tion between the genotype and the BMI among the groups (Table 5).

Table 2. Genotype distribution in NT subjects and EH patients

	Total		Males		Females	
	NT	EH	NT	EH	NT	EH
Number of subjects	262	317	184	193	78	124
Genotype						
9,11	0 (0.0%)	1 (0.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (0.8%)
11,11	203 (77.5%)	264 (83.3%)	145 (78.8%)	158 (81.9%)	58 (74.4%)	106 (85.5%)
11,12	1 (0.4%)	1 (0.3%)	1 (0.5%)	0 (0.0%)	0 (0.0%)	1 (0.8%)
11,15	1 (0.4%)	2 (0.6%)	1 (0.5%)	1 (0.5%)	0 (0.0%)	1 (0.8%)
11,16	50 (19.1%)	43 (13.6%)	31 (16.8%)	29 (15.0%)	19 (24.4%)	14 (11.3%)
11,17	4 (1.5%)	1 (0.3%)	3 (1.6%)	1 (0.5%)	1(1.3%)	0 (0.0%)
11,18	0 (0.0%)	1 (0.3%)	0 (0.0%)	1 (0.5%)	0 (0.0%)	0 (0.0%)
15,16	1 (0.4%)	0 (0.0%)	1 (0.5%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
16,16	2 (0.8%)	3 (0.9%)	2 (1.1%)	2 (1.0%)	0 (0.0%)	1 (0.8%)
16,19	0 (0.0%)	1 (0.3%)	0 (0.0%)	1 (0.5%)	0 (0.0%)	0 (0.0%)
11,11	203 (77.5%)	264 (83.3%)	145 (78.8%)	158 (81.9%)	58 (74.4%)	106 (85.5%)
11,16	50 (19.1%)	43 (13.6%)	31 (16.8%)	29 (15.0%)	19 (24.4%)	14 (11.3%)
others	9 (3.4%)	10(3.2%)	8 (4.3%)	6 (3.1%)	1(1.3%)	4 (3.2%)
P value	0.187		0.706		0.039*	
Allele						
9	0 (0.0%)	1 (0.2%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (0.4%)
11	462 (88.2%)	577 (91.0%)	326 (88.6%)	348 (90.2%)	136 (87.2%)	229 (92.3%)
12	1 (0.2%)	1 (0.2%)	1 (0.3%)	0 (0.0%)	0 (0.0%)	1 (0.4%)
15	2 (0.4%)	2 (0.3%)	2 (0.5%)	1 (0.3%)	0 (0.0%)	1 (0.4%)
16	55 (10.5%)	50 (7.9%)	36 (9.8%)	34 (8.8%)	19 (12.2%)	16 (6.5%)
17	4 (0.8%)	1 (0.2%)	3 (0.8%)	1 (0.3%)	1 (0.6%)	0 (0.0%)
18	0 (0.0%)	1 (0.2%)	0 (0.0%)	1 (0.3%)	0 (0.0%)	0 (0.0%)
19	0 (0.0%)	1 (0.2%)	0 (0.0%)	1 (0.3%)	0 (0.0%)	0 (0.0%)
16	55 (10.5%)	50 (7.9%)	36 (9.8%)	34 (8.8%)	19 (12.2%)	16 (6.5%)
others	469 (89.5%)	584 (92.1%)	332 (90.2%)	352 (91.2%)	137 (87.8%)	232 (93.5%)
P value	0.124		0.645		0.046*	

NT, normotensives; EH, essential hypertension
*, $p<0.05$.

Table 3. The association between genotype and phenotype

	Total			Males			Females		
genotype	without 16 repeat	with 16 repeat	P value	without 16 repeat	with 16 repeat	P value	without 16 repeat	with 16 repeat	P value
No. of subjects	479	100		311	66		168	34	
SBP	146.4±33.7	143.1±35.4	0.387	142.9±31.9	143.1±35.0	0.950	152.9±35.8	143.1±36.8	0.152
DBP	90.5±21.5	88.2±21.8	0.324	89.7±21.9	88.9±21.7	0.783	92.0±20.9	86.8±22.3	0.189
Pulse	76.5±14.2	77.0±14.1	0.775	76.1±14.5	76.7±15.3	0.785	77.1±13.7	77.5±11.6	0.889

SBP; systolic blood pressure, DBP; diastolic blood pressure

Table 4. Plasma BNP levels for patients with or without 16 repeat

	Total				Male				Female			
	NT		EH		NT		EH		NT		EH	
	No. of subjects	BNP (pg/ml)	No. of subjects	BNP (pg/ml)	No. of subjects	BNP(pg/ml)	No. of subjects	BNP (pg/ml)	No. of subjects	BNP(pg/ml)	No. of subjects	BNP(pg/ml)
without 16 repeat	33	5.3±5.3	31	17.0±32.2	26	4.4±5.0	18	10.8±11.6	7	8.4±5.1	13	25.5±47.6
with 16 repeat	10	4.3±3.5	2	8.2±2.6	7	3.4±2.3	2	8.2±2.6	3	6.5±5.5	0	—
p value		0.611		0.703		0.628		0.748		0.602		—

Table 5. Distribution of subjects by BMI

	Total				Males				Females			
	Lean	Normal	Obese	P value	Lean	Normal	Obese	P value	Lean	Normal	Obese	P value
without 16 repeat	22	285	134		13	186	88		9	99	46	
with 16 repeat	1	58	33		1	35	24		0	23	9	
p value				0.179				0.262				0.345

Lean;18.5>BMI, Normal;18.5<BMI<25, Obese;25<BMI

4. Discussion

Ogawa *et al.* reported that the 5'-flanking region of the 1.9 kilo-base pairs in the *NPPB* gene has a high transcriptional activity. Using the deletion mutant model, the deletion of sequences between -1288 and -1095 reduced transcriptional activity to approximately 30%. These deleted sequences contain a characteristic CT-rich region (-1248 to -1191), followed by an Alu family sequence (-1190 to -934). Thus, these results are consistent with the hypothesis that Alu repeat sequences in the 5'-flanking regions have regulatory roles in *NPPB* expression [14]. Therefore, we assessed the association between mutations or polymorphisms in the 5'-flanking region of *NPPB* and the presence of EH. In the present study, a novel VNTR was discovered, and a significant association between the VNTR and EH was found in female patients. The present study found that the number of patients with a 16 repeat allele of VNTR was lower in EH women than in NT women. It is well known that the plasma BNP level is higher in EH patients than in NT subjects, since an elevated blood pressure results in a high plasma BNP level, which is one of the protective factors for hypertension [4]. We compared the plasma BNP levels of patients with and without the 16 repeat allele and found that there was no significant difference between the two groups. In fact, there were not enough subjects to allow the association studies to be done by gender. Given our results, these limitations should be addressed in future studies. It is possible that factors other than the *NPPB* gene may have affected the BNP levels, since many factors, including cardiac function and blood pressure, are known to affect human plasma BNP levels. Thus, it is possible that the plasma BNP level is not an accurate reflection of the function of the *NPPB* gene.

In the present study, the overall distribution of the VNTR genotype and the allele frequency were significantly different in females but not in males. Gender-specific susceptibility to EH is an interesting finding, but its importance is still unclear [11]. Redfield *et al.* reported that BNP levels increased with age, and were higher in females than males among subjects with no known cardiovascular or detectable structural heart disease [18]. Maffei *et al.* reported that hormone replacement therapy increased BNP levels in postmenopausal women [19]. Although the absolute BNP value was different between these two studies, which used two different assays, the associations of the BNP levels with age and gender were consistent. Furthermore, the BNP level that had the optimal sensitivity and specificity for detecting systolic dysfunction in the overall population increased with age and was higher in women. This underscores the clinical relevance of the relationship of age, gender, and BNP. In both studies that used different assays, the effect of gender on BNP was substantial and independent of other factors [18]. Unfortunately, we were not able to obtain samples to measure plasma BNP and ANP levels, due to the difficulty in obtaining written informed consent for blood examinations from subjects not receiving medications.

In the Japanese population, it has been reported that plasma BNP levels are positively associated with age, urinary salt excretion, higher blood pressure, a high R-wave voltage in the 12-lead ECG (Code 3-1 or 3-3), and female gender [20,21]. On the other hand, Freitag *et al.*, based on multivariate models adjusting for known risk factors, showed that elevated plasma BNP levels were associated with an increased risk of blood pressure progression in males but not in females. However, there was no significant trend of an increasing incidence of hypertension among BNP categories in either males or females. In a community-based sample, higher plasma BNP levels were found to be associated with an increased risk of BP progression in males, but not in females [22]. Further studies are needed to resolve these conflicting results.

