A DOCTORAL DISSERTATION

Polymorphisms of Candidate Genes for Essential Hypertension in the Jeju Population

Department of Life Science

Graduate School
Cheju National University

Ju-Hyung Oh

December, 2005

제주집단에서 본태성 고혈압 후보 유전자에 대한 유전적 다형현상

지도교수 : 오 문 유

오 주 형

이 논문을 이학 박사학위 논문으로 제출함

2005년 12월

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2005년 12월

Polymorphisms of candidate genes for essential hypertension in the Jeju population

Ju-Hyung Oh

(Supervised by Professor **Moon-You Oh**)

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

Date Approved:

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Department of Life Science
GRADUATE SCHOOL, CHEJU NATIONAL UNIVERSITY
December, 2005

Abstract

Hypertension is a major cause of the morbidity and mortality associated with coronary heart disease, renal failure, and stroke. The etiology is unclear for approximately 90% of all cases of human hypertension, which are referred to as primary or essential hypertension. The development of essential hypertension involves both environmental and heritable factors.

The present study examined genetic polymorphisms of candidate genes for essential hypertension in the Jeju population. The genes for aldosterone synthase, a-adducin, angiotensin-converting enzyme, kallikrein, bradykinin receptor, angiotensin II type I receptor, and angiotensinogen have been implicated in essential hypertension. These genes have functions that are associated with modulation of blood pressure, and mutations in these genes lead to dysfunction of kidney and elevation of blood pressure. Thus, these mutations represent risk factors for the development of hypertension or disease linked to hypertension. In studies of candidate genes associated with essential hypertension, the focus has been on genetic variations linked to renal damage, as first reported in 1947 and debated ever since. The present study examines mutations in candidate genes associated with hypertension to probe the theoretical linkage between essential hypertension and renal dysfunction and to search for genetic markers that are

linked to hypertension.

The aldosterone synthase gene showed different DNA band patterns for the exon sites of the *CYP11B1* and *B2* genes, and different types of band patterns for the promoter region. The *a-adducin* gene showed variable DNA fragmentation patterns for the exon and T-variant allele, and these patterns differed from those of other ethnic groups. The angiotensin-converting enzyme (ACE) gene showed significant differences in I/D polymorphisms of DCP-1 ($P=0.630$, $P>0.05$; Kruskal-Wallis). The polymorphisms examined in the present study exerted effects on blood pressure in both hypertensive patients and normotensive subjects. The results show that mutations in the *a-adducin* gene are more frequent in hypertensive and normotensive subjects by affect of environmental and genetic factors, a finding that is not consistent with studies conducted in a Japanese population which showed weak associations between these factors. This study shows similarities with studies of Hispanics and Black-Americans in terms of a strong association between blood pressure and mutations in the *a-adducin* gene. Analysis of the gene for kallikrein using *Mva*1 enzyme digestion showed the existence of various alleles ($P=0.000$, $P<0.05$; Kruskal-Wallis). The band patterns for the angiotensin II type I receptor gene showed differences for hypertensive patients and normotensive subjects, although these differences were not statistically significant. The bradykinin receptor gene showed recombinant hybrids

by crossing over. We found polymorphisms of the promoter region of the bradykinin receptor gene, which was not different from result suggested of till now.. These polymorphisms may account for the increasing hypertension rate in the population over time. Numerous studies have described correlations between increases the numbers of hypertensive patients and increased frequencies of mutation in genes linked to hypertension. This is appeared by action of over two factors, thus, it could be caused genetic mutation of different type with time.

Polymorphisms upstream of the aldosterone gene promoter, particularly -344C/T, have been shown to be genetic markers of essential hypertension. The *angiotensinogen* gene revealed a banding pattern of mobility shift by mutation, which appeared to represent a C→G substitution in most of the hypertensive subjects. Several mutations were discovered in genes associated with essential hypertension and differences were noted among the racial groups. These results indicate the potential value of polymorphisms as risk factors for the development of essential hypertension with complex traits. In addition, these results suggest to apparent proof about association between renal failure and essential hypertension, although further studies are needed to test this hypothesis.

Key words: essential hypertension, polymorphism, candidate gene, etiology, coronary heart disease, renal failure, stroke, aldosterone synthase, alpha-adducin,

angiotensin converting enzyme, kallikrein, bradykini receptor, angiotensin II type1 receptor, angiotensinogen, blood pressure, kruskal-wallis, recombinant hybrid, mobility shift, substitution, genomic marker, risk factor.

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Introduction

Blood pressure is a complex trait that is determined by an array of interlocking homeostatic systems with feedback mechanisms that maintain homeostasis in the face of highly variable environmental factors. Although hypertension is defined by arbitrary limits, it represents the upper end of the distribution of blood pressure levels determined by a combination of genetic and environmental factors, and in which renal mechanisms seem to be involved, even at the earliest stages. A useful strategy in investigating the heredity of complex traits is the analysis of intermediate phenotypes, as opposed to simply focusing on the ultimate disease phenotype.

Several hypotheses have been proposed to explain the mechanisms responsible for defective sodium handling in the kidneys. A favored hypothesis is that the renal dysfunction is the result of a polygenic defect that leads to alterations in the regulation or expression of the tubular transport systems involved in sodium reabsorption and excretion. The recent discovery that many forms of genetic hypertension are associated with enhanced sodium reabsorption provides support for this hypothesis (Johnson *et al*., 2005).

Although essential hypertension was originally described as hypertension that occurs in the absence of clinical renal disease (Dahl *et al*., 1975), it has been

subsequently shown in transplantation studies with experimental models of hypertension that the kidney is ultimately responsible for the elevation in blood pressure, a finding that has been verified in humans. It is now generally believed that the mechanism by which the kidney causes hypertension involves a physiological defect in sodium excretion (Guyton *et al*., 1972).

The development of essential hypertension involves both environmental and heritable factors. Experimental animals with inherited forms of hypertension, such as spontaneously developing hypertension, have been used for the development of antihypertensive drugs. The observation that renal microvascular disease is present in the majority of subjects with hypertension led Goldblatt (1947) to postulate that microvascular disease is a primary causative factor that produces hypertension *via*a mechanism that involves renal ischemia. However, this hypothesis has been discarded due to the lack of a scheme to show how microvascular disease would develop in this case, and because it has become apparent that microvascular disease is a consequence of hypertensive renal damage.

Numerous candidate genes have been associated with hypertension. Candidate genes that determine blood pressure variation include those whose products have a direct impact on blood pressure, e.g., aldosterone synthase, adducin, bradykinin, kallikrein, angiotensinogen, angiotensin-converting enzyme, and angiotensin II type 1 receptor (AGT1R).

Adducin, which is a cytoskeleton protein, regulates transmembrane ion transport and is a substrate for protein kinase C in intact cells as well as under *in vitro* conditions. Since adducin is present in many tissues, genetic variants may affect the functions of many tissues, which in turn may modify renal function (including tubular reabsorption) through some extra-renal mechanism (Manunta *et al*., 1999). Adducin has been purified from brain tissue membranes and immunologically related isoforms are present in other tissues, including the liver, kidneys, and lungs, as well as in various cultured cells (Joshi *et al*., 1991).

The gene for adducin was first characterized in the Milan hypertensive (MHS) rat, which is an animal model of salt-sensitive hypertension. In the rat model, the 316Tyr single nucleotide polymorphism (SNP) is associated with the hypertensive phenotype. The a-adducin protein is linked to blood pressure, and mutations in the a-adducin gene have been reported to be strongly associated with altered blood pressure and sodium intake levels; increased mutation of this gene has pronounced effects on the blood pressure levels of hypertensive patients. Studies in the Milan hypertension rat and in human beings with essential hypertension suggest that alterations in tubular reabsorption as well as changes in cytoskeleton proteins are involved in this effect. Known point mutations, one each in the a- and b-adducin subunits, account for up to 50% of the difference in blood pressure between the Milan hypertensive (MHS) rat and Milan

normotensive (MNS) rat strain. Thus, this polymorphism accounts for only a portion of the blood pressure differences observed between MHS and MNS rats.

Therefore, other genes appear to be involved, as suggested by genetic crossing experiments (Bianchi *et al*., 1994). Cross-transplantation studies in rat models of primary hypertension have shown that hypertension is associated, at least in part, with renal function. Similar findings have been obtained for humans. Although abnormalities in kidney function and cell membrane ion transport have been described in cases of hypertension, a link with modifications of kidney function is difficult to prove (Manunta *et al*., 1999). In humans, the progression from a genetic abnormality that causes an increase in plasma ACE level is widely used for the diagnosis and follow-up of sarcoidosis, since elevated levels of this enzyme are often observed in this disease. More recently, attention has been focused on mutations in the adducin genes that affect renal function.

Aldosterone synthase is expressed in the human adrenal cortex, which is regulated positively by corticotrophin. The activation of this gene is required in the final step of aldosterone biosynthesis. This enzyme is encoded by two genes on chromosome 8q22. These two genes are called *CYP11B1* and *CYP11B2*, each of which contains nine exons and is more than 8,000 bp in length.

Mutations in *CYP11B1* are linked to the hypertensive form of congenital

adrenal hyperplasia, which is due to the accumulation of 11-deoxycorticosterone. On the other hand, mutations in the *CYP11B2* gene are linked to aldosterone synthase deficiency, which is characterized by salt wasting and hypertension. Moreover, unequal recombination between *CYP11B1* and *CYP11B2* induces duplicate hybrid *CYP11B* genes, which causes the hypertensive disorder to be inherited dominantly. The condition, which is known as aldosteronism, is an autosomal dominant form of hypertension caused by the inheritance of a hybrid gene composed of the regulatory sequence from the *CYP11B1* gene and the coding sequence from the *CYP11B2* gene (Lifton *et al*., 1992). In addition, other mutations in this gene may be attributed to genetic susceptibility for hypertension.

Cytochrome p450, CYP11B1, and the steroid 11b-hydroxylase catalyze the terminal step of cortical biosynthesis. CYP11B1 is expressed at high levels throughout the human adrenal cortex and is positively regulated by corticotrophin. A related enzyme, CYP11B2 (aldosterone synthase), has steroid 11b-hydroxylase activity as well the 18-hydroxylase and 18-oxidase activities required for the terminal steps of aldosterone biosynthesis. The two genes that encode the CYP11B1 and CYP11B2 components are located 40 kb apart on chromosome 8q22 (Brand *et al*., 1998).

Mutations in the *CYP11B1* gene that elevate the level of aldosterone synthesis may be linked to hypertensive disease by increasing therate of binding to the

promoter regions of these genes. Moreover, mutations in this region increase four-fold the binding rate of the steroidogenic transcription factor (SF-1) to the enzyme promoter in essential hypertension patients.

A recent study has shown that the T allele of the C344T polymorphism, which involves a cytosine/thymine substitution at the putative binding site for SF-1, is significantly associated with hypertension, in that it shows a positive correlation with plasma aldosterone levels (Mitsunobu *et al*., 2001). A significant association between the -344T allele and essential hypertension has been reported in white subjects in some studies, although other investigators have failed to find any relationship between blood pressure and the presence of the -344T allele (Zhu *et al*., 2003).

Mutations in the aldosterone synthase genes have been studied with respect to high blood pressure in essential hypertension patients. Our hypothesis regarding gene polymorphisms has three elements: (1) the -344C/T substitution affects gene transcription by increasing the binding rate of transcription factor SF-1; (2) the *CYP11B2* gene has a functional activity that is related to gene conversion caused by replacement of *CYP11B1* (3) a point mutation in exon 3 causes an Arg→Lys substitution at position 173. The association with hypertension may be based on activity due to reciprocal recombination or the activity of a single gene. In particular, differences have been noted for -344 C/T (promoter region) between

different population groups.

Studies of *CYP11B1* gene polymorphisms in Japanese subjects have provided conflicting results owing to differences in regional and genetic factors. Thus, it seems likely that different types of mutations exist among the different ethnic groups. Therefore, the polymorphisms described here highlight the boundary between hypertensive subjects and normotensive controls, and may represent genetic markers of either essential hypertension or complicated disease linked to secondary hypertension.

Angiotensin-converting enzyme (ACE) plays a key role in the rennin-angiotensin system. ACE cleaves angiotensin I, which convert into angiotensin II, stimulates aldosterone, which influences blood pressure. ACE is encoded by a 21-kb, 26-exon gene located on chromosome 17 at q23. A deletion and insertion (I/D) polymorphism of the ACE gene has been described that represents the absence or presence of a 287-bp DNA fragment located near the 3'-end of intron 16. The ACE I/D polymorphism accounts for almost half the variability in ACE plasma levels. Thus, it is hypothesized that the ACE I/D polymorphism is associated with blood pressure levels and hypertension.

