Original Article

Association of Genetic Polymorphisms of Endothelin-Converting Enzyme-1 Gene with Hypertension in a Japanese Population and Rare Missense Mutation in Preproendothelin-1 in Japanese Hypertensives

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Endothelin-1 (EDN1), a 21-amino acid peptide, is a potent vasoconstrictor with various pharmacological responses. EDN1 is synthesized from a 212-amino acid precursor protein, preproEDN1, through multiple proteolytic steps. Endothelin-converting enzyme (ECE) cleaves a Trp73–Val74 peptide bond in big-EDN1 to give rise to mature EDN1. In this study, we examined the possible association of genetic variations in *ECE1* with hypertension in a general Japanese population and searched for missense mutations in and around the EDN1 polypeptide. We genotyped 5 single nucleotide polymorphisms (SNPs) in the *ECE1* gene in 1,873 individuals from a general Japanese population and identified one SNP associated with hypertension in women (rs212528: TT vs. TC+CC: odds ratio=1.40; 95% confidence intervals: 1.04–1.89; $p=0.026$), after adjusting for confounding factors. The systolic blood pressure in women with the CC genotype was 6.44 mmHg higher than that in those with the TT genotype ($p=0.007$), after adjusting for the same factors. Next, to identify the missense mutations that may influence the biological activity of EDN1, we sequenced the genomic region that encodes EDN1 in 942 Japanese hypertensive patients. We identified a novel missense mutation, G36R, in one hypertensive patient, but no mutations were observed in EDN1. A gene polymorphism in *EDN1*, Lys198Asn, has been reported to be associated with hypertension in obese subjects. Taken together, these findings reveal that the EDN-ECE pathway is an important system involved in essential hypertension in Japanese. (*Hypertens Res* 2007; 30: 513–520)

Key Words: endothelin, endothelin-converting enzyme, gene variants, hypertension, general population

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Introduction

The endothelin (EDN) system is comprised of 4 active EDNs, with EDN1 being the predominant isoform in the cardiovascular system (1). Because of the potent vasoconstricting and mitogenic effects of EDN1 and its involvement in various cardiovascular diseases, biosynthesis of EDN1 has received considerable attention. EDN1 is synthesized from a 212-amino acid precursor protein, preproEDN1, through multiple proteolytic steps. In the first step, preproEDN1 is cleaved by a signal peptidase, resulting in the formation of proEDN1, which is then cleaved by a furin-like enzyme to yield the 38-amino acid protein known as big-EDN1 (amino acids 53–92) or other intermediates. Big-EDN1 is subsequently cleaved by a unique type II metalloprotease, EDN-converting enzyme-1 (ECE1), to yield EDN1 (amino acids 53–73) (2).

The EDN system is a promising target for the genetic analysis for hypertension. The missense mutation Lys198Asn has been identified in preproEDN1, and several reports have described that this polymorphism showed a positive association with blood pressure elevation in overweight people (3–5), although no significant difference in the EDN1 levels between the Asn-type and Lys-type transfectant was observed in an expression analysis (6). As for *ECE1*, an association between the –338C>A polymorphism in *ECE1* and blood pressure levels in women but not in men has recently been reported (7). This C>A polymorphism is associated with increased promoter activity, as demonstrated in a promoter assay analysis (8).

Complex traits such as hypertension, diabetes mellitus, and hyperlipidemia are suggested to be caused by common sequence variants that may have a small to moderate phenotypic effect (9–11). On the other hand, accumulating data has shown that most Mendelian disorders are caused by a set of different mutations that often reside in coding regions. These rare variants tend to have strong phenotypic effects. Several recent studies have shown that rare genetic variations in *ABCA1*, *APOA1*, and *LCAT* collectively contribute to the variation in plasma levels of high-density lipoprotein (HDL) cholesterol in the general population (12, 13). We hypothesized that rare genetic variations in hypertension candidate genes could collectively contribute to hypertension. To investigate this hypothesis, we have been identifying such mutations in Japanese hypertensive subjects; to date, we have identified missense mutations in the β - or γ -subunit of the amiloride-sensitive epithelial sodium channel encoded by *SCNN1B* and *SCNN1G* (14), a causative gene for pseudohypoaldosteronism type II encoded by serine-threonine kinase *WNK4* (15), the regulator of G-protein signaling 2 (*RGS2*) (16), and the mineralocorticoid receptor encoded by *NR3C2* (17). As the next hypertension candidate gene, we have begun to sequence the *EDN1* gene and to search for missense mutations (18).

