DVT for genetic variation. By genotyping three polymorphisms (−202G>A, 2487A>T, 2729A>G) and two missense mutations (R385K, D468Y) in a Japanese general population, we assessed the prevalence of these genetic variations. We then evaluated the association of sTM levels with genetic variations. We finally compared the genotype prevalence of these genetic variations in DVT patients with those in population-based controls to test whether these mutations are associated with DVT in the Japanese.

Patients and methods

DVT patients

A total of 118 Japanese DVT patients (59 men and 59 women, mean age: 52.3 ± 16.1 years old) were recruited from Osaka University Hospital from 2000 to 2004 and the National Cardiovascular Center from 2002 to 2004. All patients examined in this study were unselected patients diagnosed with DVT. Clinical diagnosis of DVT was confirmed by imaging analysis including computerized tomography and ultrasonography.

Screening of genetic variations in TM gene

Blood samples were obtained from DVT patients and genomic DNA was isolated from peripheral blood leukocytes [17]. All the putative promoter, exon, and 3′-UTR regions in 118 Japanese DVT patients were directly sequenced with an ABI PRISM3700DNA analyzer (Applied Biosystems, Foster City, CA) using seven sets of primers. Primer sequences are available upon request. The obtained sequences were examined for the presence of variations using Sequencher software (Gene Codes Corporation, Ann Arbor, MI), followed by visual inspection [18]. The A of ATG of the initiator Met codon is denoted nucleotide +1, and the initial Met residue is denoted amino acid +1 [19]. The nucleotide sequence (GenBank Accession ID: AF-495471) was used as a reference sequence.

General population (Suita Study)

The sample selection and study design of the Suita Study have been described previously [20–22]. Briefly, the subjects visited the National Cardiovascular Center every 2 years for general health checkups, underwent a routine blood examination that included lipid profiles and glucose levels, and underwent blood pressure measurements. The basic characteristics of the individuals have been reported previously [23,24]. sTM levels of 2247 population-based samples were measured by an enzyme-linked immunosorbent assay (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan).

Genotyping of mutations and single nucleotide polymorphisms (SNPs) in the general population

Two common SNPs with a minor allele frequency of greater than 5% and all of the missense mutations we detected were tried for genotyping by the

Table 1 Clinical profiles of 118 DVT patients

Clinical profiles		Clinical profiles	
Age, years ± S.D.	52.3 ± 16.1	Nephrotic syndrome, *n* (%)	0 (0.0)
Women, *n* (%)	59 (50.0)	Chronic heart failure, *n* (%)	17 (14.4)
BMI, kg/m^2, mean ± S.D.	23.7 ± 3.2	Diabetes Mellitus, *n* (%)	47 (39.8)
DVT family history, *n* (%)	8 (6.8)	Hyperlipidemia, *n* (%)	48 (40.7)
Previous DVT, *n* (%)	12 (10.2)	Autoimmue disease, *n* (%)	11 (9.3)
		Inflammatory bowel disease, *n* (%)	2 (1.7)
Pregnancy, *n* (%)	5 (4.2)	Estrogen use, *n* (%)	3 (2.5)
Stroke, *n* (%)	1 (1.5)	Steroid use, *n* (%)	9 (7.6)
Prolonged immobility, *n* (%)	14 (11.9)	Paralysis, *n* (%)	5 (4.2)
Malignancy, *n* (%)	16 (13.6)	Myeloproliferative disease, *n* (%)	1 (0.8)
Major surgery (abd, hip, leg), *n* (%)	21 (17.8)	Reduced plasminogen activity, *n* (%)	7 (5.9)
Trauma (pelvis, hip, leg), *n* (%)	3 (2.5)	Reduced antithrombin activity, *n* (%)	7 (5.9)
Stasis due to compression, *n* (%)	6 (5.1)	Reduced protein C activity, *n* (%)	8 (6.8)
Central venous catheter, *n* (%)	0 (0.0)	Reduced protein S antigen, *n* (%)	10 (8.5)
		Lupus anticoagulant (cardiolipin,ACLb2), *n* (%)	3 (11.0)

BMI, body mass index; DVT, deep vein thrombosis; Diabetes mellitus indicates fasting plasma glucose ≧126 mg/dl or non-fasting plasma glucose ≧200 mg/dl or HbA1c ≧6.5% or use of antidiabetic medication; Hypertension, systolic blood pressure ≧140 mm Hg and/or diastolic blood pressure ≧90 mm Hg or use of antihypertensive medication; Hyperlipidemia, total cholesterol ≧220 mg/dl or use of antihyperlipidemia medication; Myeloproliferative disease, Plt. $>5\times10^5$ and Ht. >55%; Reduced plasminogen activity, plasminogen activity <70%; Reduced antithrombin activity, antithrombin activity <80%; Reduced protein C activity, protein C activity <70%; Reduced protein S antigen, protein S antigen <60%.

TaqMan-PCR method [25]. Among three missense mutations, genotyping for 1418C>T (A455V) was failed. Additionally, another common SNP (2729A>C) which was in linkage disequilibrium (r-square>0.9) with A455V mutation was genotyped instead of A455V mutation. Thus, five genetic variations were successfully genotyped in 2247 subjects (1032 men and 1215 women). The sequences of PCR primers and probes for the TaqMan-PCR method are available upon request. All clinical data and sequencing and genotyping results were anonymous. The study protocol was approved by the Ethical Review Committee of Osaka University Hospital and National Cardiovascular Center. Gene analyses were performed after informed consent had been obtained in written.

Statistical analysis

Values are means ± S.E. The distributions of basic characteristics in men and women in the Japanese general population were examined using the Student's t-test or X^2 analysis. The correlations of two missense mutations and three common SNPs with sTM levels were examined by logistic analysis, with adjustment for confounding factors, including age, body mass index (BMI), present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). Odds ratios for each mutation are presented both adjusted for age and age-BMI. All analyses were performed using SAS (release 8.2, SAS Institute Inc.). Statistical significance was established at $p<0.05$. Linkage disequilibrium was calculated using SNPAlyze version 4.0 (DYNACOM Co., Ltd., Mobara, Japan).

Results

Characteristics of DVT patients

The clinical profiles of the 118 Japanese DVT patients (59 men, 59 women aged 52.3 ± 16.1) are summarized in Table 1. Eight patients (6.8%) had a DVT family history and 12 patients (10.2%) had previous DVT. Sixteen patients (13.6%) suffered from cancer and 21 (17.8%) had undergone major surgery of the abdomen, hip or leg. Seven patients (5.9%) had reduced plasminogen activity (<70%) and 7 (5.9%) had reduced antithrombin activity (<80%). Eight patients (6.8%) had reduced PC activity (<70%), and 10 patients (8.5%) had reduced PS antigen (<60%). To eliminate effects of warfarin on PS/PC activities, we did not count numbers of patients having reduced PC activity (PC<70%) and PS antigen (PS<60%) when they had taken warfarin.

Screening of TM gene for sequence variation in DVT patients

On sequencing the TM gene in 118 DVT patients, we identified 17 genetic variants (Table 2). Three of 17

Table 2 Genetic variations in TM gene identified in 118 Japanese DVT patients

SNPs	LD	Region	Amino acid substitution	Allele 1 frequency (%)	Allele 2 frequency (%)	Flanking sequence	db SNP ID
*−832C>A		Promoter		99.6	0.4	gggcagagggcg [c/a] tggtgttaggcc	
*−754G>C		Promoter		99.1	0.9	caagcgcgctcc [g/c] ctggtttcctga	
*−265C>A		Exon(5′ UTR)		99.6	0.4	aatccgagtatg [c/a] ggcatcagccct	
−202G>A	A	Exon(5′ UTR)		89.2	10.8	ggagggagggcc [g/a] ggcacttataaa	
*−58G>C		Exon(5′ UTR)		98.3	1.7	ctgctccggcac [g/c] gccctgtcgcag	
*1197C>T		Exon(EGF4)	H381	99.6	0.4	gcccattcccca [c/t] gagccgcacagg	
1208G>A		Exon(EGF4)	R385K	99.1	0.9	acgagccgcaca [g/a]gtgccagatgtt	
1418C>T	B	Exon(EGF6)	A455V	65.1	34.9	actcggcccttg [c/t] ccgccacattgg	rs1042579
1456G>T		Exon(Ser/Thr-rich)	D468Y	99.1	0.9	tccggcaaggtg [g/t] acggtggcgaca	
1754C>T		Exon(3′ UTR)		98.7	1.3	aggagcctggct [c/t] cgtccaggagcc	rs13306852
2005G>A	A	Exon(3′ UTR)		89.2	10.8	gtcctcactacc [g/a]ggcgcaggaggg	rs3176134
*2230T>C		Exon(3′ UTR)		99.6	0.4	tcttggtgaatt [t/c] ttttttcctagc	
*2487A>T		Exon(3′ UTR)		93.1	6.9	ttcccagagcaa [a/t] ataattttaaac	
2521A>G		Exon(3′ UTR)		79.8	20.2	gatgtaaaaggt [a/g] ttaaattgatgt	rs1042580
2729A>C	B	Exon(3′ UTR)		65.0	35.0	tgctctagattg [a/c] gagaagagacaa	rs3176123
*3521−3522insT		3′ flanking		99.6	0.4	ctcgggttgtgt [−/t] gtctgttcactt	
*3559T>A		3′ flanking		99.6	0.4	gccctcatttta [t/a] gtcattaaatgg	

