Table 1. Characteristics of Study Participants

	Total					Men				
	NT1	NT2	EH	*p* value *vs.* NT1	*p* value *vs.* NT2	NT1	NT2	EH	*p* value *vs.* NT1	*p* value *vs.* NT2
Number of subjects	279	285	281			180	138	185		
Age (years)	51.9±9.6	77.8±4.2	50.6±5.9	0.055	<0.001	51.5±6.3	78.0±4.6	50.4±6.3	0.104	<0.001
BMI (kg/m^2)	22.7±3.2	22.6±2.9	24.6±3.8	<0.001	<0.001	22.9±3.2	22.8±2.7	24.8±3.6	<0.001	<0.001
SBP (mmHg)	112.6±10.9	135.1±16.5	173.4±19.7	<0.001	<0.001	113±10.3	135.1±16.5	171.2±18.3	<0.001	<0.001
DBP (mmHg)	69.6±8.3	78.1±10.7	105.4±13.4	<0.001	<0.001	70.3±7.8	78.3±9.8	105.5±13.4	<0.001	<0.001
Pulse (beats/min)	74.2±14.6	69.9±10.9	77.7±15.3	0.019	<0.001	73.4±15.8	68.9±11.9	77.6±16.0	0.027	<0.001
Creatinine (mg/dL)	0.8±0.2	0.8±0.2	0.9±0.2	0.303	0.696	0.9±0.2	0.9±0.2	0.9±0.2	0.102	0.902
Total cholesterol (mg/dL)	199.1±46.0	218.4±44.0	210.8±41.7	0.002	0.038	193.9±45.5	205.3±32.4	205.3±43.0	0.018	0.990
HDL cholesterol (mg/dL)	56.7±17.2		56.5±17.4	0.891		54.8±16.0		53.1±16.7	0.377	
Uric acid (mg/dL)	5.6±4.2		5.7±1.7	0.839		5.83±1.4		6.2±1.5	0.019	
Alcohol consumption (%)	38.7	37.0	63.0	0.070	<0.001	48.3	44.2	77.3	0.030	0.006
Smoking (%)	25.8	26.7	51.2	0.003	<0.001	33.3	41.5	62.2	0.018	<0.001

	Women				
	NT1	NT2	EH	*p* value *vs.* NT1	*p* value *vs.* NT2
Number of subjects	99	147	96		
Age (years)	53.8±1.5	77.7±3.8	51.1±0.5	0.091	<0.001
BMI (kg/m^2)	22.5±3.4	22.3±3.0	24.3±3.4	0.001	<0.001
SBP (mmHg)	112.0±12.0	135.8±17.7	177.7±21.6	<0.001	<0.001
DBP (mmHg)	68.2±9.0	77.9±11.5	105.1±13.5	<0.001	<0.001
Pulse (beats/min)	75.7±12.3	70.9±9.9	77.8±14.0	0.350	<0.001
Creatinine (mg/dL)	0.7±0.2	0.8±0.2	0.7±0.2	0.308	0.003
Total cholesterol (mg/dL)	208.6±45.5	230.7±49.6	220.9±37.5	0.045	0.106
HDL cholesterol (mg/dL)	60.2±18.8		62.6±17.2	0.389	
Uric acid (mg/dL)	5.3±6.8		4.7±1.5	0.440	
Alcohol consumption (%)	21.2	28.9	35.4	0.589	0.090
Smoking (%)	12.1	17.9	30.2	0.065	0.318

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; NT, normotension; EH, essential hypertension.

The threshold value of the frequencies of the haplotypes included in the analysis was set to $1/2n$ (n: numbers of subjects in each group), as suggested by Excoffier and Slatkin (*25*). All haplotypes below the threshold value were excluded from the analysis. The overall distribution of haplotypes was analyzed using $2 \times m$ contingency tables with a value of $p<0.05$ considered to indicate statistical significance. The p value for each haplotype was determined by the χ^2 analysis and permutation method using the software SNPAlyze version 3.2.3 (*18*, *26*).

Results

Table 1 shows the clinical features for the EH patients and the NT controls. The SBP and DBP were significantly higher in the EH group as compared to those seen in the NT group. Age and serum concentrations of creatinine and uric acid did not differ significantly between the two groups.

We performed an association study using four SNPs. Table 2 shows the genotype and allele distributions of the four SNPs. The overall distributions of the genotypes and alleles did not differ significantly between the EH and NT1 groups. Almost all of the data were duplicated between the groups with the exception of the genotype distribution of rs1047047 between the EH and NT2 group. However, the allelic distribution of rs1047047 was not significantly different between the EH and NT2 group.

LD analysis showed that the four SNPs were located in one haplotype block, because all D' values were beyond 0.5. Because the r^2 between rs883062 and rs885846 was beyond 0.5, the combination of rs883062 and rs885846 was not adequate when using these simultaneously in the one haplotype-based case-control study (Tables 3, 4). These results were completely the same for the NT1 and NT2 groups.

Table 2. Genotype Distribution in Normotensives (NT) and Patients with Essential Hypertension (EH)

	Total subjects					Male subjects					Female subjects				
	NT1	NT2	EH	*p* value *vs.* NT1	*p* value *vs.* NT2	NT1	NT2	EH	*p* value *vs.* NT1	*p* value *vs.* NT2	NT1	NT2	EH	*p* value *vs.* NT1	*p* value *vs.* NT2
Number of participants	279	285	281			180	138	185			99	147	96		
Variants															
rs883062															
Genotype															
C/C	24 (0.086)	24 (0.084)	22 (0.078)			17 (0.094)	13 (0.094)	15 (0.081)			7 (0.070)	11 (0.075)	7 (0.073)		
C/T	111 (0.398)	105 (0.368)	121 (0.431)			73 (0.406)	45 (0.326)	79 (0.427)			38 (0.384)	60 (0.408)	42 (0.438)		
T/T	144 (0.516)	156 (0.547)	138 (0.491)	0.727	0.318	90 (0.500)	80 (0.580)	91 (0.492)	0.861	0.182	54 (0.545)	76 (0.517)	47 (0.490)	0.727	0.901
Allele															
C	159 (0.285)	153 (0.268)	165 (0.294)			107 (0.297)	71 (0.257)	109 (0.295)			52 (0.263)	82 (0.279)	56 (0.292)		
T	399 (0.715)	417 (0.732)	397 (0.706)	0.750	0.346	253 (0.703)	205 (0.743)	261 (0.705)	0.938	0.295	146 (0.737)	212 (0.721)	136 (0.708)	0.522	0.761
rs1047047															
Genotype															
A/A	93 (0.333)	85 (0.298)	90 (0.320)			60 (0.333)	40 (0.290)	61 (0.330)			33 (0.333)	45 (0.306)	29 (0.302)		
A/G	132 (0.473)	146 (0.512)	115 (0.409)			85 (0.472)	68 (0.493)	73 (0.395)			47 (0.475)	78 (0.531)	42 (0.438)		
G/G	54 (0.194)	54 (0.190)	76 (0.270)	0.085	0.023*	35 (0.194)	30 (0.217)	51 (0.276)	0.147	0.200	19 (0.192)	24 (0.163)	25 (0.260)	0.519	0.154
Allele															
A	318 (0.570)	316 (0.554)	295 (0.525)			205 (0.569)	148 (0.536)	195 (0.527)			113 (0.571)	168 (0.571)	100 (0.521)		
G	240 (0.430)	254 (0.446)	267 (0.475)	0.131	0.320	155 (0.431)	128 (0.464)	175 (0.473)	0.250	0.817	85 (0.429)	126 (0.429)	92 (0.479)	0.323	0.273
rs2297566															
Genotype															
A/A	12 (0.043)	13 (0.046)	10 (0.036)			5 (0.028)	4 (0.029)	7 (0.038)			7 (0.070)	9 (0.061)	3 (0.031)		
A/G	94 (0.337)	99 (0.347)	87 (0.310)			63 (0.350)	48 (0.348)	55 (0.297)			31 (0.313)	51 (0.347)	32 (0.333)		
G/G	173 (0.620)	173 (0.607)	184 (0.655)	0.676	0.478	112 (0.622)	86 (0.623)	123 (0.665)	0.516	0.598	61 (0.616)	87 (0.592)	61 (0.635)	0.456	0.530
Allele															
A	118 (0.211)	125 (0.219)	107 (0.190)			73 (0.203)	56 (0.203)	69 (0.186)			45 (0.227)	69 (0.235)	38 (0.198)		
G	440 (0.789)	445 (0.781)	455 (0.810)	0.379	0.228	287 (0.797)	220 (0.797)	301 (0.814)	0.578	0.601	153 (0.773)	225 (0.765)	154 (0.802)	0.479	0.339
rs885846															
Genotype															
G/G	30 (0.108)	26 (0.091)	28 (0.100)			21 (0.117)	17 (0.123)	19 (0.103)			9 (0.091)	9 (0.061)	9 (0.094)		
G/A	125 (0.448)	126 (0.442)	123 (0.438)			82 (0.456)	51 (0.370)	84 (0.454)			43 (0.434)	75 (0.51)	39 (0.406)		
A/A	124 (0.444)	133 (0.467)	130 (0.463)	0.896	0.943	77 (0.428)	70 (0.507)	82 (0.443)	0.899	0.311	47 (0.475)	63 (0.429)	48 (0.500)	0.923	0.245
Allele															
G	185 (0.332)	178 (0.312)	179 (0.319)			124 (0.344)	85 (0.308)	122 (0.330)			61 (0.308)	93 (0.316)	57 (0.297)		
A	373 (0.668)	392 (0.688)	383 (0.681)	0.641	0.822	236 (0.656)	191 (0.692)	248 (0.670)	0.674	0.558	137 (0.692)	201 (0.684)	135 (0.703)	0.810	0.650

*Significant difference in distribution.

