of hypertensive (HT) and normotensive (NT) subjects. We also investigated the genotype distribution and the gene frequency for angiotensin I-converting enzyme in Korean population, and examined an association between hypertension and genetic variance of ACE.

MATERIALS AND METHODS

Subjects

We studied 316 Korean adults (258 men, 58 women); 189 in normotensive group and 127 hypertensive group, 63 of which has complications with hypertension. On the basis of classifying hypertension with SBP/DBP over 140/90 mmHg, this group consisted of 17 men and 46 women.

Measurement of Blood Pressure

All subjects in this study had their blood pressure measured 3 times. Measurements were in the morning, with subjects seated, after 5 min relaxation, and with the mercury sphygnomanometer at heart level. Measurements by the two different researchers agreed well when simultaneous recording were made with right and left arms of the subjects.

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Isolation of Genomic DNA

Genomic DNA was extracted from human blood using Blin and Stafford's method (1976). Briefly, 500 μl of blood was diluted in 1.0 ml of PBS buffer, vortexed, and centrifuged. The pellets were resuspended in 600 μl of lysis buffer (10 mM Tris-HCl, 100 mM EDTA; pH 8.0), 15 μl of sodium dodecyl sulfate (20%), and 30 μl of proteinase K (10 mg per milliliter of solution) were added. Overnight digestion with proteinase K at 55°C was followed by centrifugation and precipitation of the supernatant in ethanol. The solution of TE (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) buffer was added to DNA pellet.

Polymerase Chain Reaction for Detection of ACE Insertion/Deletion Polymorphism

The sequences of the sense primer and the antisense primer were 5'-CTG GAG ACC ACT CCC ATC CTT TCT-3' and 5'-GAT GTG GCC ATC ACA TTC GTC AGA T-3', respectively. PCR was performed in a final volume of 50 μl which contained 100 ng of genomic DNA, 20 pmol of each primers, 250 μM each of the four dNTP, 1.5 mM MgCl, 50 mM KCl, 10 mM Tris-HCl, pH 8.4 and 0.4 unit of Taq polymerase. Amplication was carried out in an Perkin Elmer PCR (U.S.A.) for 30 cycles with steps of denaturation at 95°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1 min. The PCR products were electrophoresed in 1.5% agarose gels, and DNA was visualized directly with ethidium bromide staining.

Subcloning of PCR Product

PCR was performed in a final volume of 50 μl which contained 100 ng of genomic DNA and then agarose gel (1.5%) electrophoresis was performed in a horizontal slob gel in 1x TAE buffer (90mM Tris-acetate, 1mM EDTA reaction at 4°C, for 1h at 100V. After electrophoresis the DNA band (190 bp, 490 bp) was cut from agarose gel with a sterile blade. The gel containing the DNA fragment was purified in a Wizard PCR column. For ligating PCR products, pGEM -T vector is prepared by adding a 3' terminal thymidine to both ends. PCR products and pGEM-T vector were mixed in the ratio of 1:1, and then T_4 DNA ligase (3 units) and 10x ligase buffer were added. After an overnight, reaction at 4°C, the ligation sample was transformed to a competent cell, *E. coli* JM 109.

Transformation

Using a chilled, sterile pipette tip 100 μl of competent cells were transfered to a sterile microfuge tube and added DNA to each tube. The contents of the tubes were mixed by tapping gently and then storing the tubes on ice for 30 min. The tubes were placed in a circulating water bath at 42°C for 1 min. The tubes were transfered to ice rapidly, and the cells allowed to cool for 2 min. SOC medium (950 μl) was added to each tube. Incubation was performed at 37°C for 1 h with vigorous shaking to allow the bacteria to be recovered and to express the antibiotic resistance markers encoded by the plasmid. The appropriate volume of transformed competent cell was spread on to a LB plate containing X-gal, IPTG and ampicillin, using a sterile bent glass rod. The plate was inverted and then incubated at 37°C for 16-24 h.