Since there are many loci with a high degree of polymorphism in the number of tandemly repeated nucleotide sequence units, VNTR polymorphisms, also called minisatellites, were originally studied for linkage-mapping purposes. VNTRs have a highly polymorphic nature that makes them very useful as markers, both in linkage studies to map disease loci in families and in forensic applications. Recent reports indicate that some VNTR sequences may function as transcriptional or translational regulators, and that they may modify the function of a protein when the tandemly repeated region lies within the coding region of the gene [23]. Although no clear effect on transcription has been shown, it has been reported that a VNTR in the second intron of the serotonin transporter gene is associated with susceptibility to major depression [24].

We previously determined the structural organization of human natriuretic peptide receptor genes [25-28] and identified an insertion/deletion mutation in the 5'-untranslated region of NPRA [12]. The deletion encompasses eight nucleotides and alters the binding sites for the AP2 and zeste transcription factors. Transcriptional activity of the deletion allele was less than 30% that of the wild-type allele. The deletion allele was significantly more common in the EH group than in the NT group. These findings suggest that in Japanese individuals, this deletion in the NPRA gene reduces receptor activity and may confer increased susceptibility for the individual to develop EH or left ventricular hypertrophy (LVH). Animal models with a deletion of this gene develop disorders that resemble the symptoms of subjects with a deleted allele in the 5'-untranslated region of NPRA. We previously isolated a missense mutation of the NPRA gene [29] and a VNTR polymorphism upstream of the NPRC gene; this VNTR influences blood pressure levels in obesity-associated hypertension [30]. Since the sampling of the above reports was different from the present experiment, it was impossible to analyze the relationship between systemic natriuretic peptide genes and EH.

Wang *et al.* reported that obese individuals have low circulating natriuretic peptide levels, which may contribute to their susceptibility to hypertension and

hypertension-related disorders. The mechanisms linking obesity to hypertension have not been established, but sodium retention and excessive sympathetic tone are key contributors. Natriuretic peptides are important regulators of sodium homeostasis and neurohormonal activation; this raises the possibility that obese individuals have an impaired natriuretic peptide response [31]. Therefore, we examined the relationship between the genotype and BMI and found no significant association.

In conclusion, a novel VNTR in the 5'-flanking region of the *NPPB* gene was discovered. This polymorphism was associated with EH in female subjects. However, this finding does not necessarily imply that there is a relationship between the *NPPB* gene and EH. Further studies of other polymorphisms are needed to determine whether there is an association between the *NPPB* gene and EH.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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Association between prostaglandin E2 receptor gene and essential hypertension

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Abstract

Background: Essential hypertension (EH) is a complex multifactorial polygenic disorder that is thought to result from an interaction between an individual's genetic makeup and various environmental factors. In the kidney, prostaglandins (PGs) are important mediators of vascular tone and salt and water homeostasis, and are involved in the mediation and/or modulation of hormonal action. In previous studies, mice deficient in the prostaglandin E2 (PGE_2) EP2 receptor had resting systolic blood pressure (BP) that was significantly lower than that of wild-type controls. The BP of those mice increased when they were put on a high-salt diet, suggesting that the EP2 receptor is involved in sodium handling by the kidney. In the present study, we investigated the association between EH and nucleotide polymorphisms in the gene encoding the prostaglandin E2 receptor subtype EP2 (PTGER2).
Methods: We selected three single-nucleotide polymorphisms (SNP) in the human PTGER2 gene (rs1254601, rs2075797, and rs17197), and we performed a genetic association study of 266 EH patients and 253 age-matched normotensive (NT) controls.
Results: There was no significant difference in overall distribution of genotypes or alleles of any of the SNP between the EH and NT groups. However, among men, the A/A type of the SNP rs17197 (rs17197, A/G in 3′UTR) was significantly more frequent in EH subjects than in NT subjects ($P = 0.041$).
Conclusion: The present findings suggest that rs17197 is useful as a genetic marker of EH in men.

1. Introduction

Prostaglandins (PGs) are ubiquitous lipid mediators derived from cyclooxygenase metabolism of arachidonic acid. PGs have a broad range of physiologic activities, including modulation of inflammation, ovulation and arterial blood pressure. In the kidney, PGs are important mediators of vascular tone and salt and water homeostasis, and are involved in the mediation and/or modulation of hormonal action [1]. PGs comprise a diverse family of autacoids derived from cyclooxygenase (COX)-mediated metabolism of arachidonic acid to prostaglandin G/H_2 (PGG/H_2), which generates five primary bioactive prostanoids: PGE_2, $PGF_2\alpha$, PGD_2, thromboxane A_2 (TXA_2), and PGI_2 [2,3]. PGE_2, a major product of cyclooxygenase activity, affects blood pressure and fertility, although the specific G protein-coupled receptors that mediate these effects are poorly defined. PGE_2 exerts biological effects via four receptor subtypes: EP1, EP2, EP3 and EP4 [4]. The human EP2 (PTGER2) gene is located at chromosome 14q22, spans approximately 18.1 kilo-base pairs (kbp), and contains 2 exons [5,6]. Recent studies indicate that targeted disruption of PTGER2 interferes with fertility and may result in hypertension [7]. These findings

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suggest that mutations in the human PTGER2 gene can cause human hypertension. Thus, PTGER2 is considered a candidate causal gene of EH [7].

Hypertension affects 25% of adults in most populations, and is a major risk factor for death from stroke, myocardial infarction, and congestive heart failure [8]. The most prevalent form of hypertension is essential hypertension (EH). EH is considered to be a multifactorial disease [9]. Several reports indicate that the angiotensinogen gene [10] or the angiotensin-converting enzyme gene [11] is involved in susceptibility to EH. Population-based case-control studies are a useful method of testing for genetic association between a trait and a marker [12].

The aim of the present study was to investigate the relationship between EH and human PTGER2 gene single-nucleotide polymorphisms (SNPs) in a Japanese population.

2. Materials and methods

2.1. Subjects

The EH subjects were 266 patients who were diagnosed with EH according to the following criteria: seated systolic blood pressure (SBP) $\geq$160 mmHg or diastolic BP (DBP) $\geq$100 mmHg, on three occasions within 2 months after the first medical examination. None of the EH subjects was using antihypertensive medication or Cox inhibitors (NSAIDs). Patients with secondary forms of hypertension were excluded based on the results of clinical and laboratory examinations: (1) measurement of fasting blood sugar, glycosylated hemoglobin A_{1C}, plasma aldosterone, plasma renin activity and plasma catecholamine; and (2) computed tomography and magnetic resonance imaging to assess the condition of adrenal glands and check for pituitary tumors. For comparison, we included 255 healthy normotensive (NT) controls. None of the NT subjects had a family history of hypertension, and all NT subjects had a SBP of $<$130 mmHg and a DBP of $>$85 mmHg. A family history of hypertension was defined as prior diagnosis of hypertension in a grandparent, uncle, aunt, parent, or sibling. Both groups were recruited from the northern area of Tokyo, Japan. Informed consent was obtained from each subject according to a protocol approved by the Human Studies Committee of Nihon University [13].

2.2. Biochemical analysis

Blood samples were obtained from subjects in the morning after a rest in the sitting position after at least 30 min without eating. In the clinical laboratory department of our university hospital, these blood samples were used to measure the plasma concentration of total cholesterol, the serum concentrations of creatinine and uric acid, and plasma renin activity (PRA) and aldosterone concentration (PAC) [14].

2.3. Genotyping

Using information about allelic frequencies of SNP registered on the website of the National Center for Biotechnology Information (NCBI) and Celera Discovery System–Applied Biosystems, we selected three SNP in the human PTGER gene with minor allele frequencies $>$20%. We examined association between EH and these three SNP. All three SNP were confirmed using the NCBI website; their accession numbers were rs1254601, rs2075797, and rs17197 (Fig. 1). Genotypes were determined using assays-on-demand kits (Applied Biosystems, Branchburg, NJ) together with TaqMan polymerase chain reaction (PCR) (Applied Biosystems) [13].

2.4. Statistical analysis

Data are shown as means$\pm$SD. Hardy–Weinberg equilibrium was assessed using two methods of analysis. The overall distribution of alleles was analyzed using $2*2$ contingency tables, and the distribution of the genotypes between EH patients and NT control subjects was analyzed using a two-sided Fisher exact test. To assess the contribution of confounding factors, multiple logistic regression analysis was performed. A probability level of $P<0.05$ was considered to indicate statistical significance. Differences in clinical data between the EH and NT groups were assessed using analysis of variance followed by Fisher's protected least-significant-difference test [15].

