

Assessment of Genetic Effects of Polymorphisms in the MCP-1 Gene on Serum MCP-1 Levels and Myocardial Infarction in Japanese

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Background Recently, the Framingham Heart Study reported that genetic variations in *CCL2* influence serum levels of monocyte chemoattractant protein-1 (MCP-1) and the incidence of myocardial infarction (MI). The purpose of the present study was to investigate the possible involvement of *CCL2* in the pathogenesis of atherosclerosis and MI in Japanese.

Methods and Results Multiple regression analysis indicated that the MCP-1 levels were significantly influenced by various factors including age, body mass index, smoking, alcohol intake, high density lipoprotein-cholesterol, and systolic blood pressure. Moreover, the serum MCP-1 level was significantly correlated with intima-media thickness ($p < 0.0001$). However, this association disappeared when other clinical confounding factors were included in the analyses. Comprehensive analysis of common polymorphisms of *CCL2* in a large community-based population and in subjects with MI found that the A(-2138)T polymorphism affected the serum MCP-1 level in a subgroup of subjects 65 years and older. However, no significant differences in the frequencies of any of the polymorphisms or haplotypes were found between subjects with and without MI. None of the polymorphisms in *CCL2* affected carotid atherosclerosis.

Conclusions The serum MCP-1 level was a good surrogate marker of atherosclerosis in the present study population. Although genetic variations in *CCL2* may have some influence on MCP-1 production, their influence does not seem to contribute appreciably to atherosclerosis in Japanese. The present results did not support the recently published findings from the Framingham Heart Study. The discrepancy between the 2 studies may be related to differences in confounding factors that contribute to MCP-1 levels and in the haplotype structure of the 2 populations. (Circ J 2006; 70: 805–809)

Key Words: Atherosclerosis; Epidemiology; Monocyte chemoattractant protein-1; Myocardial infarction; Polymorphisms

Monocyte chemoattractant protein-1 (MCP-1; gene name *CCL2*) has been suggested to play an important role in the initiation of atherosclerosis by recruiting monocytes to sites of injured endothelium. MCP-1 promotes monocyte differentiation to lipid-laden macrophages, and also contributes to the proliferation of arterial smooth muscle cells.^{1–4}

In various murine models of atherosclerosis, deletion of *CCL2* has resulted in large reductions in atherosclerotic plaque size⁵ but conversely, overexpression of MCP-1 in the leukocytes of susceptible mice resulted in increased plaque size⁶

Several human epidemiological studies have also suggested links between MCP-1 levels and atherosclerotic disease.^{7–10} Higher MCP-1 levels have been associated with increased risks of myocardial infarction (MI), sudden death, coronary angioplasty, and stent restenosis. Very recently, the Framingham Heart Study reported that *CCL2* polymor-

Table 1 Characteristics of the Study Population

	Suita	MI	p value
n	2,266	342	
M (%)	46.0	87.1	<0.0001
Age	65.2 (11.0)	57.9 (9.9)	<0.0001
BMI	22.8 (3.1)	23.9 (2.9)	<0.0001
HTN (%)	38.7	53.4	<0.0001
DM (%)	9.4	40.4	<0.0001
TG	107 (71)	125 (69)*	0.0007
TC	209 (33)	197 (37)*	<0.0001
HDL-C	60 (16)	43 (13)*	<0.0001
Smoking	16.3	61.1	<0.0001
MCP-1	243 (958)**	–	
log (MCP-1)	5.23 (0.42)**	–	
IMT	0.79 (0.13)***	–	
MI	34 (1.5%)	342 (100%)	

Values are expressed as mean (SD).

*n=235, **n=2,180, ***n=2,035.

MI, myocardial infarction; M, male subjects; BMI, body mass index (kg/m^2); HTN, hypertensive subjects; DM, diabetes mellitus; TG, triglycerides (mg/dl); TC, total cholesterol (mg/dl); HDL-C, high density lipoprotein cholesterol (mg/dl); Smoking, current smokers; MCP-1, serum MCP-1 level (ng/ml); log (MCP-1), logarithmic transformation of MCP-1 level; IMT, intima media thickness (mm).