Table 3. Pairwise Linkage Disequilibrium in GUCA2B Gene, Evaluated by D' and r^2

NT1

		D'			
	SNP	rs883062	rs1047047	rs2297566	rs885846
	rs883062		0.917	0.937	0.950
r^2	rs1047047	0.253		1.000	0.967
	rs2297566	0.094	0.202		1.000
	rs885846	0.725	0.350	0.133	

NT2

		D'			
	SNP	rs883062	rs1047047	rs2297566	rs885846
	rs883062		0.850	1.000	0.909
r^2	rs1047047	0.213		0.973	0.983
	rs2297566	0.103	0.214		1.000
	rs885846	0.668	0.353	0.128	

|D'|>0.5
r^2 >0.5

Table 4. Distribution of Individual Haplotypes

Combination of SNPs	Overall distribution		Distribution of individual haplotypes					
	p value *vs.* NT1	*p* value *vs.* NT2	Haplotype	NT1	NT2	EH	*p* value *vs.* NT1	*p* value *vs.* NT2
rs883062-rs1047047	<0.001*	<0.001*	T-A	0.426	0.439	0.447	0.482	0.780
			C-G	0.279	0.255	0.266	0.603	0.698
			T-G	0.295	0.306	0.259	0.186	0.084
			C-A	0.000	0.000	0.028	0.00006*	0.00007*
rs883062-rs1047047-rs2297566	<0.001*	<0.001*	T-A-G	0.427	0.432	0.438	0.685	0.837
			C-G-G	0.275	0.258	0.260	0.601	0.942
			T-G-A	0.211	0.222	0.185	0.291	0.126
			T-G-G	0.088	0.088	0.082	0.677	0.715
			C-A-G	0.000	0.000	0.035	0.00001*	0.00001*
rs883062-rs2297566	0.629	0.361	T-G	0.507	0.512	0.520	0.677	0.806
			C-G	0.282	0.268	0.293	0.699	0.359
			T-A	0.210	0.219	0.188	0.337	0.184
rs1047047-rs2297566-rs885846	0.656	0.703	A-G-A	0.428	0.442	0.464	0.234	0.462
			G-G-G	0.329	0.313	0.318	0.697	0.843
			G-A-A	0.212	0.219	0.191	0.377	0.244
			G-G-A	0.031	0.027	0.027	0.743	0.934
rs1047047-rs2297566	0.356	0.422	A-G	0.430	0.444	0.471	0.166	0.353
			G-G	0.358	0.338	0.341	0.530	0.929
			G-A	0.212	0.218	0.188	0.331	0.210
rs1047047-rs885846	0.378	0.516	A-A	0.428	0.444	0.468	0.186	0.424
			G-G	0.329	0.312	0.316	0.637	0.877
			G-A	0.243	0.245	0.217	0.300	0.264
rs2297566-rs885846	0.484	0.474	G-A	0.457	0.468	0.491	0.253	0.445
			G-G	0.332	0.312	0.319	0.641	0.822
			A-A	0.212	0.219	0.190	0.379	0.229

*Significant difference in distribution. SNP, single nucleotide polymorphism; NT, normotension; EH, essential hypertension.

The results of the LD analysis provided an estimate of seven combinations of SNPs for the haplotype-based case-control studies (Table 4). Two combinations, rs883062-rs1047047 and rs883062-rs1047047-rs2297566, showed significant differences in overall distributions in the haplotype-based case-control studies. The occurrence of the C-A haplotype at rs883062-rs1047047 was significantly higher in the EH group (2.8%) than in the NT1 group (0.0%) (p=0.00006).

The occurrence of the C-A haplotype was not significantly different between EH males (2.9%) and EH females (2.7%) (p=0.803). The occurrence of the C-A-G haplotype at rs883062-rs1047047-rs2297566 was significantly higher in the EH group (3.5%) than in the NT group (0.0%) (p=0.00001). The occurrence of the C-A-G haplotype was not significantly different between EH males (3.3%) and EH females (4.0%) (p=0.575). The results for the second case-control study were the same as those found for the haplotype-based case-control study (Table 4).

As estimated based on the diplotype analysis, there were 10 patients with the C-A haplotype and 16 patients with the C-A-G haplotype in the EH group. The SBP in patients with and without the C-A haplotype were 183.1±34.6 and 170.5±19.7 mmHg (p=0.053), respectively. The DBP in patients with the C-A haplotype (114.2±17.1 mmHg) was significantly higher than that in patients without the C-A haplotype (103.3±15.2 mmHg) (p=0.027). Other clinical parameters were not significantly different between the patients with and without the C-A haplotype (data not shown). There were no significant differences in any of the clinical parameters between the patients with and without the C-A-G haplotype (data not shown).

Discussion

Initially, it was considered that the physiologic role of uroguanylin was to regulate the secretion of fluid and electrolytes in the intestinal epithelium. While there may be some residual belief that guanylin peptides influence cellular functions *via* the intracellular second messenger, cyclic GMP (cGMP), uroguanylin appears to cause no abnormalities of intestinal fluid secretion in mice lacking GC-C (*1*). However, it should be noted that GC-C, the so-called uroguanylin receptor, is not identical to the natriuretic peptide type C receptor (NPR3). The gene encoding human GC-C (GUCY2C) is located on 12p12, while the human NPR3 gene is located on 5p14-p13. Recently, it was reported that uroguanylin regulates transport in the cortical collecting duct that is independent of GC-C (*27*). These findings suggest that there may be other physiologic roles for uroguanylin in addition to signal transduction *via* GC-C. Although there have been some reports showing that natriuretic peptides and their receptors are related to the pathophysiology of hypertension, there have been no previous association studies concerning the EH and the GUCA2B gene. Our study is the first to attempt to elucidate this relationship. We examined the association between the GUCA2B gene and EH using four SNPs of this gene. The use of SNPs is a valuable tool for association studies examining genomic markers. Since the SNPs consisted of three genotypes and two alleles, we were able to investigate the association between EH and the frequency of the genotype and the allele.

Since the completion in 2001 of the draft sequence of the human genome, the methodology and strategy for doing genetic research have changed dramatically. SNPs are now used for the positional cloning of susceptibility genes by performing whole genome-wide scanning (*28*). However, one of the biggest changes involves haplotype analysis. Recent studies have shown that the human genome has a haplotype block structure that can be divided into discrete blocks of limited haplotype diversity. In each block, a small fraction of SNPs, referred to as "tag SNPs," can be used to distinguish a large fraction of the haplotypes. These tag SNPs have the potential to be extremely useful in association studies, since they make it unnecessary to have to genotype all of the SNPs. The online information of the HapMap projects indicate that the rs883062 and rs885846 in the four SNPs used in our experiment were registered, and the r^2 between rs883062 and rs885846 was 0.631. Therefore, a combination of the two SNPs is not adequate when using these simultaneously in a one haplotype-based case-control study, as was determined in our study.

Haplotype-based analysis is considered to be much more powerful than a marker-by-marker analysis (*29*). In genes with multiple susceptibility alleles, in particular when the LD between the polymorphisms is weak, a haplotype-based association study has advantages over an analysis that is just based on individual polymorphisms (*30*). In the present study, we estimated haplotypes using three polymorphisms (rs883062, rs1047047, rs2297566), which were based on the results of the LD analysis. We used these to conduct a haplotype-based case-control study between the EH and NT groups. Statistically, the two haplotypes were significantly more frequent in the EH group than in the control group. Furthermore, the DBP in patients with the C-A haplotype was significantly higher than that in patients without the haplotype. This is very interesting because a possible functional mutation in the GUCA2B gene linked to the C-A haplotype may be involved in the pathophysiology of EH. Blood pressure may be higher in patients with than in those without the C-A haplotype. Thus, this phenotype could be one of the characteristics of the GUCA2B gene–related hypertension. However, the frequency of the C-A-G haplotype at rs883062-rs1047047-rs2297566 in the EH group was only 3.5%. This is not surprising given the low frequency of the susceptibility haplotype, since EH is thought to be a multifactorial disorder. Uroguanylin belongs to a group of depressor factors that include natriuretic peptides, the nitric oxide system, the adrenomedullin system and prostacyclin. It is thought that each susceptibility gene is related to the development of EH even though the contribution of each gene is small and that these genes may interact in a complex manner. At the present time, it is impossible to predict the contribution of SNPs or haplotypes in the GUCA2B gene in regard to EH. However, the accumulation of additional data on the genotyping of multiple susceptibility genes for EH may ultimately lead to the ability to determine the individual contributions.

In conclusion, the present haplotype-based case-control study showed that the GUCA2B gene could be the susceptibility gene of EH. The present data indicate that the GUCA2B

gene is a promising candidate for use as a genetic marker for EH. Further studies are needed to clarify the causal/susceptibility mutation of the GUCA2B gene and/or neighboring genes in EH.

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Research Paper

Association Study of Aromatase Gene (CYP19A1) in Essential Hypertension

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Background: As aromatase-deficient mice, which are deficient in estrogens, reportedly have reduced blood pressure, the aromatase gene (CYP19A1) is thought to be a susceptibility gene for essential hypertension (EH). The aim of the present study was to investigate the relationship between CYP19A1 and EH by examining single nucleotide polymorphisms (SNPs).

Methods: Five SNPs in the human CYP19A1 gene (rs1870049, rs936306, rs700518, rs10046 and rs4646) were selected, and an association study was performed in 218 Japanese EH patients and 225 age-matched normotensive (NT) individuals.

Results: There were significant differences between these groups in the distribution of genotypes rs700518 and rs10046 in male subjects, and genotypes rs700518, rs10046 and rs4646 in female subjects. On multiple logistic regression analysis, a significant association between rs700518 ($p=0.023$) and rs10046 ($p=0.036$) in male subjects and rs700518 in female subjects ($p=0.018$) was noted. Interestingly, the risk genotypes of rs700518 and rs10046 showed a sex-dependent inverse relationship. Both SBP and DBP levels were higher in total (cases and controls) male subjects with the G/G genotype with rs700518 or the T/T genotype with rs10046 than in male subjects without the G/G genotype or T/T genotype. SBP levels were lower in female subjects with the G/G genotype with rs700518 than in female subjects without G/G. The A-T haplotype constructed with rs1870049 and rs10046 was a susceptibility marker for EH.