3. Results

Table 1 shows the clinical features of the EH patients and NT control subjects. There were no significant differences in age, serum concentration of creatinine, or plasma concentration of total cholesterol or HDL cholesterol between the two groups.

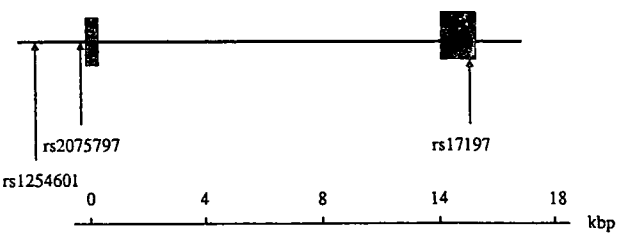

Fig. 1. Organization of the gene encoding the human prostaglandin E2 receptor subtype EP2 (PTGER2), and location of single-nucleotide polymorphisms (SNP) used in present association study. Closed boxes indicate exons, and lines represent introns.

Table 1
Characteristics of study participants

	Total			Men			Women		
	NT	EH	*p*-value	NT	EH	*p*-value	NT	EH	*p*-value
Number of subjects	253	266		171	167		82	99	
Age (years)	51.8±9.2	51.9±8.0	0.896	52.3±6.9	50.9±7.0	0.057	50.8±12.8	53.7±9.3	0.078
BMI (kg/m^2)	22.8±3.1	24.6±3.7	<0.001*	22.9±3.1	24.8±3.6	<0.001*	22.4±3.1	24.2±3.9	0.001*
SBP (mmHg)	112.4±10.7	172.2±20.7	<0.001*	113.0±10.4	171.0±19.0	<0.001*	111.2±11.3	174.3±23.2	<0.001*
DBP (mmHg)	69.3±8.5	105.8±13.7	<0.001*	69.8±8.1	107.5±13.1	<0.001*	68.1±9.3	103.0±14.4	<0.001*
Pulse (beats/min)	72.0±10.4	76.8±14.5	<0.001*	71.8±10.9	76.5±14.7	0.005*	72.5±9.6	77.3±14.2	0.033*
Creatinine (mg/dl)	0.83±0.20	0.84±0.24	0.575	0.90±0.19	0.93±0.22	0.996	0.68±0.13	0.68±0.17	0.186
Total cholesterol (mg/dl)	202.3±40.2	210.7±36.2	0.015*	198.6±38.1	205.5±34.2	0.090	209.8±43.3	219.3±37.7	0.123
HDL cholesterol (mg/dl)	56.8±17.1	56.2±17.5	0.729	54.7±16.0	52.5±16.5	0.242	61.3±18.7	62.6±17.6	0.649
Uric acid (mg/dl)	5.51±1.70	5.67±1.58	0.293	5.84±1.36	6.26±1.51	0.010*	4.76±2.12	4.60±1.05	0.530
PRA (ng/ml/h)	3.1±7.9	2.4±6.2	0.554	4.2±10.1	2.7±6.9	0.360	1.3±1.0	1.8±4.5	0.643
PAC (pg/ml)	94.4±50.2	116.9±54.9	0.054	90.6±53.5	116.6±55.6	0.141	97.3±49.3	117.5±53.9	0.203

NT, normotensives, EH, essential hypertensives.
BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein.
PRA, plasma renin activity; PAC, plasma aldosterone concentration.

Table 2 shows the distribution of genotypic and allelic frequencies of the 3 SNP in each group. The observed and expected genotypic frequencies of each SNP in the total study group and among both men and women in the NT group were in good agreement with predicted Hardy–Weinberg equilibrium values (data not shown). There was no significant difference in overall distribution of genotype in the additive model of all three SNP between the total EH and total NT groups. Among men, the genotypic frequency of the A/A type of the SNP rs17197 (A/G in 3′UTR) was significantly greater for EH subjects than for NT subjects ($P = 0.041$). Also among men, the frequency of the G allele of the SNP rs17197 (A/G and G/G genotypes) was significantly greater for EH subjects than for NT subjects ($P = 0.029$). Multiple logistic regression analysis revealed that the G /G genotype of the SNP rs17197 was significantly associated with EH in men (odds ratio = 1.59; 95% confidence interval = 1.02 to 2.48), even after adjustment for age.

4. Discussion

PGs are bioactive lipids that modulate a broad spectrum of biologic processes including reproduction and circulatory homeostasis [16,17]. Previous studies have assessed the relationship between hypertension and PGs associated with vascular constriction/dilation such as prostacyclin, thromboxane and PGE_2 [18,19].

We previously reported that mutations of the human prostacyclin synthase gene are associated with EH. In a study of 300 subjects (150 EH subjects, and 150 healthy controls), a nonsense mutation in exon 2 of the prostacyclin synthase gene was found in one female patient. Three of that patient's five living siblings had the same mutation, and all three of them were hypertensive [20]. Furthermore, we discovered a T-to-C mutation of the second base pair in the splicing region of intron 9. *In vitro* transcription of a minigene construct including this mutation resulted in the skipping of exon 9. Three-dimensional structural analysis suggests that this mutation results in the loss of the heme-binding site, which is critical for enzymatic activity. In a study of 400 subjects (200 EH subjects, and 200 control NT subjects), this mutation was found in only one subject, who was in the EH group. Every relative of that patient who had the same mutation exhibited hypertension, and had significantly reduced urinary PGI_2 metabolites, compared to normal levels [21]. These observations suggest that the risk of EH varies with prostacyclin activity.

For several years, it has been known that PGE_2 is involved in BP homeostasis, but its actions are complex and involve regulation of vascular tone and sodium balance [22]. PGE_2 is unique in that it acts via four different receptors (EP1, EP2, EP3 and EP4), which have distinct but overlapping tissue distributions and activate different intracellular signaling pathways [23]. Prostanoids are abundantly produced in the kidney [2]. EP1, EP3 and EP4 receptors are present in the collecting duct. EP4 has also been shown to be expressed in the glomerulus, and EP2 mRNA was found to be restricted to the outer and inner medulla in the rat kidney. Reverse transcription-PCR analysis of microdissected resistance vessels and nephron segments has revealed EP2 expression in the descending thin limb of Henle's loop and in the vasa recta of the outer medulla [24,25]. All four PGE_2 receptors bind PGE_2 with a higher affinity than they exhibit for other endogenous prostanoids. In terms of amino acid homology, they are not as closely related to each other as they are to other prostanoid receptors

Table 2
Genotype and allele distributions in normotensives and patients with EH

Number of participants			Total			Men			Women		
			NT	EH	*p*-value	NT	EH	*p*-value	NT	EH	*p*-value
			253	266		171	167		82	99	
Variants											
rs1254601	Genotype	C/C	120(0.474)	119(0.447)	0.703	79(0.461)	74(0.443)	0.819	41(0.500)	45(0.454)	0.826
		C/T	106(0.418)	113(0.424)		76(0.444)	7(0.443)		30(0.365)	39(0.393)	
		T/T	27(0.106)	34(0.127)		16(0.093)	19(0.113)		11(0.134)	15(0.151)	
		C/C and C/T	226(0.893)	232(0.872)	0.538	155(0.906)	148(0.886)	0.542	71(0.865)	84(0.848)	0.740
		T/T	27(0.106)	34(0.127)		16(0.935)	19(0.113)		11(0.134)	15(0.151)	
		C/C	120(0.474)	119(0.447)	0.538	79(0.461)	74(0.443)	0.727	41(0.50)	45(0.454)	0.542
		T/T and C/T	133(0.525)	147(0.552)		92(0.538)	93(0.556)		41(0.50)	54(0.545)	
	Allele	C	346(0.683)	351(0.659)	0.410	234(0.829)	155(0.775)	0.133	112(0.682)	129(0.651)	0.528
		T	160(0.316)	181(0.340)		48(0.170)	45(0.225)		52(0.317)	69(0.348)	
rs17197	Genotype	A/A	88(0.347)	112(0.421)	0.120	55(0.321)	73(0.437)	0.041*	33(0.402)	39(0.393)	0.993
		A/G	134(0.529)	117(0.439)		98(0.573)	73(0.437)		36(0.439)	44(0.444)	
		G/G	31(0.122)	37(0.139)		18(0.105)	21(0.125)		13(0.158)	16(0.161)	
		A/A and A/G	222(0.874)	229(0.860)	0.576	153(0.894)	146(0.874)	0.556	69(0.841)	83(0.838)	0.955
		G/G	31(0.122)	37(0.139)		18(0.105)	21(0.125)		13(0.158)	16(0.161)	
		A/A	88(0.347)	112(0.421)	0.087	55(0.321)	73(0.437)	0.029*	33(0.402)	39(0.393)	0.907
		G/G and A/G	165(0.652)	154(0.578)		116(0.678)	94(0.562)		49(0.597)	60(0.6061)	
	Allele	A	310(0.612)	341(0.640)	0.346	208(0.608)	219(0.655)	0.201	102(0.621)	122(0.616)	0.910
		G	196(0.387)	191(0.359)		134(0.391)	115(0.344)		62(0.378)	76(0.383)	
rs2075797	Genotype	C/C	83(0.328)	93(0.349)	0.871	53(0.309)	62(0.539)	0.316	30(0.365)	31(0.0.313)508	0.239
		C/G	130(0.513)	133(0.500)		99(0.561)	80(0.454)		34(0.414)	53(0.535)	
		G/G	40(0.158)	40(0.150)		22(0.128)	25(0.531)		18(0.219)	15(0.151)	
		C/C and C/G	213(0.841)	226(0.849)	0.808	149(0.871)	142(0.539)	0.576	64(0.780)	84(0.848)	0.238
		G/G	40(0.158)	40(0.150)		22(0.128)	25(0.149)		18(0.219)	15(0.151)	
		C/C	83(0.328)	93(0.349)	0.604	53(0.309)	62(0.371)	0.234	30(0.365)	31(0.313)	0.455
		G/G and C/G	170(0.671)	173(0.650)		118(0.690)	105(0.628)		52(0.634)	68(0.686)	
	Allele	C	296(0.584)	319(0.599)	0.631	205(0.589)	204(0.610)	0.563	94(0.573)	115(0.580)	0.884
		G	210(0.415)	213(0.400)		143(0.410)	130(0.389)		70(0.426)	83(0.419)	