LD, mutations in linkage disequilibrium (group A; r-square=0.84, group B r-square=0.93); allele 1, major allele; allele 2, minor allele; *, novel mutation; EGF, epidermal growth factor like domain; Ser/Thr-rich, serine/threonine-rich domain; UTR, untranslated region.

Table 3 Basic characteristics of subjects in general population

	Women ($n=1215$)	Men ($n=1032$)	p
Age, years ± S.D.	64.6 ± 10.7	67.1 ± 10.9	<0.0001
Systolic blood pressure, mm Hg ± S.D.	123.5 ± 19.8	126.1 ± 17.9	0.0008
Diastolic blood pressure, mm Hg ± S.D.	74.3 ± 10.4	77.2 ± 10.4	<0.0001
Body mass index, kg/m^2 ± S.D.	22.4 ± 3.2	23.4 ± 3.0	<0.0001
Total cholesterol, mg/dl ± S.D.	215.9 ± 31.6	198.7 ± 31.5	<0.0001
HDL-cholesterol, mg/dl ± S.D.	64.4 ± 15.1	55.2 ± 14.0	<0.0001
Current smokers, %	4.4	27.2	<0.0001
Current drinkers, %	26.0	67.0	<0.0001
Present illness, %			
Hypertension	35.3	42.8	0.0003
Hyperlipidemia	55.7	34.3	<0.0001
Diabetes mellitus	6.1	13.2	<0.0001

Hypertension indicates systolic blood pressure ≧140 mm Hg and/or diastolic blood pressure ≧90 mm Hg or use of antihypertensive medication; Hyperlipidemia, total cholesterol ≧220 mg/dl or use of antihyperlipidemia medication; Diabetes mellitus, fasting plasma glucose ≧126 mg/dl or non-fasting plasma glucose ≧200 mg/dl or HbA1c ≧6.5% or use of antidiabetic medication. The distributions of basic characteristics in men and women in general population were analyzed using the Student's t-test or X^2 analysis.

mutations were missense mutations (R385K; $n=2$, A455V; $n=53$ heterozygous, $n=14$ homozygous, D468Y; $n=2$). Four mutations within the TM promoter region and the 5′-untranslated region (5′-UTR) (−832C>A, −754G>C, −265C>A, −58G>C) were rare. Twenty-five patients were heterozygous carriers for the −202G>A mutation within the promoter region, which was reported as a −33G>A mutation. This mutation has been reported to decrease TM promoter activity in vitro [26]. It was in linkage disequilibrium (r-square>0.8) with 2005G>A in the 3′-UTR. No patients were carriers for previously reported mutations in the lectin-like domain [A25A (847G>C), E61A (954G>C)] [27,28]. One patient was heterozygous for a novel neutral mutation within the fourth EGF-like domain [H381 (1197C>T)]. Two patients were heterozygous carriers for the previously described R385K mutation (1208G>A) in the fourth EGF-like domain [28]. The previously reported A455V mutation (1418C>T) was found within the sixth EGF-like domain ($n=53$ heterozygous, $n=14$ homozygous), an important region for thrombin binding and activation of PC [13]. This mutation was in linkage disequilibrium (r-square>0.9) with the 2729A>C mutation within the 3′-UTR. Within the serine/threonine-rich domain,

Table 4 Genotype distribution of two missense mutations and three common single nucleotide polymorphisms (SNPs) of TM gene in DVT patients and in individuals in general population

SNPs (amino acid change)	Genotypes	Individuals in general population			DVT patients		
		Women	Men	Total	Women	Men	Total
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
−202 G>A	GG	1009 (83.1)	855 (82.9)	1864 (83.0)	45 (76.3)	46 (80.7)	91 (78.5)
	GA	192 (15.8)	157 (15.2)	349 (15.5)	14 (23.7)	11 (19.3)	25 (21.6)
	AA	14 (1.2)	19 (1.8)	33 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1215	1031	2246	59	57	116
1208 G>A (R385K)	GG	1207 (99.3)	1023 (99.1)	2230 (99.2)	57 (98.3)	56 (98.3)	113 (98.3)
	GA	8 (0.7)	9 (0.9)	17 (0.8)	1 (1.7)	1 (1.8)	2 (1.7)
	AA	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1215	1032	2247	58	57	115
1456 G>T (D468Y)	GG	1181 (97.3)	1015 (98.5)	2196 (97.7)	57 (96.6)	57 (100.0)	114 (98.3)
	GT	33 (2.7)	16 (1.6)	49 (2.2)	2 (3.4)	0 (0.0)	2 (1.7)
	TT	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1214	1031	2245	59	57	116
2487 A>T	AA	1001 (82.4)	873 (84.6)	1874 (83.4)	41 (83.7)	47 (87.0)	94 (86.2)
	AT	206 (17.0)	155 (15.0)	361 (16.1)	8 (16.3)	7 (13.0)	15 (13.8)
	TT	8 (0.7)	4 (0.4)	12 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1215	1032	2247	49	54	109
2729 A>C	AA	707 (58.2)	570 (55.2)	1277 (56.8)	24 (43.6)	22 (40.0)	46 (41.8)
	AC	419 (34.5)	393 (38.1)	812 (36.1)	26 (47.3)	25 (45.5)	51 (46.4)
	CC	89 (7.3)	69 (6.7)	158 (7.0)	5 (9.1)	8 (14.6)	13 (11.8)
	Total	1215	1032	2247	55	55	110

Table 5 Comparison of sTM levels by genetic variations of TM gene in general population

SNPs (amino acid change)	Genotypes	Women				Men			
		Age-adjusted		Multi-adjusted		Age-adjusted		Multi-adjusted	
		Mean ± SE U/ml	p	Mean ± SE U/ml	p	Mean ± SE U/ml	p	Mean ± SE U/ml	p
−202 G>A	GG	16.9 ± 1.6		17.0 ± 1.6		19.2 ± 1.9		19.6 ± 1.9	
	GA+AA	17.4 ± 0.2	0.73	17.4 ± 0.2	0.77	19.9 ± 0.2	0.68	19.9 ± 0.2	0.87
1208 G>A	GG	17.4 ± 0.2		17.4 ± 0.2		19.9 ± 0.2		19.9 ± 0.2	
(R385K)	GA+AA	16.2 ± 2.4	0.62	16.0 ± 2.3	0.54	20.5 ± 2.2	0.79	20.4 ± 2.2	0.84
1456 G>T	GG	17.4 ± 0.2		17.4 ± 0.2		19.9 ± 0.2		19.9 ± 0.2	
(D468Y)	GT+TT	18.1 ± 1.0	0.51	18.1 ± 1.0	0.52	22.2 ± 1.7	0.20	22.6 ± 1.7	0.11
2487 A>T	AA	17.6 ± 0.2		17.6 ± 0.2		20.0 ± 0.2		20.0 ± 0.2	
	AT+TT	16.7 ± 0.4	0.04	16.7 ± 0.4	0.04	19.6 ± 0.6	0.54	19.5 ± 0.6	0.40
2729 A>C	AA	17.9 ± 0.2		17.9 ± 0.2		20.4 ± 0.3		20.3 ± 0.3	
	AC+CC	16.7 ± 0.3	<0.01	16.8 ± 0.3	<0.01	19.4 ± 0.3	0.03	19.5 ± 0.3	0.07

The correlations of five genetic variations with sTM level were examined by logistic analysis, adjusting for age and multiple factors, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking).

two patients were heterozygous carriers for the previously described D468Y mutation (1456G>T) [29].