Conclusions: We confirmed that rs700518 and rs10046, as well as a haplotype constructed with rs1870049 and rs10046, in the human CYP19A1 gene can be used as genetic markers for gender-specific EH.

Key words: Essential hypertension, aromatase, CYP19A1, single nucleotide polymorphism, genetic

Introduction

High blood pressure or hypertension affects about 25% of adults and is an important risk factor for death from stroke, myocardial infarction and congestive heart failure. The main cause of hypertension is a primary condition known as essential hypertension (EH). EH is thought to be a multifactorial disease [1]. Several reports have indicated that there are susceptibility genes for EH, including those for estrogen, estrogen receptor [2] and aromatase [3]. The final stage of estrogen synthesis is catalyzed by aromatase.

There are numerous proposed mechanisms by which estrogen may bring about beneficial effects on the cardiovascular system. However, the precise role of estrogens has been difficult to establish, perhaps due to their wide variety of actions. In humans, estrogen facilitates vasodilation by stimulating prostacyclin and nitric oxide synthesis, as well as decreasing the production of vasoconstrictor substances, such as cyclooxgenase-derived products, reactive oxygen species, angiotensin II and endothelin-1 [4]. Estrogen also reduces the number of angiotensin type I (AT1) receptors [5]. Furthermore, men are at higher risk of developing cardiovascular disease than premenopausal women, and age-matched women have been shown to have lower blood pressure than men [6].

The aromatase enzyme complex catalyzes the

conversion of androgens to estrogens in a variety of tissues, including the ovary and placenta [7,8], brain [9] and adipose tissue [10]. It was recently demonstrated that both estrogens and aromatase are produced in vascular tissue, particularly in smooth muscle cells [11] and endothelial cells [12]. It has been reported that aromatase-deficient (ArKO) mice, which are deficient in estrogens due to deletion of the aromatase gene, exhibit reduced blood pressure (BP)[3]. Thus, we hypothesized that aromatase is one of the factors affecting BP, and that the aromatase gene is a susceptibility gene for hypertension, as single nucleotide polymorphisms (SNPs) in this gene are associated with differences in estrogen levels in human [13].

The human CYP19A1 gene, which encodes aromatase, consists of 503 amino acids and is located on chromosome 15q21.1 [14]. The gene is very unique; it contains 11 exons, with 9 exons being translated, interrupted by 10 introns (about 80 kb, exon 2a to exon 2), and consists of approximately 130 kilobase pairs (kb).

The aim of the present study was to investigate the relationship between the human CYP19A1 gene and EH by examining 5 SNPs in the human CYP19A1 gene (Figure 1) in Japanese individuals.

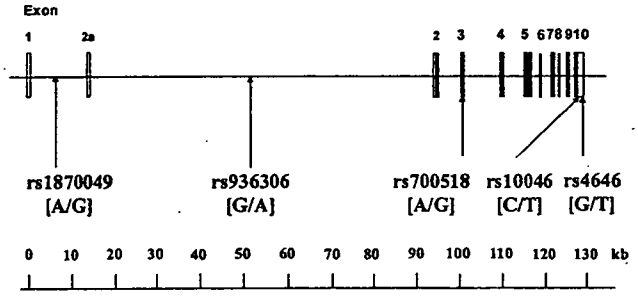

Figure 1. Organization of the human CYP19A1 gene and location of SNPs. The gene is approximately 130 kilobase pairs (kb) in length, and has a total of 11 exons. Boxes indicate exons, and lines indicate introns and intergenic regions. Filled boxes indicate coding regions. There are two transcript variants; variant 1 does not include exon 2a, and thus has a shorter 5'-UTR than transcript variant 2; variant 2 includes exon 2a. Both variants encode the same protein. Polymorphisms were expressed as nucleotide number on the sense strand of the CYP19A1 gene.

Subjects and Methods

Subjects

EH subjects were 218 patients diagnosed with EH according to the following criteria: seated systolic blood pressure (SBP) above 160 mmHg and/or diastolic blood pressure (DBP) above 100 mmHg, on 3 occasions within 2 months after the first medical examination. None of the EH subjects were using anti-hypertensive medication. Patients diagnosed with secondary hypertension were excluded. Control subjects were 225 healthy, normotensive (NT) individuals. None of the controls had a family history of hypertension, and they all had SBP and DBP below 130 and 85 mmHg, respectively. A family history of hypertension was defined as prior diagnosis of hypertension in grandparents, uncles, aunts, parents or siblings. Both groups were recruited from the northern area of Tokyo, Japan, and informed consent was obtained from each individual according to a protocol approved by the Human Studies Committee of Nihon University [15].

Biochemical analysis

Plasma concentration of total cholesterol, and serum concentrations of creatinine and uric acid were measured using the methods of the Clinical Laboratory Department of Nihon University Hospital [16].

Genotyping

Using information regarding allelic frequencies of SNPs registered with the National Center for Biotechnology Information (NCBI) and Celera Discovery System-Applied Biosystems, 5 SNPs with minor allele frequencies greater than 20% were selected. SNPs with relatively high minor allele frequencies have been shown to be useful as genetic markers for genetic association studies.

We selected 5 SNPs in the human CYP19A1 gene as markers for the genetic association experiment (Fig. 1). All 5 SNPs were confirmed using the NCBI website (accession numbers rs1870049, rs936306, rs700518, rs10046 and rs4646). rs1870049 and rs936306 are located in introns, rs700518 is a synonymous SNP that does not result in a change in amino acids, and rs10046 and rs4646 are located in the 3'-untranslated region. Genotypes were determined using Assays-on-Demand kits (Applied Biosystems, Branchburg, NJ) together with TaqMan® PCR. When allele-specific fluorogenic probes hybridize to the template during polymerase chain reaction (PCR), the 5'-nuclease activity of Taq polymerase is able to discriminate between alleles [17].

Linkage disequilibrium (LD) analysis and haplotype-based case-control analysis

LD analysis and haplotype-based case-control analysis were performed with SNPAlyze version 3.2.3 (Dynacom Co., Ltd., Yokohama, Japan) using 5 SNPs. The software is available from the following website: http://www.dynacom.co.jp/products/package/snpalyze/index.html. We used |D'| values of >0.5 to assign SNP locations to 1 haplotype block. SNPs with an r^2 value of <0.5 were selected as tagged. In the

haplotype-based case-control analysis, the frequency distribution of the haplotypes was calculated by performing a chi-squared test using the contingency table method.

Statistical analysis

Data are shown as means ±SD. Hardy-Weinberg equilibrium was assessed by chi-squared analysis in NT controls. The overall distribution of alleles was analyzed using 2 × 2 contingency tables, and the distribution of genotypes between EH patients and NT controls was tested using a 2-sided Fisher exact test and multiple logistic regression analysis, as the results of multiple logistic regression analyses after adjusting for confounding factors are known to be highly reliable. Statistical significance was established at $p < 0.05$. Differences in clinical data between the EH and NT groups were assessed by student t-test. Statistical analyses were performed using SPSS software for Windows, version 12 (SPSS Inc., Chicago, IL, USA).

Results

Table 1 shows the clinical features of the EH patients and NT controls. SBP, DBP, body mass index (BMI) and pulse rate were significantly higher in the EH group than in the NT group. Age, serum concentrations of creatinine, and plasma concentrations of total cholesterol and uric acid did not significantly differ between the two groups.

Table 1. Characteristics of study participants.

	Total				Men				Women			
	NT	EH	p Value		NT	EH	p Value		NT	EH	p Value	
Number of subjects	225	218			144	142			81	76		
Age (years)	50.3±9.01	50.5±6.2	0.812		50.0±5.6	50.2±6.7	0.809		50.9±13.1	51.1±5.3	0.899	
BMI (kg/m^2)	22.8±3.1	24.8±3.8	<0.001	*	23.0±2.9	24.8±3.6	<0.001	*	22.4±3.2	24.8±4.1	<0.001	*
SBP (mmHg)	111.8±11.0	173.1±19.0	<0.001	*	112.4±10.6	171.2±16.9	<0.001	*	110.9±11.6	176.7±22.0	<0.001	*
DBP (mmHg)	68.8±8.6	106.0±12.0	<0.001	*	69.6±8.2	106.8±10.9	<0.001	*	67.4±9.2	104.3±13.6	<0.001	*
Pulse (beats/min)	72.1±9.9	77.5±15.6	<0.001	*	76.9±9.5	77.8±16.0	<0.001	*	74.3±10.2	77.0±14.8	0.257	
Creatinine (mg/dl)	0.8±0.2	0.8±0.2	0.550		0.9±0.2	0.9±0.2	0.505		0.7±0.1	0.7±0.2	0.842	
Total cholesterol (mg/dl)	203.3±41.1	209.9±40.6	0.103		199.1±39.0	204.2±39.9	0.290		210.8±44.0	220.2±40.0	0.177	
Uric acid (mg/dl)	5.4±1.5	5.6±1.5	0.123		6.0±1.3	6.2±1.5	0.093		4.5±1.3	4.6±1.0	0.609	
Alcohol consumption (%)	70.5	69.0	0.761		77	85	0.084		38.8	37.7	0.904	
Smoking (%)	40.7	51.9	0.038	*	52.1	62.3	0.114		21.6	31.9	0.117	

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high density lipoprotein; NT, normotension; EH, essential hypertension. *Significant difference

Table 2 shows the distribution of genotypic and allelic frequencies of the 5 SNPs in each group. The genotype distribution of the each SNP in NT controls did not differ significantly from the Hardy-Weinberg equilibrium values (data not shown). The overall distributions of genotype and allele frequencies of all 5 SNPs did not significantly differ between the EH and total NT groups. However, some distributions showed significant gender-based differences between the groups. Among men, there were significant differences between the EH and NT groups in the distribution of rs700518 (P=0.012) and rs10046 genotypes (P=0.005). In the dominant model, the G/G genotype was significantly more frequent than the A/A&A/G genotypes of rs700518 (P=0.009), and the T/T genotype was significantly more frequent than the C/C&C/T genotypes of rs10046 (P=0.003) in EH men. Furthermore, the genotype distribution showed reciprocal findings in women when compared to men; in EH women, the G/G genotype was significantly less frequent than the A/A&A/G genotypes of rs700518 (P=0.021), and the T/T genotype was significantly less frequent than the C/C&C/T genotypes of rs10046 (P=0.030). The T allele of SNP rs4646 (p=0.046) and the GT&T/T genotype (p=0.032) were significantly more frequent in EH women than in NT women.