In present study, we genotyped the genetic polymorphisms

of one of the EDN-converting enzymes, the *ECE1* gene, in a general Japanese population to examine whether the *ECE1* gene is a susceptibility gene for hypertension. Secondly, to evaluate the EDN system in essential hypertension in Japanese, we re-sequenced the EDN1 polypeptide in the *EDN1* gene in Japanese hypertensive patients to identify missense mutations that may deleteriously affect EDN1 function.

Methods

General Population

The selection criteria and design of the Suita study have been described previously (19, 20). Only those who gave written informed consent for genetic analyses were included in this study. The study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center. In this study, the genotypes of 1,873 samples were determined. The characteristics of the 1,873 participants (863 men and 1,010 women) are shown in Table 1. Routine blood examinations that included total serum cholesterol, HDL cholesterol, triglyceride, and glucose levels were performed. A physician or nurse interviewed each patient regarding smoking and alcohol drinking habits and personal history of cardiovascular disease, including angina pectoris, myocardial infarction, and/or stroke. Blood pressure was measured after at least 10 min of rest in a sitting position. Systolic and diastolic blood pressures (SBP and DBP) were the means of two measurements by well-trained doctors (recorded >3 min apart). Hypertension was defined as SBP of ≥ 140 mmHg, DBP of ≥ 90 mmHg, or the current use of antihypertensive medication (20). Diabetes mellitus was defined as fasting plasma glucose ≥ 7.0 mmol/L (126 mg/dL), non-fasting plasma glucose ≥ 11.1 mmol/L (200 mg/dL), current use of antidiabetic medication, or HbA1c $\geq 6.5\%$. Hyperlipidemia was defined as total cholesterol ≥ 5.68 mmol/L (220 mg/dL) or antihyperlipidemia medication. Body mass index (BMI) was calculated as weight (in kg) divided by height (in m) squared.

Hypertensive Subjects

A total of 942 hypertensive subjects (518 men and 424 women; average age: 65.1 ± 10.5 years) were recruited from the Division of Hypertension and Nephrology at the National Cardiovascular Center. Ninety-two percent of study subjects (870 subjects) were diagnosed with essential hypertension, and the rest had secondary hypertension, including renal hypertension (36 subjects), renovascular hypertension (23 subjects), primary aldosteronism (11 subjects) and hypothroid-induced hypertension (2 subjects) (14–17). The hypertension criteria were blood pressure above 140 and/or 90 mmHg or the use of antihypertensive agents. Blood pressure was the average of three measurements taken in a sitting position after at least 5 min of rest on each occasion. About one-third of the hypertensive subjects had hypertensive cardiovas-

Table 1. Basic Characteristics of Subjects in Japanese General Population (Suita Study)

	Women (n=1,010)	Men (n=863)
Age (years old)	63.3±11.0	66.3±11.1*
Systolic blood pressure (mmHg)	128.0±19.6	131.9±19.5*
Diastolic blood pressure (mmHg)	76.6±9.8	79.7±10.7*
Body mass index (kg/m ²)	22.3±3.2	23.3±3.0*
Total cholesterol (mmol/L)	5.57±0.79*	5.10±0.78
HDL-cholesterol (mmol/L)	1.67±0.40*	1.42±0.36
Current smokers (%)	6.3	30.1 [†]
Current drinkers (%)	29.3	67.0 [†]
Present illness (%)		
Hypertension	38.2	47.4 [†]
Hyperlipidemia	55.2 [†]	27.4
Diabetes mellitus	5.2	12.6 [†]

Values are mean±SD or percentage. Hypertension: systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg or antihypertensive medication; hyperlipidemia: total cholesterol ≥220 mg/dL or antihyperlipidemia medication; diabetes: fasting plasma glucose ≥126 mg/dL or non-fasting plasma glucose ≥200 mg/dL or HbA1c ≥6.5% or antidiabetic medication. * $p < 0.05$ between women and men by Student's *t*-test. [†] $p < 0.05$ between women and men by χ^2 test. HDL, high-density lipoprotein.

cular complications. The clinical features of the patients in this study are summarized in Table 2.