NT, normotensives, EH, essential hypertensives.
*$p<0.05$.

that use similar signaling mechanisms [26,27]. For example, the relaxant/cyclic AMP (cAMP)-coupled EP2 receptor is more closely related to other relaxant prostanoid receptors such as the IP and DP receptors. Similarly, the constrictor/Ca^{2+}-coupled EP1 receptor is more closely related to other Ca^{2+}-coupled prostanoid receptors such as the TP and FP receptors [27]. In a study by Hebert and Breyer, activation of the EP1 receptor increased intracellular calcium levels and inhibited Na^+ and water reabsorption in microperfused collecting ducts *in vitro* [28,29], suggesting that renal EP1 receptor activation contributes to natriuretic and diuretic effects of PGE_2. There have been no reported studies of expression of EP receptors in hypertensive model mice, or of relationships between EP subtypes and EH. In a preliminary report, the data indicated that EP1 receptor knockout mice exhibited hypotension and hyper-reninemia, supporting the theory that the EP1 receptor plays a role in maintenance of blood pressure [30]. Unfortunately, in the present study, we were not able to obtain samples for measurement of sodium intake, sodium excretion, or plasma or urinary prostaglandins, because of the difficulty in obtaining informed consent for examination from subjects not receiving medication.

Recently, the role of the PTGER2 gene in blood pressure control has been evaluated using targeted disruption of this gene (EP2–/–) [7]. EP2–/– mice have slightly elevated baseline systolic blood pressure. When EP2–/– mice were fed a high-salt diet, they developed profound systolic hypertension, whereas wild-type mice fed the same diet showed no change in systolic blood pressure [7,31]. These findings suggest that in individuals with a high-salt diet under otherwise normal conditions, the EP2 receptor facilitates sodium excretion as a means of preventing elevation of blood pressure. Such findings prompted us to conduct the present study of the association between PTGER2 and EH.

In the present study, in light of the known effects of age on BP, we selected closely age-matched subjects for the NT and EH groups. Additionally, we strictly defined NT subjects and omitted anyone whose family history was EH-positive, because such individuals may become hypertensive later in life. We also omitted borderline hypertensive subjects. In the present results, there were no significant differences in the overall distribution of genotypes or alleles of any of the three SNPs between the EH and NT groups. However, among men, the genotype frequency of the SNP rs17197 (A/G in 3′UTR) significantly differed between EH and NT subjects ($P = 0.0414$). In a previous study, male EP2–/– mice (107 ± 2 mmHg) had higher systolic blood pressure than female EP2–/– mice (99 ± 3 mmHg) [4]. Although there have been reports of sexual dimorphism in production of PGE_2 in mice [32,33], none of the available evidence explains the above-mentioned difference in blood pressure levels between those male and female PTGER2 knockout mice. Stapleton et al. reported that expression levels of EP2, EP3 and EP4 receptors were higher in female pro-estrus mice than in male mice, and that expression of all four receptors decreased after trauma in female estrus mice, compared with control estrus mice. These findings suggest that responses of EP receptor expression to traumatic injury are gender-related, and that alterations in specific EP receptor subtypes are involved in immune dysfunction after injury [34]. Genetic association studies have identified genes associated with gender-specific susceptibility to EH [35,36]. The reason for the present finding of a positive association between EH and the SNP rs17197 in men is unclear. Further studies are needed to clarify why there is an association between the PTGER2 gene and EH in men.

In conclusion, SNP rs17197 in the PTGER2 gene was associated with EH, and appears to be a genetic marker for Japanese men.

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Original Article

Common Single Nucleotide Polymorphisms in Japanese Patients with Essential Hypertension: Aldehyde Dehydrogenase 2 Gene as a Risk Factor Independent of Alcohol Consumption

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Essential hypertension (EH) is a multifactorial disorder determined by the interaction of environmental and genetic factors. EH patients' responses to these factors may vary, depending on differences in their genes that determine the physiological systems that mediate the response. The purpose of this investigation was to clarify the contributions of genetic background and lifestyle to EH through an association study using some common single nucleotide polymorphisms (SNPs) that should have functional effects on EH phenotypes. We studied the associations between common SNPs of some causal genes related to EH and lifestyle in a Japanese population. The variants of the causal genes were selected based on their functions, including: obesity (adrenergic, β-3-, receptor: ADRB3), alcohol consumption (aldehyde dehydrogenase 2: ALDH2), water-electrolyte metabolism (guanine nucleotide binding protein [G protein], β polypeptide 3: GNB3), glycometabolism (peroxisome proliferator-activated receptor γ: PPARG), lipometabolism (cholesteryl ester transfer protein, plasma: CETP), atherosclerosis (5,10-methylenetetrahydrofolate reductase [NADPH]: MTHFR), and cellular behavior (gap junction protein, α 4, 37 kD: GJA4). Case-control association analysis showed a significant association between EH and both the ALDH2 (Lys487Glu) and GNB3 (C825T) variants. Logistic regression analysis indicated that body mass index (BMI) is an important risk factor for EH, and that the GG (Glu/Glu) genotype of ALDH2 was an independent risk factor for EH overall and especially for EH in males. There was no interaction between the ALDH2 genotype and alcohol consumption overall or in male subjects. Our results suggest that the ALDH2 genotype is associated with EH independently of alcohol consumption. (*Hypertens Res* 2007; 30: 585–592)

***Key Words*: single nucleotide polymorphisms, essential hypertension, gene-environment interaction, common variants**

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Introduction

Hypertension affects 1 billion people worldwide and is implicated in 7.1 million deaths each year due to ischemic heart disease and stroke (www.who.int/en/index.html). Essential hypertension (EH) is a multifactorial disorder caused by the interaction of environmental and genetic factors. It is most likely that there are several causal genes, which together account for 30% to 50% of the blood pressure variation found among individuals (*1*). It is clear from familial and epidemiological studies that hypertension occurs as a result of a complex interplay between genetic and environmental lifestyle exposures (*2*). EH subjects happen to have inherited an aggregate of genes related to hypertension and/or have been exposed to exogenous factors that predispose them to hypertension.

It is quite remarkable that obesity and salt intake have consistently been shown to be risk factors for hypertension worldwide. Some of the other well-recognized risk factors are alcohol intake, inactivity, and psychosocial stress (*3*). Lifestyle factors have long been recognized as playing an important role in the pathogenesis of EH. Individuals may vary in their responses to these factors depending on differences in their genes that determine the way their physiological systems mediate responses.

During the last several years, many genetic susceptibility variants have been reported to be associated with multifactorial diseases. A few causal variants have been proven to have a functional effect on gene expression or protein structure, resulting in phenotypic differences. Furthermore, among the variants mentioned above, very few are considered common variants. To the best of our knowledge, the human aldehyde dehydrogenase 2 (ALDH2) gene (*4*) is the most famous variant proven to have a relationship between a genetic variant and alcohol consumption as a phenotype. No reports have examined the relationship between multiple common variants and EH.