Renal kallikrein is a serine protease that recognizes the substrate kininogen,

from which it cleaves the kinin product bradykinin, a multifunctional peptide that is involved in the regulation of local blood flow and sodium balance. The kallikrein gene (*hklk1*) is located on chromosome 19 (19q13.2-q13.4), together with several homologous genes that code for non-kinin-forming serine proteases or a short amino-terminal peptide (Slim *et al*., 2002). A polymorphic microsatellite repeat sequence has been identified at the 3'-end of the human tissue kallikrein gene (Richards *et al*., 1991). Although this is a potentially useful marker, it has not yet been linked to a high blood pressure phenotype (Song *et al*., 1997).

Several polymorphisms of the *hklk1*gene have been identified. In addition to a microsatellite marker at the *hklk1* gene locus, a complex multiallelic polymorphism has been identified in the 5'-flanking region of the gene, with two alleles displaying lower *in vitro* promoter activities. A *TaqI* polymorphism and three additional diallelic polymorphisms, including and leading to an amino acid substitution, have also been described (Slim *et al*., 2002). Renal kallikrein secretion is accelerated by potassium in kidney slices and *in vivo*, as well as by ATP-sensitive potassium channel blockers, such as glibenclamide (Katori and Majima, 2004).

The B2 bradykinin receptor has been implicated as a candidate gene for the

complex genetic mechanisms underlying common chronic disorders, such as hypertension and ischemic heart disease. Recently, the gene structure has been elucidated (Kammerer *et al*., 1995) and three well-defined polymorphisms, located in each of the three exons, have been characterized (Braun *et al*., 1995; 1996a; 1996b).

The search for genetic determinants has focused primarily on genes that are involved in the renal control of sodium balance. Although rare genetic mutations in the renal epithelial sodium channel genes and a chimeric gene duplication that involves the genes for 11b-hydroxylase and aldosterone synthase have been described, these affect only a small fraction of the hypertensive syndrome.

Most of the known actions of angiotensin II are mediated by AGT1R, including vascular contraction, pressure responses, proximal tubule sodium transport, and aldosterone secretion. This receptor is expressed on the surfaces of various cell types, such as vascular smooth muscle cells, myocardial cells, vascular smooth muscle cells of the afferent and efferent arterioles, proximal tubule cells of the kidney, and the glomerulosa cells of the adrenal gland (Miller *et al*., 1999).

The A1166C polymorphism has been described for the AGT1R promoter and is considered to be a genetic marker for diseases associated with hypertension, e.g., arteriosclerosis. Recently, the 158-nucleotide promoter region adjacent to the exon has been reported to contain an A→G substitution.

After binding to renin, angiotensinogen is cleaved to angiotensin I. Thus, angiotensinogen acts as a substrate for renin, which, upon further cleavage by the angiotensin-converting enzyme, yields angiotensin II, a peptide hormone that promotes vasoconstriction and sodium retention.

The angiotensinogen gene has been implicated in essential hypertension through both genetic linkageand allelic association studies. The M235T polymorphism, which encodes a threonine instead of a methionine at residue 235 of the mature angiotensinogen protein, has been found to be significantly more common in hypertensive subjects than in normotensive controls, in studies of Utah residents and French Caucasians (Jeunemaitre *et al.,*1997; Jeunemaitre, 1998). This association was confirmed in unselected French hypertensives who participated in a clinical trial (Jeunemaitre *et al*., 1997; Jeunemaitre, 1998) and in a study of selected hypertensive patients in Germany with age at onset of <50 years (Schmidt *et al*., 1995). Two other studies have found an association with the T174M polymorphism, which is in complete linkage disequilibrium with M235T, in a Japanese population (Hata *et al*., 1994; Kamitani *et al*., 1994; Iwai *et al*., 1995).

In a systematic search performed using SSCP analysis of the region that contains an enhancer core element from positions +2170 to +2230 with 20 T235T homozygous French and 20 M235M homozygous individuals, no electrophoretic variants were observed (Jeunemaitre *et al.,* 1997).

Purpose of the present study

The rising numbers of hypertensive individuals implicate environmental and genetic factors, which are not consistent with regional and genetic backgrounds. Furthermore, ethnicity-based differences have come to fundamental root for case-control study in population genetics. Each of the candidate genes represents a discrete and specific contribution to the association with essential hypertension. This study focuses on the relevance of specific candidate genes in hypertensive and normotensive groups in the Jeju population of Korea, the genetic differences between these two groups, and whether polymorphisms of the candidate genes affect hypertension.

The genetic variations and gene frequencies of these candidate genes were investigated to elucidate the genetic profiles of the Jeju population, and to corroborate the fundamental genetic diagnosis of the Jeju population.

Materials and Methods

1. Subjects and materials

Blood samples (10 ml) were collected into vaccumtainer tubes that contained anticoagulants from the punctured veins of the hypertensive patients and normotensive subjects who agreed to participate in this study. The collected blood samples were transferred to the Genetics Laboratory of Cheju National University and were used for DNA isolation.

At the Department of Cardiac Medicine, Halla General Hospital, blood samples were collected randomly from outpatients and/or hospitalized patients with essential hypertension and from normotensive subjects without essential hypertension or secondary hypertension. The patients were not chosen on the basis of heritability for pedigree. Blood pressure was determined over the range of 90 mmHg of diastolic blood pressure (DBP) to 140 mmHg of systolic blood pressure (SBP).

2. Methods

2-1. DNA extraction from human blood samples

DNA was isolated from the blood samples using a slightly modified DNA extraction method (Sambrook *et al.*, 1989) and a DNA extraction kit (Bioneer, USA). The isolated DNA was dissolved in TE buffer and stored at 4℃. The buffy-coats were suspended in 10 ml of NaCl (0.2%) solution. Then, 10 ml of a 1.5% NaCl solution was added and the samples were resuspended. The mixture was centrifuged at 13,000 rpmfor 5 min. This step was repeated until the pellet became clear. The final pellet was suspended in 3.6 ml WCLB [10 mM Tris-HCl (pH 7.6), 10 mM EDTA, 50 mM NaCl], and 20 ml of proteinase K (20 mg/dl) and 0.4 ml of a 10% SDS solution were added. This mixture was then incubated at 55℃ for 3 h or at 37℃ overnight. After the addition of 1 ml of 5 M NaCl, the mixture was centrifuged at 13,000 rpm. Two volumes of cold ethanol were added to the supernatant. The isolated DNA was air-dried, and dissolved in TE buffer.

2-2. Polymerase chain reaction (PCR)

DNA amplification was carried out in a volume of 15-20 ml that contained 0.1 mM each of dCTP, dATP, dGTP, and dTTP, 20 pmol of each oligonucleotide primer, 1.25 U *Taq* DNA polymerase, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl, and 0.01% gelatin. PCR was performed under the following conditions: for the *CYP11B2* promoter region, 34 cycles of denaturation at 94℃ for 45 sec, annealing at 58℃ for 45 sec, and extension at 72℃ for 45 sec; for exon 1 of *CYP11B2*, 35 cycles of denaturation at 94℃ for 30 sec, annealing at 57℃ for 40 sec, and extension at 72℃ for 40 sec; for exon 2 of *CYP11B1*, 35 cycles of denaturation at 94℃ for 40 sec, annealing at 56℃ for 50 sec, and extension at 72℃ for 50 sec; for exon 1 of *CYP11B1*, 35 cycles of denaturation at 94℃ for 30 sec, annealing at 52℃ for 40 sec, extension at 72℃ for 40 sec; for exon 10 of *a-adducin*, 35 cycles of denaturation at 94℃ for 20 sec, annealing at 60℃ for 30 sec, and extension at 72℃ for 30 sec; for exon 1 of *a-adducin*, 35 cycles of denaturation at 94℃ for 20 sec, annealing at 60℃ for 30 sec, and extension at 72℃ for 30 sec; for the *B2R* promoter, 35 cycles of denaturation at 94℃ for 1 min, annealing at 53℃ for 45 sec, and extension at 72℃ for 30 sec; for the exon of the angiotensin receptor gene, 30 cycles of denaturation at 94℃ for 45 sec, annealing at 58℃ for 45 sec, and extension at 72℃ for 45 sec; for *DCP*-1, 30 cycles of denaturation at 94℃ for 1 min,

extension at 58℃ for 1 min, and extension at 72℃ for 1 min; for *kallikrein*, 35 cycles of denaturation at 94℃ for 45 sec, annealing at 58℃ for 45 sec, and extension at 72℃ for 45 sec; and for the exon of the angiotensinogen gene, 35 cycles of denaturation at 94℃ for 45 sec, annealing at 62℃ for 45 sec, and extension at 72℃ for 45 sec.

MS (mutagenetically separated) PCR was also carried out with forward and reverse primers that contained over 30 bp of the nucleotide sequences of the variant types. Sense and antisense allele-specific primers were designed for the uptake-specific regions of the genes. The primers used for PCR amplification are shown in Table 1.

Table 1. List of primers used in PCR

Name		sequence (5'-3')	reference
CYP11B1	exon1	GTG CAA CTT GGG AGG ACC ACG C TTG TGC GGT GTA AAC ACG GGT GC	This study
	exon2	ATG GCA CTC AGG GCA AAG GC CCG GGG TCA AGG ACC TCC CA	This study
CYP11B2	exon1	GTA CAA CTT GGG AGG ACC ACG C TTG TGC GGT GTA AAC ACG GGT GC	This study
Alpha adducin		FG: GGG GCG ACG AAG CCT AGG TAG FT: GCT GAA CTC TGG CCC AGG CGA CGA AGC TTC CGA GGA TT R CCT CCG AAG CCC CAG CTA CCC A	Castejon et al.,2003
	exon1	TGA GGG GCG GAG AGG CCT GG GAG TCT TCG CTT AGC ACC CG	This study
AGT1R		AAC GCT GAT CTG ATA GTT GAC ACG CTC TGT TTT GCA TTC CCT CCT C	Matayoshi et al.,2004
Bradykinin Receptor		GGT CAC TGA TTC CTC CGT CTT C TTG CCC CCC CGC CTC CGA TC	Braun et al.,1996
ACE(DCP-1)		CTG GAG ACC ACT CCC ACT CTT TCT GAT GTG GCC ATC ACA TTC GTC AGA T	Rigat et al.,1992
Kallikrein	intron	GGT CAC TGA TTC CTC CGT CTT C TTG CCC CCC CGC CTC CGA TC	Slim et al., 2002
Angiotensinogen		ACA GAT GTA TAC AAT TCA GCA G CAC CTA AAA CTT CAA AGG ACT G	Jeunemaitre et al., 1997

2-3. Restriction digestion analysis of the PCR products

Approximately 7 ml of each PCR product was digested with restriction enzymes, and genotyping was performed on ethidium bromide-stained agarose gels. The enzymes used for digestion differed with respect to the optimal temperature for enzyme activity (37℃ or 55℃). After enzyme treatment, the samples were incubated at the optimal temperature for more than 1 h. The samples were electrophoresed in a 2% agarose gel, and the band patterns were visualized by ethidium bromide staining and UV illumination.

2-4. PAGE and silver staining

The amplified DNA fragments were detected by using 10% or 20% polyacrylamide gel electrophoresis (PAGE) with silver staining or ethidium bromide staining. The polyacrylamide gel was prepared by adding 10% ammonium persulphate and TEMED to a mixture of 30% acrylamide, distilled water, and 5×TBE. Silver staining was carried out by washing with 10% ethanol, oxidization for 6 min with 1% nitric acid, washing twice with distilled water, followed by silver staining with 0.012 M silver nitrate for 30 min. The gels were rinsed briefly with distilled water and then reduced with a solution that contained 0.28 M anhydrous sodium carbonate and 0.019 M formalin until the DNA bands were visualized. Finally, the bands were fixed by the addition of a stop solution of 10% acetic acid.

2-5. Single strand conformation polymorphism (SSCP)

SSCP was carried out with slight modification for the analysis of single-stranded DNA mobility shifts caused by point mutations. Each PCR product was denatured by boiling for 4 min, and was then placed on ice for 1 min, to prevent DNA annealing. Each PCR product was loaded onto a polyacrylamide gel and electrophoresis was carried out at 120 V for 20 hrs.