Multiple logistic regression analysis revealed significant associations between rs700518 G/G and EH in men (p=0.023) and between rs10046 T/T and EH in men (p=0.036), even after adjustment for confounding factors such as age, BMI, creatinine, total cholesterol and uric acid. The calculated odds ratios were 2.48 (95%CI: 1.11-5.53) and 2.10 (95%CI: 1.04-4.23), respectively. Multiple logistic regression analysis revealed a significant association between rs700518 A/A&A/G and EH in women (p=0.018), even after adjustment for confounding factors such as age, BMI, creatinine, total cholesterol and uric acid. The calculated odds ratio was 3.31 (95%CI: 1.16-3.40). Multiple logistic regression analysis for rs10046 and rs4646 in women showed no significant associations (data not shown). The opposite direction of the association of rs700518 and rs10046 in men and women was confirmed by multiple logistic regression analysis (p=0.001, <0.001, respectively).

Table 2. Genotype and allele distributions among NT subjects and patients with EH.

		Total subjects						Men						Women					
		NT	EH	chi-square p Value	Odss ratio	95%CI		NT	EH	chi-square p Value	Odss ratio	95%CI		NT	EH	chi-square p Value	Odss ratio	95%CI	
Number of paticipants		225	218					144	142					81	76				
Variants																			
rs1870049	Genotype																		
	A/A	144 0.640	134 0.615					89 0.618	87 0.613					55 0.679	47 0.618				
	A/G	72 0.320	75 0.344					48 0.333	50 0.352					24 0.296	25 0.329				
	G/G	9 0.040	9 0.041	0.856	1.11	0.76-1.64		7 0.049	5 0.035	0.826	1.40	0.43-4.52		2 0.025	4 0.053	0.561	2.19	0.39-12.3	
	Allele																		
	A	360 0.800	343 0.787					224 0.783	224 0.789					134 0.827	119 0.783				
	G	90 0.200	93 0.213	0.625	1.08	0.78-1.50		62 0.217	60 0.211	0.907	1.03	0.69-1.54		28 0.173	33 0.217	0.322	1.32	0.76-2.33	
rs936306	Genotype																		
	G/G	88 0.391	89 0.408					56 0.392	60 0.423					31 0.383	29 0.382				
	G/A	102 0.453	104 0.477					63 0.441	66 0.465					39 0.481	38 0.500				
	A/A	35 0.156	25 0.115	0.454				24 0.168	16 0.113	0.420				11 0.136	9 0.118	0.942			
	Allele																		
	G	278 0.618	282 0.647					177 0.615	186 0.655					101 0.623	96 0.632				
	A	172 0.382	154 0.353	0.371	1.13	0.86-1.49		111 0.385	98 0.345	0.316	1.19	0.85-1.67		61 0.377	56 0.368	0.882	1.04	0.66-1.64	
rs700518	Genotype																		
	A/A	82 0.364	88 0.404					55 0.382	58 0.408					27 0.333	30 0.395				
	A/G	111 0.493	95 0.436					77 0.535	57 0.401					34 0.420	38 0.500				
	G/G	32 0.142	35 0.161	0.478	1.18	0.80-1.73		12 0.083	27 0.190	0.012 *	2.58	1.25-5.33 * #		20 0.247	8 0.105	0.068	2.79	1.14-6.79 * #	
	Allele																		
	A	275 0.611	271 0.622					187 0.649	173 0.609					88 0.543	98 0.645				
	G	175 0.389	165 0.378	0.749	1.05	0.80-1.37		101 0.351	111 0.391	0.320	1.19	0.85-1.67		74 0.457	54 0.355	0.067	1.53	0.97-2.40	
rs10046	Genotype																		
	C/C	66 0.293	66 0.303					44 0.306	46 0.324					22 0.272	20 0.263				
	C/T	116 0.516	103 0.472					83 0.576	60 0.423					33 0.407	43 0.566				
	T/T	43 0.191	49 0.225	0.591	1.23	0.77-1.94		17 0.118	36 0.254	0.005 *	2.54	1.35-4.77 * #		26 0.321	13 0.171	0.061	2.29	1.07-4.89 *	
	Allele																		
	C	248 0.551	235 0.539					171 0.594	152 0.535					77 0.475	83 0.546				
	T	202 0.449	201 0.461	0.717	1.05	0.81-1.37		117 0.406	132 0.465	0.158	1.27	0.91-1.77		85 0.525	69 0.454	0.210	1.32	0.85-2.07	
rs4646	Genotype																		
	G/G	119 0.529	114 0.523					71 0.493	82 0.577					48 0.593	32 0.421				
	G/T	85 0.378	83 0.381					55 0.382	44 0.310					30 0.370	39 0.513				
	T/T	21 0.093	21 0.096	0.990	1.02	0.71-1.49		18 0.125	16 0.113	0.347	1.41	0.88-2.24		3 0.037	5 0.066	0.094	2.00	1.06-3.78 *	
	Allele																		
	G	323 0.718	311 0.713					197 0.684	208 0.732					126 0.778	103 0.678				
	T	127 0.282	125 0.287	0.883	1.02	0.76-1.37		91 0.316	76 0.268	0.203	1.26	0.88-1.81		36 0.222	49 0.322	0.046 *	1.67	1.01-2.75 *	

NT, normotension; EH, essential hypertension.
95%CI, 95% confidence interval
Odds ratios and 95%CIs were calculated as the risks of the susceptibility allele or genotype(s) for EH.
*Significant differences by chi-square analysis
#Significant differences by multiple logistic regression analysis

Clinical characteristics of the study participants by genotype are shown in Table 3. Genotypes showing significant differences in distribution on multiple logistic regression analysis were selected for analysis. Both SBP and DBP levels were higher in total (EH plus NT) male subjects with the G/G genotype in rs700518 than in male subjects without the G/G genotype. Furthermore, both SBP and DBP levels were higher in total male subjects with the T/T genotype in rs10046 than in male subjects without the T/T genotype. In contrast, SBP levels were higher in total female subjects with the A/A&A/G genotype in rs700518than in female subjects without the A/A&A/G genotype.

LD patterns in the CYP19A1 gene are illustrated by their |D'| values in NT groups (Table 4). The |D'| values indicate that all 5 SNPs are located in 1 haplotype block, as most |D'| values were over 0.5, except for rs1870049-rs700518, rs1870049-rs10046 and rs936306-rs10046. All pair-wise SNPs, except rs700518-rs10046, were available for the performance of a haplotype-based case-control study because all r^2 values were below 0.5. Because r^2 values calculated for the rs700518 and rs10046 SNPs were large, we did not perform a haplotype-based association study using the 2 SNPs in the same analysis. All 18 combinations of pair-wise SNPs were analyzed in men and women. Significant differences in overall distribution were only seen for the rs1870049 and rs10046 combination in men. Thus, the A-C haplotype is a resistance marker for EH, while the A-T haplotype is a susceptibility marker for EH. There is no overall distribution showing a significant difference in women (Table 5).

Table 3. Clinical characteristics of the study partipants in each genotype.

Men	rs700518				rs10046			
	A/A&A/G	G/G	p Value		C/C&C/T	T/T	p Value	
Number of subjects	247	39			233	53		
Age (years)	50.2±6.1	49.7±6.1	0.666		50.1±6.2	49.9±5.8	0.812	
BMI (kg/m^2)	23.9±3.5	24.1±3.1	0.820		23.9±3.5	24.2±3.1	0.511	
SBP (mmHg)	139.4±32.1	155.6±33.1	0.004	*	138.7±32.1	154.2±32.5	0.002	*
DBP (mmHg)	86.7±20.8	96.3±20.2	0.008	*	86.3±20.7	95.5±20.8	0.004	*
Pulse (beats/min)	75.3±14.6	73.1±11.0	0.453		75.3±15.0	73.7±10.1	0.522	
Creatinine (mg/dl)	0.9±0.2	1.0±0.2	0.193		0.9±0.2	0.9±0.2	0.262	
Total cholesterol (mg/d	202.2±39.6	197.9±38.3	0.557		201.0±39.7	204.6±38.4	0.555	
Uric acid (mg/dl)	6.1±1.4	6.0±1.4	0.795		6.1±1.4	6.0±1.3	0.835	
Alcohol consumption ('	82.8	76.7	0.415		82.2	81.0	0.848	
Smoking (%)	59.3	44.1	0.819		60.0	51.1	0.383	

Table 4. Pairwise LD in CYP19A1 gene of each NT group.

SNP	rs1870049	rs936306	rs700518	rs10046	rs4646
rs1870049		0.934	0.176	0.257	0.653
rs936306	0.352		0.567	0.384	0.764
rs700518	0.005	0.127		0.967	1.000
rs10046	0.013	0.074	0.730		0.976
rs4646	0.042	0.142	0.250	0.305	

LD, linkage disequilibrium; SNP, single-nucleotide polymorphism.
Upper right triangle shows absolute D' values. D' > 0.5 are shown as shaded values.
Lower left triangle shows r^2 values. $r^2 > 0.5$ are shown as shaded values.