The purpose of this investigation was to clarify the contributions of genetic background and lifestyle to EH through an association study using common variants that are known to have functional effects in the phenotypes of multifactorial disorders. In particular, we studied the associations between common variants of some causal genes related to lifestyle and EH in a Japanese population. The candidate genes were selected based on their functions, and included: alcohol consumption (aldehyde dehydrogenase 2: ALDH2) (*4*), obesity (adrenergic, β-3-, receptor: ADRB3) (*5*), atherosclerosis (5,10-methylenetetrahydrofolate reductase [NADPH]: MTHFR) (*6*), glycometabolism (peroxisome proliferator–activated receptor γ: PPARG) (*7*), water-electrolyte metabolism (guanine nucleotide binding protein [G protein], β polypeptide 3: GNB3) (*8*), lipometabolism (cholesteryl ester transfer protein, plasma: CETP) (*9*), and cellular behavior (gap junction protein, α 4, 37 kD: GJA4) (*10*). Based on their effects on gene expression or protein structure (Table 1), we chose seven common variants of these causal genes.

Methods

Subjects

The EH group consisted of 261 EH patients diagnosed according to the following criteria: sitting systolic blood pressure (SBP) >160 mmHg and/or diastolic blood pressure (DBP) >100 mmHg on three occasions within 2 months after the first blood pressure reading. None of the subjects were using antihypertensive medications. Subjects diagnosed as having secondary hypertension were excluded. We also studied 271 normotensive (NT) healthy subjects as controls. None of the NT subjects had a family history of hypertension, and they all had an SBP <130 mmHg and a DBP <85 mmHg. A family history of hypertension was defined as a prior diagnosis of hypertension in grandparents, uncles, aunts, parents, or siblings. Daily alcohol intake was assessed by an interviewer. The frequency of drinking during a typical week and the alcohol intake on each occasion were determined and used to calculate the alcohol intake per week, which was then divided by 7 to obtain the average alcohol intake per day. Subjects were asked to estimate their alcohol intake based on "gou" (180 mL), a traditional Japanese drinking unit; a "gou" of Japanese sake contains 20 g of ethanol, while a similar amount (180 mL) of Japanese "shochu" contains 50 g of ethanol, a medium-sized bottle of beer (550 mL) contains 22 g of ethanol, two single shots of whiskey (60 mL) contain 20 g of ethanol, and a glass (120 mL) of wine contains 12 g of ethanol. Both the EH patients and the NT control subjects were recruited from the northern part of Tokyo, and informed consent was obtained from each individual according to a protocol approved by the Human Studies Committee of Nihon University.

Biochemical Analysis

Plasma total cholesterol and high-density lipoprotein (HDL) cholesterol concentrations and serum creatinine and uric acid concentrations were measured at the Clinical Laboratory Department of Nihon University Hospital using previously described methods (*11*).

Genotyping of Single Nucleotide Polymorphisms

After consulting public databases, including PubMed and Online Mendelian Inheritance in Men (OMIM), we selected 7 causal genes that have been characterized and whose association with alcoholism, obesity, diabetes, lipid levels, salt intake, and other metabolic factors has been suggested. We further selected 7 common variants of these genes located in the exons or splice donors that might be expected to affect the function or expression of the encoded protein (Table 1). We

Table 1. Gene Polymorphisms Examined for Association

Gene	Symbol	mRNA position	Polymorphism	Amino acid change	Popular name	Region	dbSNP ID	Assay ID	Locus	Reference
Aldehyde dehydrogenase 2 family (mitochondrial)	ALDH2	1951	G→A	Lys504Glu	Lys504Glu	Exon 12	rs671	C_11703892_10	12q24.2	(*4*)
Adrenergic, β-3-, receptor	ADRB3	387	T→C	Trp64Arg	Trp64Arg	Exon 1	rs4994	C_2215549_20	8p12-p11.2	(*5*)
5,10-Methylenetetrahydrofolate reductase (NADPH)	MTHFR	716	C→T	Ala222Val	C677T	Exon 5	rs1801133	C_1202883_20	1p36.3	(*6*)
Peroxisome proliferator–activated receptor γ	PPARG	132	C→G	Pro12Ala	Pro12Ala	Exon 2	rs1805192	C_1129864_10	3p25	(*7*)
Guanine nucleotide binding protein (G protein), β polypeptide 3	GNB3	1230	C→T	41amino acids deletion	C825T	Exon 10	rs5443	C_2184734_10	12p13	(*8*)
Cholesteryl ester transfer protein, plasma	CETP	1506	A→G	Asp459Gly	D442G	Exon 15	rs2303790	C_790072_1_	16q21	(*9*)
Gap junction protein, α 4, 37 kD (connexin 37)	GJA4	1043	C→T	Ser 319 Pro	C1019T	Exon 2	rs1764391		1p35.1	(*10*)

Table 2. Characteristics of Study Participants

	Total			Men			Women		
	NT	EH	*p* value	NT	EH	*p* value	NT	EH	*p* value
Number of subjects	271	261		182	170		89	91	
Age (years)	51.5±8.6	51.1±5.6	0.525	52.0±6.7	51.0±5.8	0.145	50.4±11.5	51.1±5.3	0.563
BMI (kg/m^2)	22.7±3.6	24.4±4.4	<0.001*	22.8±3.6	24.4±4.6	<0.001*	22.5±3.5	24.4±4.1	0.002*
SBP (mmHg)	112.8±10.8	173.6±20.0	<0.001*	113.2±10.4	171.7±19.0	<0.001*	112.1±11.5	177.1±21.4	<0.001*
DBP (mmHg)	69.7±8.5	105.4±13.4	<0.001*	70.5±8.0	105.4±13.4	<0.001*	68.2±9.2	70.5±8.0	<0.001*
Pulse (beats/min)	73.9±14.3	77.6±15.4	0.011*	73.2±15.6	77.4±16.1	0.032	75.2±11.1	78.2±13.9	0.181
Creatinine (mg/dL)	0.8±0.2	0.9±0.3	0.434	0.9±0.2	0.9±0.2	0.114	0.7±0.2	0.7±0.2	0.569
Total cholesterol (mg/dL)	201.4±42.1	210.6±35.8	0.011*	196.8±39.0	204.9±32.9	0.057	210.8±46.6	220.5±38.5	0.133
HDL cholesterol (mg/dL)	56.2±17.4	57.4±17.8	0.459	53.9±15.7	55.8±17.0	0.973	61.0±19.6	63.6±17.6	0.372
Uric acid (mg/dL)	5.4±1.5	5.6±1.6	0.157	5.8±1.4	6.1±1.5	0.057	4.6±1.3	4.7±1.6	0.676
Hyperlipidmia (%)	19.2	25.3	0.096	15.4	22.4	0.102	27.0	30.8	0.624
Diabetes mellitus (%)	3.3	9.2	0.006*	3.9	10.6	0.021*	2.3	6.6	0.278
Alcohol frequency (%)	58.9	67.8	0.061	71.1	83.3	0.015*	33.9	33.9	0.570
Alcohol consumption (g/day)	21.7±37.3	32.7±45.5	0.010*	29.0±43.2	43.6±50.8	0.013*	7.2±11.5	12.5±22.3	0.101
Smoking (%)	41.7	53.8	0.013*	52.5	63.8	0.056	20.0	34.5	0.057

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

examined the relationship between the genotypes of these genes and hypertension in the study's 532 participants.