Characteristics of individuals in the general population

The characteristics of the 2247 subjects of the Japanese general population group (1032 men, 1215 women) are shown in Table 3. Age, systolic blood pressure, diastolic blood pressure, BMI, percentage current smokers, percentage current drinkers, and frequencies of hypertension and diabetes mellitus were significantly higher in men than in women, while total cholesterol, HDL-cholesterol, and percentage of subjects with hyperlipidemia were significantly higher in women than in men.

Genotyping of two missense mutations (R385K, D468Y) and three common SNPs (−202G>A, 2487A>T, 2729A>C) and association of sTM levels with TM genotypes in the general population

In the general population of 2247 subjects, five mutations were successfully genotyped (Table 4). Plasma levels of sTM were measured in all subjects. As shown in Table 5, sTM levels were significantly lower in C-allele carriers of the 2729A>C mutation than in non-carriers in the general population (women: 16.7 ± 0.3 U/ml vs. 17.9 ± 0.2 U/ml, $p<0.01$, men: 19.4 ± 0.3 U/ml vs. 20.4 ± 0.3 U/ml, $p=0.03$), when adjusted for age. Additionally, in male patients, the CC genotype group was associated with significantly higher DVT risk than the combined AA/AC genotype after adjustment for age and age-BMI (odds ratio = 2.76, 95% confidence interval = 1.14–6.67; $p=0.02$ and odds ratio = 2.98, 95% confidence interval = 0.21–7.33; $p=0.02$, respectively) (Table 6). This mutation was in linkage disequilibrium (r-square > 0.9) with the A455V mutation (Table 2).

Discussion

Several mutations within the TM gene have been reported in small numbers of patients with DVT [27,30–33]. However, it was reported that polymorphisms within the TM gene were not common risk factors for incidental DVT in a recent Caucasian population-based case-control study [34]. Because the factor V-Leiden mutation is not detected in Japanese DVT patients [7], while PS Tokushima mutation (K196E) is a risk factor for DVT in a

Table 6 Odds ratios and 95% confidence intervals for DVT in relation to 2729A>C in TM gene

Genotypes	Women				Men			
	Age-adjusted		Age, BMI-adjusted		Age-adjusted		Age, BMI-adjusted	
	Odds ratio (95% CI)	p	Odds ratio (95% CI)	p	Odds ratio (95% CI)	p	Odds ratio (95% CI)	p
AA+AC	1 (reference)		1 (reference)		1 (reference)		1 (reference)	
CC	0.97 (0.35–2.70)	0.95	0.96 (0.34–2.70)	0.93	2.76 (1.14–6.67)	0.02	2.98 (0.21–7.33)	0.02

CI, confidence interval.

Japanese population [9,10], we suspected that frequencies of the TM mutations in Japanese DVT patients might differ from those in Caucasians. We therefore performed a case-control study to test TM polymorphisms for associations with DVT in Japanese. In this study, we found that sTM levels were lower in those with 2729C and 2729C was more common in DVT patients than in the general population. It is a reasonable assumption that the low sTM levels in plasma reflect the decreased TM expression on endothelial cells. If so, the capacity of the PC anticoagulant system, which is comprised of TM, PC and PS, would be decreased to thrombosis-prone.

We first screened the TM putative promoter, exon, and 3′ -UTR regions for sequence variations in a random sample ($n=118$) of DVT patients, and identified one novel neutral mutation (1197C>T; H381) and three previously described missense mutations (1208G>A; R385K, 1418C>T; A455V, 1456G>T; D468Y) (Table 2). As shown in previous report showing A455V mutation within the sixth EGF-like domain, an important region for thrombin binding and activation of PC, was a common missense mutation [13], the frequency of A455V mutation was also higher than the other mutation found in this study. The 1197C>T (H381, $n=1$) mutation and 1208G>A (R385K, $n=2$) mutation within the fourth EGF-like domain were rare. Although the fourth EGF-like domain serves as the binding site for PC, the functional consequences of the Arg-to-Lys substitution at position 385 are not known. D468Y mutation lies in the serine/threonine-rich domain. An in vitro study showed that this mutation did not cause any abnormality in levels of production or functional activity of TM [31]. In our study, patients carrying this mutation were rare ($n=2$).

We genotyped five genetic variants in the 2247 population-based controls (Table 4). We failed in genotyping for the A455V mutation, so the 2729A>C mutation in linkage disequilibrium with the A455V mutation was genotyped. In the Japanese general population, the frequency of 2729A>C mutation (36.1% heterozygous, 7.0% homozygous) was higher than that of A455V mutation in Caucasians (24.0% heterozygous, 4.3% homozygous) and African-Americans (15.9% heterozygous, 2.2% homozygous) [33]. Since the frequency of A455V mutation in the Chinese population has been reported to be 45% heterozygous and 9% homozygous [35], the frequency of the 2729A>C mutation in our study was similar to the result in the Chinese population. This difference in genotype frequency may be associated with differences in ethnical genetic background.

The extracellular region of endothelial TM is cleaved and the cleaved fragments are called sTM. sTM processes anticoagulant properties, and sTM levels reported to have a statistically significant correlation with sTM cofactor activity in healthy individuals [36,37]. The LITE Study reported that sTM levels tended to exhibit gene dosage effects, with AA-genotype of A455V mutation carriers exhibiting approximately 10% higher sTM levels than VV-genotype of A455V mutation carriers, and values for the AV-genotype carriers were intermediate, with no significant differences among these three groups [33]. In our study, particularly in women, sTM levels in individuals carrying 2729A>C mutation were lower than those in noncarriers (Table 5). Since the 2729A>C mutation and the A455V missense mutation are in linkage disequilibrium, our findings might support those of these previous reports. For the other mutations, there was no significant difference in sTM level among the genotypes. Despite much interest in sTM as a marker of endothelial injury, few studies have investigated the relationship between sTM and DVT. The findings of previous studies are conflicting or difficult to judge, partly because of small sample sizes or cross-sectional design [33,38–40]. However, systemic infusion of recombinant sTM has been shown to have antithrombotic potential and dose-dependent effects in the prevention of venous thrombosis after total hip replacement [41,42]. Moreover, the ARIC Study, performed in the United States, reported that high levels of sTM are associated with a lower risk of incidental coronary heart disease [43].

Finally, we compared the genotype frequencies in the population-based controls with those in the DVT patients. In male DVT patients, the frequency of 2729A>C mutation was higher than in the population-based controls (Table 6). The LITE Study reported no difference in the frequency of A455V mutation between DVT patients and controls among Caucasians and African-Americans [33]. This discrepancy might come from the difference of sample size, ethnical genetic background or study design. Especially, in our study, difference of mean ages between DVT patients (52.3 ± 16.1 years old) and general population (women: 64.6 ± 10.7 years old, men: 67.1 ± 10.9 years old) may affect the results, although all analysis has been done in age-adjusted manner.

Additionally, significant decrease of sTM levels in the C-allele carriers of 2729A>C mutation was found in women, whereas not much in men in our study (Table 5). However, the incidence of DVT was associated with only men, but not women (Table 6). The mechanisms by which 2729A>C mutation might

contribute to DVT in only men are unknown. This inconsistency might be derived from gender differences or a lack of statistical power due to the sample size. Regarding the gender differences, TM proteins are known to be modulated by estrogens [44]. 17β-estradiol is known to reduce the anticoagulant properties of endothelial cells by decreasing thrombomodulin expression. This can well explain the gender difference of sTM levels, where men showed higher sTM levels than women. The anticoagulant activity of TM was destroyed by oxidation caused by chloramine T, H_2O_2, or hypochlorous acid generated from H_2O_2 by myeloperoxidase [45]. Activated neutrophil, the primary in vivo source of biological oxidants, also rapidly inactivate TM. Oxidation of Met388 in the sixth EGF-like domain was critical for inactivation. Men are supposed to have greater oxidative stress than women. If so, men might be exposed more for DVT risk. Thus, we suppose that the cause of gender difference in relationship between TM polymorphism and DVT may be via the influences of hormonal and environmental effects.