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Table 2 Probes and Primers in TaqMan

Polymorphisms	Probe		Primer	
	VIC	FAM	Forward	Reverse
G(-2581)A	acagctGtcacttc	agacagctAtcacttc	ttccactcacttctctcagc	gacttggcctttgcatatcaga
A(-2138)T	ctctctctaatcTgttagtgcac	ctctctctaatcAgttagtgcac	cccgaaagcagctgagttat	cctaggccatctcaccctcatct
A(-1811)G	aaatggccActccatag	aaatggccGctccata	caaagcagggctcagttg	cttgggactagacttgaigtcca
C(-972)G	cttagctgtCtgcccat	ttagctgtGtgcccat	gcctcttaactacataatgacttagcc	ctctgtctcagcatcttccaa
G(-928)C	aagcaGgcaactagt	ccaagcacgCaacta	tggaagaigtgaggacagaga	ggaaacggigtacaagctcccaa
C(7320112)G	atgagctcttCcttct	tgagctcttGcttct	tgaggataggcaguagcactgg	uagcaaaaggcaggcagga

Table 3 Summary of CCL2 Polymorphisms

Polymorphism	Sequence	Region	Mi-AF
G(-2581)A	GACAGCT G/A TCACTTT	Promoter	0.332
G(-2411)C	CAAAGCT G/C GGAAGTT	Promoter	0.082
A(-2138)T	CACTAAC T/A GATTAGA	Promoter	0.049
A(-1811)T	AATGGCC A/T CTCCATA	Promoter	0.082
C(-972)G	TAGCTGT C/G TGCCCAT	Promoter	0.005
G(-928)C	CCAAGCA G/C GCAACTA	Promoter	0.049
C(-362)G	CGCTTCA C/G AGAAAGC	Promoter	0.332
C(7320112)G	GCTCTTT C/G TCTTCTC	Intron1	0.086
T(7320249)C	CCTGCTG T/C TATAACT	Exon2	0.044
C(7320891)T	AGACACC C/T TGTTTTA	Exon3	0.332

Mi-AF (minor allele frequency) was calculated based on the sequencing data of 93 subjects.

phisms are associated with serum MCP-1 levels and MI.¹¹ In genetic association studies, validation in other study populations is very important to confirm that the observed effects are not statistical errors, so the purpose of the present study was to assess the genetic effects of CCL2 polymorphisms on serum MCP-1 levels and atherosclerosis in Japanese subjects.

Methods

Study Population

The selection criteria and design of the Suita study have been described previously.¹²⁻¹⁴ The genotypes were determined in 2,266 subjects (including 34 MI subjects) recruited from the Suita study between September 2003 and March 2005. Serum MCP-1 levels were measured in 2,180 subjects. The MI group consisted of 342 randomly selected inpatients and outpatients with documented MI who were enrolled in the Division of Cardiology at the National Cardiovascular Center between May 2001 and April 2003.^{15,16} All the subjects enrolled in the present study gave written informed consent. The present study was approved by the Ethics Committee of the National Cardiovascular Center and by the Committee on Genetic Analysis and Gene Therapy of the National Cardiovascular Center. The characteristics of the study population are shown in Table 1. Subjects with systolic blood pressure (SBP) ≥ 140 mmHg, diastolic blood pressure ≥ 90 mmHg, and/or who were taking antihypertensive medication were categorized as having hypertension. Subject with fasting blood glucose ≥ 126 mg/dl, hemoglobin A1c $\geq 6.5\%$, and/or who were being treated for diabetes mellitus was categorized as having the disease.

Fasting serum samples were collected and stored at -80°C . MCP-1 levels were measured in duplicate with a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The inter- and intra-assay variabilities were 6.3% and 6.2%, respectively. Because the distribution of serum MCP-1 levels was skewed, the values were logarithmically

transformed in the statistical analysis.

The details of the method used for the carotid ultrasonic examination have been reported previously.¹⁴ We used a high-resolution B-mode ultrasonic machine with 7.5-MHz transducers, which gave an axial resolution of 0.2 mm. The regions between 30 mm proximal from the beginning of the dilation of the bifurcation bulb and 15 mm distal from the flow divider of both common carotid arteries (CCAs) were scanned. All measurements were made at the time of scanning with the instrument's electronic caliper and were recorded as photocopies. The intima-media thickness (IMT) was measured on a longitudinal scan of the CCA at a point 10 mm proximal from the beginning of the dilation of the bulb.

DNA Study

The promoter (up to -2.8 kb) and exons 1, 2, and 3 (including 3' UTR) regions were sequenced in 93 subjects, which included the top 12 subjects with high serum MCP-1 levels and the bottom 12 subjects with low serum MCP-1 levels. The sequence primers will be provided on request. The genotypes were determined by the TaqMan method (Table 2). The success rate of genotyping was greater than 96%.