Blood samples were collected from all participants, and genomic DNA was extracted from the peripheral blood mononuclear cells by standard procedures. Genotyping was performed using an Assays-on-Demand® and Assays-on-Design kit (Applied Biosystems, Branchburg, USA) (*12*). Both kits included TaqMan PCR. In the 5′ nuclease assay, discrimination occurs during the polymerase chain reaction (PCR) because allele-specific fluorogenic probes, when hybridized to the template, are cleaved by the 5′ nuclease activity of Taq polymerase. The cleavage leads to increased emission of a reporter dye that otherwise is quenched by the dye TAMRA. Each 5′ nuclease assay requires two unlabeled PCR primers and two allele-specific probes. Each probe is labeled with a reporter dye at the 5′ end and TAMRA at the 3′ end. Both VIC and FAM were used as reporter dyes. The PCR method was done using the TaqMan Universal Master Mix (Applied Biosystems) in a 25 μL final reaction volume containing (final concentrations) 50 ng DNA, 700 nmol/L primer,

Table 3. Genotype Distribution in Normotensives (NT) and Patients with Essential Hypertension (EH)

	Total			Men			Women		
	NT	EH	*p* value	NT	EH	*p* value	NT	EH	*p* value
Number of participants	271	261		182	170		89	91	
Variants									
ALDH2									
Genotype									
AA	21 (0.077)	14 (0.054)	0.008*	14 (0.077)	7 (0.041)	0.001*	7 (0.079)	7 (0.077)	0.233
AG	114 (0.421)	81 (0.310)		78 (0.429)	45 (0.265)		36 (0.404)	48 (0.527)	
GG	136 (0.502)	166 (0.636)		90 (0.494)	118 (0.694)		46 (0.517)	36 (0.396)	
Allele									
A	156 (0.288)	109 (0.209)	0.003*	106 (0.291)	59 (0.174)	<0.001*	50 (0.281)	62 (0.341)	0.221
G	386 (0.712)	413 (0.791)		258 (0.709)	281 (0.826)		128 (0.719)	120 (0.659)	
ADRB3									
Genotype									
TT	198 (0.731)	170 (0.651)	0.11	136 (0.747)	110 (0.647)	0.093	62 (0.697)	60 (0.659)	0.853
TA	66 (0.243)	85 (0.326)		42 (0.231)	57 (0.335)		24 (0.270)	28 (0.308)	
AA	7 (0.026)	6 (0.023)		4 (0.022)	3 (0.018)		3 (0.033)	3 (0.033)	
Allele									
T	462 (0.852)	425 (0.814)	0.094	314 (0.863)	277 (0.815)	0.083	148 (0.831)	148 (0.813)	0.65
A	80 (0.148)	97 (0.186)		50 (0.137)	63 (0.185)		30 (0.169)	34 (0.187)	
MTHFR									
Genotype									
CC	104 (0.384)	83 (0.318)	0.275	70 (0.385)	61 (0.359)	0.875	34 (0.382)	22 (0.242)	0.121
CT	123 (0.454)	129 (0.494)		81 (0.445)	78 (0.459)		42 (0.472)	51 (0.560)	
TT	44 (0.162)	49 (0.188)		31 (0.170)	31 (0.182)		13 (0.146)	18 (0.198)	
Allele									
C	331 (0.611)	295 (0.565)	0.131	221 (0.607)	200 (0.588)	0.609	110 (0.618)	95 (0.522)	0.512
T	211 (0.389)	227 (0.435)		143 (0.393)	140 (0.412)		68 (0.382)	87 (0.478)	
PPARG									
Genotype									
CC	261 (0.963)	245 (0.939)	0.192	177 (0.973)	158 (0.929)	0.059	84 (0.944)	87 (0.956)	0.707
CG	10 (0.037)	16 (0.061)		5 (0.027)	12 (0.071)		5 (0.056)	4 (0.044)	
GG	0	0		0	0		0	0	
Allele									
C	532 (0.982)	506 (0.969)	0.198	359 (0.986)	328 (0.965)	0.063	173 (0.972)	178 (0.978)	0.71
G	10 (0.018)	16 (0.031)		5 (0.014)	12 (0.035)		5 (0.028)	4 (0.022)	
GNB3									
Genotype									
CC	72 (0.266)	78 (0.299)	0.036*	50 (0.275)	57 (0.335)	0.022*	22 (0.247)	21 (0.231)	0.732
CT	148 (0.546)	115 (0.441)		100 (0.549)	69 (0.406)		48 (0.539)	46 (0.505)	
TT	51 (0.188)	68 (0.260)		32 (0.176)	44 (0.259)		19 (0.214)	24 (0.264)	
Allele									
C	292 (0.539)	271 (0.519)	0.522	200 (0.549)	183 (0.538)	0.765	92 (0.571)	88 (0.484)	0.527
T	250 (0.461)	251 (0.481)		164 (0.451)	157 (0.462)		86 (0.483)	94 (0.516)	
CETP									
Genotype									
AA	256 (0.945)	240 (0.920)	0.247	170 (0.934)	153 (0.900)	0.321	86 (0.966)	87 (0.956)	0.612
AG	15 (0.055)	19 (0.073)		12 (0.066)	16 (0.094)		3 (0.034)	3 (0.033)	
GG	0	2 (0.007)		0	1 (0.006)		0	1 (0.011)	
Allele									
A	527 (0.972)	499 (0.956)	0.454	352 (0.967)	322 (0.947)	0.02*	175 (0.983)	177 (0.973)	0.494
G	15 (0.055)	23 (0.044)		12 (0.033)	18 (0.053)		3 (0.017)	5 (0.027)	

Table 3. Continued

	Total			Men			Women		
	NT	EH	*p* value	NT	EH	*p* value	NT	EH	*p* value
GJA4									
Genotype									
CC	164 (0.605)	170 (0.651)	0.271	110 (0.604)	112 (0.659)	0.382	54 (0.607)	58 (0.637)	0.672
CT	106 (0.391)	91 (0.349)		71 (0.039)	58 (0.341)		35 (0.393)	33 (0.363)	
TT	1 (0.004)	0		1 (0.006)	0		0	0	
Allele									
C	434 (0.801)	431 (0.826)	0.297	291 (0.799)	282 (0.829)	0.307	143 (0.803)	149 (0.819)	0.711
T	108 (0.199)	91 (0.174)		73 (0.201)	58 (0.171)		35 (0.197)	33 (0.181)	

*Indicates significant difference.

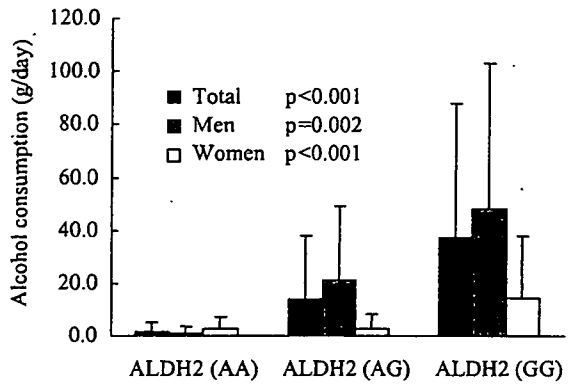

Fig. 1. *Comparison of alcohol consumption between the different ALDH2 genotype groups.*

and 100 nmol/L probe. The thermal cycling conditions were: 95°C for 10 min, then 50 cycles of 92°C for 15 s, and finally 60°C for 1 min. Thermal cycling was performed using the GeneAmp 9700 system.

Each 96-well plate contained 80 samples of unknown genotype and four reactions with reagents but no DNA. The homozygote and no-DNA control samples were necessary for the SDS 7700 signal processing, as outlined in the TaqMan Allelic Discrimination Guide (Applied Biosystems). Direct sequencing or single-stand conformation polymorphism (SSCP) was used to confirm control sample genotypes. The PCR plates were read on the ABI 7700 instrument using the end-point analysis mode of the SDS version v16.3 software package (Applied Biosystems). Genotypes were determined visually based on the dye-component fluorescent emission data depicted in SDS's *X-Y* scatter-plot. Genotypes were also determined automatically by the software's signal processing algorithms. The results of each scoring method were saved in two separate output files and compared later.

Statistical Analysis

The data are presented as means±SD. The Hardy-Weinberg equilibrium was assessed using χ^2 analysis. The overall distribution of alleles was analyzed using 2×2 contingency tables, and the distributions of the genotypes between EH patients and NT subjects were tested using a two-sided Fisher's exact test. Statistical significance was established at $p<0.05$. Differences in clinical data between the EH and NT groups were assessed by analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test.

To assess the contributions of the confounding factors, we performed logistic regression analysis with hypertension as a dependent variable and the following independent variables: body mass index (BMI), alcohol consumption status (0=nondrinker, 1=drinker), metabolic variables (0=no history of either diabetes mellitus or hyperlipidemia; 1=positive history of either), and genotype of each single nucleotide polymorphism (SNP; no susceptibility homozygote + heterozygote=0, susceptibility homozygote=1). The *p* value, odds ratios, and 95% confidence intervals (CIs) were calculated. Differences in alcohol consumption as continuous variables between genotypes were analyzed by one-way ANOVA. A *p* value of less than 0.05 was considered statistically significant. Statistical analyses were done using SPSS software for Windows, version 12 (SPSS Inc., Chicago, USA).

Results

The clinical characteristics of the EH patients and the NT subjects are shown in Table 2. The SBP, DBP, BMI, plasma total cholesterol concentrations, and pulse rate were significantly higher in the EH group than in the NT group. No significant differences in age, serum creatinine concentration, or serum uric acid concentration were observed between the two groups. Male subjects with EH had a higher prevalence of diabetes mellitus and were more likely to drink alcohol, but the prevalence of hyperlipidemia was not significantly different between EH and NT in men.