Table 5. Haplotypes showing significant differences in overall distribution between NT controls and EH patients in men.

Combination of SNPs	Overall distribution		Distribution of Individual haplotypes					
	Chi-square	p value	Haplotype	NT 288	EH 284	Chi-square	p-value	
rs1870049-rs10046	8.1	0.044	A-C	0.464	0.381	4.232	0.040	*
			A-T	0.320	0.408	4.895	0.027	*
			G-C	0.129	0.155	0.823	0.364	
			G-T	0.086	0.057	1.995	0.158	

* significant difference

Discussion

Human aromatase deficiency was first reported in 1995. The disorder is very rare, and only a few cases have been reported [18-20]. Male patients with aromatase deficiency exhibit eunuchoid skeletal proportions, macroorchidism, sexually precocity. In contrast, female patients with the disease develop progressive signs of virilization, pubertal failure with no signs of estrogen action, hypergonadotropic hypogonadism, polycystic ovaries on pelvic sonography, and tall stature. Common clinical data in men and women with aromatase deficiency are high levels of plasma testosterone, androsterone, FSH and LH, and low estradiol and estrone [18,19]. They also have homozygous or compound heterozygous mutations in the CYP19A1 gene. Interestingly, male patients with aromatase deficiency exhibit hypertension [19,20].

In the present study, the findings regarding genotype and allele distributions were particularly interesting from the viewpoint of gender differences. The gender differences in genotype and allele distributions were similar between rs700518 and rs10046, while the overall distribution of genotypes was significantly different between the EH and the NT groups. Blood pressure values for each genotype were

similar between rs700518 and rs10046. These results were consistent with those of LD analysis showing that rs700518 and rs10046 were closely linked with a large r^2.

Although systolic BP in ArKO female mice was similar to that in age- and weight-matched wild-type (WT) mice, diastolic and mean BP were lower in ArKO mice (-6.3 ± 1.9 and -4.6 ± 2.1 mmHg, respectively). The baroreflex sensitivity of ArKO mice was 46% that observed in WT mice [3]. However, there have been no previous studies on male ArKO mice or comparing data between male and female ArKO mice.

Some investigators have been reported the CYP19A1 gene variants associated with hypertension. Peter et al. found suggestive evidence of gender-specific contributions of rs4646 to DBP variation in women in the Framingham Heart Study [21]. DBP in patients with T/T genotype was significantly higher than in those without this genotype. This is very interesting because the frequencies of EH women with T/T genotype or T alleles were significantly higher in the present study when compared to NT women. In addition, our data for rs4646 also showed no significant results in men, which is also in agreement the report by Peter et al. Recently, Ramirez-Lorca et al. reported that DBP in subjects with C/C genotype in rs10046 was significantly higher than in those without C/C genotype [22]. This corresponds with our data, as the frequency of EH patients with the T/T genotype was significantly lower than that of NT subjects. However, the opposite direction of the association in men found in our study was not detected in men in their study. There are several reasons for this discrepancy between the results in our study and those of previous studies. Our study used a case-control design with patients clearly diagnosed by EH criteria, while Ramirez-Lorca et al. used a population-based cohort in the general population. Therefore, the data on blood pressure in each genotype from their study were within normal ranges. This discrepancy may be attributed to both the different criteria used in subject selection, and to racial differences in the populations studied.

In the present study, none of the SNPs were thought to have functional consequences. Possible functional mutations in the CYP19A1 gene with quantitative effects on genomic transcription, posttranslational processing or amino acid sequence have a strong linkage with genetic markers such as rs10046, and subsequently reduce the activity of aromatase associated with EH. Unfortunately, we were not able to obtain samples to measure plasma sex hormones levels and aromatase activity, due to the difficulty in obtaining written informed consent for blood examinations from subjects not receiving medications.

In conclusion, the present study was the first to examine correlations between the human CYP19A1 gene (encoding aromatase) and EH. The present data indicate that the CYP19A1 gene is a gender-specific candidate genetic marker for EH.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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Original Article

Identification of Hypertension-Susceptibility Genes and Pathways by a Systemic Multiple Candidate Gene Approach: The Millennium Genome Project for Hypertension

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A multiple candidate-gene approach was used to investigate not only candidate genes, but also candidate pathways involved in the regulation of blood pressure. We evaluated 307 single nucleotide polymorphisms (SNPs) in 307 genes and performed an association study between 758 cases and 726 controls. Genes were selected from among those encoding components of signal transduction pathways, including receptors, soluble carrier proteins, binding proteins, channels, enzymes, and G-proteins, that are potentially related to blood pressure regulation. In total, 38 SNPs were positively ($p<0.05$) associated with hypertension. Replication of the findings and possible polygenic interaction was evaluated in five G-protein–related positive genes (GNI2, GNA14, RGS2, RGS19, RGS20) in a large cohort population (total $n=9,700$, 3,305 hypertensives and 3,827 normotensive controls). In RGS20 and GNA14, dominant models for the minor allele were significantly associated with hypertension. Multiple dimension reduction (MDR) analysis revealed the presence of gene–gene interaction between GNA14 and RGS20. The MDR-proved combination of two genotypes showed a significant association with hypertension ($\chi^2=9.93$, $p=0.0016$) with an odds ratio of the high-risk genotype of 1.168 (95% confidence interval [CI] [1.061–1.287]). After correction for all possible confounding parameters, the MDR-proved high-risk genotype was still a risk for hypertension ($p=0.0052$). Furthermore, the high-risk genotype was associated with a significantly higher systolic blood pressure (133.08±19.46 *vs.* 132.25±19.19 mmHg, $p=0.04$) and diastolic blood pressure (79.65±11.49 *vs.* 79.01±11.32 mmHg, $p=0.019$) in the total population. In conclusion, a systemic multiple candidate gene approach can be used to identify

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not only hypertension-susceptibility genes but also hypertension-susceptibility pathways in which related genes may synergistically collaborate through gene–gene interactions to predispose to hypertension. (*Hypertens Res* 2008; 31: 203–212)

Key Words: **hypertension, candidate gene, association study, gene–gene interaction, pathways**

Introduction

Hypertension is one of the most common complex genetic disorders affecting a large population, with a genetic heritability ranging from 15% to 35% (*1–5*). Like other multifactorial human traits, hypertension is caused by the interaction of multiple risk genes and environmental risk factors (*1–5*).

Vigorous efforts have been made to identify genes for hypertension. The candidate gene approach is a cornerstone for identifying the hypertension-susceptibility gene(s). Several candidate genes have been selected on the basis of known biochemical or physiologic components related to blood pressure regulation. However, the results of these studies have not been consistent, and the positive findings have rarely been replicated. Several underlying causes have been postulated to explain the failure of these studies to reproduce the data on candidate gene(s), including the heterogeneity of cases and controls, population stratification, and gene–gene and gene–environment interactions (*1–5*). In addition, it has been demonstrated that genetic interactions at multiple loci rather than a variant of a single gene underlie the genetic basis of hypertension (*1–5*).

Hypertension is polygenic (*6–8*), with alleles at many different loci being suggested to contribute to the ultimate disease trait, and specific combinations of causative alleles may differ between individuals (*6–9*). Although several candidate genes have been associated with the development of hypertension, the mechanisms and genetic network underlying this disease remain unknown (*3*). It has been persuasively argued that the gene being assessed increases susceptibility for the disease, but its effect is not sufficient to cause the disease (*3*).

Accordingly, the strategy for the candidate gene approach needs to be changed to detect polygenetic factors with weak effects. There has been a trend towards the simultaneous evaluation of several polymorphisms in separate genes (*4*, *10*, *11*). Combinations of polymorphisms at several of these loci could steadily increase the odds ratio for predicting hypertension or a hypertensive intermediate phenotype (*12–16*). However, the above-referenced studies were directed at genes for which there was a published association with hypertension. To search for multiple novel genes interacting with each other, it would be rational to find a specific pathway in which the genes are involved. Dominiczak *et al.* (*17*) referred to "pathwayomics," meaning a cardiovascular continuum leading from the investigation of interrogation of multiple single nucleotide polymorphisms (SNPs) in genes for a specific pathway. No study has ever evaluated susceptibility pathways in which multiple genes interact with each other to cause hypertension.

In the present study, we evaluated multiple genes to identify not only candidate genes but also candidate pathways in which multiple genes are synergistically involved in the regulation of blood pressure. The study was performed one of the Millennium Genome Project. In 2000, a series of national cooperative projects were begun under the auspices of the Prime Minister's Millennium Project (*18*). The projects focus on bold technological innovations in three areas: informatization, the aging society and the environment. The identification of genetic variations linked to the development of hypertension is one of the leading missions of the Millennium Project. Four other diseases—diabetes mellitus, cancer, asthma, and Alzheimer's disease—have also been chosen as targets for gene search.

In the present study, we evaluated 307 SNPs in 307 genes, and performed an association study between 758 cases and 726 controls. Genes were selected from those related to signal transduction pathways that included receptors, soluble carrier proteins, binding proteins, channels, enzymes, and G-proteins, and that were possibly related to blood pressure regulation. A replication study of positive G-protein–related SNPs including possible gene–gene interactions was further performed in a larger, independent general population.

Methods

Subjects

Case-Control Study

The present study was performed as part of the Millennium Genome Project for Hypertension under the auspices of the National Millennium Project in Japan. Four centers throughout Japan, located in Asahikawa, Tokyo, Osaka, and Hiroshima, participated in the case-control study. Cases and controls were recruited at each center, using the same criteria.