Extraction of Recombinant DNA

Extraction of recombinant DNA was performed using the method of Birnboim and Doly. A single bacterial colony was incubated in 5 ml of LB medium containing the ampicillin (100 μγ /ml) and cultured at 37°C for 14 h with vigorous shaking. Cultured bacteria was transferred to a microfuge tube and the cells were collected by centrifuging at 12,000 rpm for 5 min at 4°C. The medium was removed by aspiration and the bacterial pellet was dried as much as possible. For the digestion of bacterial cell membranes, the bacterial pellet was resuspended in 100 μl of ice cold solution I by vigorous vortexing. The freshly prepared 200 μl of solution II was added by tapping gently and 150 μl of ice cold solution III was added to the sample. The tube was placed in ice for 3-5 min. Centrifuge was performed at 12,000 rpm for 5 min at 4°C and the supernatant was transferred to a fresh tube. To remove soluble proteins, an equal volume of phenol:Chloroform:Isoamylalcohol = (25:24:1) was added to the supernatant, vortexed vigorously, and the sample was centrifuged the sample at 12,000 rpm for 5 min. DNA-containing aqueous phase was transferred to a new tube, being careful to avoid the proteins at the interface. The dsDNA was precipitated by adding 2 volumes of cold ethanol and placed in -20°C freezer for 1h. The sample was centrifuged at 12,000 rpm at 4°C for 15 min to the pellet of DNA. The suspernatant was removed by gentle aspiration and the pellet allowed to dry at room temperature. The DNA pellet was washed with 70% ethanol and recentrifuged. After removing the 70% ethanol, the pellet was allowed to dry at room temperature. The DNA was resuspended in 100 μl TE and RNA was removed by digestion with 3 μl of DNase-free RNase (10 mg /ml).

DNA Sequence Determination

DNA sequence was determined by the dideoxy chain termination method using Sequenase version 2.0 kit (United States Biochemical Co). All the steps were carried out according to the manufacture's protocol and reaction mixtures were heated at 90°C for 3 min, quickly chilled and immediately loaded on a 6% polyacrylamide sequencing gel containing 8 M urea in TBE buffer.

Statistical Analysis

Statistical analysis was performed with the Statistical Analysis System (version 6.04; SAS Institute, Inc). Allele frequencies

in different groups were compared by use of gene counting and χ^2 analysis (SAS, 1989).

RESULTS

PCR products were electrophoresed on 2% agarose gels, and DNA was visualized by ethidium bromide staining. Polymorphism detected by PCR was as a 490 bp product in the presence of the insertion and as a 190 bp fragment in the absence of the insertion (Fig. 1). The base sequence of the 490 bp, 190 bp DNA fragments prepared by PCR method was analyzed by the dideoxy chain termination method (Fig. 2). ACE gene revealed a 287 bp insertion/ deletion polymorphism in intron 16 (Hubert *et al.*, 1991).

As shown in Table 1, distributions of ACE genotype and frequencies of gene were quite different between normotensive Koreans and Caucasians. Distributions of normotensive Koreans ACE genotypes were II (37.6%) : ID (41.3%) : DD (21.1%), thus ID genotype was most numerous, the frequency was I:D = 0.582:0.418. In comparison with Caucasian distributions (Cambien *et al.*, 1992), there were significant differences between the genotypes ($p<0.001$). They were different from Japanese distributions (Kario *et al.*, 1996), quoted in this study ($p<0.01$).

More important fact was that genotype distributions between male and female were different hereditarily in Korean population. According to Table 2, the distributions of ACE genotype of the subjects (n=316) showed II (39.2%):ID (40.2%) :DD (20.6%), thus, was showed II:ID:DD = 34.5%: 43.4%:22.1% in male group, and II (60.3%): ID (25.9%):DD (13.8%) in the female group. The difference was significant between the male and female groups ($p<0.001$). It showed that the Korean male group had more DD genotype and ID genotype than the Korean female group. Further analysis showed that 60% of female had II genotype. There was notable difference between genotype II to genotype DD of male and female ($p<0.05$). The D allele frequency in Korean male group was higher than that of female group and the frequency of I allele was 0.733. Table 3 shows the distribution of ACE genotype and allele frequency between male and female group in hypertension group classified by sex. The

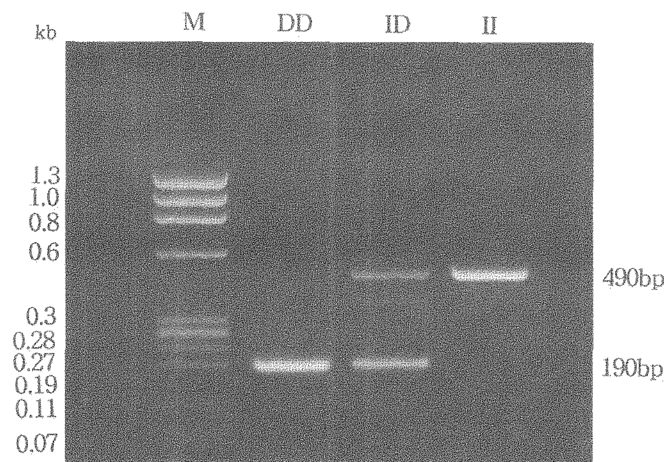

Fig. 1. PCR products from amplification of the polymorphic region in intron 16 of the ACE gene. Lane DD contains the 190-bp product from a DD homozygote; lane ID, the products from an ID heterozygote with both the 490- and 190-bp fragments; lane II, the 490-bp product from an II homozygote; and lane M, markers ($\phi\times 174$/*Hae* III).