The distributions of the genotypes and alleles of each SNP

Table 4. Odds Ratios (OR) and 95% Confidence Intervals (CI) for Each Risk Factor and SNP Genotype Associated with Essential Hypertension

Risk factor	Total			Men			Women		
	OR	95% CI	*p* value	OR	95% CI	*p* value	OR	95% CI	*p* value
BMI	1.12	1.06–1.19	<0.001*	1.11	1.04–1.19	0.004*	1.14	1.04–1.25	0.004*
Total cholesterol	1.00	1.00–1.01	0.285		n.u.			n.u.	
Diabetes mellitus	1.81	0.73–4.50	0.204	1.83	0.67–5.04	0.241		n.u.	
Alcohol consumption	1.00	1.00–1.01	0.323	1.01	1.00–1.01	0.146		n.u.	
Smoking	1.60	1.02–2.51	0.041*		n.u.			n.u.	
ALDH2	1.60	1.02–2.51	0.039*	1.93	1.12–3.31	0.018*	0.98	0.53–1.79	0.934
GNB3	1.53	0.91–2.57	0.106	1.38	0.74–2.56	0.310	1.10	0.53–2.26	0.797
ALDH2 and alcohol consumption#	1.01	0.99–1.02	0.257	1.00	0.98–1.02	0.999	0.98	0.93–1.04	0.182

*Indicates significant difference. SNP, single nucleotide polymorphism; BMI, body mass index; n.u., not used. #The interaction between the ALDH2 genotype and alcohol consumption were analyzed.

in the 261 EH patients and 271 NT control subjects are displayed in Table 3. The overall genotype distributions of the ALDH2 (Lys504Glu) and GNB3 (C825T) variants were significantly different between the groups. The overall genotype distributions of other SNPs did not differ significantly. Among men, the allelic distributions of ALDH2 and CETP genes were significantly different between the groups. The distribution of the ALDH2 genotype was also significantly different between drinkers and those who drank rarely or never ($p<0.001$). The ALDH2 genotype was significantly associated with alcohol consumption overall among both males and females (Fig. 1).

A logistic regression analysis was done using variables that showed significant differences in the association studies: BMI, a history of diabetes mellitus, level of total cholesterol, smoking, ALDH2 and GNB3 genotypes, *etc.* The odds ratios, 95% CIs, and *p* values are shown in Table 4. Overall, BMI, smoking, and the GG (Glu/Glu) genotype of ALDH2 were independent risk factors for EH. There was no interaction between the ALDH2 genotype and alcohol consumption overall among either males or females. In men, the odds ratio for the presence of hypertension for the GG (Glu/Glu) genotype of ALDH2 compared with the other genotype was 1.93 (95% CI=1.12–3.31). In women, only BMI was significantly associated with EH.

Discussion

Hypertension is a common phenotype that is considered a multifactorial trait. In concert with environmental or biological factors, genetic factors are thought to raise or lower blood pressure (*3*).

Approximately 50% of hypertensive patients are salt-sensitive; their blood pressure increases in response to sodium intake or volume expansion. The mechanisms that underlie salt sensitivity have not been completely elucidated, although there is evidence that they may be genetically determined. The C825T genetic variant of the GNB3—which is the C-to-T base substitution in exon 9 of the gene that results in the protein lacking 41 amino acids—is considered a genetic risk for developing salt-sensitive hypertension (*13*). The frequency of GNB3/825T has been found to be significantly higher in the Japanese population than in the Caucasian population (*8*). Our results showed that the C825T genotype of GNB3 was significantly different between the EH and control groups ($p<0.05$). This suggests that the GNB3 gene variant is associated with EH in the Japanese population.

The relationship between alcohol consumption and blood pressure elevation is well documented (*14*). Although the mechanism is not clear, it may be mediated partly by the speed of alcohol metabolism, the types of alcoholic beverages consumed, the regularity of drinking, and nutritional status. In the present study, the prevalence of a drinking habit was significantly higher in the EH group in men than in the NT group in men.

ALDH2, the second enzyme of the ethanol metabolic pathway, converts acetaldehyde to acetic acid and plays a major role in acetaldehyde detoxification. A deficiency of ALDH2 activity results from a single nucleotide (G-to-A) substitution at codon 504, which produces a Glu→Lys change at position 504 on the β-subunit and causes the isozyme to be inactive (*15*). ALDH2 enzyme inactivation plays a major role in producing unpleasant symptoms after drinking, such as facial flushing, palpitations, headache, vomiting, and sweating. A study of racial differences in alcohol sensitivity demonstrated that about 50% of Japanese and Chinese populations had a defect in ALDH2 enzyme activity (*16*).

Those with the AA genotype of ALDH2 have a high intolerance to alcohol and do not generally drink alcoholic beverages. In contrast, ALDH2 heterozygotes have an intermediate tolerance and drink about half as much as GG (Glu/Glu) homozygotes overall. However, ALDH2 heterozygotes attain substantially higher blood concentrations of acetaldehyde if they drink alcohol (*17*). Thus, the ALDH2 variant can affect drinking behavior by affecting alcohol metabolism. In our experiment, the ALDH2 genotype actually affected the

amount of alcohol consumed (Fig. 1). Few drinkers had the AA genotype of ALDH2. Although our data showed that subjects with the GG (Glu/Glu) genotype were more likely to have a drinking habit and a higher prevalence of hypertension, the logistic regression analysis revealed that the GG (Glu/Glu) genotype was an independent risk factor for EH overall and especially for EH in males. There was no interaction between the ALDH2 genotype and alcohol consumption overall or in male subjects. Finally, our results suggest that the ALDH2 genotype is associated with EH independently of alcohol consumption.

Several studies in Japan have examined alcohol drinking in relation to hypertension (*18–20*). Two recent reports examined the relationship between ALDH2 genotypes and hypertension in the general population. Amamoto *et al.* found no causal relationship between hypertension and the ALDH2 genotypes *per se* after excluding some confounding factors, particularly alcohol drinking, in the general population (*21*). Takagi *et al.* determined the influence of the ALDH2 genotypes on blood pressure in a large cohort in a population-based study (the Suita study) (*22*). The results of that study also revealed that the GG (Glu/Glu) genotype was a potent risk factor for high blood pressure among men, and that the ALDH2 genotype does not affect sensitivity to alcohol's effect on blood pressure. Both investigations were performed in a general population in Japan, while our study design was a case-control association analysis using EH cases. Although many case-control studies have used logistic regression analysis (*23*), such analysis in case-control studies using population stratification can sometimes yield highly significant results (*24*). Therefore, in our study, it would be unwise to hastily conclude that the GG (Glu/Glu) genotype is a powerful factor independent of alcohol consumption for EH. Unexpectedly, the effect of the ALDH2 genotype on blood pressure or hypertension was almost the same in Takagi's results (*22*) as in our study. Thus, this result is very interesting for identifying EH susceptibility genes beyond lifestyle factors such as drinking.

Recently, the enzyme activity of ALDH2 has been reported to prevent acetaldehyde-induced cell injury *via* extracellular signal–regurated kinase (ERK)1/2 and p38 mitogen-activated protein (MAP) kinase in human vein endothelial cells (*25*). Moreover, it was reported that ALDH2 catalyzes mitochondrial bioactivation of nitroglycerin by the formation of a reactive nitric oxide–related intermediate that activates soluble guanylate cyclase (*26*). This may explain why ALDH2's effect on blood pressure or vessel dilation is independent of alcohol consumption.

Genetic association studies have identified genes associated with gender-specific susceptibility to EH (*27*). The underlying reason for the present study's finding of a positive association between EH and the ALDH2 genotypes in men is unclear. Female hormones may act to protect women from developing high blood pressure (*28*).

Further research involving studies with more detailed data may help clarify the unresolved interaction between alcohol consumption levels and patterns, the relevant ALDH2 genotypes, and hypertension.