We observed that 2729A>C mutation and A455V mutation are in linkage disequilibrium and 2729A>C mutation is associated with sTM levels and DVT. At present, the causative genetic mutations for this association are not known. A455V mutation may directly affect the expression of TM molecule. 2729A>C mutation in the 3′-UTR may affect the mRNA stability. TM mRNA is known to be unstable [46], and C-allele may create more unstable mRNA. Two polymorphisms may be in linkage disequilibrium with another genetic variation in the region that was not examined by sequencing. Therefore, additional in vitro studies are required for the identification of the functional genetic variation. Since association studies are not consistently reproducible due to false-positives, false-negatives or true variability in association between different populations [47], the association of TM polymorphism to sTM levels and DVT must be reexamined in other populations.

In summary, TM mutations, especially those with a haplotype consisting of 2729A>C and A455V, affect sTM levels, and may be associated with DVT in Japanese.

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References

[1] Souto JC, Almasy L, Borrell M, Blanco-Vaca F, Mateo J, Soria JM, et al. Genetic susceptibility to thrombosis and its relationship to physiological risk factors: the GAIT study. Genetic Analysis of Idiopathic Thrombophilia. *Am J Hum Genet* 2000;**67**:1452-9.

[2] Heit JA, Phelps MA, Ward SA, Slusser JP, Petterson TM, De Andrade M. Familial segregation of venous thromboembolism. *J Thromb Haemost* 2004;**2**:731-6.

[3] Rosendaal FR, Bovill EG. Heritability of clotting factors and the revival of the prothrombotic state. *Lancet* 2002;**359**: 638-9.

[4] de Lange M, Snieder H, Ariens RA, Spector TD, Grant PJ. The genetics of haemostasis: a twin study. *Lancet* 2001;**357**:101-5.

[5] Ariens RA, de Lange M, Snieder H, Boothby M, Spector TD, Grant PJ. Activation markers of coagulation and fibrinolysis in twins: heritability of the prethrombotic state. *Lancet* 2002;**359**:667-71.

[6] Martinelli I. Risk factors in venous thromboembolism. *Thromb Haemost* 2001;**86**:395-403.

[7] Fujimura H, Kambayashi J, Monden M, Kato H, Miyata T. Coagulation factor V Leiden mutation may have a racial background. *Thromb Haemost* 1995;**74**:1381-2.

[8] Miyata T, Kawasaki T, Fujimura H, Uchida K, Tsushima M, Kato H. The prothrombin gene G20210A mutation is not found among Japanese patients with deep vein thrombosis and healthy individuals. *Blood Coagul Fibrinolysis* 1998;**9**:451-2.

[9] Kinoshita S, Iida H, Inoue S, Watanabe K, Kurihara M, Wada Y, et al. Protein S and protein C gene mutations in Japanese deep vein thrombosis patients. *Clin Biochem* 2005;**38**:908-15.

[10] Kimura R, Honda S, Kawasaki T, Tsuji H, Madoiwa S, Sakata Y, et al. Protein S-K196E mutation as a genetic risk factor for deep vein thrombosis in Japanese patients. *Blood* 2006;**107**:1737-8.

[11] Esmon CT, Owen WG. Identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. *Proc Natl Acad Sci U S A* 1981;**78**:2249-52.

[12] Weiler H, Isermann BH. Thrombomodulin. *J Thromb Haemost* 2003;**1**:1515-24.

[13] Sadler JE, Lentz SR, Sheehan JP, Tsiang M, Wu Q. Structure–function relationships of the thrombin–thrombomodulin interaction. *Haemostasis* 1993;**23(Suppl 1)**:183-93.

[14] Dittman WA, Majerus PW. Structure and function of thrombomodulin: a natural anticoagulant. *Blood* 1990;**75**: 329-36.

[15] Weiler-Guettler H, Christie PD, Beeler DL, Healy AM, Hancock WW, Rayburn H, et al. A targeted point mutation in thrombomodulin generates viable mice with a prethrombotic state. *J Clin Invest* 1998;**101**:1983-91.

[16] Isermann B, Hendrickson SB, Zogg M, Wing M, Cummiskey M, Kisanuki YY, et al. Endothelium-specific loss of murine thrombomodulin disrupts the protein C anticoagulant pathway and causes juvenile-onset thrombosis. *J Clin Invest* 2001;**108**:537-46.

[17] Kamide K, Tanaka C, Takiuchi S, Miwa Y, Yoshii M, Horio T, et al. Six missense mutations of the epithelial sodium channel β and γ subunits in Japanese hypertensives. *Hypertens Res* 2004;**27**:333-8.

[18] Okuda T, Fujioka Y, Kamide K, Kawano Y, Goto Y, Yoshimasa Y, et al. Verification of 525 coding SNPs in 179 hypertension candidate genes in the Japanese population: identification of 159 SNPs in 93 genes. *J Hum Genet* 2002;**47**:387-94.

[19] Antonarakis SE. Recommendations for a nomenclature system for human gene mutations. Nomenclature Working Group. *Hum Mutat* 1998;**11**:1-3.

[20] Mannami T, Konishi M, Baba S, Nishi N, Terao A. Prevalence of asymptomatic carotid atherosclerotic lesions detected by high-resolution ultrasonography and its relation to cardiovascular risk factors in the general population of a Japanese city: the Suita study. *Stroke* 1997;**28**:518-25.

[21] Mannami T, Baba S, Ogata J. Potential of carotid enlargement as a useful indicator affected by high blood pressure in a large general population of a Japanese city: the Suita study. *Stroke* 2000;**31**:2958-65.

[22] Mannami T, Baba S, Ogata J. Strong and significant relationships between aggregation of major coronary risk factors and the acceleration of carotid atherosclerosis in the general population of a Japanese city: the Suita Study. *Arch Intern Med* 2000;**160**:2297-303.

[23] Kokubo Y, Kamide K, Inamoto N, Tanaka C, Banno M, Takiuchi S, et al. Identification of 108 SNPs in TSC, WNK1, and WNK4 and their association with hypertension in a Japanese general population. *J Hum Genet* 2004;**49**:507-15.

[24] Kamide K, Kokubo Y, Yang J, Tanaka C, Hanada H, Takiuchi S, et al. Hypertension susceptibility genes on chromosome 2p24-p25 in a general Japanese population. *J Hypertens* 2005;**23**:955-60.

[25] Tanaka C, Kamide K, Takiuchi S, Miwa Y, Yoshii M, Kawano Y, et al. An alternative fast and convenient genotyping method for the screening of angiotensin converting enzyme gene polymorphisms. *Hypertens Res* 2003;**26**:301-6.

[26] Li YH, Chen CH, Yeh PS, Lin HJ, Chang BI, Lin JC, et al. Functional mutation in the promoter region of thrombomodulin gene in relation to carotid atherosclerosis. *Atherosclerosis* 2001;**154**:713-9.

[27] Ohlin AK, Norlund L, Marlar RA. Thrombomodulin gene variations and thromboembolic disease. *Thromb Haemost* 1997;**78**:396-400.

[28] Franchi F, Biguzzi E, Cetin I, Facchetti F, Radaelli T, Bozzo M, et al. Mutations in the thrombomodulin and endothelial protein C receptor genes in women with late fetal loss. *Br J Haematol* 2001;**114**:641-6.

[29] Faioni EM, Merati G, Peyvandi F, Bettini PM, Mannucci PM. The G1456 to T mutation in the thrombomodulin gene is not frequent in patients with venous thrombosis. *Blood* 1997;**89**:1467.

[30] Ohlin AK, Marlar RA. The first mutation identified in the thrombomodulin gene in a 45-year-old man presenting with thromboembolic disease. *Blood* 1995;**85**:330-6.

[31] Kunz G, Ohlin AK, Adami A, Zoller B, Svensson P, Lane DA. Naturally occurring mutations in the thrombomodulin gene leading to impaired expression and function. *Blood* 2002;**99**:3646-53.

[32] Thude H, Wilkens A, Anders O, Barz D. Analysis of the thrombomodulin gene in patients with venous thrombosis. *Thromb Res* 2002;**107**:109-14.

[33] Aleksic N, Folsom AR, Cushman M, Heckbert SR, Tsai MY, Wu KK. Prospective study of the A455V polymorphism in the thrombomodulin gene, plasma thrombomodulin, and incidence of venous thromboembolism: the LITE Study. *J Thromb Haemost* 2003;**1**:88-94.

[34] Heit JA, Petterson TM, Owen WG, Burke JP, De Andrade M, Melton III LJ. Thrombomodulin gene polymorphisms or haplotypes as potential risk factors for venous thromboembolism: a population-based case-control study. *J Thromb Haemost* 2005;**3**:710-7.