Sense primer ⇒

TGGAGACCACTCCCATCCTTTCTCCCATTTCTCTAGACCTGCTGCCTATA
CAGTCACTTTTTTTTTTTTT**TTGAGACGGAGTCTCGCTCTGTCGCCCAGGCTGGA**
GTGCAGTGGCGGGATCTCGGCTCACTGCAACGTCCGCCTCCCGGGTTCACGCC
ATTCTCCTGCCTCAGCCTCCCAAGTAGCTGGGACCACAGCGCCCGCCACTACG
CCCGGCTAATTTTTTGTATTTTTAGTAGAGACGGGGTTTCACCGTTTTAGCCGGG
ATGGTCTCGATCTCCTGACCTCGTGATCCGCCCGCCTCGGCCTCCCAAAGTGCT
GGGATTACAGGCGTGATACAGTCACTTTTATGTGGTTTCGCCAATTTTATTCCAG
CTCTGAAATTCTCTGAGCTCCCCTTACAAGCAGAGGTGAGCTAAGGGCTGGAGC
TCAAGCCAT
TCAACCCCCTACCAGATCTGACGAATGTGATGGCCACATC

← anti-sense primer

Fig. 2. Nucleotide sequence of PCR product
190bp fragment in the absense of the insertion (287bp) and a 490bp fragment in the presence of the insertion (287bp) are underlined. Sense primer 5'-TGGAGACCACTCCCATCCTTTCT-3'
Anti-sense primer 5'-GATGTGGCCATCACATTCGTCAGAT-3'

Table 1. ACE genotype distributions and gene frequencies in normal populations between Korean and Caucasian

	Korean (n=189)	Japanese (n=104)	Caucasian (n=733)
ACE genotype, n(%)			
I I	71(37.6)	41(39.4)	143(19.5)
ID	78(41.3)	55(52.9)	390(53.2)
DD	40(21.1)	8(7.7)	200(27.3)
P-value		0.009[a]	0.0001[b]
Allele frequence			
I	0.582	0.659	0.461
D	0.418	0.341	0.539

ACE: angiotensin converting enzyme, I: insertion allele,
D: deletion allele
[a] Comparisons involving the genotype Korean vs Japanese.
[b] Comparisons involving the genotype Korean vs Caucasian.

Table 2. ACE genotype distributions and gene frequencies in Korean classified by sex

	Total (n=316)	Male (n=258)	Feamle (n=58)
ACE genotype, n(%)			
I I	124(39.2)	89(34.5)	35(60.3)
ID	127(40.2)	112(43.4)	15(25.9)
DD	65(20.6)	57(22.1)	8(13.8)
P-value		0.001[a]	0.013[b]
Allele frequence			
I	0.593	0.562	0.733
D	0.407	0.438	0.267

[a] Comparisons involving the genotype all.
[b] Comparisons involving the genotype I I vs DD..

Table 3. ACE genotype distributions and gene frequencies in hypertensive group classified by sex

	Male (n=81)	Feamle (n=46)
ACE genotype, n(%)		
I I	26(32.1)	27(58.7)
ID	37(45.7)	12(26.1)
DD	18(22.2)	7(15.2)
P-value	0.013[a]	0.057[b]
Allele frequence		
I	0.549	0.717
D	0.451	0.283

[a] Comparisons involving the genotype all.
[b] Comparisons involving the genotype I I vs DD..

Table 4. ACE genotype distributions and gene frequencies in hypertensive group and in normotensive control group

	Normotensive control group (n=189)	Hypertensive control group (n=127)
ACE genotype, n(%)		
I I	71(37.6)	53(41.7)
ID	78(41.3)	49(38.6)
DD	40(21.1)	25(19.7)
P-value	0.758[a]	0.570[b]
Allele frequence		
I	0.582	0.610
D	0.418	0.390

[a] Comparisons involving the genotype all.
[b] Comparisons involving the genotype I I vs DD..