All of the participants for the genetic analysis in the present study gave their written informed consent. The study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center.

Genotyping of Mutations of Single Nucleotide Polymorphisms of the *ECE1* Gene in the General Population

We obtained genetic polymorphisms in the *ECE1* gene using the database of Japanese Single Nucleotide Polymorphisms (JSNP) (<http://snp.ims.u-tokyo.ac.jp/>) (21, 22) and genotyped the following 5 single nucleotide polymorphisms (SNPs) by the TaqMan-PCR system: rs212548-TC (IMS-JST017298 in intron 4), rs212528-TC (IMS-JST004319 in intron 5), rs212526-CT (IMS-JST009090 in intron 6), rs2038090-AC (IMS-JST004325 in intron 17), and rs2038089-AG (IMS-JST004324 in intron 17). The primers and probes of the TaqMan-PCR system are available on request. Hereafter, SNPs are described according to the RS nomenclature system.

Screening of Mutations in Exon 2 of the *EDN1* Gene

Blood samples were obtained from each subject and genomic

Table 2. General Characteristics of Patients with Hypertension and/or Renal Failure

Number	942
Age (years)	65.1±10.5
Gender (M/F)	518/424
Body mass index (kg/m ²)	24.2±3.3
Systolic blood pressure (mmHg)	145.5±19.2
Diastolic blood pressure (mmHg)	84.8±13.4
Essential hypertension	870
Secondary hypertension	72
Renal hypertension	36
Renovascular hypertension	23
Primary aldosteronism	11
Hypothyroid-induced hypertension	2
Renal impairment*	110
Ischemic heart disease	102
Stroke**	145

Values are expressed as mean±SD. *Patients who had serum creatinine ≥1.4 mg/dL. **Silent cerebral infarction was included. M, male; F, female.

DNA was isolated from peripheral blood leukocytes using an NA-3000 nucleic acid isolation system (KURABO, Osaka, Japan). The region of exon 2 was amplified by polymerase chain reaction (PCR) using a pair of specific primers, 5'-CTGATGGCAGGCTGTGTGCTT-3' and 5'-CCCCATCAGATGCCACTGTGA-3', which flank the 612-bp region containing exon 2. The PCR products were directly sequenced on an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, USA) as described previously (23, 24). The obtained sequences were examined for the presence of mutations using Sequencher software (Gene Codes Corporation, Ann Arbor, USA), followed by visual inspection (25).

Statistical Analysis

Analysis of variance was used to compare mean values between groups, and if overall significance was demonstrated, the intergroup difference was assessed by means of a general linear model. Frequencies were compared by χ^2 analysis. Association analyses between genotypes and blood pressure in each sex were performed through logistic regression analysis with consideration for potential confounding risk variables, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), lifestyle (smoking and drinking), and antihypertensive medication. For multivariate risk predictors, the adjusted odds ratios were given with the 95% confidence intervals. The relationship between genotypes and risk of hypertension was expressed in terms of the odds ratios adjusted for possible confounding effects, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). Odds ratios were calculated as a measure of the association between each genotype