2-6. Plasmid transformation

SOB medium that contained ampicillin was added to cells that were transformed with the PCR product and vector. After incubation at 37℃ for 24 h, white colonies were placed in LB medium in 15-ml conical tubes and cultured on a shaking incubator at 37℃ for 24 h. After plasmid isolation, the DNA was digested with restriction enzymes and electrophoresed on an agarose gel.

2-7 DNA sequencing

The DNA samples were electrophoresed on a 1% low-melting-agarose gel. DNA fragment slices were removed and eluted using an elution kit or *via*the manual method. The DNA samples were dissolved with EB (elution buffer) with an approximate yield of 80% of the original product. DNA sequencing was performed using the ALFexpress DNA Sequencing System (Pharmacia, Sweden)

with Cy5-labelled vector inner primers (M13-40 and M13 reverse) and the Cy5-AutoCycle Sequencing Kit (Pharmacia, Sweden).

Results

Mutations in the a-adducin gene have been reported to be strongly associated with blood pressure and sodium intake; increased levels of mutation of this gene show increasing influence on the blood pressure levels of hypertensive patients.

The present study investigated the effects of polymorphisms on the blood pressure levels of hypertensive patients and normotensive subjects. The results show that mutations in the *a-adducin* gene are more frequent in hypertensive and normotensive subjects as a result of environmental and genetic factors, a finding that is not consistent with studies conducted in a Japanese population, which showed weak associations between these factors. This study shows similarities with studies of Hispanics and Black-Americans in terms of a strong association between blood pressure and mutations in the *a-adducin* gene.

Exon 1 of the *a-adducin* gene exhibited multiple DNA fragments in both hypertensive patients and normotensive subjects. The G/T polymorphism of exon 10 was found to be of the T type in 89 out of 100 (89%) patients and in 77 out of 190 (40.5%) normal subjects (Table 2).T variation appeared in most of the hypertensive patients. The genotype distribution was in concordance with Hardy-Weinberg equilibrium for the total subject population. The ratio was higher in the Gly/Trp hypertensive patient group than in the Gly/Gly hypertensive patient

Table 2. Alpha adducin gene polymorphism in Jeju population

Genes	Types	Hypertensive	normotensive
-adducin	total	100	190
	G type	11	108
	T type	89	77
	G/T type	-	5
		X^2	0.925
		p	0.630

group (P=0.630). The adducin genotype was differed significantly between the hypertensives and normotensives (Fig. 1).

The association between *a*-adducin and blood pressure appeared to be due to mutations in the *a-adducin* gene, which have been associated with blood pressure and sodium intake; increased mutation of this gene has pronounced effects on the blood pressure levels of hypertensive patients. Based on these results, the *a-adducin* gene appears to be a genetic marker for essential hypertension in specific ethnic groups.

PCR amplification of exon 1 of *CYP11B1* gave multiple bands, with a PCR product that was shorter than 140bp of the original nucleotide sequence, which suggests a deletion in minor subject. Figure 2 shows the band pattern of the *CYP11B1* gene. The mutation was confirmed by direct sequencing of the deletion bands of exon 1 of the *CYP11B1* gene (Fig. 3). In addition, multiple bands were observed for the PCR products of exon 2 of the *CYP11B1* gene from essential hypertension patients (Fig. 3, 4). Figure 5 shows the band pattern of exon 2 of the *CYP11B2* gene. This mutation appears to belinked to a failure to modulate the biosynthesis of aldosterone by aldosterone synthase (*CYP11B1*), since the function of aldosterone synthase, a functional enzyme that combines *CYP11B1* and *CYP11B2*, is affected by changes in the *CYP11B1* gene. The promoter region of the *CYP11B2* gene may be affected by the adjacent exon, as the mutation

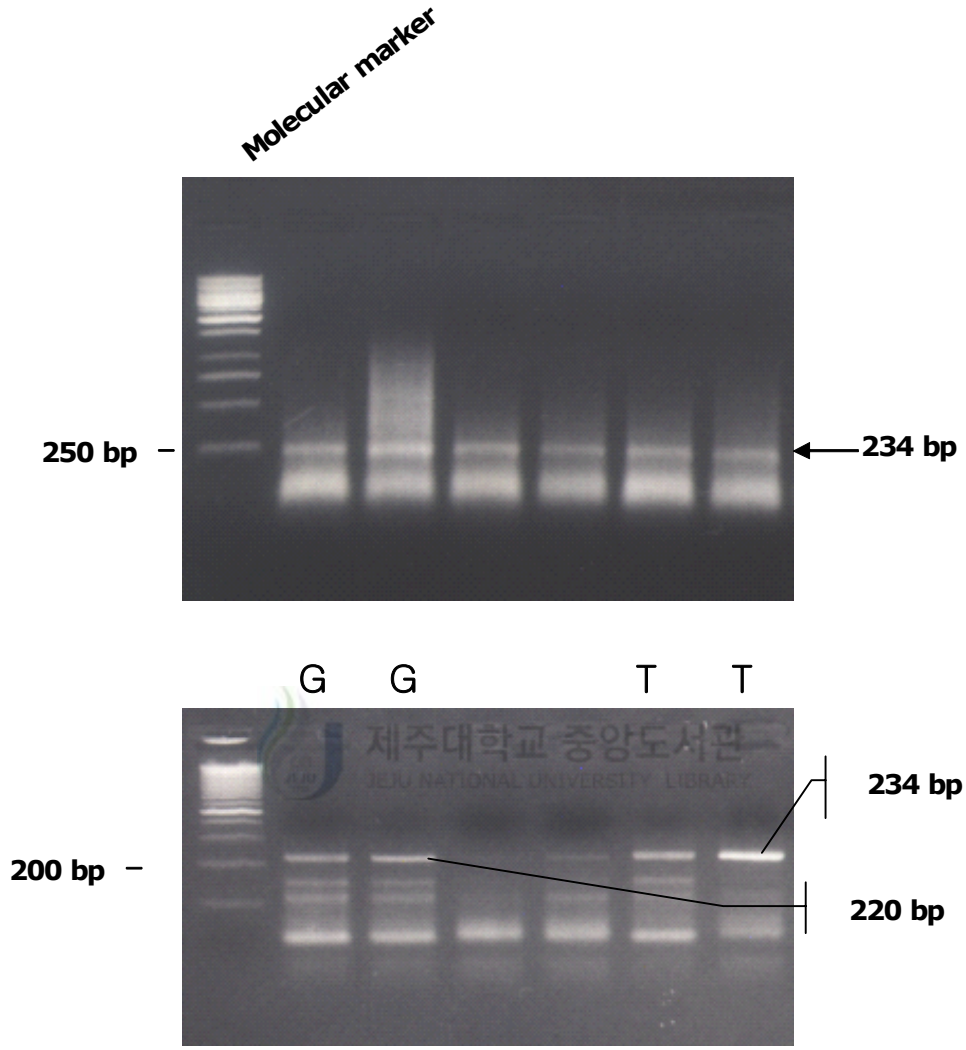


Figure 1. G/T polymorphism of exon 10 of alpha-adducin gene in hypertensives; upper (234 bp), all T types, and lower, T (234 bp) and G (220 bp) types.

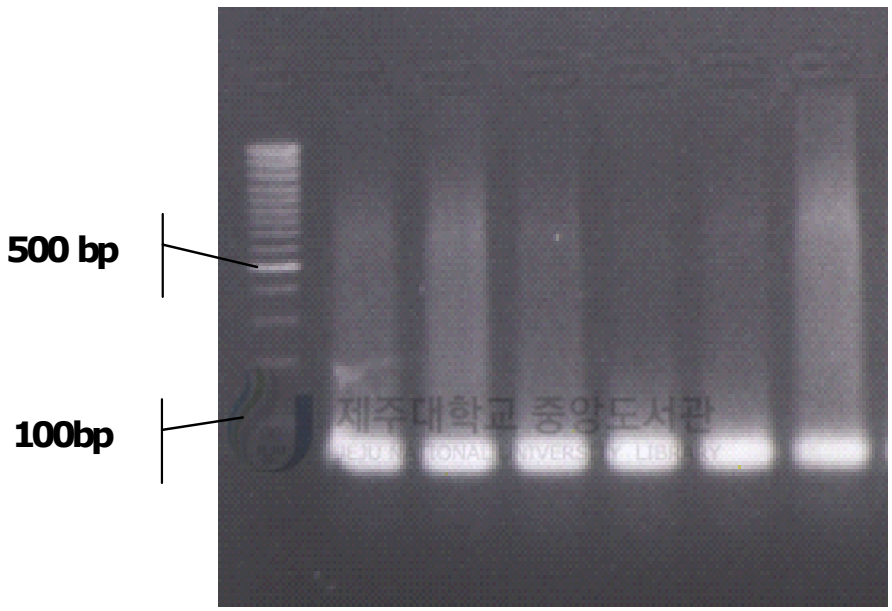


Figure 2. Deletion band patterns of PCR products of promoter region of CYP11B1 gene in normotensives.

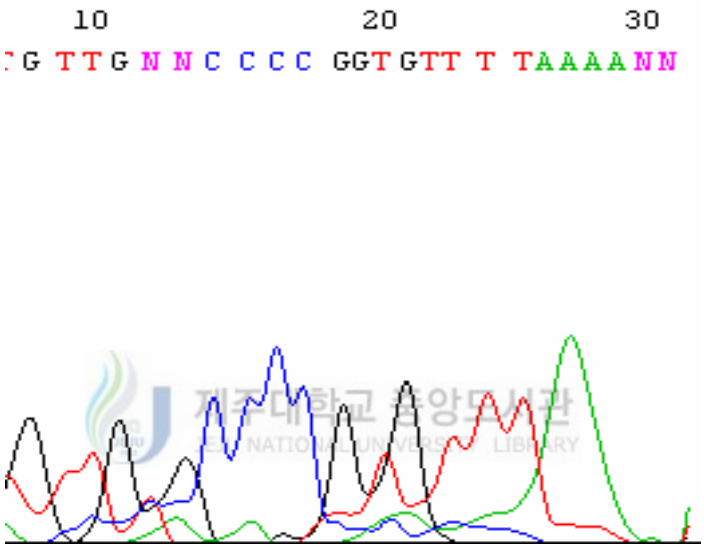
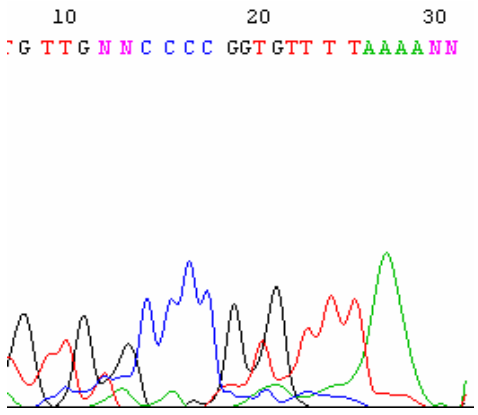


Figure 3. Nucleotide sequence with deletion variation of CYP11B1 gene in normotensives. From base 30, all were deleted in normotensives and hypertensives, also (see Figure 4).



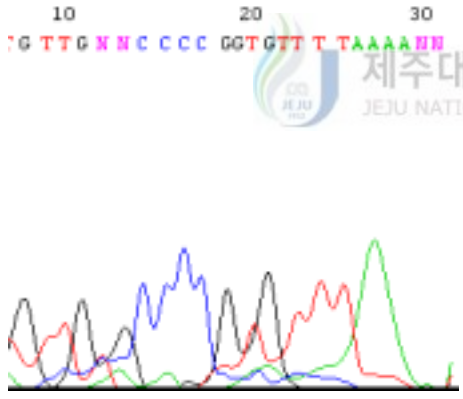


Figure 4. Nucleotide sequence of deletion variation of CYP11B1 gene in essential hypertensives.