Statistical Analysis

Values are expressed as mean \pm standard deviation (SD). All statistical analyses were performed with the JMP statistical package (SAS Institute Inc, Cary, NC, USA). Multiple regression analysis was performed to obtain predictors of the serum MCP-1 level and to assess the contribution of polymorphisms of CCL2 to the serum MCP-1 level. Multiple logistic analysis was performed to obtain predictors for MI. Residuals of the serum MCP-1 level and IMT were calculated by adjusting for appropriate confounding factors. R-square values between polymorphisms and haplotype frequencies in the control and MI groups were analyzed using the SNPalyze Pro statistical package (version 3.2, Dynacom Inc). A statistical power calculation was per-

Table 4 Linkage Disequilibrium Among the Polymorphisms of CCL2

	G(-2581)A	A(-2138)T	A(-1811)T	C(-972)G	G(-928)C	C(7320112)G
G(-2581)A						
A(-2138)T	0.12356					
A(-1811)T	0.16035	0.00565				
C(-972)G	0.02467	0.00086	0.00034			
G(-928)C	0.12411	0.97084	0.00582	0.00089		
C(7320112)G	0.15605	0.00546	0.00716	0.00108	0.00562	

Linkage disequilibrium (LD) among the polymorphisms of CCL2 was calculated from the TaqMan data of the Suiita subjects. R-square values between polymorphisms are shown. Tight LD was observed between the A(-2581)T and G(-928)C polymorphisms.

Table 5 Predictors of Serum MCP-1 Level

Predictor	t-ratio	p value
Age	7.9	<0.0001
BMI	-3.21	0.0014
SBP	2.42	0.0155
Alcohol	2.71	0.0067
Smoking	3.36	0.0008
HDL-C	-2.59	0.0096

Predictors of serum MCP-1 levels were identified by multiple regression analysis (n=2,180). Alcohol, ethanol consumption per day (g/day); Smoking, number of cigarettes per day X years.

SBP, systolic blood pressure. See Table 1 for other abbreviations.

Table 6 Predictors of Intima-Media Thickness

Predictor	t-ratio	p value
log (MCP-1)	0.13	0.7191
Age	353.82	<0.0001
SBP	29.67	<0.0001
Sex	33.21	<0.0001
BMI	33.45	<0.0001

n=2,034, F=128.197, p<0.0001.

The serum MCP-1 levels were assessed in 2,034 of the 2,035 subjects assessed by carotid sonography.

See Tables 1,5 for abbreviations.

Table 7 Influence of the Polymorphisms of CCL2 on Serum MCP-1 Level

	AA	Aa	aa	p value
G(-2581)A	0.002 (0.399)	0.004 (0.418)	-0.020 (0.387)	0.692
n	936	961	270	
A(-2138)T	-0.006 (0.402)	0.049 (0.436)	-0.054 (0.211)	0.122 (0.052)
n	1,909	253	7	
A(-1811)T	0.006 (0.407)	-0.031 (0.406)	-0.079 (0.292)	0.268 (0.117)
n	1,839	313	13	
C(-972)G	-0.001 (0.406)	0.045 (0.404)	-	0.409
n	2,111	54		
G(-928)C	-0.006 (0.403)	0.048 (0.430)	-0.054 (0.211)	0.123 (0.052)
n	1,896	262	7	
C(7320112)G	0.004 (0.401)	-0.013 (0.023)	-0.163 (0.108)	0.259 (0.349)
n	1,840	311	14	
A(-2138)T	-0.012 (0.351)	0.081 (0.462)	-0.051 (0.153)	0.0126 (0.0041)
Age ≥65 years	1,041	154	4	
G(-928)C	-0.012 (0.351)	0.081 (0.500)	-0.051 (0.153)	0.0124 (0.0040)
Age ≥65 years	1,035	156	4	

Residuals of log (MCP-1) were calculated by adjusting for Age, BMI, SBP, alcohol, smoking, and HDL-C. Values are expressed as mean (SD). p values calculated by grouping AA/Aa+aa are shown in parentheses. The effects of the A(-2138)T and G(-928)C polymorphisms on the MCP-1 level were more significant in subjects aged 65 years and older.

See Tables 1,5 for abbreviations.

formed with the statistical package SamplePower (version 2.0, SPSS, Chicago, IL, USA).