Hypertensive subjects (n=758) had a previous diagnosis of hypertension at between 30 and 59 years of age and were being treated with antihypertensive medication or had a systolic blood pressure (SBP) ≥160 mmHg and/or diastolic blood pressure (DBP) ≥90 mmHg. They had a family history of hypertension in their parents and/or siblings. They were not obese (body mass index [BMI] <25 kg/m^2). Normotensive controls (n=726) aged more than 45 years were recruited from the same regions. They had never been treated with antihypertensive medication and their SBP was <120 mmHg and DBP <80 mmHg. They had no family history of hypertension.

Table 1. Clinical Characteristics of Cases and Controls

	Hypertensive cases	Normotensive controls
Number of subjects	758	726
Asahikawa	192	192
Tokyo	159	153
Osaka	238	189
Hiroshima	169	192
Male (*n* (%))	564 (74.4)	550 (75.8)
Age (years old)	59.0±11.0*	62.8±9.4
Body mass index (kg/m^2)	23.6±3.0*	22.7±2.9
Systolic blood pressure (mmHg)	163.5±24.6	115.9±12.0
Diastolic blood pressure (mmHg)	100.3±15.7	72.0±7.6
Antihypertensive medication (*n* (%))	499 (65.8)	

*$p<0.05$ *vs.* normotensive controls.

Table 1 summarizes the background characteristics of the cases and controls.

Replication Study

Replication of positive findings including gene–gene interactions was evaluated in a large, independent general population recruited from five cohorts throughout Japan (Ohasama, Yokohama, Shigaraki, Ehime 1 and Ehime 2) (*19–22*). Subjects aged ≥30 years were enrolled in the analysis. Measurement of blood pressure, an anthropometric and biochemical parameters was performed during a medical checkup. Table 2 summarizes the clinical characteristics of the population. In this population sample, hypertension was defined as an SBP ≥160 mmHg and/or DBP ≥90 mmHg, or current use of antihypertensive medication. Normotension was defined as SBP <140 mmHg and DBP <90 mmHg and no current use of antihypertensive medication. Among the total 9,700 subjects, 3,305 were defined as hypertensives and 3,827 were classified as normotensive controls.

All of the participants in the study were native Japanese. All participants received a full explanation of the purpose of the study, and written informed consent for the procedures was obtained. The entire protocol followed the guidelines for genomic research of the Japanese government and was approved by the ethical committees of all institutes participating in the project.

SNP Selection

Three hundred and seven SNPs of the following types were selected. 1) SNPs in genes encoding the components of signal transduction systems, including enzymes, channels, receptors, solute carriers, G-proteins, and binding proteins, that are potentially related to blood pressure regulation. SNPs in other genes of particular interest, such as collagens, growth factors, adhesion molecules, and hormones, were also evaluated. 2) From among these 307 SNPs, we selected one SNP from one gene, preferably in the promoter region or exons with the highest minor allele frequency in the Japanese population, was selected based on information published in the JSNP website (*23*) (http://snp.ims.u-tokyo.ac.jp/index.html). 3) To find novel susceptible genes, unpublished SNPs were selectively studied. All SNPs examined in the present study are listed in the Online Data Supplement 1 (it is up-loaded to J-STAGE).

Genotyping

DNA was extracted from white blood cells by the standard method, at each institute. The whole genome was amplified by degenerated oligonucleotide primer (DOP)–polymerase chain reaction (PCR) in the Core Laboratory of the Department of Geriatric Medicine, Ehime University. DOP-PCR was performed according to a previous report, with slight modifications (*24*, *25*). In brief, 10 ng genomic DNA was used as a template in a 100-μL reaction mixture containing 5 U polymerase (TaKaRa LA Taq; Takara Biomedicals, Tokyo, Japan), 2.5 mmol/L $MgCl_2$, 400 μmol/L dNTP, and 4 μmol/L DOP primer (5′-CCGACTCGAGNNNNNNATGTGG-3′; Sigma Genosys Japan, Ishikari, Japan). The amplification conditions consisted of an initial incubation at 93°C for 1 min; followed by 8 cycles of 93°C for 1 min, 30°C for 1 min, and 72°C for 3 min; followed by 28 cycles of 93°C for 1 min, 60°C for 1 min, and 72°C for 3 min. The initial denaturation for 8 min at 96°C was omitted. The quality of amplified DNA obtained by DOP-PCR was checked in all samples.

Genotyping for the case-control study was performed in the Human SNP Typing Center of Tokyo University (Department of Human Genetics) using automated fluorescence correlation spectroscopy (*26*), and in the Core Lab of the Department of Geriatric Medicine, Ehime University, by the TaqMan PCR method.

Genotyping for the replication population study was performed at Ehime University using a TaqMan SNP genotyping assay system. The structures of primers and probes are listed in the Online Data Supplement 2 (it is up-loaded to J-STAGE).

Statistical Analysis

For comparison of the baseline characteristics between cases and controls, Student's unpaired *t*-test for continuous data and χ^2 test for categorical data were used. Differences in genotype and allele frequency between cases and controls were evaluated by χ^2 test. In the present study, both recessive and dominant models for the minor allele were considered.

Gene–gene interaction was evaluated by the multiple dimension reduction (MDR) method developed by Ritchie *et al.* (*27*). Genes were evaluated with a specific model, either dominant or recessive. This method included a combined cross-validation and permutation-testing procedure that mini-

Table 2. Clinical Characteristics of General Population

	Total sample	Hypertensives	Normotensives	*p*
Number of subjects	9,700	3,305	3,827	
Ohasama	1,663	523	542	
Yokohama	2,196	425	1,201	
Shigaraki	2,190	831	842	
Ehime 1	848	335	339	
Ehime 2	2,803	1,191	903	$\chi^2=253, p<0.0001$
Male (%)	52.2	53.5	50.0	
Age (years old)	56.8±12.6	62.8±10.6	50.5±11.6	<0.0001
Body mass index (kg/m^2)	23.1±3.2	24.0±3.2	22.2±2.9	<0.0001
Systolic blood pressure (mmHg)	132.5±19.3	148.6±17.3	115.3±9.6	—
Diastolic blood pressure (mmHg)	79.3±11.4	88.0±11.0	71.3±7.9	—
Antihypertensive medication (%)	19.6	60.0	—	
Total cholesterol (mmol/L)	5.21±0.90	5.29±0.88	5.13±0.90	<0.0001
HDL cholesterol (mmol/L)	1.53±0.40	1.52±0.41	1.57±0.40	<0.0001
Triglyceride (mmol/L)	1.37±0.94	1.52±1.06	1.22±0.84	<0.0001
Blood glucose (mmol/L)	5.8±1.6	6.1±1.8	5.5±1.3	<0.0001
Current smoker (%)	34.9	35.5	34.5	
History of stroke (%)	2.2	4.3	0	
History of heart disease (%)	5.8	9.3	0	

HDL, high-density lipoprotein.

mizes false-positive results by multiple examinations of the data. Cross-validation divides the data into a training set and a testing set. In 10-fold cross-validation, the data are divided into 10 equal parts, and the model is developed based on 9/10 of the data (training set) and then tested on the remaining 1/10 of the data (testing set). This is repeated for each possible training set, and the resulting 10 prediction errors are averaged (*27*).

The genotype combination obtained by the MDR method was then re-evaluated with an χ^2 test in the total population. Furthermore, differences in SBP and DBP as quantitative traits between MDR-defined genotypes were evaluated by ANOVA. Values are expressed as the means±SD. Values of $p<0.05$ were defined as statistically significant.

Results

Case-Control Study

Sixty-five percent of hypertensive cases were receiving antihypertensive medication. There were no region-specific differences in the parameters examined.

Systemic Multiple Candidate Gene Analysis

Of the 307 SNPs, 13 showed *p* values less than 0.05 in genotype frequency, 23 showed a significantly different allele frequency between cases and controls, and 32 showed significant associations with hypertension in either a dominant or recessive model for the minor allele. In total, 38 SNPs were found to be positively associated with hypertension (Table 3).

In the subcategorized analysis, the positive rate was high in genes related to G-proteins and genes of solute carriers (Table 4). The positive rate of SNPs in genes encoding the Gα-subunit or RGS (5/12), which could possibly interact with each other, was significantly higher than those in other classes of genes (5/12 *vs.* 33/295, $\chi^2=6.2, p=0.013$).

Replication in a Large Cohort Population

Replication of the findings of the case-control study and possible polygenic interaction in the susceptible pathway was evaluated in G-protein–related genes in a large cohort population.

Table 2 summarizes the clinical characteristics of the cohort population sample, from which 3,305 hypertensive subjects and 3,827 normotensive controls were recruited. Allele and genotype frequencies of the five G-protein–related SNPs in the cohort population are summarized in Table 5. The genotypes of GNA14 showed significant associations with hypertension. In RGS20 and GNA14, dominant models for the minor allele were significantly associated with hypertension. Accordingly, the gene–gene interaction of G-protein–related genes was evaluated in GNA14 and RGS20.