[35] Chao TH, Li YH, Chen JH, Wu HL, Shi GY, Tsai WC, et al. Relation of thrombomodulin gene polymorphisms to acute myocardial infarction in patients < or =50 years of age. *Am J Cardiol* 2004;**93**:204-7.

[36] Takahashi Y, Hosaka Y, Imada K, Adachi T, Niina H, Mochizuki H. Species specificity of the anticoagulant activity of human urinary soluble thrombomodulin. *Thromb Res* 1998;**89**:187-97.

[37] Ohlin AK, Larsson K, Hansson M. Soluble thrombomodulin activity and soluble thrombomodulin antigen in plasma. *J Thromb Haemost* 2005;**3**:976-82.

[38] Yamada N, Wada H, Nakase T, Minamikawa K, Nagaya S, Nakamura M, et al. Hemostatic abnormalities in patients with pulmonary embolism compared with that in deep vein thrombosis. *Blood Coagul Fibrinolysis* 1995;**6**:627-33.

[39] Trifiletti A, Scamardi R, Pizzoleo MA, Soraci S, Nevoso A, Bagnato L, et al. Haemostatic changes in patients with deep vein thrombosis. *Panminerva Med* 1997;**39**: 21-23.

[40] Smith A, Quarmby JW, Collins M, Lockhart SM, Burnand KG. Changes in the levels of soluble adhesion molecules and coagulation factors in patients with deep vein thrombosis. *Thromb Haemost* 1999;**82**:1593-9.

[41] Mohri M, Suzuki M, Sugimoto E, Sata M, Yamamoto S, Maruyama I. Effects of recombinant human soluble thrombomodulin (rhs-TM) on clot-induced coagulation in human plasma. *Thromb Haemost* 1998;**80**:925-9.

[42] Kearon C, Comp P, Douketis J, Royds R, Yamada K, Gent M. Dose–response study of recombinant human soluble thrombomodulin (ART-123) in the prevention of venous thromboembolism after total hip replacement. *J Thromb Haemost* 2005;**3**:962-8.

[43] Salomaa V, Matei C, Aleksic N, Sansores-Garcia L, Folsom H, Juneja H, et al. Soluble thrombomodulin as a predictor of incident coronary heart disease and symptomless carotid artery atherosclerosis in the Atherosclerosis Risk in Communities (ARIC) Study: a case-cohort study. *Lancet* 1999;**353**:1729-34.

[44] Richardson MA, Berg DT, Calnek DS, Ciaccia AV, Joyce DE, Grinnell BW. 17β-estradiol, but not raloxifene, decreases thrombomodulin in the antithrombotic protein C pathway. *Endocrinology* 2000;**141**:3908-11.

[45] Glaser CB, Morser J, Clarke JH, Blasko E, McLean I, Kuhn I, et al. Oxidation of a specific methionine in thrombomodulin by activated neutrophil products blocks cofactor activity. A potential rapid mechanism for modulation of coagulation. *J Clin Invest* 1992;**90**:2565-73.

[46] Lentz SR, Tsiang M, Sadler JE. Regulation of thrombomodulin by tumor necrosis factor-alpha: comparison of transcriptional and posttranscriptional mechanisms. *Blood* 1991;**77**:542-50.

[47] Lohmueller KE, Pearce CL, Pike M, Lander ES, Hirschhorn JN. Meta-analysis of genetic association studies supports a contribution of common variants to susceptibility to common disease. *Nat Genet* 2003;**33**:177-82.

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REGULAR ARTICLE

Genotypes of vitamin K epoxide reductase, γ-glutamyl carboxylase, and cytochrome P450 2C9 as determinants of daily warfarin dose in Japanese patients

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Abstract The dose required for the anticoagulant effect of warfarin exhibits large inter-individual variations. This study sought to determine the contribution of four genes, vitamin K epoxide reductase (*VKORC1*), γ-glutamyl carboxylase (*GGCX*), calumenin (*CALU*), and cytochrome P450 2C9 (*CYP2C9*) to the warfarin maintenance dose required in Japanese patients following ischemic stroke. We recruited 93 patients on stable anticoagulation with a target International Normalized Ratio (INR) of 1.6–2.6. We genotyped eleven representative single nucleotide polymorphisms (SNPs) in the three genes involved in vitamin K cycle and the 42613A>C SNP in *CYP2C9*, known as *CYP2C9*3*, and then examined an association of these genotypes with warfarin maintenance doses (mean±SD=2.96±1.06 mg/day). We found an association of effective warfarin dose with the −1639G>A ($p=0.004$) and 3730G>A genotypes ($p=0.006$) in *VKORC1*, the 8016G>A genotype in *GGCX* ($p=0.022$), and the 42613A>C genotype in *CYP2C9* ($p=0.015$). The model using the multiple regression analysis including age, sex, weight, and three genetic polymorphisms accounted for 33.3% of total variations in warfarin dose. The contribution to inter-individual variation in warfarin dose was 5.9% for *VKORC1* −1639G>A, 5.2% for *CYP2C9*

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42613A>C, and 4.6% for *GGCX* 8016G>A. In addition to polymorphisms in *VKORC1* and *CYP2C9*, we identified *GGCX* 8016G>A, resulting in the missense mutation R325Q, as a genetic determinant of warfarin maintenance dose in Japanese patients.

Warfarin is the most widely prescribed anticoagulant for long-term prevention of thromboembolic events. The dose of warfarin required to achieve target levels of anticoagulation varies dependent on dietary intake and individual variations in pharmacokinetics. Management of warfarin therapy is difficult because of significant inter-individual and intra-individual variability and the narrow therapeutic range. The effectiveness and safety of warfarin must be monitored by serial determinations of prothrombin time using the standardized international normalized ratio (INR).

Warfarin exerts an anticoagulant effect by interfering with the regeneration of reduced vitamin K from the epoxide form, which is required for the enzymatic activity of vitamin K epoxide reductase subunit 1 (VKORC1) [1,2]. γ-Carboxylation of a wide variety of proteins, including numbers of factors in the clotting cascade, is catalyzed by γ-glutamyl carboxylase (GGCX), a vitamin K-dependent enzyme. This reaction incorporates a carbon dioxide molecule into specific glutamic acid residues with the help of the reduced form of vitamin K and oxygen, generating γ-carboxylglutamic acid and vitamin K 2,3-epoxide. When reduced vitamin K cannot be regenerated, the biosynthesis of vitamin K-dependent coagulation/anticoagulation factors, including prothrombin, factors VII, IX, and X, and proteins C and S, is suppressed. The endoplasmic reticulum resident protein calumenin (CALU) associates with γ-glutamyl carboxylase, inhibiting its activity [3]. Recent studies on the genetic aspects of the inter-individual variability of warfarin have demonstrated that single nucleotide polymorphisms (SNPs) in the *VKORC1* gene influence warfarin responses [4–15]. Haplotype analysis demonstrated that individuals who can be controlled by the low dose of warfarin showed the low hepatic expression of *VKORC1* mRNA [6].

The inter-individual variability of warfarin can also be explained by the genetic variability of the warfarin metabolizing enzyme, *CYP2C9*. The missense mutations R144C and I359L in the *CYP2C9* gene known as *CYP2C9*2* and *CYP2C9*3* are known to associate with warfarin dose [16]. These two genetic variations exhibited ethnic specificity. Asian population does not have the *CYP2C9*2* allele but carries the *CYP2C9*3* allele [17].

In this study, we investigated the influence of SNPs in four genes controlling γ-carboxylation (*VKORC1*, *GGCX*, *CALU*, and *CYP2C9*) on the inter-individual variability of warfarin dose requirements in Japanese patients. We identified SNPs in *VKORC1*, *GGCX*, and *CYP2C9* associated with the inter-individual differences in warfarin dosage.

Materials and methods

Subjects

The study population consisted of 93 unrelated Japanese patients admitted to the Cerebrovascular Division of the National Cardiovascular Center between November 2003 and March 2004. The patients had all experienced an ischemic stroke within the 7 days prior to admission. Stroke subtype consisted of cardioembolic infarction ($n=48$) and the embolic infarction of unknown origin with non-valvular atrial fibrillation ($n=45$). Anticoagulation of all patients was stably controlled with a target INR of 1.6–2.6 for the prevention of stroke recurrence [18,19]. Inclusion criteria were a confirmed date of initial exposure to warfarin, and current anticoagulation therapy. Data collection consisted of inpatient and outpatient medical records. The anticoagulant database was used to obtain information on daily warfarin doses. This study was approved by the Ethical Review Committee of the National Cardiovascular Center. All patients who participated in the study provided written informed consent for genetic analysis.