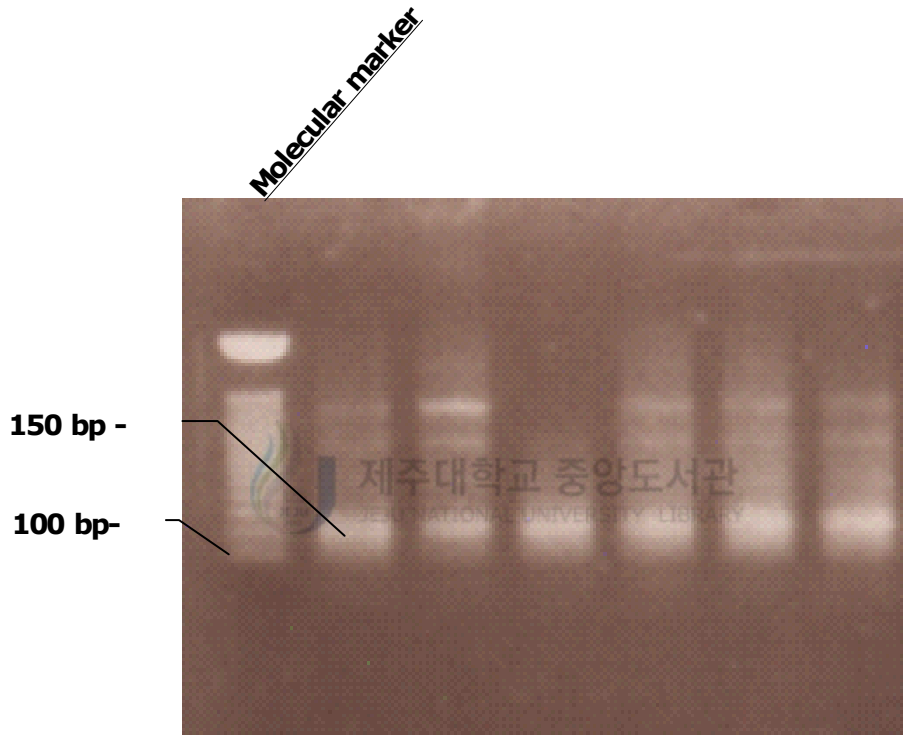


Figure 5. Band patterns of PCR products of exon 1 of *CYP11B2* gene in normotensives.

probably led to conversion of the transcriptional start site in the promoter-exon region (Figure 6).

ACE plays a key role in the rennin-angiotensin system. ACE is encoded by a 21-kb, 26-exon gene that is located on chromosome 17q23. A insertion and deletion (I/D) polymorphism of the ACE gene has been described that represents the absence or presence of a 287-bp DNA fragment located near the 3'-end of intron 16. The ACE D/I polymorphism accounts for almost half the variability in ACE plasma levels. Thus, it is hypothesized that the ACE D/I polymorphism is associated with blood pressure levels and hypertension.

Recently, evidence has been presented to support the hypothesis that the mutation in intron 16 of the ACE gene correlates with the development of essential hypertension. It has been shown that the D allele is associated with higher levels of ACE, which suggests a strong association of the ACE I/D polymorphism with increasing blood pressure levels. Nevertheless, evidence to support this notion is lacking. Jeunemaitre *et al*. (1997) carried out an extensive study on a sample of Caucasian patients from Utah but did not find any significant association of ACE locus with essential hypertension. The present study examined the influence on blood pressure of I/D polymorphisms under presumption which study about association of hypertension and ACE gene has a

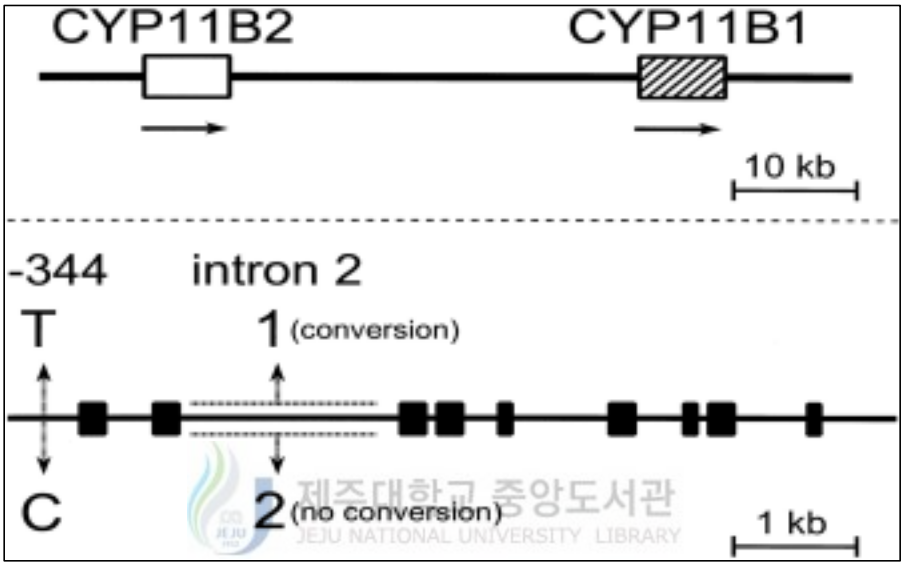


Figure 6. Top, Relative orientations of the *CYP11B2* and *CYP11B1* genes.
Arrows indicate the direction of transcription, and a scale of 10 kb is marked. Bottom, schematic of the *CYP11B2* gene showing the locations of polymorphisms at the -344 position in the promoter and in the second intron (an intervening region that is spliced out of mRNA). Protein encoding regions in the gene are indicated by boxes. 1 kb is marked (Kupari *et al.*, 1998).

product to conflicted consequence.

Gulf Bedouins have the highest frequency of the D allele, but they do not show an association between carriage of this allele and hypertension. African-Americans have a higher frequency of the D allele than either Caucasians or Japanese, but do not show an association with hypertension. Persons living in Nigeria, Jamaica, and the USA have a similar prevalence of the D allele but exhibit large differences in hypertension prevalence (16-33%) (Zaman *et al*., 2001). The results of O'Donnell *et al*. (1998) regarding the prevalence of the DD genotype are consistent with the results of prior studies, as the D allele was found in approximately 30% of men in whom there was a 59% increased risk of hypertension after adjustment for common hypertension risk factors. Previously, a weak association was noted between hypertension and ACE I/D genotype. The ACE I/D genotype has not been proven to lead to increased blood pressure with attenuation of urinary excretion or sodium sensitivity. In the present study, insertion and heteroduplex types were identified in most of the population, and the D allele was observed in only a small fraction of the population. The results described here are similar to those obtained in an Asian study for the frequency of the ACE I/D genotype (Table 3). Polymorphisms of the *DCP-1* gene (Fig. 7) in an essential hypertension population were observed as insertions (15%), deletions (32%), and I/D (52%). In a normal population, insertions, deletions, and I/D were observed at rates of 18.9%, 21.7%, and 59.4%, respectively (Table 3).

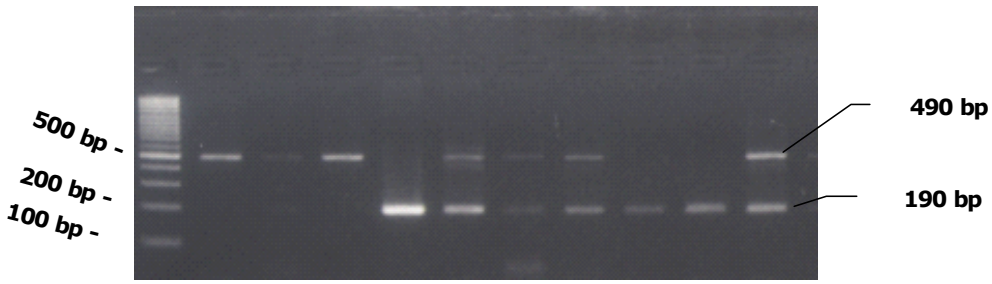


Figure 7. I/D polymorphism of intron 16 of *DCP-1* gene in essential hypertensives.

Table 3. ACE (DCP1) gene polymorphism in Jeju population

Genes	Types	Hypertensive	normotensive
ACE (DCP1)	total	100	180
	I	15	4
	D	32	39
	I/D	52	107
	X^2	2.398	1.332
	p	0.302	

In general, polymorphism of the *kallikrein* gene has been described as a *Taq* I site polymorphism and three additional diallelic polymorphisms, which include one that leads to an amino acid substitution (Arg53His). The identification of SNPs of candidate genes is a powerful strategy for the identification of genetic factors associated with quantitative traits and complex diseases.

The SNPs in the downstream region of the promoter have been shown to be multiallelic polymorphisms that involve nucleotide substitution and transition. The present study showed the existence of different types of *kallikrein* gene mutations in the Jeju population, which is known to be different from other ethnic groups. In comparisons of the normal and hypertensive patient populations, a tendency towards differential band patterns (mobility shifting) was noted for most of the hypertensive patients.The *Mva*I-digested PCR products of the *kallikrein* gene showed differences in the band patterns (mobility shifts) between essential hypertension patients (Fig. 8, Fig. 9) and normal subjects (Fig. 10, 11, 12). The distributions of the kallikrein genotype frequencies were as follows: 20.7% (type 1); 79.3% (type 2); 81.1% (type 3); and 18.9% (type 4) (Table 4). The difference between the normotensive and hypertensive subjects was statistically significant ($P<0.0001$).

Present study identified a polymorphism of the promoter region of the gene for the bradykinin receptor (Fig. 13).This polymorphism may be implicated in the

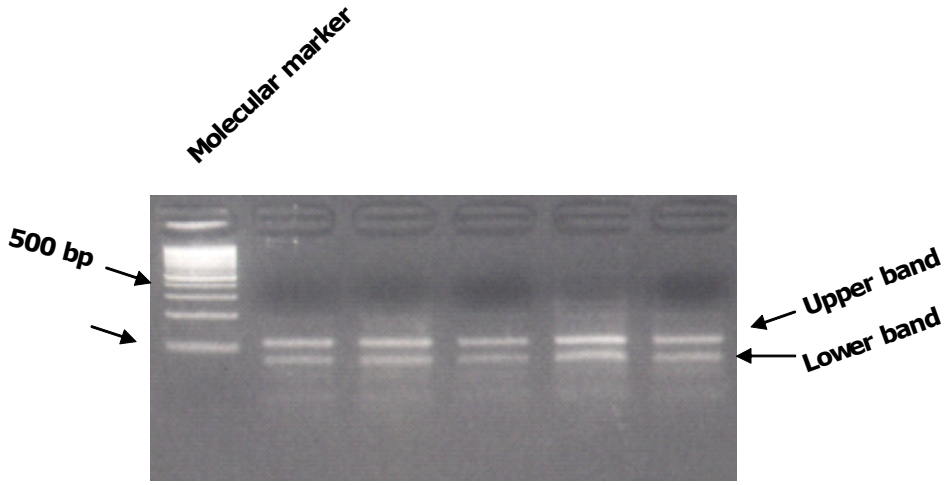


Figure 8. Genotypes of kallikrein gene in promoter region digested with *Mva I* in essential hypertensives

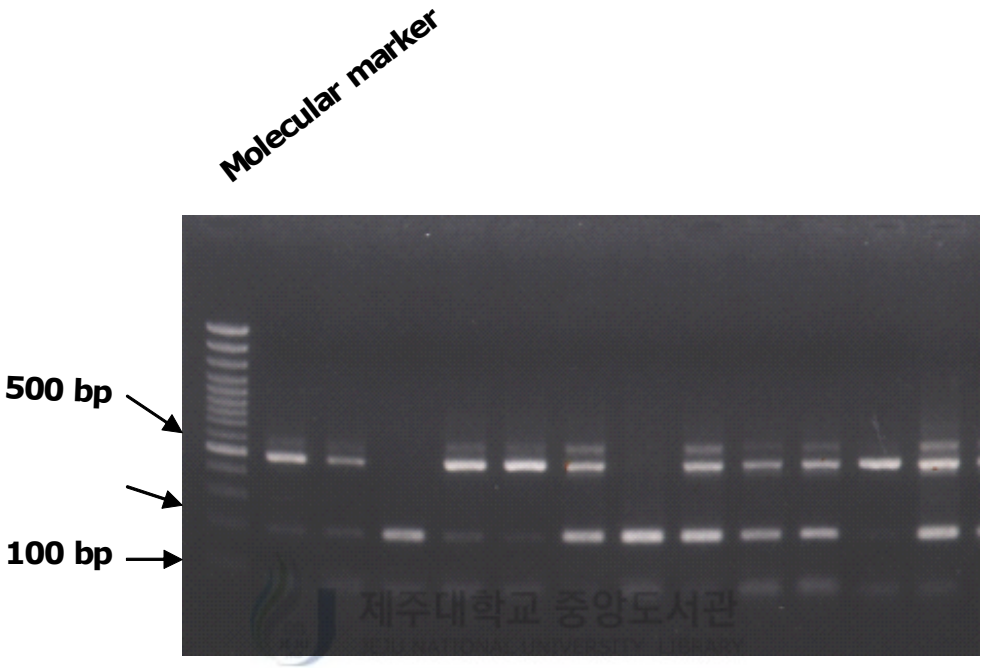


Figure 9. PCR product digested with *Mva I* of kallikrein gene in hypertensives.