Table 5. ACE genotype distributions and gene frequencies in the patients with hypertension

	Total (n=63)	Male (n=17)	Feamle (n=46)
ACE genotype, n(%)			
I I	33(52.4)	6(35.3)	27(58.7)
ID	20(31.7)	8(47.1)	12(26.1)
DD	10(15.9)	3(17.6)	7(15.2)
P-value		0.216[a]	0.421[b]
Allele frequence			
I	0.682	0.588	0.717
D	0.318	0.412	0.283

[a] Comparisons involving the genotype all.
[b] Comparisons involving the genotype I I vs DD..

different distribution of the three kinds of genotype between male and female group in hypertension group was significant ($p<0.05$), however, genotype II to genotype DD was not significant.

Table 4 shows ACE genotype distributions and allele frequencies between the hypertension and normal control groups. The analysis showed that there is no signigicance, it showed that there are no correlations between hypertension and ACE genotypes. Table 5 shows frequencies of alleles and distributions of ACE genotype for patients of cadiovascular disease in the hyper-tension group. Different from total group, it did not show significant differences between male and female. Especially, in the case of DD genotype, there was no different distributions between male and female. However, gene frequency is similar between male and female the subjects of the total group.

DISCUSSION

ACE is a key component of the renin-angiotensin system that has long been considered to play a role in the pathogenesis of hypertension and cardiovascular disease. Analysis by PCR of 287 bp insertion/deletion polymorphism in intron 16 of the 21 kb, 26 exon human angiotensin I-converting enzyme gene has demonstrated a statistically significant association of this polymorphism with essential hypertension. Insertion/Deletion polymorphism in intron 16 of the angiotensin converting enzyme gene has been reported to control plasma ACE activity and to be a risk factor for myocardial infarction. The genotype DD, which results in high ACE activity, has also been linked with dilated and hypertrophic cardiomyopathy, i.e., with conditions characterized by left ventricular hypertrophy and systolic or diastolic dysfunction (Rigat *et al.*, 1990, 1992).

Tiret *et al.* (1993) and Raynolds *et al.* (1993) reported that the D allele of the ACE gene has been linked with an increased incidence of myocardial infarction and increased mortality. The present study describes results of a cross-sectional study in which we use a polymerase chain reaction (PCR) technique to detect the 287 bp insertion/deletion polymorphism located in intron 16 of ACE in groups of hypertension and normotensive subjects.

We ascertained the truth of same DNA sequencing results of this study and Hubert *et al.* (1991)'s.

We also investigated the genotype distribution and the gene frequency of angiotensin I-converting enzyme in Korean population. Aspects of gene frequency and genotype distribution between Korean and Caucasian group showed significant differences. It was similar to the finding which was reported by Lee *et al.* (1996) and Park *et al.* (1996). As compared with Japanese group acknowledged significantly quoted from materials data this study (Kario *et al.*, 1996). There are no significant differences compared to among Japanese (Higashimori *et al.*, 1993). Compared between Orientals and Caucasians, we could conclude that Orientals have more genotype II (Table 1).

It became especially clear that the distribution of ACE genotype and frequency of alleles are different between Korean men and women (Table 2). Namely the female group had a lower distribution of ID and II genotype than male. In case of frequency of alleles, the female group had D allele frequency 0.267 and frequency of I allele showed a high value 0.733. It is of our interest whether the frequency of ACE genes is related so with the presence of X chromosome. Due to differences between man and woman group, the different frequency of alleles and distribution of ACE genotype between male and female in the hypertension group (Schunkert *et al.*, 1994), also showed significant difference (Table 3).

Thus, we should study to classify male group and female group about concerning the ACE gene in Korean subjects.

Somewhat, as compared to normal Korean adult group with hypertension group, it judged no mutual relation (Table 4). We should consider as we investigated to classify male and female patients with cadiovascular diseases with hypertension, no significant differences between male and female groups, and especially the distribution of DD genotype showed like male and female group. The distribution of allele frequency of female showed I:D = 0.717:0.283, in the subject of this study complication patient group, female group and male group were different from hereditary differences.

Thus, in the future, we must draw up an experiment plan, classify male and female groups, and need synthetic analysis with various environmental factors (Yang and Moon, 1996 ; Yang *et al.*, 1996), association of cadiovascular disease, and a research of Angiotensin II receptor gene.

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