DNA analyses

We previously performed DNA sequence analyses of 3 genes (*VKORC1*, *GGCX*, and *CALU*) involved in vitamin K cycling in 96 Japanese stroke patients; that study identified genetic polymorphisms and pair-wise linkage disequilibrium (LD) [20]. Using the minor allele frequency (over 4%), LD (r^2 more than 0.5), and possible functional change (missense mutation) as guidance, we selected nine representative SNPs for genotyping: 523G>A, 1338A>G (H68R), and 3730G>A in *VKORC1*, 412G>A, 8016G>A (R325Q), and 8445C>T in *GGCX*, and 11G>A (R4Q), 344G>A, and 20943T>A in *CALU*. In *CYP2C9*, only the 42613A>C (I359L) SNP,

known as the *CYP2C9*3* genotype, was analyzed. In addition, recent studies have demonstrated the significant association of the *VKORC1* polymorphisms −1639G>A and 1173C>T with warf polymorphisms. We adopted the numbering standards of the Nomenclature Working Group, wherein the A of the initiator Met codon (ATG) is denoted nucleotide +1 [21].

The genotypes of the 12 SNPs in our subjects were identified by the TaqMan-PCR system. TaqMan genotyping methodology has been described previously [22]. The PCR primers and probes used for the TaqMan system are available on request.

Statistical analysis

The significance level for all statistical tests was set at $P<0.05$. Pair-wise LD between two polymorphisms was evaluated by r^2 using SNPAlyze v4.0 software (DYNACOM, Kanagawa, Japan). Statistical analyses were performed using JMP v 5.1 software and the SAS release 8.2 (SAS Institute Inc., Cary, NC). Associations between genotypes and warfarin daily doses were examined by one-way analysis of variance or univariate regression analysis. In addition, the relative contributions of age, sex, weight, and selected genetic variations to inter-individual variations in warfarin dose were estimated by using the multiple regression analysis. An index P_i, for estimating the relative contribution of a specific independent variable, x_i, was employed and given by

$$P_i = R^2 - R^2_{-i},$$

where R was the multiple correlation coefficient from the model with all of the selected independent variables (x_1, x_2,..., x_p) and R^2_{-i} was that of the model excluding x_i from the independent variables.

Results

We analyzed the frequency of 11 SNPs in three genes involved in the vitamin K cycle and one polymorphism in *CYP2C9* 42613A>C (*CYP2C9*3*) in 93 stroke patients under stable anticoagulation with warfarin. Characteristics of the patients are summarized in Table 1. The mean±SD daily warfarin dose was 2.96±1.06 mg/day (1.00–5.50 mg/day).

Table 1 Characteristics of patients

Number	93
Number of men (%)	66 (71.0)
Age (years)	68.1±10.6
Weight (kg)	59.8±9.7
Warfarin dose (mg/day)	2.96±1.06
Warfarin dose range (mg/day)	1.00–5.50

Age, weight, and warfarin dose are shown as mean±SD.

Table 2 Differences in daily warfarin dose for each genotype of the *VKORC1*, *GGCX*, and *CYP2C9* genes

Gene	SNP	Genotype	*n*	Mean±SD (mg/day)	*P*
VKORC1	−1639 G>A*	AA	79	2.83±1.00	
		GA	14	3.70±1.11	0.004
		GG	0	–	
VKORC1	1173 C>T*	TT	79	2.83±1.00	
		CT	14	3.70±1.11	0.004
		CC	0	–	
VKORC1	3730 G>A*	GG	79	2.84±1.00	
		GA	14	3.68±1.12	0.006
		AA	0	–	
GGCX	8016 G>A	GG	48	3.25±1.19	
	(R325Q)	GA	39	2.63±0.77	0.022
		AA	6	2.79±1.07	
CYP2C9	42613 A>C	AA	83	3.06±1.05	
	(CYP2C9*3)	AC	9	2.17±0.84	0.015
	(I359L)	CC	0	–	

P values were calculated by one-way ANOVA. *These SNPs were in linkage disequilibrium. Rieder et al. reported that the hepatic expression levels of *VKORC1* mRNA were significantly decreased in the carriers with the *VKORC1* −1639A allele [6]. As for the *GGCX* R325Q mutation, there were no available data on its function. CYP2C9 mutant carrying the missense mutation, I359L (*CYP2C9*3*), showed a markedly high *Km* for the 7-hydroxylation of *S*-warfarin [28].

We examined the association of the genotype data with maintenance warfarin doses by one-way analysis of variance (ANOVA). Of the 12 SNPs examined, five SNPs, −1639G>A, 1173C>T, and 3730G>A in *VKORC1*, 8016G>A (R375Q) in *GGCX*, and *CYP2C9*3* exhibited a significant association with daily warfarin dose (Table 2). The *VKORC1* 1338G>A allele could not be evaluated due to the low minor allele frequency. None of the other SNPs demonstrated a significant association with warfarin dosage.

The mean warfarin dose was higher ($p=0.004$) in patients with the *VKORC1* −1639GA or 1173CT genotypes (3.70 mg/day) than in those with the −1639AA or 1173TT genotypes (2.83 mg/day). The mean warfarin dose was higher ($p=0.006$) in patients with the *VKORC1* 3730GA genotype (3.68 mg/day) than in those with the 3730GG genotype (2.84 mg/day). For *CYP2C9*, the mean warfarin dose was higher ($p=0.015$) in patients with the *CYP2C9*1*1* (*CYP2C9* 42613AA) genotype (3.06 mg/day) than in those with the **1*3* (42613AC) genotype (2.17 mg/day).

A significant association was observed between warfarin dosage and the 8016G>A SNP of *GGCX*. The mean warfarin dose was higher ($p=0.022$) among patients with the *GGCX* 8016GG genotype (3.25 mg/day) than in those with the GA (2.84 mg/day) or AA (2.79 mg/day) genotypes. The *GGCX* 8016G>A SNP,

rs699664, leads to the substitution of Gln for Arg at amino acid 325.

We previously genotyped three SNPs, −1639G>A, 1173C>T, and 3730G>A in *VKORC1*, in 3652 population-based individuals [20]. This analysis obtained a minor allele frequency of 0.086 for all SNPs. Three SNPs were in tight LD with a pair-wise r^2 value of 0.98. Two SNPs in particular, −1639G>A and 1173C>T, were in complete LD in the study population. Therefore, −1639G>A and 3730G>A were used for additional analysis to estimate the influence of *VKORC1* genotypes of warfarin dosage.

To estimate the contribution of each SNP to variabilities in warfarin dosages, we performed univariate regression analyses for four SNPs, *VKORC1* −1639G>A and 3730G>A, *GGCX* 8016G>A, and *CYP2C9* 42613A>C (*CYP2C9*3*) (Table 3). The R^2 values determined for *VKORC1* −1639G>A and 3730G>A were 0.086 and 0.082, respectively. The equivalent R^2 value observed in the model of *GGCX* 8016G>A ($R^2 = 0.081$) was higher than that of *CYP2C9* 42613A>C ($R^2 = 0.064$).

Multiple regression analysis was performed to estimate the relative contributions of age, sex, weight, and three genetic polymorphisms to the inter-individual variations in warfarin dose. These results were shown in Table 4. The model included age, sex, weight, and three genetic polymorphisms, 6 variables in total, as the independent variables and accounted for 33.3% of total variations in warfarin dose. The contribution, P_i, to inter-individual variation in warfarin dose was 5.9% for *VKORC1* −1639G>A, 5.2% for *CYP2C9* 42613A>C, and 4.6% for *GGCX* 8016G>A.

Discussion

In this study, we have examined the contribution of four genes to the warfarin maintenance dose required in Japanese patients following ischemic stroke. The patients were controlled in the target INR of 1.6–2.6. A previous study on the optimal intensity of warfarin therapy for secondary prevention of stroke in patients with non-valvular atrial fibrillation showed that the low-intensity warfarin (INR 1.5 to 2.1) treatment seemed to be safer than the conventional-intensity (INR 2.2 to 3.5) treatment [18]. The annual rate of ischemic stroke was low in both groups (1.1% per year in the conventional-intensity group and 1.7% per year in the low-intensity group) and did not differ significantly. Based on this result and the guideline of the Japanese Circulation Society for the treatment of atrial fibrillation, we adopted the target INR of 1.6–2.6. Daily warfarin dose of each patient was properly controlled to meet target INR. As a result, the range of the warfarin dose was between 1 and 10 mg.