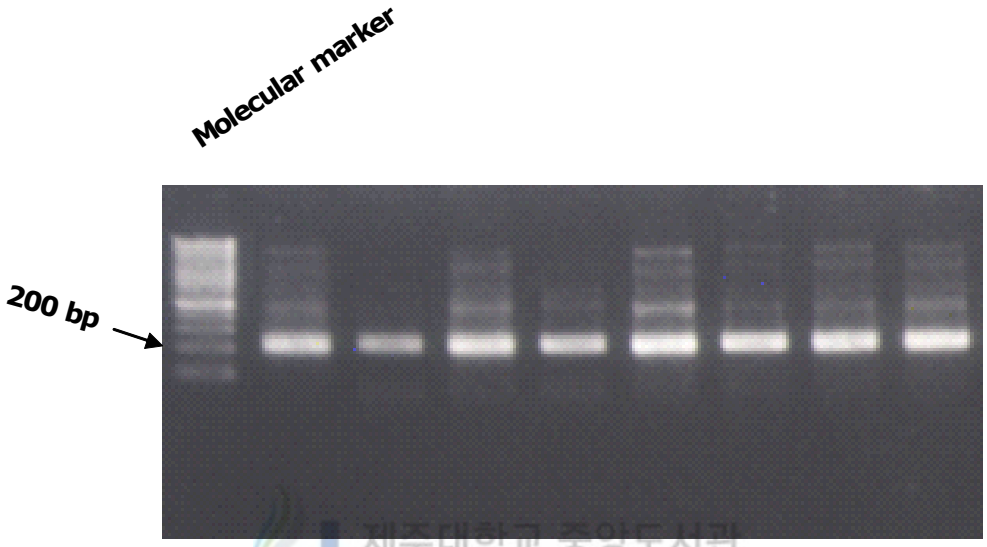


Figure 10. PCR product of promoter region of kallikrein gene

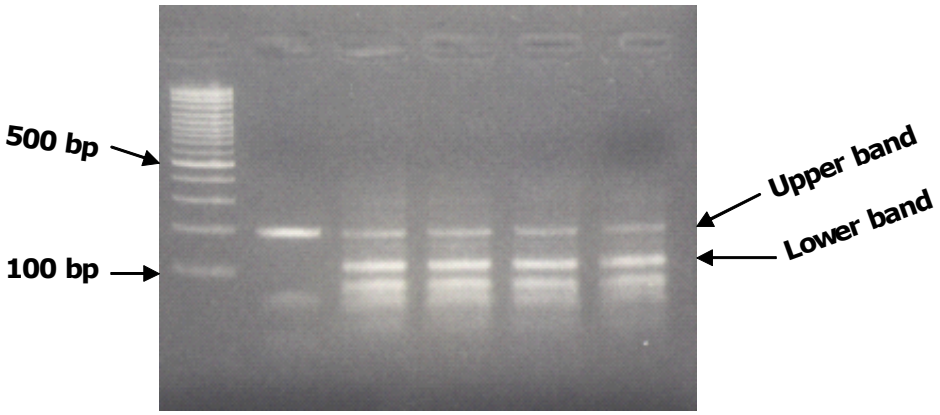


Figure 11. Genotypes of kallikrein gene in promoter region digested with *Mva I* in normotensives.

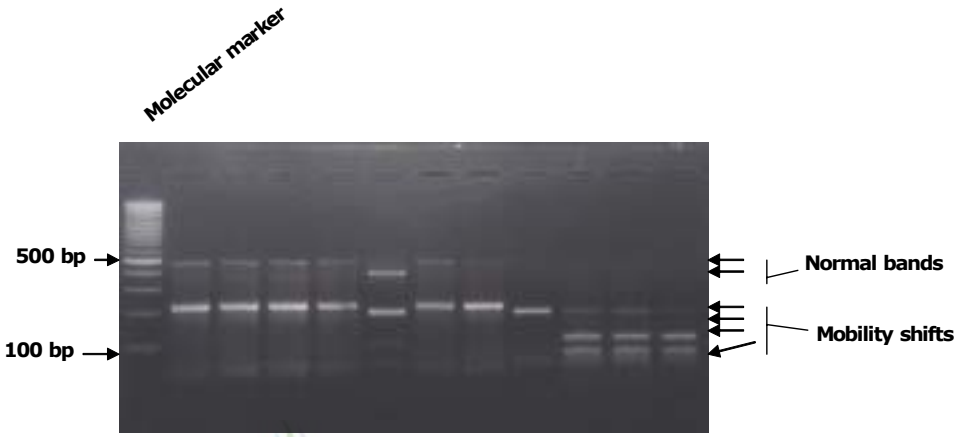


Figure 12. PCR product digested with *Mva I* of kallikrein gene in normotensives. Lower two bands and middle two bands are mobility shift. Upper two bands are normal bands.

Table 4. Klk1 gene polymorphism in Jeju population

Genes	Types	Hypertensive	normotensive
klk1	total	87	106
	type1	18	-
	type 2	69	-
	type 3	-	86
	type 4	-	20
	X^2	2.400	165.070
	p	0.12	0.000

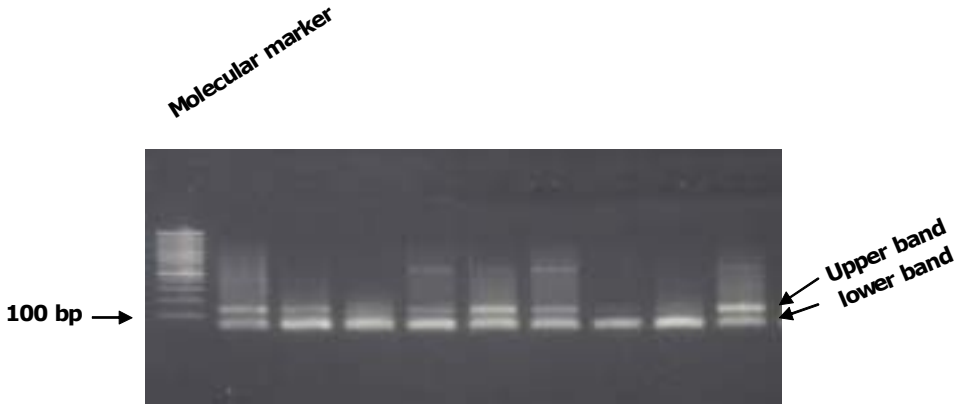


Figure 13. Polymorphism of B2R gene digested with *Rsa I* in hypertensives.

increasing hypertension rate in the population over time. Numerous reports have shown that increases in the number of hypertension patients are linked to increases in the number of mutations in genes linked to hypertension. This appears to be due to two factors, and may be caused by the activities of different types of genetic mutation over time.

Polymorphisms upstream of the promoter, particularly those in the -344C/T region, have been confirmed as genetic markers for diagnosing the development of essential hypertension.

Polymorphisms of the promoter region of the bradykinin receptor gene were reflected in the recombination of PCR products from most of the hypertensive and normotensive subjects (Fig. 13, 14). Thissuggests that mutations caused by crossing-over of genes in the patient and non-patient groups, which were not revealed in other population groups, are found in the promoter region as well as the translated region of this gene.

The present study focused on the correlation between blood pressure and mutations in the promoter region of the *AGT1R* gene that affect enzyme-binding. Mutation of the promoter region of AGT1R (Fig. 15) may influence the transcription of this gene. *Fok*I digestion of the downstream regions of the AGT1R promoter showed differences between the hypertensive (Fig. 16) and

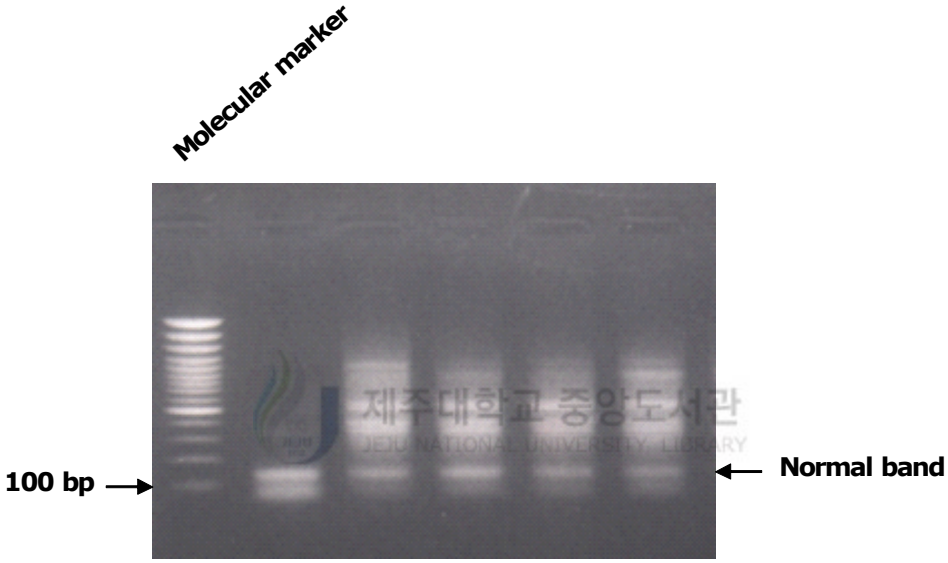


Figure 14. Band patterns of PCR product of promoter region of B2R gene in normotensives.

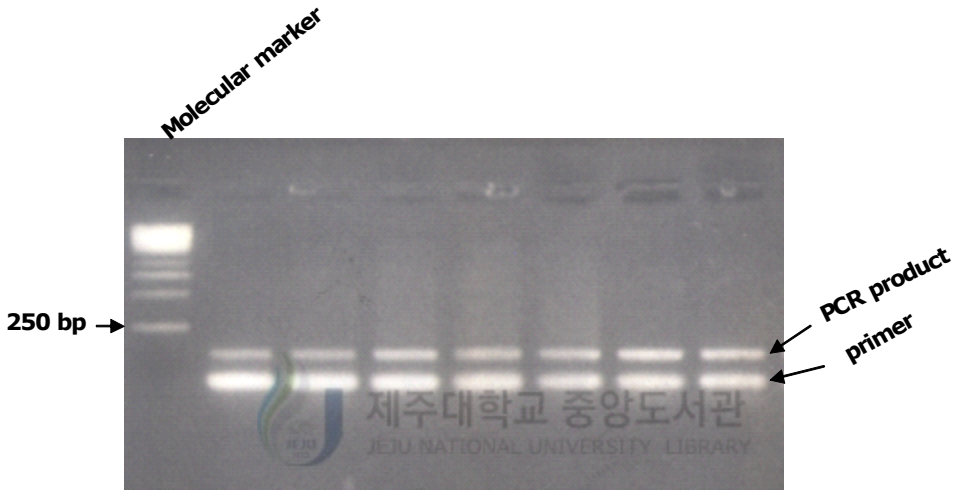


Figure 15. PCR product of AGT1R gene in essential hypertensives.

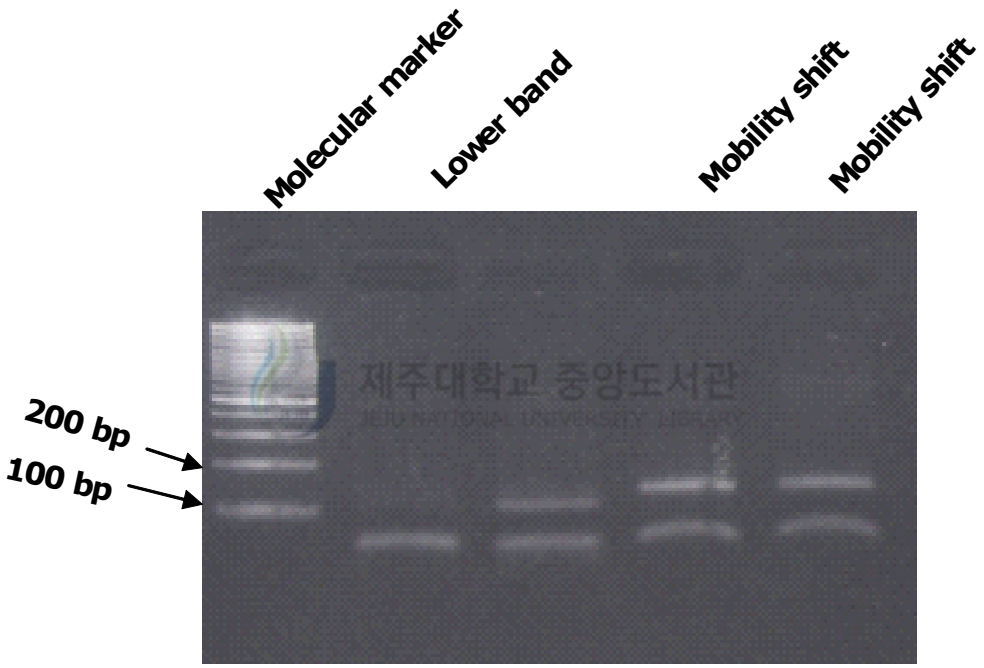


Figure 16. Genotypes digested with *Fok* 1 of A153G of AGT1R gene in essential hypertensives.

normotensive groups. Two band patterns appeared above and below the 100-bp marker in most of the hypertensive patients (Fig. 16), and a single band pattern below the 100-bp marker was observed for the normotensive population (Fig.16). Present study concludes that it is possible to distinguish between hypertensive and normotensive subjects based on polymorphisms of the promoter region of the *AGT1R* gene. The frequencies were 98% for type 1 and 2% for type 2 among all the subjects (P=0.325, χ^2=0.970) (Table 5).