Gene–Gene Interaction

MDR analysis showed that the combination of two SNPs, GNA14 and RGS20, had a testing accuracy of 0.526 and

Table 3. Genotype and Allele Frequencies and Odds Ratio of Dominant and Recessive Models of Minor Allele in 38 Positive SNPs

Gene symbol	A (minor)/ B	A frequency	Case (*n*)				Control (*n*)				Allele (A *vs.* B)		A dominant		A recessive		Genotype *p*
			AA	AB	BB	H-W *p*	AA	AB	BB	H-W *p*	Odds ratio	*p*	Odds ratio	*p*	Odds ratio	*p*	
ATP2B1	C/A	0.4654	151	379	216	0.515	177	341	186	0.409	0.862	0.045	0.88	0.281	0.76	0.026	0.078
ATP10C	A/G	0.3574	115	342	291	0.382	80	333	295	0.335	1.159	0.056	1.122	0.283	1.426	0.023	0.069
ATP2A3	G/A	0.3359	81	324	345	0.705	52	301	366	0.354	1.223	0.013	1.217	0.06	1.553	0.017	0.028
ATP10D	C/T	0.4296	132	347	271	0.253	161	326	232	0.024	0.838	0.018	0.842	0.119	0.74	0.022	0.052
PRKWNK1	G/A	0.4918	185	342	214	0.040	182	356	158	0.523	0.863	0.049	0.723	0.008	0.934	0.607	0.026
DLGAP2	A/C	0.4907	150	385	219	0.412	192	346	182	0.299	0.81	0.004	0.826	0.104	0.683	0.002	0.007
GUCA1C	T/C	0.33	71	318	338	0.762	102	322	294	0.362	0.8	0.005	0.798	0.034	0.654	0.009	0.014
CYP17	T/C	0.4678	149	340	251	0.085	156	364	187	0.399	0.827	0.011	0.7	0.002	0.891	0.368	0.008
PTPRT	C/T	0.4925	144	362	249	0.543	165	348	205	0.459	0.845	0.023	0.812	0.066	0.79	0.066	0.08
PPP1R1B	G/A	0.4479	144	362	231	0.919	172	334	182	0.449	0.812	0.006	0.728	0.013	0.729	0.013	0.021
KCNN1	A/G	0.339	73	303	367	0.369	77	316	302	0.677	0.848	0.039	0.787	0.024	0.875	0.437	0.078
HCN4	G/T	0.4795	158	342	244	0.063	160	356	195	0.918	0.875	0.073	0.774	0.026	0.929	0.559	0.081
KCNIP2	A/G	0.4571	162	332	238	0.025	156	355	186	0.588	0.885	0.103	0.756	0.016	0.986	0.909	0.041
KCNMB4	T/G	0.41	136	370	242	0.793	133	300	263	0.005	1.096	0.226	1.27	0.0305	0.941	0.65	0.042
CACNA2D2	G/A	0.4845	185	401	166	0.065	158	356	206	0.857	1.202	0.013	1.415	0.004	1.161	0.228	0.015
CACNA1E	T/A	0.4693	160	331	245	0.016	179	318	201	0.020	0.845	0.024	0.811	0.066	0.805	0.082	0.097
ACCN1	G/A	0.4877	204	367	180	0.553	162	344	208	0.387	1.213	0.009	1.304	0.025	1.271	0.048	0.036
PTHR1	A/G	0.433	143	364	209	0.495	134	302	233	0.047	1.12	0.139	1.296	0.025	0.996	0.979	0.056
CALCR	A/G	0.4483	170	350	222	0.157	131	350	229	0.893	1.147	0.066	1.115	0.337	1.314	0.036	0.108
ADORA1	T/C	0.4558	188	361	186	0.632	152	337	205	0.542	1.172	0.035	1.237	0.073	1.226	0.103	0.111
SLC13A1	G/A	0.4043	144	337	255	0.088	104	347	239	0.229	1.097	0.224	1	0.997	1.371	0.025	0.06
SLC2A11	T/C	0.4037	125	342	254	0.592	97	304	266	0.503	1.169	0.045	1.22	0.074	1.232	0.156	0.139
SLC21A6	T/C	0.3851	129	289	283	0.000	93	285	271	0.200	1.123	0.144	1.059	0.605	1.348	0.044	0.127
SLC26A8	C/T	0.2602	59	276	412	0.184	63	295	347	0.979	0.841	0.037	0.788	0.024	0.874	0.476	0.077
SLC22A7	A/G	0.3443	101	339	296	0.802	75	299	325	0.615	1.228	0.009	1.292	0.017	1.323	0.084	0.034
RGS19IP1	G/A	0.3804	102	306	291	0.143	122	299	242	0.082	0.828	0.017	0.806	0.053	0.758	0.058	0.066
RGS2	G/A	0.438	140	341	255	0.173	135	365	208	0.262	0.898	0.15	0.785	0.032	0.997	0.982	0.077
RGS20	C/G	0.3973	93	346	298	0.631	115	316	268	0.183	0.881	0.101	0.916	0.417	0.733	0.039	0.118
GNAI2	A/G	0.454	177	375	187	0.682	132	350	228	0.909	1.278	0.001	1.396	0.004	1.379	0.013	0.004
GNA14	T/C	0.4042	105	351	283	0.819	122	347	241	0.879	0.858	0.044	0.828	0.085	0.798	0.12	0.13
RAC2	T/C	0.435	157	340	207	0.439	126	304	228	0.172	1.186	0.028	1.273	0.038	1.212	0.152	0.087
FGF2	C/T	0.4713	143	361	240	0.727	163	312	209	0.029	0.88	0.09	0.924	0.489	0.761	0.034	0.105
COL4A1	G/A	0.4633	165	372	205	0.878	143	309	226	0.052	1.148	0.067	1.31	0.02	1.07	0.6	0.062
HLA-DMB	A/C	0.4947	159	362	217	0.727	189	331	176	0.201	0.823	0.009	0.813	0.081	0.737	0.013	0.03
CAST	A/G	0.4695	155	368	210	0.791	174	308	198	0.015	0.923	0.29	1.023	0.846	0.78	0.048	0.091
EXOSC3	G/A	0.485	150	362	218	0.99	178	322	187	0.033	0.852	0.013	0.878	0.2712	0.74	0.0168	0.055
ERCC1	A/C	0.4443	145	362	231	0.882	170	322	204	0.056	0.873	0.069	0.91	0.413	0.757	0.029	0.092
CHGA	T/C	0.3482	105	371	268	0.194	94	323	291	0.770	1.135	0.1	1.24	0.047	1.073	0.643	0.1366

H-W, Hardy-Weinberg equilibrium.

cross-validation consistency of 10/10 (p=0.025), by sign test (Table 6). An empirical permutation test with 1,000 replications showed that the combination was statistically significant (p=0.01). Therefore, we concluded the presence of a gene–gene interaction between GNA14 and RGS20.

The MDR-proved combination of GNA14 and RGS20 was further verified in the cohort population (Table 7). The combination of the genotypes of GNA14 and RGS20 showed significant association with hypertension in the population (χ^2=9.93, p=0.0016). The odds ratio of the risk genotype (GNA14: CC+TC and RGS20: CC+CG) to the non-risk genotypes defined by the MDR method was 1.168 (95% confidence interval [CI] [1.061–1.287], p=0.0016). Since the prevalence of hypertensives and normotensives was signifi-

Table 4. Positive Rates

Classification	Positive/total	Positive rate
Enzymes	10/71	14.1
ATPase	4/12	33.3
Kinase	2/12	16.7
Hcy	0/3	0
Metalloproteinase	0/3	0
Guanylate cyclase	1/3	33.3
NO synthase	0/3	0
Channels	7/55	12.7
K channel	4/27	14.8
Ca channel	2/10	20.0
Cl channel	0/8	0
Receptors	3/36	8.3
Serotonin	0/4	0
Thyroid	0/3	0
Cholinergic	0/2	0
Solute carriers	5/31	16.1
Na related	1/8	12.5
Neurotransmitter	0/6	0
Glucose	1/3	33.3
G-protein	6/27	22.2
α-Subunit	2/7	28.6
RGS	3/5	60.0
rho	1/9	11.1
ras	0/4	0
Growth factors	1/13	7.7
VEGF	0/3	0
FGF	1/3	33.3
IGF	0/3	0
Cytokines	0/12	0
IL1	0/6	0
TNF	0/3	0
Binding proteins	0/9	0
Collagen	1/4	25.0
Coagulation	0/4	0
Adhesion molecules	0/3	0
HLA	1/2	50.0
Miscellaneous	4/39	10.3
Total	38/307	12.4

Hcy, homocysteine; RGS, regulator of G-protein signaling; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; IL1, interleukin 1; TNF, tumor necrosis factor; HLA, human leukocyte antigen.

cantly different among regions, gene–gene interaction was further evaluated by a general linear model with the following parameters; sex, region, age, BMI, total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, and blood glucose. After correction for sex, region, and age, the MDR-proved high-risk genotype had a significantly higher risk for hypertension (odds ratio 1.16 [1.035–1.294], $p=0.01$). After correction for all these confounding parameters, the MDR-proved risk genotype was still a risk for hypertension (odds ratio 1.19 [1.053–1.340], $p=0.0052$).

The blood pressure levels of the combined genotype were further evaluated in the total population of the cohort ($n=9,700$) (Table 8). The risk genotype was associated with a significantly higher SBP and DBP. Even after correction for other confounding parameters, including region, sex, age, BMI, use of antihypertensive medication, total cholesterol, HDL cholesterol, triglycerides and fasting glucose, DBP in the high-risk genotype was still significantly higher compared with that in the non-risk genotype ($p=0.0294$).

Discussion

Hypertension is a polygenic disease in which individual genes contribute only a very weak genetic effect (*1–5*). Furthermore, epigenetic effects could play pivotal roles. Accordingly, a conventional candidate gene approach may limit the possibility of detecting the susceptible genes for hypertension, since the effect of a single gene might not always be detected in different populations. Several studies have evaluated multiple genes as candidate hypertension genes and evaluated gene–gene interactions among candidate genes (*10–16*). However, many of these genes evaluated so far were already reported as positive genes in a previous candidate gene study. The strategy we took in the present study was to search for not only susceptibility genes, but also susceptibility pathways for hypertension using a candidate gene approach. The results raised the possibility that many susceptibility genes play an indirect role and exist in numerous pathways involved in blood pressure regulation.

The candidate genes evaluated thus far include genes within the renin-angiotensin system, α-adrenergic and β-adrenergic receptors, and growth factors, as well as genes encoding enzymes and peptides involved in endothelial function and vasoactivity (*17*). In the present study, we chose genes in signal transduction pathways possibly related to blood pressure regulation. The results revealed that none of the genes showed a high odds ratio or small p value.