Table 3. Odds Ratio of Polymorphisms in *ECE1*

SNP	Sex	Genotype	<i>n</i>	Odds ratio	(95% CI)	<i>p</i>	Genotype	<i>n</i>	Odds ratio	(95% CI)	<i>p</i>
rs212548	Women	TT	328	1	(reference)		TT+TC	821	1	(reference)	
		TC+CC	686	1.28	(0.94–1.74)	0.116	CC	193	1.21	(0.85–1.72)	0.293
	Men	TT	275	1	(reference)		TT+TC	692	1	(reference)	
rs212528	Women	TC+CC	590	1.10	(0.82–1.50)	0.520	CC	173	0.98	(0.69–1.40)	0.924
		TT	663	1	(reference)		TT+TC	980	1	(reference)	
	Men	TC+CC	347	1.40	(1.04–1.89)	0.026	CC	30	1.63	(0.74–3.58)	0.227
rs212526	Women	TT	528	1	(reference)		TT+TC	827	1	(reference)	
		TC+CC	335	0.83	(0.62–1.11)	0.198	CC	36	0.75	(0.37–1.53)	0.428
	Men	CC	734	1	(reference)		CC+CT	996	1	(reference)	
rs2038090	Women	CT+TT	280	0.76	(0.55–1.05)	0.099	TT	18	0.77	(0.25–2.35)	0.650
		CC	615	1	(reference)		CC+CT	842	1	(reference)	
	Men	CT+TT	251	0.95	(0.70–1.30)	0.751	TT	24	1.40	(0.58–3.38)	0.455
rs2038089	Women	AA	774	1	(reference)		AA+AC	999	1	(reference)	
		AC+CC	239	1.17	(0.84–1.64)	0.348	CC	14	1.05	(0.30–3.61)	0.939
	Men	AA	676	1	(reference)		AA+AC	856	1	(reference)	
rs2038089	Women	AC+CC	189	1.00	(0.71–1.40)	0.989	CC	9	3.32	(0.67–16.45)	0.142
		AA	414	1	(reference)		AA+AG	880	1	(reference)	
	Men	AG+GG	598	1.19	(0.89–1.59)	0.240	GG	132	1.21	(0.80–1.84)	0.358
rs2038089	Women	AA	380	1	(reference)		AA+AG	788	1	(reference)	
		AG+GG	486	1.12	(0.84–1.49)	0.450	GG	78	1.33	(0.81–2.18)	0.264
	Men	AG+GG	486	1.12	(0.84–1.49)	0.450	GG	78	1.33	(0.81–2.18)	0.264

*Conditional logistic analysis, adjusted for age, body mass index, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). SNP, single nucleotide polymorphism; CI, confidence interval.

Table 4. Association of Genotypes with Blood Pressure Variation

SNP	Genotype	Women				Men			
		<i>n</i>	DBP (mmHg)	<i>p</i> *	SBP (mmHg)	<i>p</i> *	<i>n</i>	DBP (mmHg)	SBP (mmHg)
rs212528	TT	663	76.49±0.37		126.89±0.64		528	79.98±0.43	131.94±0.75
	TC	317	76.55±0.53		129.21±0.93		299	79.48±0.57	131.18±1.00
	CC	30	77.57±1.72	0.698	133.33±3.02	0.007	36	80.93±1.66	133.83±2.89
	TT	663	76.49±0.37		126.89±0.64		528	79.98±0.43	131.94±0.75
	TC+CC	347	76.63±0.51	0.823	129.56±0.89	0.016	335	79.64±0.54	131.47±0.94
	TT+TC	980	76.51±0.30		127.64±0.53		827	79.67±0.34	131.66±0.60
rs212526	CC	734	76.56±0.35		128.07±0.61		615	79.41±0.40	131.67±0.69
	CT	262	76.90±0.58		127.51±1.03		227	80.15±0.66	131.39±1.15
	TT	18	70.08±2.19	0.344	120.04±3.87	0.175	24	84.13±2.06	138.16±3.59
	CC	734	76.56±0.35		128.07±0.61		615	79.41±0.40	131.67±0.69
	CT+TT	280	76.45±0.56	0.874	127.02±0.99	0.371	251	80.52±0.63	132.03±1.09
	CC+CT	996	76.65±0.30		127.92±0.52		842	79.61±0.34	131.59±0.59
rs212526	TT	18	70.08±2.19	0.003	120.04±3.87	0.044	24	84.13±2.06	138.16±3.59
	TT	18	70.08±2.19	0.003	120.04±3.87	0.044	24	84.13±2.06	138.16±3.59

Values are mean±SEM. *Conditional logistic analysis, adjusted for age, body mass index (BMI), present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). SNP, single nucleotide polymorphism; DBP, diastolic blood pressure; SBP, systolic blood pressure.

and hypertension under the assumption of a dominant (with scores of 0 for patients homozygous for the major allele and 1

for carriers of the minor allele) or recessive (with scores of 0 for carriers of the major allele and 1 for patients homozygous