Present study also shows that the detection of SNPs of the angiotensinogen gene reveal the presence of various types of mutation.

The band patterns of PCR product of angiotensinogen gene is showed in Figure 17. SSCP analysis (Fig. 18) revealed mutations (mobility shifts) in the exon of the angiotensinogen gene in hypertensive patients. Figures 17 shows the band patterns in polyacrylamide gel and the DNA sequence data showing a G/C substitution (Fig. 17, 18, 19, 20, 21, 22). These data indicate a novel mutation that may be useful as a specific marker for essential hypertension in different ethnic groups

Table 5. AGT1R gene polymorphism in Jeju population

Genes	Types	Hypertensive	normotensive
AGT1R	total	97	200
	type 1	97	194
	type 2	-	6
	X^2		0.970
	p		0.325

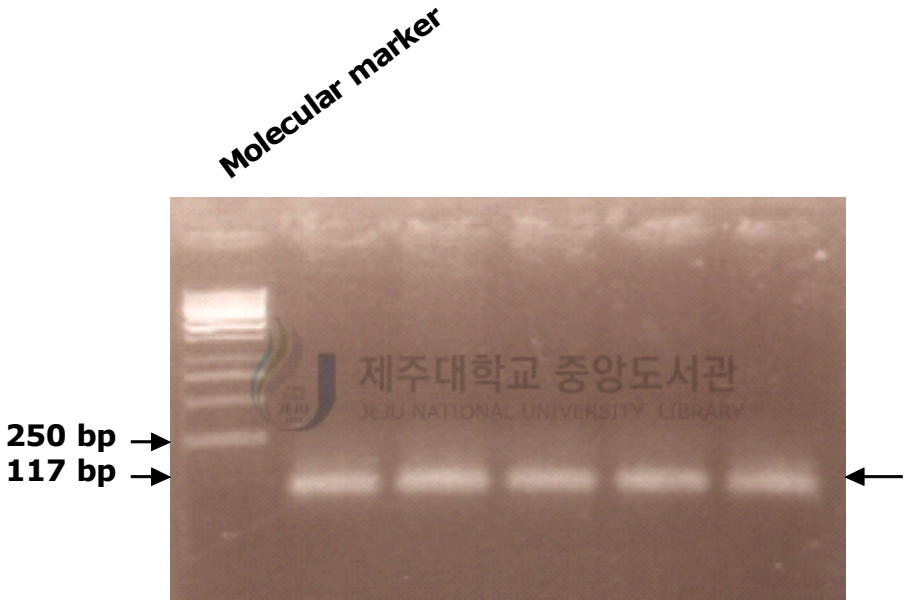


Figure 17. Band patterns of PCR product of angiotensinogen gene.

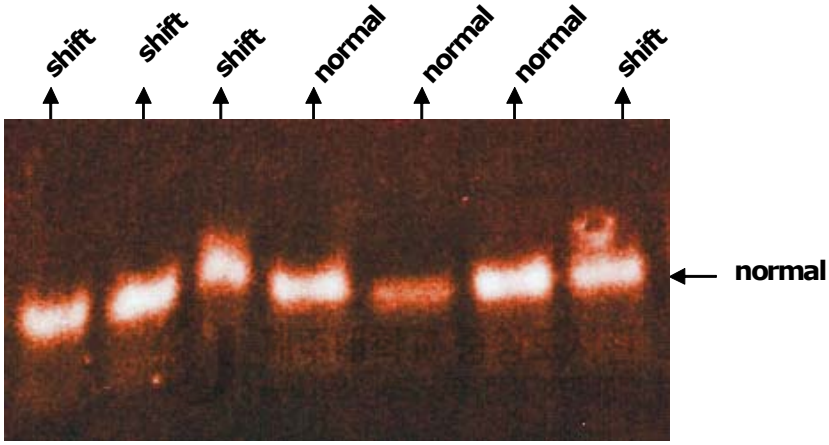


Figure 18. SSCP band patterns show mobility shifts in angiotensinogen gene in essential hypertensives. SSCP was carried out for analysis of mobility shift of single-stranded DNA caused by point mutation. PCR product was denatured with iling for 4 minutes placed on ice. After PCR was loaded on polyacrylamide gel, electrophoresis was carried out for 20 hours at 100 V.

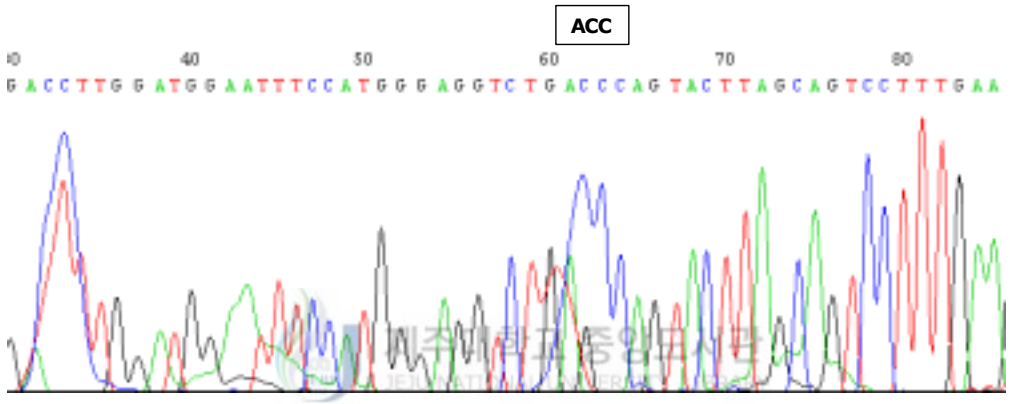


Figure 19. Nucleotide sequence of intron 3 of angiotensinogen gene in normotensives.

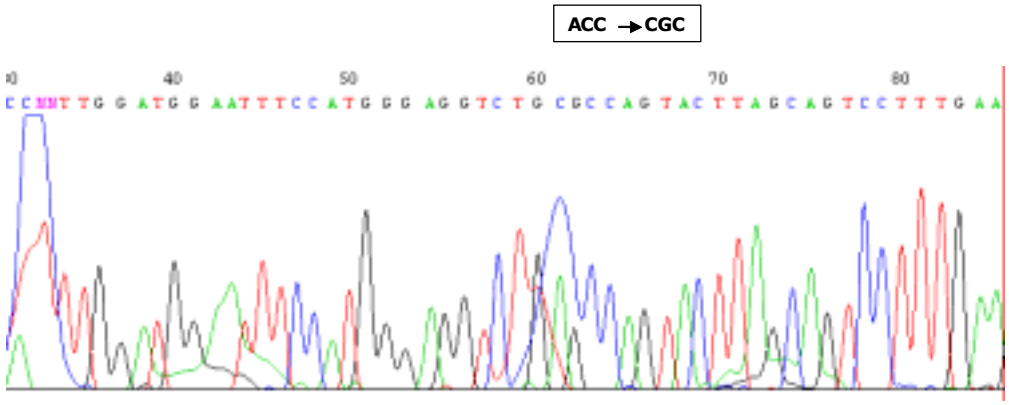


Figure 20. The A→C, C→G substitution of intron 3 of angiotensinogen gene in essential hypertensives. In sequence numbers 61 to 63, ACC (normal) were changed to CGC.

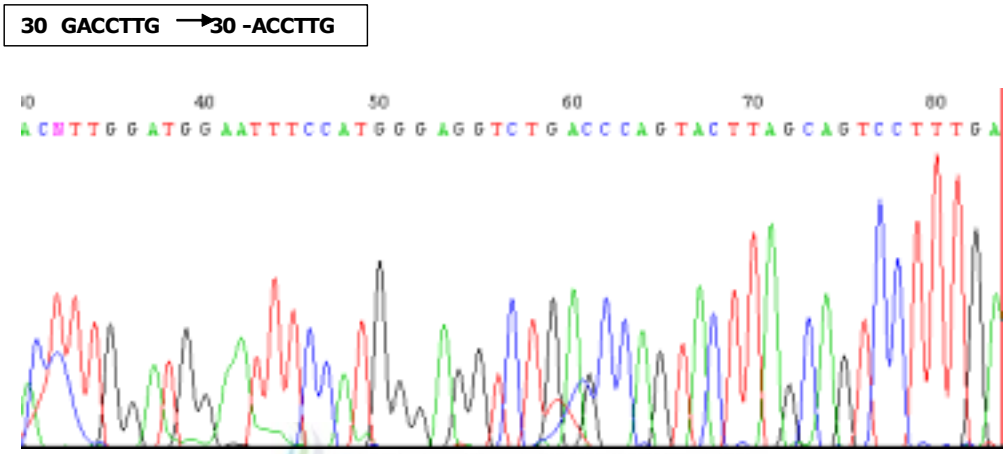


Figure 21. Nucleotide sequence of intron 3 of angiotensinogen gene in hypertensives, showing deletion in base number 10, which is G in normal (see Figure 19), but this shows A instead G; the following sequences are all the same.

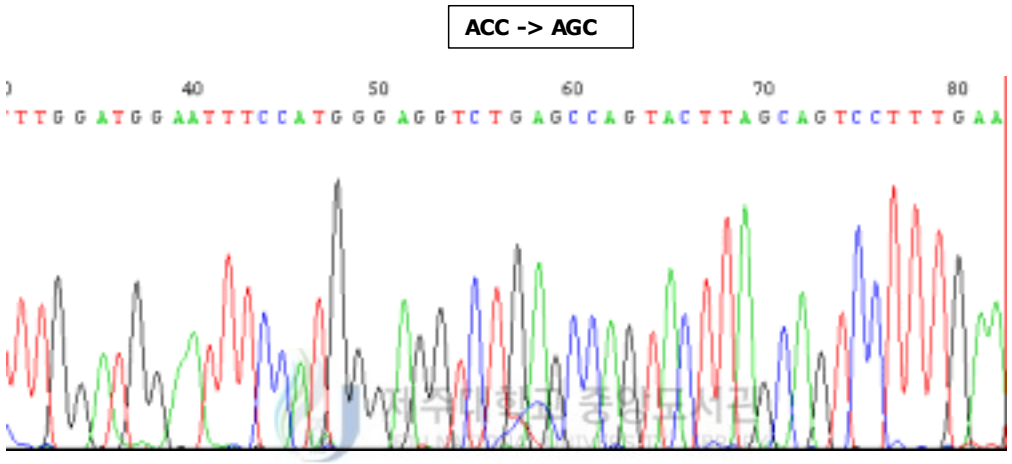


Figure 22. The C→G substitution of intron 3 of angiotensinogen gene in essential pertensives. Base number 58 - 60 ACC (normal) changes to AGC

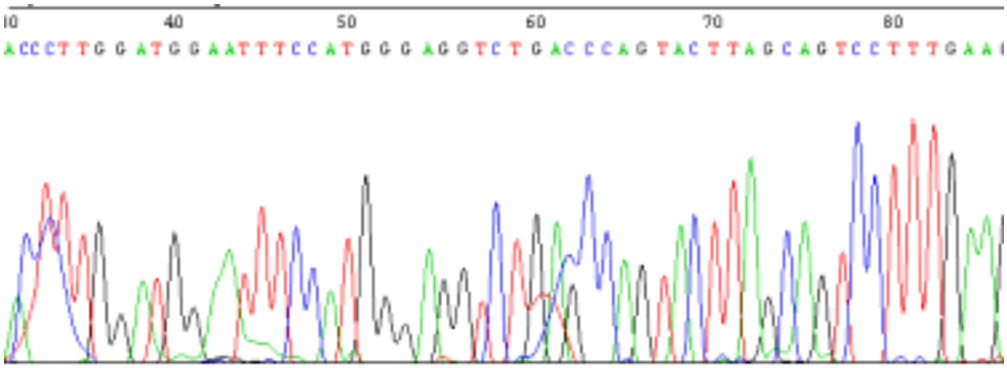


Figure 23. Nucleotide sequence with deletion/insertion variation of angiotensinogen gene in essential hypertensives. Base number 30 G was deleted and C was inserted in 31, as GACCTTG ACCCTTG.