Table 3 Univariate regression analyses for warfarin daily dosage

Variables	R^2	P
VKORC1-1639G>A*	0.086	0.004
VKORC1 3730G>A*	0.082	0.006
GGCX 8016G>A	0.081	0.022
CYP2C9 42613A>C	0.064	0.015

R^2 and P values were calculated by univariate regression analyses. *These two SNPs were in linkage disequilibrium.

Table 4 Multiple regression analysis for estimating the relative contributions of age, sex, weight, and selective genetic variations with warfarin dose

Independent	Std $\beta^{\dagger}$	$P_i \times 100$
Age	−0.141	1.69
Sex	0.786	8.12*
Weight	0.374	7.78*
VKORC1 −1639G>A	0.735	5.88**
GGCX 8016G>A	−0.451	4.60**
CYP2C9 42613A>C	−0.847	5.19**

†: Standardized regression coefficient.
*: $P<0.01$, **: $0.01 \le P<0.05$.

Warfarin is the most prescribed oral anticoagulant. Warfarin targets VKORC1 and antagonizes vitamin K, an essential cofactor for the modification of specific glutamic acid to γ-carboxyglutamic acid in coagulation factors II, VII, IX and X. Warfarin is metabolized by CYP2C9. Patients with *CYP2C9*2* and *CYP2C9*3* alleles have lower mean daily warfarin doses and a greater risk of bleeding [16,23]. Recent studies on *VKORC1* showed that SNPs in *VKORC1* have a more important function than the *CYP2C9* variations in terms of inter-individual variability of warfarin. It has been reported that the *VKORC1* haplotype accounted for 21% of inter-individual variability of warfarin and the *CYP2C9* genotype explained 6% [6]. Subsequent studies reached the similar conclusion that the *VKORC1* genotype affects inter-individual variability of warfarin more greatly than the *CYP2C9* genotype [5,8–11]. Inclusion of non-genetic factors such as age, sex, body surface area, body weight, and drug interaction with genotype information accounted for up to 60% of inter-individual variability of warfarin [5,8–11]. The remaining 40% of warfarin dosing variability remains unexplained.

In our study, *VKORC1* −1639G>A explained 5.9% of the inter-individual variabilities in warfarin dose, while CYP2C9*3 explained 5.2% (Table 4). We also detected a significant association between *GGCX* 8016G>A (R325Q) and warfarin dosage, which explained 4.6% of the variability seen in our subjects (Table 4). We have recently reported that *GGCX* 8016G>A influences the inter-individual variations in

protein C activity in the general population of Japan; women with the GG genotype exhibit approximately 5% higher plasma protein C activity ($p=0.002$) than those with either the GA or AA genotypes [20]. The R325Q mutation is predicted by the topological model to reside within the cytoplasmic domain of GGCX [24]. In this domain, amino acids 343–355 mediate GGCX enzyme/substrate interactions; residues 343–345 of CVY are necessary for both substrate binding and γ-carboxylase activity [25].

Recent studies reported the association of a microsatellite marker in intron 6 of *GGCX* with warfarin dose [26,27]. In 45 warfarin-treated Japanese patients, 10, 11, and 13 CAA repeats were detected. Three individuals heterozygous for the 13 repeat allele required higher maintenance doses than patients with fewer repeats [26]. In 183 warfarin-treated Swedes, a group of individuals bearing both alleles with 13 repeats or those with 14–16 repeats required significantly higher maintenance doses than patients with fewer repeats. Taken together, GGCX is a promising candidate influencing warfarin maintenance doses significantly. Further studies with larger populations and additional ethnic groups are required to elucidate the association between variations in warfarin dosages and the *GGCX* 8016G>A genotype.

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References

[1] Rost S, Fregin A, Ivaskevicius V, Conzelmann E, Hortnagel K, Pelz HJ, et al. *VKORC1* cause warfarin resistance and multiple coagulation factor deficiency type 2. *Nature* 2004;**427**:537–41.

[2] Li T, Chang CY, Jin DY, Lin PJ, Khvorova A, Stafford DW. Identification of the gene for vitamin K epoxide reductase. *Nature* 2004;**427**:541–4.

[3] Wajih N, Sane DC, Hutson SM, Wallin R. The inhibitory effect of calumenin on the vitamin K-dependent γ-carboxylation system. Characterization of the system in normal and warfarin-resistant rats. *J Biol Chem* 2004;**279**:25276–83.

[4] D'Andrea G, D'Ambrosio RL, Di Perna P, Chetta M, Santacroce R, Brancaccio V, et al. A polymorphism in the *VKORC1* gene is associated with an interindividual variability in the dose-anticoagulant effect of warfarin. *Blood* 2005;**105**:645–59.

[5] Bodin L, Verstuyft C, Tregouet DA, Robert A, Dubert L, Funck-Brentano C, et al. Cytochrome P450 2C9 *(CYP2C9)* and vitamin K epoxide reductase *(VKORC1)* genotypes as determinants of acenocoumarol sensitivity. *Blood* 2005;**106**: 135–40.

[6] Rieder MJ, Reiner AP, Gage BF, Nickerson DA, Eby CS, McLeod HL, et al. Effect of *VKORC1* haplotypes on transcriptional regulation and warfarin dose. *N Engl J Med* 2005;**352**:2285–93.

[7] Yuan HY, Chen JJ, Lee MT, Wung JC, Chen YF, Charng MJ, et al. A novel functional VKORC1 promoter polymorphism is associated with inter-individual and inter-ethnic differences in warfarin sensitivity. *Hum Mol Genet* 2005;**14**: 1745–51.

[8] Sconce EA, Khan TI, Wynne HA, Avery P, Monkhouse L, King BP, et al. The impact of *CYP2C9* and *VKORC1* genetic polymorphism and patient characteristics upon warfarin dose requirements: proposal for a new dosing regimen. *Blood* 2005;**106**:2329–33.

[9] Veenstra DL, You JH, Rieder MJ, Farin FM, Wilkerson HW, Blough DK, et al. Association of Vitamin K epoxide reductase complex 1 *(VKORC1)* variants with warfarin dose in a Hong Kong Chinese patient population. *Pharmacogenet Genomics* 2005;**15**:687–91.

[10] Wadelius M, Chen LY, Downes K, Ghori J, Hunt S, Eriksson N, et al. VKORC1 and GGCX polymorphisms associated with warfarin dose. *Pharmacogenomics J* 2005;**5**:262–70.

[11] Takahashi H, Wilkinson GR, Nutescu EA, Morita T, Ritchie MD, Scordo MG, et al. Different contributions of polymorphisms in VKORC1 and CYP2C9 to intra- and inter-population differences in maintenance dose of warfarin in Japanese, Caucasians and African-Americans. *Pharmacogenet Genomics* 2006;**16**:101–10.

[12] Vecsler M, Loebstein R, Almog S, Kurnik D, Goldman B, Halkin H, et al. Combined genetic profiles of components and regulators of the vitamin K-dependent γ-carboxylation system affect individual sensitivity to warfarin. *Thromb Haemost* 2006;**95**:205–11.

[13] Mushiroda T, Ohnishi Y, Saito S, Takahashi A, Kikuchi Y, Saito S, et al. Association of *VKORC1* and *CYP2C9* polymorphisms with warfarin dose requirements in Japanese patients. *J Hum Genet* 2006;**51**:249–53.

[14] Lee SC, Ng SS, Oldenburg J, Chong PY, Rost S, Guo JY, et al. Interethnic variability of warfarin maintenance requirement is explained by *VKORC1* genotype in an Asian population. *Clin Pharmacol Ther* 2006;**79**:197–205.

[15] Montes R, Ruiz de Gaona E, Martinez-Gonzalez MA, Alberca I, Hermida J. The c.–1639G>A polymorphism of the *VKORC1* gene is a major determinant of the response to acenocoumarol in anticoagulated patients. *Br J Haematol* 2006;**133**: 183–7.