Candidate SNPs were selected from among those in the gene of interest with the highest minor allele frequencies based on the JSNP data (*23*). The rationale behind this strategy is the common-disease/common-variant (CD/CV) hypothesis (*28, 29*). Although several authors have argued that the CD/CV hypothesis has not been sufficiently validated (*5, 30, 31*), detection of susceptibility genes with low frequency is beyond the power of a conventional case-control approach, and at least this approach has the necessary statistical power to detect an association.

We demonstrated that positive SNPs were more common in genes related to G-proteins. We found that SNPs in genes that encoded ATPase, channels and solute carrier proteins also showed a higher positive rate. Among the positive pathways, replication of G-protein–related genes, including gene–gene interaction, was evaluated. Two of five positive G-protein–

Table 5. Five G-Protein Related SNPs in a General Population

Gene	Case (*n*)				Control (*n*)				Allele (A *vs.* B)		A dominant		A recessive		Genotype
	AA	AB	BB	H-W *p*	AA	AB	BB	H-W *p*	Odds ratio [95% CI]	*p*	Odds ratio [95% CI]	*p*	Odds ratio [95% CI]	*p*	*p*
RGS19	GG	GA	AA		GG	GA	AA								
	529	1,498	1,220	0.056	591	1,751	1,396	0.283	1.005 [0.94–1.08]	0.88	0.99 [0.90–1.09]	0.82	1.03 [0.91–1.17]	0.65	0.829
RGS2	GG	GA	AA		GG	GA	AA								
	603	1,520	1,104	0.049	663	1,837	1,224	0.563	0.99 [0.93–1.06]	0.78	0.94 [0.85–1.04]	0.24	1.06 [0.94–1.20]	0.34	0.179
RGS20	CC	CG	GG		CC	CG	GG								
	456	1,578	1,190	0.066	540	1,715	1,460	0.318	1.04 [0.97–1.12]	0.23	1.11 [1.004–1.22]	0.04	0.97 [0.85–1.11]	0.64	0.061
GNAI2	AA	AG	GG		AA	AG	GG								
	724	1,557	965	0.044	836	1,847	1,044	0.725	0.96 [0.90–1.03]	0.28	0.92 [0.83–1.02]	0.11	1.01 [0.88–1.14]	0.93	0.261
GNA14	TT	TC	CC		TT	TC	CC								
	542	1,611	1,111	0.302	629	1,757	1,386	0.072	1.06 [0.99–1.13]	0.11	1.13 [1.02–1.24]	0.018	0.99 [0.88–1.13]	0.94	0.040

H-W, Hardy-Weinberg equilibrium; CI, confidence interval.

Table 6. Multiple Dimensionality Reduction

	Gene(s)	Training accuracy	Testing accuracy	CV	Sign test consistency (*p*)	*p* value for testing accuracy by permutation test
One gene	GNA14	0.5044	0.491	6/10	3 (0.109)	0.93
Two genes	RGS20+GNA14	0.526	0.526	10/10	9 (0.0215)	0.014

Permutation test count 1,000, affected, ratio threshold was 0.868. CV, cross-validation.

related SNPs in the case-control study, GNA14 and RGS20, were also positively associated with hypertension in a replicated study in a large general population. Furthermore, possible gene–gene interaction was found between the two genes.

Heterotrimeric G-proteins are central components of the primary mechanism to receive, interpret and respond to a wide range of structurally and chemically diverse extracellular stimuli (*32*, *33*). Agonist binding of G-protein–coupled receptors (PCRs) promotes G-protein activation. This activation is achieved by catalyzing the GDP–GTP exchange on the α-subunit. A conformational change in the GTP-bound α-subunit leads to dissociation of Gα from the β- and γ-subunits. GTP-bound Gα-subunits and dissociated βγ-dimers regulate downstream effectors. These signaling events are terminated as a consequence of intrinsic GTPase activity of the Gα-subunit, which hydrolyzes bound GTP to GDP, resulting in reassociation of the G-protein heterotrimer (*34*). The intrinsic GTPase activity of α-subunits is generally insufficient to correlate with the physiological rate of G-protein inactivation, but this activity can be accelerated by the presence of GTPase-activating proteins (GAPs), such as regulator of G-protein–signaling (RGS) proteins (*35*).

Although the present study showed there is a statistically significant gene–gene interaction between GNA14 and RGS20, the details of this interaction remain to be clarified. Although GNA14 is much less promiscuous than GNA15 or GNA16, it has the ability to interact with several selective Gi-coupled receptors, such as α2-adrenergic, δ-opioid, ORL1 and SSTR3, to increase IP formation (*36*). On the other hand, it has also been shown that transfected RGSZ1 blocked mitogen-activated protein kinase activity induced by an α2-adrenergic receptor agonist (*37*). Taken together, these findings may indicate that GNA14 and RGS20 can interact through α2-adrenergic receptor. However, how the gene–gene interaction observed in the present study is functionally related to hypertension needs to be biologically determined.

In the present study, we used the MDR method to evaluate gene–gene interactions. The MDR method has been widely used for statistical mining of gene–gene and gene–environmental interactions (*38*, *39*). The dimensionality reduction approach seeks to identify combinations among multilocus genotypes that are associated with high risk of disease as well as combinations associated with low risk. Thus, MDR defines a single variable that incorporates information from several loci and/or environmental factors that can be divided into high-risk and low-risk combinations. This new variable can

Table 7. Multiple Dimensionality Reduction Method–Proved Risk Genotype and Hypertension in a General Population

	Risk genotype	Non-risk genotype
NT	1,401	2,277
HT	1,335	1,857

$\chi^2=9.93$, $p=0.0016$, odds ratio 1.168 [1.061–1.287]. Risk genotype: RGS20: CC+CG; GNA14: CC+TC. NT, normotensives; HT, hypertensives.

Table 8. MDR-Determined Genotype and Blood Pressure Values in General Population

	Non-risk genotypes	Risk genotype	*p*
n	5,625	3,667	
SBP (mmHg)	132.25±19.19	133.08±19.46	0.042
DBP (mmHg)	79.01±11.32	79.65±11.49	0.019

Model 2 (corrected for age and sex): *p* value of genotype for SBP is 0.131, and for DBP is 0.0295. Model 3 (further corrected for region, body mass index, use of antihypertensive drugs, total cholesterol, high-density lipoprotein cholesterol, triglyceride, and fasting glucose): *p* value of genotype for SBP is 0.187, and for DBP is 0.0294. MDR, multiple dimensionality reduction method; SBP, systolic blood pressure; DBP, diastolic blood pressure.

be evaluated for its ability to classify and predict disease risk status using cross-validation and permutation testing (*27*). The findings obtained by the MDR method need to be further confirmed not only in a separate population, but also by functional analysis.

The present study has several important limitations, including the threshold that was used to identify a positive gene (*40, 41*). We used the statistical threshold of a *p* value less than 0.05. However, since we evaluated more than 300 genes at the same time, it would be have been more appropriate to use the probability threshold of Bonferroni correction—*i.e.*, $p<0.00016$ (=0.05/307) instead of 0.05. With this strict definition, there were no significant SNPs related to hypertension in the case-control study. However, since we used the present findings to further extend the research, it could be appropriate to adopt a weaker criterion to pick up positive SNPs. The statistics should be used as a guide to make decisions on whether further study would be necessary with the given probability (*40*).

To determine whether a finding with an observed *p*-value is noteworthy for publication, it has also been postulated that false-positive report probability should be evaluated based on the prior probability in the case-control association study with candidate genes (*42, 43*). False-positive report probability has been shown to be influenced by statistical power, sample size, odds ratio, minor allele frequency, and *p* value of the association. In the present replication study, we had set moderate to low prior probability, *i.e.*, 0.1–0.01, since associations between genes and hypertension were already observed in the case-control study. The risk genotype obtained by the MDR method showed a false-positive report probability ranging from 0.02–0.2 with moderate to low prior probability, indicating that the observed positive findings are worthy of reporting (*43*).

The CD/CV hypothesis and common disease rare variant hypothesis have been debated (*28–41*). In the present study, candidate SNPs were selected on the basis of the common variant hypothesis. A multiple candidate gene approach for directing "pathwayomics" revealed that the effect of each SNP was small. Although exact replication of the original findings was not observed in the general population study, we did detect a gene–gene interaction which could have furthered increased the power of the effect of genes not only on hypertension but also on blood pressure variability. Again, however, the odds ratio was not high. Accordingly, the present findings were not consistent with the CD/CV hypothesis, since this hypothesis requires that the common variant should have at least a moderate effect on the phenotype (*28*). However, we could not eliminate the possibility that the common variants have only small effects and hypertension consists of numerous combinations of common variants with small effects.

In a study evaluating the effects of inbreeding on blood pressure as a quantitative trait within Croatian island isolates, a model of the distribution of locus effects suggested that the 8–16 quantitative trait loci (QTLs) of largest effect together account for a maximum of 25% of the dominance variation, while the remaining 75% of the variation is mediated by QTLs of very small effect, unlikely to be detectable using current technologies and sample sizes (*44*). The small but significant and independent effect on blood pressure variability observed in the high-risk genotype in the present study may support their findings. There is a possibility that numerous common genes with a small effect on blood pressure, either alone or in combination with other genes in the pathways, are responsible for a major part of blood pressure variation. The present findings may provide evidence that a common variant with a small effect, actually too small to influence blood pressure by itself, could be related to blood pressure variation and hypertension through combination with other common variants with a small effect allele in the same pathway. Since the effects (odds ratios) of the genes are very small, they could not be detected if their allele frequencies are low. These findings may underlie the failure of replication of candidate gene findings.

In the first case-control studies, 10 of 38 positive SNPs were found to deviate from the Hardy-Weinberg equilibrium (HWE). It has been shown that several underlying mechanisms may cause the violation of HWE (*45*). However, the first study was a screening study to pick up the possible posi-