Table 5. Haplotype Frequency (Freq) of *ECE1* Gene in Hypertensives (HT) and Normotensives (NT)

Haplotype	All				Men				Women			
	Freq (%)	χ^2	<i>p</i>		Freq (%)	χ^2	<i>p</i>		Freq (%)	χ^2	<i>p</i>	
			Asymptotic	Permutation			Asymptotic	Permutation			Asymptotic	Permutation
H1 T/T/C/A/A Overall	19.2	1.278	0.258	0.327	19.0	0.040	0.841	0.893	19.3	2.954	0.086	0.127
NT	19.8				18.8				20.4			
HT	18.4				19.2				17.3			
H2 C/C/C/A/A Overall	16.2	1.305	0.253	0.284	17.5	0.193	0.661	0.669	15.1	2.991	0.084	0.091
NT	15.5				17.9				14.0			
HT	16.9				17.1				16.9			
H3 T/T/C/A/G Overall	14.3	0.122	0.727	0.769	14.7	0.231	0.631	0.695	14.2	0.060	0.807	0.825
NT	14.1				14.4				14.0			
HT	14.5				15.2				14.4			
H4 C/T/C/A/A Overall	11.8	0.181	0.670	0.716	11.9	0.033	0.857	0.867	11.8	0.250	0.617	0.699
NT	12.0				12.1				12.1			
HT	11.5				11.8				11.4			
H5 T/T/T/A/A Overall	10.7	8.254	0.004	0.015	10.9	0.421	0.516	0.575	10.6	11.865	0.001	0.003
NT	12.0				11.4				12.4			
HT	9.0				10.4				7.5			
H6 T/T/C/C/G Overall	8.3	0.317	0.574	0.618	7.8	0.327	0.568	0.624	9.0	0.001	0.974	0.978
NT	8.1				8.1				9.0			
HT	8.7				7.3				9.0			
H7 C/T/C/A/G Overall	7.8	0.133	0.715	0.775	6.2	1.115	0.291	0.402	8.8	2.071	0.150	0.192
NT	7.6				5.5				8.2			
HT	7.9				6.7				10.0			

Haplotypes (rs212548/rs212528/rs212526/rs2038090/rs2038089) with frequencies of more than 5% are shown. One hundred thousand replicates were used for permutation test for all, men and women. Numbers of haplotypes in Overall, NT, and HT are 3,736, 2,150, 1,586 for All; 1,730, 914, 816 for men; and 2,030, 1,254, 776 for women, respectively.

for the minor allele) mode of inheritance. The *p* values were adjusted by Bonferroni correction. SAS statistical software (release 6.12; SAS Institute Inc., Cary, USA) was used for the statistical analyses. The data of linkage disequilibrium, haplotype blocks and coverage of HapMap SNPs were downloaded from the HapMap Consortium (<http://www.hapmap.org>). Haplotypes and permutation analyses were calculated using SNPalyze version 4.0 software (DYNACOM Co., Mobara, Japan).

Results

Association between SNPs in the *ECE1* Gene and Hypertension

Five genetic polymorphisms in the *ECE1* gene were genotyped in 1,873 individuals. The genotype frequencies for each polymorphism were as follows: rs212548-T>C, 0.563/0.437; rs212528-T>C, 0.800/0.200; rs212526-C>T, 0.848/0.152; rs2038090-A>C, 0.880/0.120; rs2038089-A>G, 0.655/0.345. None of the genotype frequencies were significantly different from those expected from the Hardy-Weinberg equilibrium ($p>0.05$). Multiple logistic regression analysis after

adjusting for confounding factors of age, BMI, hyperlipidemia, diabetes mellitus, smoking, and drinking revealed that one polymorphism, rs212528, in intron 5 was significantly associated with hypertension in women (rs212528-T>C: TT vs. TC+CC; odds ratio=1.40; 95% confidence interval: 1.04–1.89; $p=0.026$) (Table 3). The SBPs in women with the TT, TC, and CC genotypes were 126.89 ± 0.64 mmHg ($n=663$), 129.21 ± 0.93 mmHg ($n=317$), and 133.33 ± 3.02 mmHg ($n=30$) ($p=0.007$), after adjusting for the same confounding factors (Table 4). Thus, the difference in SBP was 6.44 mmHg between women with the CC genotype and those with the TT genotype. This association was still significant even after the Bonferroni correction.