[16] Higashi MK, Veenstra DL, Kondo LM, Wittkowsky AK, Srinouanprachanh SL, Farin FM, et al. Association between *CYP2C9* genetic variants and anticoagulation-related outcomes during warfarin therapy. *JAMA* 2002; **287**:1690–8.

[17] Nasu K, Kubota T, Ishizaki T. Genetic analysis of *CYP2C9* polymorphism in a Japanese population. *Pharmacogenetics* 1997;**7**:405–9.

[18] Yamaguchi T. Optimal intensity of warfarin therapy for secondary prevention of stroke in patients with nonvalvular atrial fibrillation: a multicenter, prospective, randomized trial. Japanese Nonvalvular Atrial Fibrillation-Embolism Secondary Prevention Cooperative Study Group. *Stroke* 2000;**31**:817–21.

[19] Chimowitz MI, Lynn MJ, Howlett-Smith H, Stern BJ, Hertzberg VS, Frankel MR, et al. Comparison of warfarin and aspirin for symptomatic intracranial arterial stenosis. *N Engl J Med* 2005;**352**:1305–16.

[20] Kimura R, Kokubo Y, Miyashita K, Otsubo R, Nagatsuka K, Otsuki T, et al. Polymorphisms in vitamin K-dependent

γ-carboxylation-related genes influence interindividual variability in plasma protein C and protein S activity in general population. Int J Hematol in press.

[21] Antonarakis SE. Recommendations for a nomenclature system for human gene mutations. Nomenclature Working Group. *Hum Mutat* 1998;**11**:1–3.

[22] Tanaka C, Kamide K, Takiuchi S, Miwa Y, Yoshii M, Kawano Y, et al. An alternative fast and convenient genotyping method for the screening of angiotensin converting enzyme gene polymorphisms. *Hypertens Res* 2003;**26**:301–6.

[23] Sanderson S, Emery J, Higgins J. *CYP2C9* gene variants, drug dose, and bleeding risk in warfarin-treated patients: a HuGEnet systematic review and meta-analysis. *Genet Med* 2005;**7**:97–104.

[24] Tie J, Wu SM, Jin D, Nicchitta CV, Stafford DW. A topological study of the human γ-glutamyl carboxylase. *Blood* 2000;**96**:973–8.

[25] Pudota BN, Hommema EL, Hallgren KW, McNally BA, Lee S, Berkner KL. Identification of sequences within the γ-carboxylase that represent a novel contact site with vitamin K-dependent proteins and that are required for activity. *J Biol Chem* 2001;**276**:46878–86.

[26] Shikata E, Ieiri I, Ishiguro S, Aono H, Inoue K, Koide T, et al. Association of pharmacokinetic *(CYP2C9)* and pharmacodynamic (*factors II, VII, IX,* and *X*; *proteins S* and *C*; and *γ-glutamyl carboxylase*) gene variants with warfarin sensitivity. *Blood* 2004;**103**:2630–5.

[27] Chen LY, Eriksson N, Gwilliam R, Bentley D, Deloukas P, Wadelius M. γ-Glutamyl carboxylase (GGCX) microsatellite and warfarin dosing. *Blood* 2005;**106**:3673–4.

[28] Sullivan-Klose TH, Ghanayem BI, Bell DA, Zhang ZY, Kaminsky LS, Shenfield GM, et al. The role of the CYP2C9-Leu359 allelic variant in the tolbutamide polymorphism. *Pharmacogenetics* 1996;**6**:341–9.

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 ≥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 hypothyroid-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 (yeasr 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.

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.

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 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′-CCCCATCAG ATGCCACTGTGA-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	*n*	Odds ratio	(95% CI)	*p*	Genotype	*n*	Odds ratio	(95% CI)	*p*
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)	
		TC+CC	590	1.10	(0.82–1.50)	0.520	CC	173	0.98	(0.69–1.40)	0.924
rs212528	Women	TT	663	1	(reference)		TT+TC	980	1	(reference)	
		TC+CC	347	1.40	(1.04–1.89)	0.026	CC	30	1.63	(0.74–3.58)	0.227
	Men	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
rs212526	Women	CC	734	1	(reference)		CC+CT	996	1	(reference)	
		CT+TT	280	0.76	(0.55–1.05)	0.099	TT	18	0.77	(0.25–2.35)	0.650
	Men	CC	615	1	(reference)		CC+CT	842	1	(reference)	
		CT+TT	251	0.95	(0.70–1.30)	0.751	TT	24	1.40	(0.58–3.38)	0.455
rs2038090	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)	
		AC+CC	189	1.00	(0.71–1.40)	0.989	CC	9	3.32	(0.67–16.45)	0.142
rs2038089	Women	AA	414	1	(reference)		AA+AG	880	1	(reference)	
		AG+GG	598	1.19	(0.89–1.59)	0.240	GG	132	1.21	(0.80–1.84)	0.358
	Men	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

*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				
		n	DBP (mmHg)	*p**	SBP (mmHg)	*p**	*n*	DBP (mmHg)	*p**	SBP (mmHg)	*p**
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	0.931	133.83±2.89	0.941
	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	0.840	131.47±0.94	0.698
	TT+TC	980	76.51±0.30		127.64±0.53		827	79.67±0.34		131.66±0.60	
	CC	30	77.57±1.72	0.545	133.33±3.02	0.066	36	80.93±1.66	0.458	133.83±2.89	0.463
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	0.048	138.16±3.59	0.422
	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	0.135	132.03±1.09	0.780
	CC+CT	996	76.65±0.30		127.92±0.52		842	79.61±0.34		131.59±0.59	
	TT	18	70.08±2.19	0.003	120.04±3.87	0.044	24	84.13±2.06	0.030	138.16±3.59	0.071

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	*p* Asymptotic	*p* Permutation	Freq (%)	χ^2	*p* Asymptotic	*p* Permutation	Freq (%)	χ^2	*p* Asymptotic	*p* 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, 0848/0.152; 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	
1910G>T		intron 2	0.473	0.527	TGTAACCCTA[G/T]TCATTCATTA	rs2070699
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.

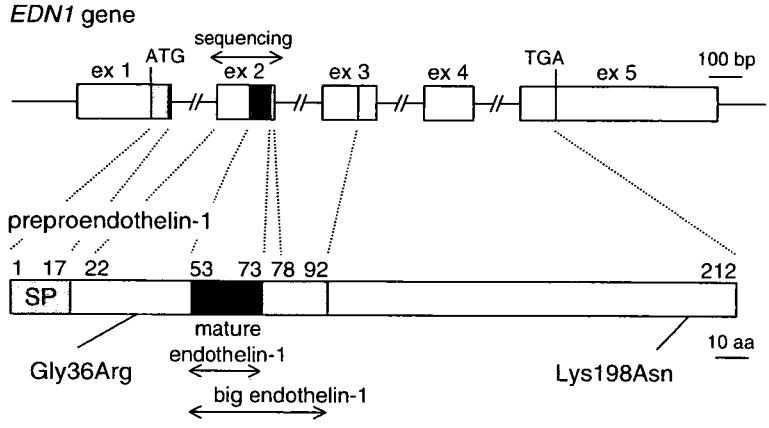

Fig. 1. *Genome and domain structure of human endothelin 1. Two missense mutations in endothelin-1, Gly36Arg (G36R) and Lys198Asn (K198N), are shown. The G36R mutation in preproendothelin-1 was identified in this study.*

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 nonsynomymous 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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References

1. Yanagisawa M, Kurihara H, Kimura S, *et al*: A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1988; **332**: 411–415.
2. Miyauchi T, Masaki T: Pathophysiology of endothelin in the cardiovascular system. *Annu Rev Physiol* 1999; **61**: 391–415.
3. Tiret L, Poirier O, Hallet V, *et al*: The Lys198Asn polymorphism in the endothelin-1 gene is associated with blood pressure in overweight people. *Hypertension* 1999; **33**: 1169–1174.
4. Asai T, Ohkubo T, Katsuya T, *et al*: Endothelin-1 gene variant associates with blood pressure in obese Japanese subjects: the Ohasama Study. *Hypertension* 2001; **38**: 1321–1324.
5. Jin JJ, Nakura J, Wu Z, *et al*: Association of endothelin-1 gene variant with hypertension. *Hypertension* 2003; **41**: 163–167.
6. Tanaka C, Kamide K, Takiuchi S, *et al*: Evaluation of the Lys198Asn and −134delA genetic polymorphisms of the endothelin-1 gene. *Hypertens Res* 2004; **27**: 367–371.