Discussion

The α-adducin G460T polymorphism

Our results show that there are significant differences between hypertensive patients with the T adducin variant and patients with the Gly/Gly genotype. Gly/Trp and Trp/Trp hypertensive patients have been characterized. In a case-control study, no association was found between the 460Trp *a-adducin* allele and hypertension in a large population from Sassari (Glorioso *et al*., 1999). Initial studies in hypertensive animals and humans have indicated that the 460Trp mutation of the *a-adducin* gene is associated with blood pressure level. However, other investigators have shown that the clinical characteristics and baseline blood pressure levels of the subjects are some of the factors that determine the existence or lack of a positive association. A previous report found no association between the *a-adducin* locus and essential hypertension in 507 Japanese subjects (Kamitani *et al.,* 1994), and no association in Scottish families between blood pressure levels and the presence of the 460Trp allele of the *a-adducin* gene using the X^2-test and ANOVA.

The frequency of the 460Trp allele in the control group was 53%, while the corresponding frequencies were 60% and 30.6% in the Japanese and Scottish populations, respectively (Kato *et al.,*1998). Some studies have reported an

association between this allele and elevated blood pressure. Impaired sodium handling was lower in persons with the T/T genotype. Consequently, T/T individuals with this alteration in renal sodium handling have increased sensitivity of blood pressure to sodium intake and are at increased risk for developing low-renin hypertension (Grant *et al*., 2002).

In another Chinese study, no linkage was detected between the two markers and essential hypertension in the TDT/S-TDT study. Although the TDT can be a much more potent system than allele-sharing methods for genes with modest effects (Morton *et al*., 1998), the power of the TDT is substantially weaker than that of case-control studies. The Korean population study showed no association between the Gly460Trp polymorphism and essential hypertension. The frequency of the 460Trp allele was 59.4% in normotensives and 61.1% in hypertensives (Shin *et al*., 2004). The present study detected the following allele frequencies: 58% for 460Trp; 41% for Gly460; and 1% for Gly460Trp. The frequency of the 460Trp allele was 89% in hypertensive patients and 40% in normotensive subjects. The 460Trp allele frequency was assessed using Kruskal-wallis (P=0.630, X^2=0.925). This studied population was not in Hardy-Weinberg equilibrium.

Polymorphisms in the aldosterone synthase gene

A highly polymorphic microsatellite marker was identified at 8 kb in the 3'-region of the *CYP11B1* gene, located 40 kb from the *CYP11B2* gene (Fig.6, 25). This marker was used to map the CYP11B gene between markers on the long arm of chromosome 8. The C344T polymorphism reported has been associated with blood pressure levels using SBP and DBP (Tamaki *et al*., 1999). The Oshasama population study (Matsubara *et al*., 2001) data, from a larger number of subjects in a general rural Japanese population, were analyzed with statistical adjustment for various confounding factors. In the present study, we examined polymorphisms that involved blood pressure changes caused by mutations in the promoter region of *CYP11B2* and the exon of *CYP11B1*. Another report has shown mutation by substitution of the exon/intron region of the *CYP11B1* gene and in the *CYP11B2* gene (Nicod *et al*., 2004). The results of the present study are similar to the results of Nicod *et a*l. (2004), in that exons 1 and 2 of *CYP11B1*, as well as exon 1 of *CYP11B2*, appear to have polymorphisms with multiple bands in the essential hypertension and normal populations. It is assumed that some factors lead to DNA fragmentation in this region. Figure 24 shows the PCR band patterns of the *CYP11B* gene. The results suggest that genetic mutations of aldosterone can be used as determinant elements

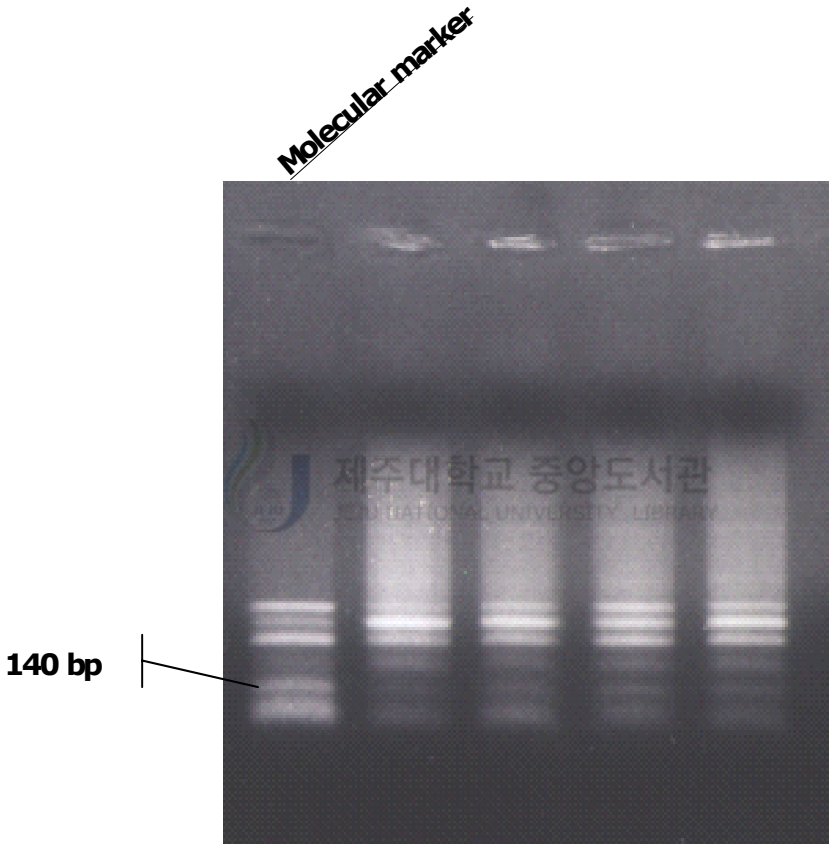


Figure 24. Band patterns of PCR product of exon 1 of CYP11B1.

of high blood pressure in essential hypertension patients. It is possible to hypothesize three reasons for this.

First, the –344C/T substitution affects gene transcription, which is manifested as increased binding of the SF-1 transcription factor (Figure 6). Second, the *CYP11B2* gene has a functional activity that is related to gene conversion by replacement of *CYP11B1* (Fig. 6). Third, a point mutation in exon 3 causes an Arg→Lys substitution at the 173 position. It has been suggested that hypertension can be associated with reciprocal recombination or a single gene. In particular, differences in -344C/T (promoter region) (Fig. 6) have been observed among different populations. Studies of *CYP11B1* gene polymorphisms in Japanese subjects have provided conflicting results, owing to differences in regional and genetic factors. Thus, it seems likely that different types of mutation exist among the different ethnic groups.

The promoter region of the *CYP11B2* gene may be affected by the adjacent exon, as the mutation probably led to conversion of the transcriptional start site in the promoter-exon region (Fig. 6, and 25). According to Adler *et al*. (2005), aldosteronism is due to unequal crossing over between the *CYP11B1* and *CYP11B2* genes, and 20% of essential hypertensive cases are due to aldosteronism. Although these patients have an increased frequency of early death from stroke and an increasedrisk for exacerbation of hypertension, they are clinically

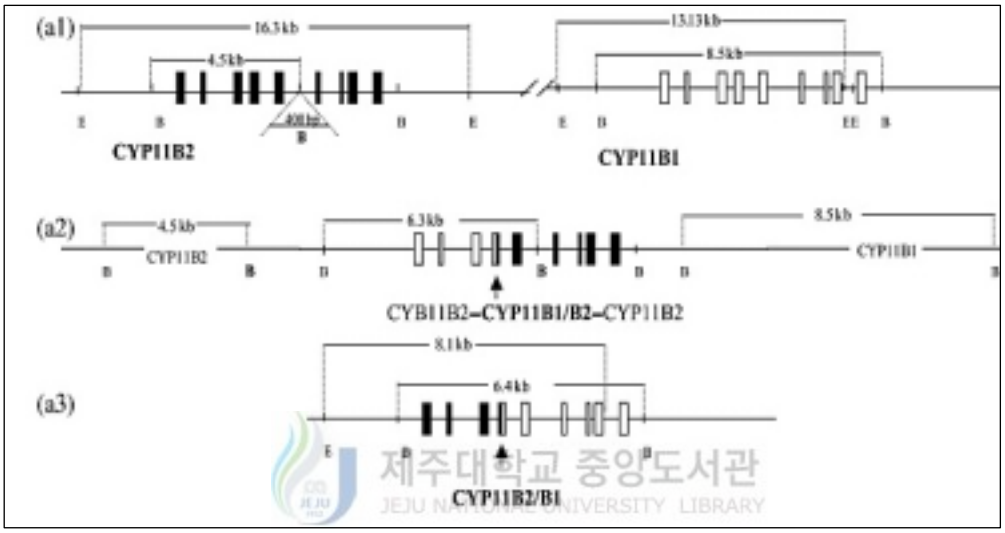


Figure 25. Diagrams of the *CYP11B2-CYP11B1* genes at locus 8q21 (a1) and the duplicated *CYP11B* hybrid involved in glucocorticoid-remediable aldosteronism (a2), and the counterpart deletion hybrid detected in the patient (a3) (Ezquieta and Luzuriaga, 2004).

indistinguishable from patients with essential hypertension (Adler *et al*. 2005). Aldosteronism caused by unequal crossing over can be discriminated from essential hypertension and diseases linked to hypertension using the mutations of different patterns described here. Figure 25 shows unequal crossing over between the *CYP11B1* and *CYP11B2*genes. This is presumed to be the functional product of the interaction between the *CYP11B1* and *CYP11B2* genes. However, the present study approached to conclusion that may be caused to other functional form of aldosterone synthase undermining results mentioned above.

Polymorphisms of the ACE gene

The relationship between the insertion/ deletion polymorphism in intron 16 of the angiotensin-converting enzyme (ACE) gene and the serum levels of ACE has been described as a significant factor in the multifactorial genetics of cardiovascular diseases.

In most published reports, the DD genotype (associated with higher serum ACE levels) is more common in groups with vascular disease (Fogarty *et al*., 1994). The frequency of the ACE deletion D allele has been found to be higher in hypertensive patients than in control subjects (X^2=20.66, P=0.0001). Comparisons of the ACE allele frequencies among the controls in this study with

those in the populations reported by Abbud *et al*. (1998), Bedir *et al*. (1999) and O'Donnell *et al*. (1998), showed that they were similar.

The I/D allelic variant is one of the most intensively investigated genetic polymorphisms in the field of cardiovascular disease research. The D allele of ACE was initially suggested to be associated with increased levels of serum ACE activity, as compared to the I variant. Subsequently, several studies tried to envisage an association of ACE I/D with various human diseases. However, many of these studies failed to show an association of the ACE I/D genotype with essential hypertension or end organ damage or were underpowered when it came to allowing valid statistical conclusions (Plassmann *et al*., 2002).

The frequency of the I/D polymorphism of the ACE gene in Black populations is similar to previously reported estimates for Europeans and is higher than that reported for Asians, but the results are not entirely consistent (Rotimi *et al*., 1996).

In the present study, it was not showed a significant association between the I/D polymorphism and blood pressure levels (P=0.302, X^2=2.398) and between allele frequency and genotype distribution. Increases in the heteroduplex allele of

I/D over the D/D allele in the Jeju population distinguish this population from other ethnic populations, such as Afro-American, Caucasians, and Japanese. Furthermore, present study found discrepancies with reports of approximately 50% of the D/D genotype inhypertensive subjects, which were influenced by blood pressure elevation due to sodium imbalance. This is meaning to required for discrimination about difference of genotype, which be able to appeared to every ethnic group. Table 3 shows the allele frequency and genotype distribution of the ACE (I/D) polymorphism. Table 6 comparesthe allele frequencies among the different groups. In the present study, we showed DNA patterns that differed among ethnic groups. Table 3 shows the allele frequency and genotype distribution of the ACE (I/D) polymorphism. Table 6 comparesthe allele frequencies of the different groups. This suggests that the frequency of the I/D genotype is higher in the Jeju population. Indeed, in the Japanese study, the high frequency of the D/D genotype coincided with those observed in Africans and Caucasians. Present study show that the frequency of the I/D genotype is higher in this case than in any other group, and that the blood pressure levels are elevated.