Another polymorphism, rs212526, was associated with a significant difference in DBP: women having the CC+CT genotype had a DBP of 76.65 ± 0.30 mmHg ($n=996$) and those with the TT genotype had a DBP of 70.08 ± 2.19 mmHg ($n=18$) ($p=0.003$) after adjusting for the same confounding factors (Table 4). This polymorphism was also significantly associated with the SBP in women (CC+CT: 127.92 ± 0.52 mmHg, $n=996$; TT: 120.04 ± 3.87 mmHg, $n=18$; $p=0.044$). However, this polymorphism did not show a significant association with hypertension. In men, this polymorphism was

Table 6. List of 5 Polymorphisms and Their Allele Frequency in Exon 2 of *EDN1* Identified by Direct Sequencing of 942 Hypertensive Japanese

Allele 1 > allele 2	Amino acid change	region	Allele frequency		Flanking sequence	rs ID
			Allele 1	Allele 2		
1753G>A	G36R	exon 2	1.000	0.000	TGAGAACGGC[G/A]GGGAGAAACC	rs2070699
1910G>T		intron 2	0.473	0.527	TGTAACCCTA[G/T]TCATTCATTA	
1918T>A		intron 2	0.999	0.001	TAGTCATTCA[T/A]TAGCGCTGGC	
2008G>A		intron 2	0.999	0.001	GTGCCTCAGT[G/A]GGGACAGTTT	
2107G>A		intron 2	0.999	0.001	TACTCATGAT[G/A]GGACAAGCAG	

The A of the ATG of the initiator Met codon is denoted nucleotide +1, as recommended by the Nomenclature Working Group (28). The nucleotide number was according to the reference sequences GenBank Accession ID: NT_007592.

Fig. 1. Genome and domain structure of human endothelin 1. Two missense mutations in endothelin-1, Gly36Arg (G36R) and Lys198Asn (K198N), are shown. The G36R mutation in preproendothelin-1 was identified in this study.

significantly associated with DBP (CC+CT: 79.61 ± 0.34 mmHg, $n=842$; TT: 84.13 ± 2.06 mmHg, $n=24$; $p=0.030$).

The haplotypes composed of the 5 SNPs genotyped in this study are shown in Table 5. Seven inferred haplotypes with frequencies of more than 5% were examined to determine their association with hypertension in all patients and in two sub-populations (men and women). In women, the frequency of haplotype H5 in the hypertensive group was significantly lower than that in the normotensive group.

A Novel Missense Mutation in the preproEDN1 Polypeptide in Japanese Hypertensives

We sequenced the region of exon 2 of *EDN1* in 942 hypertensive patients with strong genetic background and secondary hypertension. The results are shown in Table 6. In this study, we were not able to detect any missense mutations within the mature EDN1 region. However, we identified one novel missense mutation, G36R, in *EDN1* in a heterozygous form in a male patient. The prevalence of this mutation was 0.05% in our Japanese hypertensive population. We tried to screen this missense mutation, G36R in *EDN1*, in our general population by the TaqMan-PCR method, but this genotyping failed due

to technical problems.

Discussion

In this study, we used two different approaches to reveal the contribution of the EDN system to hypertension in two different populations, a general population and a hypertensive population, both from the Osaka region in Japan.

We genotyped 5 SNPs in *ECE1* and identified rs212528 as the hypertension/blood pressure susceptibility genetic variant. We used the currently available HapMap data from CHB-JPN to assess the coverage of haplotype blocks across the *ECE1* gene by 5 SNPs. The *ECE1* gene consisted of 6 haplotype blocks, in which rs212548 was present in block 2, two SNPs, rs212528 and rs212526, were present in block 3, and two SNPs, rs2038090 and rs2038089, were present in block 6, and the genotyped SNPs were estimated to cover approximately 90% of the haplotypes in block 2, 30% of those in block 3, and 90% of those in block 6, respectively. Two SNPs, rs212528 and rs212526, in block 3 had an r^2 of 0.031 and LOD score of 0.43, and rs2038090 and rs2038089 in block 6 had an r^2 of 0.163 and LOD score of 2.33.