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High-Resolution Mapping for Essential Hypertension Using Microsatellite Markers

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Abstract—During the past decade, considerable efforts and resources have been devoted to elucidating the multiple genetic and environmental determinants responsible for hypertension and its associated cardiovascular diseases. The success of positional cloning, fine mapping, and linkage analysis based on whole-genome screening, however, has been limited in identifying multiple genetic determinants affecting diseases, suggesting that new research strategies for genome-wide typing may be helpful. Disease association (case–control) studies using microsatellite markers, distributed every 150 kb across the human genome, may have some advantages over linkage, candidate, and single nucleotide polymorphism typing methods in terms of statistical power and linkage disequilibrium for finding genomic regions harboring candidate disease genes, although it is not proven. We have carried out genome-wide mapping using 18 977 microsatellite markers in a Japanese population composed of 385 hypertensive patients and 385 normotensive control subjects. Pooled sample analysis was conducted in a 3-stage genomic screen of 3 independent case–control populations, and 54 markers were extracted from the original 18 977 microsatellite markers. As a final step, each single positive marker was confirmed by individual typing, and only 19 markers passed this test. We identified 19 allelic loci that were significantly different between the cases of essential hypertension and the controls. (***Hypertension.*** **2007;49:446-452.)**

Key Words: essential hypertension ■ genome-wide ■ association study ■ Japanese ■ new candidate regions

Hypertension is a leading risk factor for cerebrovascular disease, coronary heart disease, and renal failure.[1] It is the major cause of morbidity and mortality and also the third highest risk factor for lifetime burden worldwide.[2,3] Kearney et al[4] reported that there were 972 million hypertension patients in the world, accounting for 26.4% of the adult population in 2000, and predicted that this figure will increase to 1.56 billion patients (29.2%) by 2025. The present pandemic of cardiovascular diseases has been attributed largely to the high prevalence of hypertension, suggesting that more emphasis should be placed on the prevention, detection, and treatment of hypertension.

Elucidation of the genetic etiology of hypertension has been increasingly emphasized as important for a better understanding of the pathogenesis of this disease and for ultimately improving the prevention strategies, diagnostic tools, and therapy in the new millennium.[5] Hypertension is one of the risk factors for coronary heart disease, which is a common complex human genetic disease, and its genetic variance accounts for 30% to 70% of the trait variance.[6,7] The sibling recurrent risk ratio of hypertension is reportedly to be 2 to 3.[8] Each of the hypertension-causing gene recurrent risk ratios is less than the aggregate sibling recurrent risk ratio. There are now many reports describing the results of genome-wide screens for genes controlling blood pressure (BP). The National Heart, Lung, and Blood Institute Genelink project website (https://genelink.nhlbi.nih.gov) lists the National Heart, Lung, and Blood Institute–supported genome scans for BP. The majority of these reports have described numerous chromosomal regions with suggestive evidence of linkage.[9] However, the application of linkage analysis to hypertension, with the exception of obvious Mendelian inheritance, has achieved only limited suc-

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cess thus far.[10,11] Since 2000, ≥6 large genome scans have been reported,[12] namely, an admixture mapping study,[13] a Medical Research Council Program-funded British Genetics of Hypertension Study,[14] the US National Institutes of Health-funded Family Blood Pressure Program studies,[15–18] the Victorian Family Heart Study,[19] the San Antonio Heart Study,[20] and the Quebec Family Study.[21] Except for the admixture mapping study, all of these studies were based on linkage analysis.

In many cases, complex diseases are complicated by genetic heterogeneity and small effects of each gene. In 1996, Risch and Merikangas reported[22] that numerous genetic effects in complex diseases were too weak to be identified by linkage analysis and could be better detected by genomic association studies. Thus, the new challenges to identify disease-predisposing variants in human genome research have resulted in approaches, such as the Hapmap project,[23] high-density single nucleotide polymorphism (SNP) analysis,[24] and microsatellite (MS) association analysis.[25] Disease association studies using MS markers distributed across the human genome every 100 to 150 kb have distinct advantages over linkage analysis, the candidate approach, and SNP typing in terms of linkage disequilibrium (LD).[25] The MS markers are highly polymorphic, showing a high degree of heterozygosity (on average, ≈70%) and LD lengths in the 100- to 200-kb range.[26–31] As compared with MS markers, SNPs have a low degree of genetic polymorphism (biallelic) and have a shorter, by ≈30 kb, LD range, probably because of their older age. Varilo et al[32] reported that highly polymorphic MS markers can provide much greater power for detection of intermarker LD than can either single SNPs or SNP haplotypes on chromosomes 1q and 5q. Therefore, it is possible to carry out substantial whole genome association analysis using a smaller number of MS markers than SNPs (eg, tens of thousands of MS markers versus hundreds of thousands or millions of SNPs). Recently, the usefulness of the haplotype approach and haplotype-tagging SNPs from the HapMap project has been questioned.[33]

Methods

Subjects for MS Typing

A total of 425 (stage 1: 95; stage 2: 131; stage 3: 199) patients with essential hypertension and 467 (stage 1: 103; stage 2: 132; stage 3: 232) normotensive healthy individuals participated in this study. The number of subjects for pooled DNA typing was 95 versus 95 for stage 1, 120 versus 120 for stage 2, and 170 versus 170 for stage 3. After pooled DNA typing, individual typing for the same samples was performed. The difference in number in each stage was derived from the time of sample collection. It was aimed to collect 100 volunteers for each stage each in case and control subjects, but many more subjects were collected beyond our expectations. So, we made the most of all of the subjects to increase the statistical power. The subjects were of Japanese origin from Hokkaido, Tokyo, Kanagawa, Shiga, Osaka, Kyoto, and Ehime. The subjects for the stage 1 and stage 2 screens were recruited from the Millennium Genome Project, and the subjects for the stage 3 screen were recruited from Yokohama City University School of Medicine. The diagnosis of essential hypertension was made according to the guidelines of the Japanese Society of Hypertension (declared in 2000), which include a sitting systolic BP of >140 mm Hg and/or diastolic BP of >90 mm Hg on ≥2 occasions after the first medical examination. Furthermore, subjects in this study were selected as follows, as shown in Table 1. Our criteria were classification as moderate or severe hypertension.

TABLE 1. Characteristics of Subjects

Character	Hypertensive Patients	Normotensive Control Subjects
Gender	Male	Male
Age of onset	30 to 59	≥50
Family history	Within parents and siblings	None
Body mass index, kg/m²	≤25	≤25
BP, mm Hg	Systolic BP≥160 and/or diastolic BP≥100	Systolic BP≤120 and diastolic BP≤80 and no antihypertensive treatment

We obtained informed consent from all of the patients and healthy individuals whose DNA samples were used in the analyses. Our experimental procedures were approved by the relevant ethical committee in each participating university and center. All of the personal identities associated with medical information and blood samples were carefully eliminated and replaced with anonymous identities in each recruiting institution.

Pooled DNA and Genotyping

Ninety-five subjects in stage 1, 120 in stage 2, and 170 in stage 3 were selected from each group (case and control subjects) based on the DNA quality and quantity for DNA pooling analysis. The DNA pooling method was adopted to bring down the cost and the technical burden linked to genotyping thousands of MSs without losing any significant amount of data.

The DNA pooling method for MS typing was carried out by making slight modifications[34] of the protocol of Collins et al.[35] The key factor in this methodology is the absolute equality of individual DNA quantities, so we used a highly accurate quantitative procedure to construct a pooled DNA template for PCR amplification.[35] This pool was composed of strictly measured DNA concentrations, extracted from 95 stage 1, 120 stage 2, and 170 stage 3 Japanese individuals. We checked each DNA concentration ≥3 times and equalized each DNA concentration by dilution. Multiple peak patterns in the pooled DNA showed the distribution of allele frequencies in the subjects.[35] The DNA pooling method enabled us to obtain the allele frequencies of MSs in pooled Japanese individuals by measuring the heights of multiple peaks and to apply this approach to an association study. The quality of the pooled DNA was confirmed by comparing the allelic distributions between individual and pooled typing results using 23 MS markers, unless there was the absence of any significant difference ($P \leq 0.05$) in allele frequencies between pooled DNA typing and individual. This comparison of allele frequencies for the same allele was performed by Fisher's exact test.

DNA was extracted using a QIAamp DNA blood kit (Qiagen) under standardized conditions to prevent variation in DNA quality. This was followed by 0.8% agarose gel electrophoresis to check for DNA degradation and RNA contamination. After measurement of the optical density to check for protein contamination, the DNA concentration was determined through 3 successive measurements using the PicoGreen fluorescence assay (Molecular Probes). Standardized pipetting and aliquoting of the DNA samples were robotically performed using a Biomek 2000 and Multimek 96 (Beckman). The pooled DNA template for typing with 2×18 977 MS markers (first set: case subjects; second set: control subjects) was prepared immediately after DNA quantification. After the initial tests, the 18 977 PCR reaction mixtures containing all of the components except primers were prepared and then aliquoted into 96-well reaction plates and stored until use. The MS pooled typing and individual genotyping procedures after the PCR reaction were carried out according to standard protocols using ABI3700 and 3730 DNA analyzers (Applied Biosystems). The standardized preparations allowed the reproducibility and accuracy to be maintained for the pooled DNA typing throughout the experiment. Various kinds of