However, no significant difference was detected between the hypertensives and normotensives using the Kruskal-Wallis method. This indicates that I/D is involved in regulating the blood pressure levels in the Jeju population in a

Table 6. Comparison of the ACE (I/D) allele frequencies among the different populations

Population Reference	Genotype			Allele frequency	
	D/D	I/D	I/I	D	I
African American Rotimi *et al*., 1996 (n = 260)	35	51	14	0.61	0.39
Chinese Rotimi *et al*., 1996 (n = 189)	9	41	50	0.30	0.70
Japanese Rotimi *et al.,* 1996 (n = 199)	19	43	38	0.40	0.60
Chinese (Singapore) Lee *et al*., 1994 (n = 189)	10	40	50	0.30	0.70
Caucasian Oceania Zee *et al*., 1992 (Hypertensives, n = 173)	31	42	27	0.52	0.48
Caucasian Barley *et al*., 1994a (England, n = 186)	27	48	25	0.51	0.49
Caucasian Rigat *et al*., 1990 (France, Hypertensives, n = 80)	36	46	18	0.59	0.41
Jeju (Korea)	28.5	63.9	7.6	0.60	0.40

present study
(n = 249)

manner that is different to that seen in other populations but not in specific case-control studies.

Polymorphism in the kallikrein gene promoter region

Ten alleles were detected in a GC-rich region between nucleotides -133 and -121 in Caucasians, African-Americans, and Asians. Several observations suggest that the renal kallikrein-kinin system plays an important role in blood pressure regulation (Elliot and Nuzum, 1934: Carretero and Scicli, 1971: Margolius *et al*., 1971, 1974; Carretero *et al*., 1976, 1978; Ader *et al*., 1986, 1987: Williams *et al*., 1987). It has been reported that 51% of the variance of 12-h overnight urinary kallikrein excretion can be attributed to a dominant allele, and 27% can be attributed to the combined effects of polygenes and shared family environment (Berry *et al*., 1989). The polymorphisms include those described by Berge and Berg (1993) at positions -127 to 230 (R53H) and by Song *et al.* (1997) at positions -127 to -128 and -123 in the promoter region. Figures 8, 9, 10, 11, and 12 show the variant alleles of the *kallikrein* gene detected by *Mva*I digestion. The allele frequencies were 9%, 35%, 44%, and 10%, respectively. In addition, a statistically significant difference was noted for the enzyme sites on the different alleles of the *kallikrein* gene. Two patterns were noted for the essential hypertensives and two different patterns were observed for the normotensives

(Fig. 8, 9, 10, and 11), which was dependently applied for this gene between patients and non-patients. Moreover, an additional pattern was excluded from the statistical analysis. Table 4 shows the distribution of allele frequencies according to the Kruskal-Wallis method.

B2R gene polymorphisms

The *B2R* gene has been proposed as one of the candidate genes involved in the complex mechanisms of common chronic disorders, such as hypertension (Lindpainter, 1994). Recently, experimental data have shown a protective role for B2R in the development of hypertension (Emanuelli and Madeddu, 1997).

A promoter-specific 112-bp fragment was amplified using the region homologous to nucleotides -92 to -111 and the region complementary to nucleotides +11 to -8. Thetwo polymorphic alleles were revealed by SSCP electrophoresis in polyacrylamide gels (Braun *et al*., 1996b). Kammerer *et al*. (1995) have recently reported the organization and regulatory region of the human *B2R* gene, and have subsequently identified four polymorphisms, including a promoter polymorphism 58 bp upstream of the transcript site. Bachvarov *et al*., (1998) have recently described an association between end-stage renal disease and altered frequency of the *B2R*exon 2 polymorphism. In the present study, we detected a mutation in the promoter region that could be differentiated from the

mutation identified in the *B2R* gene, and that is supposedly a recombinant hybrid of a homologous chromosome in most of the hypertensive patients and normotensive subjects. Figure 13 and 14 show the band patterns of the *B2R* gene in the hypertensive and normotensive groups.

The human *B2R* cDNA was cloned by Hess *et al.*(1992), and a study of the genomic structure showed that it is characterized by polymorphisms located in each of the exons 3 and 1 in the promoter region. Therefore, the *B2R* gene is implicated as one of the candidate genes involved in the complex genetic processes underlying essential hypertension.

The distributions of C/T genotypes were 28% for CC, 59% for TC, and 13% for TT in the hypertensive subjects, and 18% for CC, 57% for TC, 25% for TT in the normotensive subjects (Mukae *et al*., 1999).

Based on the finding that polymorphism of the promoter region might influence the transcription rate of the *B2R* gene, Braun *et al.* initiated *in vitro*transfection experiments with human embryonic kidney cells and performed luciferase reporter gene assays, to examine the effects of the different promoter alleles on the transcription rate (Braun *et al*, 1996b). SSCP of the PCR products showed a recombinant hybrid, which appeared as bands of different types. Figure 13 shows the band pattern of the PCR products. Mutation in the promoter/exon

region reduced the transcription rate of this gene.

The frequency of the C allele (variation) of the *B2R* promoter was significantly increased in the hypertensive patients in comparison with the controls. Furthermore, the functional effect was concordant with the effects of other factors (age, gender, etc.). Thus, the effect of functional variation of the *B2R*gene influences essential hypertension in combination with other factors, and predisposes for the development of essential hypertension (Gainer *et al*., 2000).

Detection of mutations in the promoter region of the AGT1R gene

This study detected two banding pattern when the PCR products of the *AGT1R* gene were digested with *Fok*1 (Fig. 16). Two band patterns were observed for the hypertensives and normotensives. Fewer subjects showed one band and mobility shifts for two bands (Fig. 15, 16). The results suggest no polymorphic site in the promoter region of *AGT1R*. Thus, this gene has a minor effect on blood pressure. Table 5shows no significant difference between hypertensives and normotensives. The recently identified polymorphism -153A/G was not in linkage disequilibrium with 1166A/C, which is located in the untranslated region (Lajemi *et al*., 2000). Thus, in the future more studies are needed on the association between blood pressure and this gene.

Mutations in the angiotensinogen gene

A common feature of negative studies is their small size and the uncertain or unusual ascertaining of either cases or controls (Bennett *et al*., 1933; Barley *et al*., 1994b; Caulfield *et al*., 1994; Fornage *et al*., 1998). In the Japanese study, the reduced heterogeneity of the pathophysiology of hypertension could be attributed to the more homogeneous genetic and environmental background of the population (Hata *et al*., 1994; Iwai *et al*., 1995). However, SSCP is a potent method for detecting mutations in specific genes associated to disease.

This study detected genetic mutations in the *Angiotensinogen* gene as mobility shifts on polyacrylamide gels. The shift types were selected ran.domly, and direct sequencing was performed to determine mutation type. The mutations appeared mainly as homozygous single band shifts in the essential hypertension patients and as the normal single band in the normotensive population. Direct sequencing revealed the mutation type of C→G substitution of the promoter region and an additional type in the promoter region in one population. Figure 17 shows the banding pattern of the *Angiotensinogen* gene, as obtained using SSCP. Thus, the promoter region of the *angiotensinogen* gene displays a number of variations among different ethnic groups. The variation of the *angiotensinoge*n promoter region may be caused by the action of genetic and environmental factors.

The results of the present study suggest that SNPs can be used for the detection

of mutations in the *angiotensinogen* gene and can reveal various types of mutation.

The mutation types in the exon of the *angiotensinogen* gene were determined by SSCP in hypertensive patients. Figures 17, 18, 19, 20, 21, 22, and 23 show the bandpatterns from polyacrylamide gel electrophoresis and the sequencing data, which indicate a G/C substitution leading to a point mutation. These findings suggest a novel mutation. In addition, this polymorphism may be useful as a specific marker for essential hypertension among ethnic groups.

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국 문 초 록

고혈압은 심혈관 질환과 신장 이상및 뇌졸증을 유발하는 원인질환 이다. 고혈압의 90% 이상이 병원론이 명백하게 규명되지않았고 때문에 본태성 고혈압이라명명된다. 본태성 고혈압은 환경적 그리고 유전적 요인으로 인하여 유발될 수있는 질환이다.

본 연구는 본태성 고혈압의 유전적, 환경적 요인의 복합적 양상과 이 질환에 관련된 유전자의 다형에 대한 연구를 수행하였다. 후보유전자로는 알도스테론 합성효소, 안지오탠신 변환 효소, 칼리크레인, 브라디키닌,어덕신, 안지오텐신 리셉터, 안지오텐시노젠으로 고혈압에 중요한 역할을 한다. 이들 유전자는 혈압의 증가와 관련된 신장 이상에 의한 고혈압의 유발 원인의 후보 유전자로 혈압 조절에 기능적인 역할을 한다. 즉, 고혈압에 연관된 질환과 고혈압 유발의 위험요소로 작용을 한다. 본태성 고혈압에 연관된 후보 유전자에 대한 연구는 1947년부터 보고되어온 신장 이상에 의해 유발되는 고혈압에 초점을 맞추었다. 따라서, 본 연구는 고혈과 연관된 후보유전자의 돌연변이에 대해 그리고 고혈압 연관 질환의 유전적 마커를 찾기 위하여 수행되었다.

본 연구에서 알도스테론 합성효소는 엑손과프로모터 영역에서 다양한 타입의 밴드패턴과 유전자의 단편화 패턴을 보여주었다. 어덕신 유전자에서도 유전자의 단편화와 증가된 변이 패턴을 보여주었다. 이는 다른 집단과는 구별되는 특이 밴드 패턴으로 추정된다. 안지오텐신 변환 효소는 삽입/결실 다

형에서 유의한 차이를 보이지 않았지만, 높은 빈도로 변이가 나타났다. 이는 타집단과 비교하여볼 때, 제주집단에서 특이적인 양상을 나타내었다.. 안지오텐신 리셉터는 서로 다른 밴드 패턴을 나타냈지만 통계적 분석은 불가하였다. 브라디키닌 리셉터에서는 대부분의 집단에서 교차에 의한 재조합 패턴을 나타냈다. 안지오텐시노젠에서는 점 돌연변이에 의하여 전기영동에서 변경된 밴드 패턴을 보였다. 이는 제주집단에서 발견된 변이 타입임을 나타내고 있다.

많은 돌연변이가 서로 다른 그룹에서 본태성 고혈압과 연관된 유전자에서 발견되고 있고,

이 결과는 고혈압에 관련된 유전적 다형현상의 연구에 많은 자극을 주고있다. 또한, 고혈압과 관련된 유전적 다형현상의 연구에서 그룹마다 서로 다른 차이를 보이며 시간이 지남에 따라 증가하는 질환의 양상을 보이고 있다.

주요어: 본태성 고혈압, 심혈관 질환, 신장 이상, 뇌졸증, 병원론, 후보 유전자, 위험요소, 혈압 조절, 알도스테론 합성효소, 어덕신, 브라디키닌 리셉터, 안지오텐신 리셉터, 안지오텐시노젠, 안지오텐신 변환 효소, 삽입/결실, 칼리크레인, 밴드 패턴, 재조합, 제주집단, 돌연변이, 유전자 마커, 유전적 다형현상, 유전자의 단편화.

감사의 글

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생물학을 배우는 학생인 저로써는 지금까지의 연구결과가 아직은 작지만, 앞으로의 발전에 많은 도움이 되기를 희망하고 있습니다. 이를 위해서는 보다 긍정적이고, 적극적인 자세로 임해야 하며, 보다 과학적 사고를 지녀야 된다고 생각하고 있습니다. 과학자로써의 태도는 저 뿐만 아니라, 저를 가르쳐주시고, 도움을 주신 많은 분들의 기대를 나타낼분만 아니라, 앞으로의 방향에 표지를 가리킨다고 할 수 있습니다. 따라서, 저는 보다 열심히 여러분의 기대에 어긋나지 않는 과학자의 길을 가겠습니다.

끝으로 저를 아껴주시고 많은 성원을 해주신 어머니께 감사드립니다. 또한, 동생 태형과 제수씨와 막내 승주에게 고맙다는 말을 전합니다.

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