In this study, the rs212528-T>C polymorphism in *ECE1* in

women was identified as the SNP conferring susceptibility for hypertension and blood pressure change. It is well known that the incidence of coronary artery disease shows a gender difference that may in part be related to the female sex hormones estrogen and progesterone. The literature provides evidence that estrogen inhibits EDN1 production (26). Furthermore, estrogen inhibits ECE-1 mRNA expression (27). These findings may explain the gender difference of *ECE1* polymorphisms for hypertension. The mean age of women in our population was 63.3 years. Despite the relatively advanced age of this population, we identified a contribution of the rs212528 polymorphism to hypertension and blood pressure change, while haplotypes containing the rs212528-C allele were not clearly associated with normotension or hypertension. The association might have been stronger if we had used a younger female population.

Another polymorphism, rs212526-C>T in intron 6, was associated with a blood pressure change in women and men. The mean DBP of the 996 women with the CC+CT genotype was 6.57 mmHg higher than that of the 18 women with the TT genotype ($p=0.003$), and the SBP change also showed the same trend—that is, women with the CC+CT genotype had higher blood pressure than women with the TT genotype ($p=0.044$) (Table 4). However, in men, the opposite trend was seen. The mean DBP of the 842 men with the CC+CT genotype was 4.52 mmHg lower than that of the 24 men with the TT genotype ($p=0.030$). Haplotype H5 containing the rs212528-T allele was significantly more prevalent in the normotensive group. This association also suggested that the T-allele of rs212528 was involved in blood pressure in women (Tables 3–5). Thus, the significance of rs212526 on blood pressure change should be evaluated using other population.

The association of SNP with hypertension and blood pressure change is at best marginally significant given the number of tests performed. All the p -values were more than 0.007. However, rs212528 is present in the *ECE1* gene, which encodes the endothelin-converting enzyme. In addition, this SNP showed a positive association with both hypertension and blood pressure change. Thus, we regarded this SNP as a hypertension candidate. SNP and blood pressure/hypertension described in the present study needs to be confirmed by another set of studies.

In the hypertensive population, we sequenced the coding region of the EDN1 polypeptide and its flanking region in 942 Japanese hypertensives and identified one novel missense mutation, G36R, that was not present in the EDN1 polypeptide but was present in the preproEDN-1 region (Fig. 1). At present, the effect of G36R mutation on the EDN1 function is not clear, because it was located far from the scissile site, the R52–C53 bond, by the furin-like enzyme. From the evolutionary point of view, G36 was conserved in humans, chimpanzees, cows, and dogs, but mice and rats have Val and chickens have Ala. The arginine residue at position 36 was not found in preproEDN1 in any species. To reveal the functional effect of this missense mutation on the processing of

preproEDN1, an expression study of the mutant preproEDN1 is needed.

We have hypothesized that rare nonsynonymous mutations in candidate genes could collectively contribute to complex traits. In this model, the extensive sequence-based approaches focusing on identification of these mutations is necessary. So far, we have sequenced several hypertension candidate genes to evaluate whether rare variants could contribute to the etiology of hypertension. At present, however, whether rare variants contribute to hypertension is not clear due to the lack of *in vitro* or *in vivo* expression studies of the mutant protein (14, 15, 17). The exception was the nonsense mutation identified in the *RGS2* gene, which has been clearly shown to produce the defective protein (16). In this study, we identified one missense mutation, G36R, in preproEDN1. The further collection of such missense mutations in hypertension candidate genes could lead to an enhanced understanding of the etiology of essential hypertension.

In summary, we revealed that the rs212528 polymorphism in *ECE1* was associated with hypertension and blood pressure change. In earlier reports, the Lys198Asn polymorphism in *EDN1* showed a positive association with blood pressure elevation in overweight people (3–5). Thus, endothelin family gene polymorphisms might play an important role in the etiology of essential hypertension.

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