Results

Sequence Analysis of CCL2

Sequence analyses in 93 subjects revealed the existence of 10 polymorphisms (Table 3) of CCL2. The G(-2581)A was in almost complete linkage disequilibrium (LD) with the C(-362)G and C(7320891)T polymorphisms. The A(-1811)G polymorphism was in almost complete LD with the G(-2411)C polymorphism. Thus, the genotypes of the C(-362)G, C(7320891)T, and G(-2411)C polymorphisms were not determined in the present study. Because the polymorphism in exon 2 [T(7320249)C] was synonymous (Cys→Cys), this polymorphism was also not determined in the present study. The genotypes of the remaining

6 polymorphisms were determined by the TaqMan method in a total of 2,570 subjects. The LD values calculated from R-square values among these SNPs are shown in Table 4.

Clinical Correlates of Serum MCP-1 Level

Multiple regression analysis indicated that the MCP-1 level was significantly influenced by various factors (p<0.0001, R-square=0.054) including age (p<0.0001), body mass index (BMI; p=0.0014), smoking (p=0.0008), alcohol intake (p=0.0067), high-density lipoprotein cholesterol (p=0.0096), and SBP (p=0.0155) (Table 5).

Many studies have reported that the serum MCP-1 level is an excellent indicator of atherosclerosis and in our study population the serum MCP-1 level significantly correlated with IMT (p<0.0001, R-square=0.009). However, this association disappeared when other clinical confounding factors were included in the multiple regression analyses (Table 6).

Table 8 CCL2 Polymorphisms and Incidence of MI

	MI (-)			MI			p value
	AA	Aa	aa	AA	Aa	aa	
G(-2581)A (%)	946 (43.35)	966 (44.13)	274 (12.52)	149 (40.93)	176 (48.53)	39 (10.71)	0.2857
A(-2138)T (%)	1,931 (88.25)	250 (11.43)	7 (0.32)	218 (87.36)	45 (12.36)	1 (0.27)	0.8686 [0.6289]
A(-1811)T (%)	1,861 (85.02)	314 (14.34)	14 (0.64)	304 (83.29)	56 (15.34)	5 (1.37)	0.3337 [0.3999]
C(-972)G (%)	2,130 (97.53)	54 (2.47)		357 (98.08)	7 (1.92)		0.5548
G(-928)C (%)	1,918 (87.82)	259 (11.86)	7 (0.32)	319 (87.40)	45 (12.33)	1 (0.27)	0.9578 [0.8200]
C(7320112)G (%)	1,855 (84.94)	315 (14.42)	14 (0.64)	302 (82.74)	61 (16.71)	2 (0.55)	0.5229 [0.2880]

Genotype frequencies between subjects with and without MI are shown. p values calculated by grouping AA/Aa + aa are shown in square parentheses.

See Table 1 for abbreviation.

Table 9 Influence of CCL2 Polymorphisms on IMT

	AA	Aa	aa	p value
G(-2581)A	-0.003 (0.104)	0.001 (0.105)	0.006 (0.115)	0.421
n	865	908	255	
A(-2138)T	0.000 (0.106)	-0.001 (0.103)	0.065 (0.118)	0.319
n	1,784	237	6	(0.958)
A(-1811)T	0.000 (0.105)	0.003 (0.112)	-0.049 (0.068)	0.227
n	1,717	294	12	(0.752)
C(-972)G	0.000 (0.106)	0.000 (0.113)	-	0.964
n	1,970	53		
G(-928)C	0.000 (0.106)	-0.003 (0.103)	0.065 (0.118)	0.291
n	1,771	246	6	(0.802)
C(7320112)G	-0.001 (0.106)	0.005 (0.101)	0.039 (0.159)	0.275
				(0.278)

Residuals of IMT were calculated by adjusting for sex, age, BMI, and SBP. Values are expressed as mean (SD). p values calculated by grouping AA/Aa + aa are shown in parentheses.

See Tables 1,5 for abbreviations.

Table 10 Haplotype Analysis of the 2 Study Populations

Suita	Framingham	G(-2581)A	A(2138)T	A(-1811)G	G(-928)C	C7320112G	MI (-)	MI	Framingham
Haplo1	H1	G	A	A	G	C	65.2	65.0	27.0
Haplo2	H4 + H5	A	A	A	G	C	13.2	10.9	26.9
Haplo3	H6	A	A	G	G	C	7.8	9.0	4.2
Haplo4	-	A	A	A	G	G	7.5	8.7	-
Haplo5	-	A	T	A	C	C	6.1	6.5	-
-	H2	A	T	A	G	G	<0.01	<0.01	20.3
-	H3	A	A	A	C	C	<0.01	<0.01	18.6

Haplotype frequencies in the MI (-) and MI groups were calculated. Haplotype frequencies reported in the Framingham study are also shown for reference. See Table 1 for abbreviation.

Thus, the serum MCP-1 level was only a surrogate marker of atherosclerosis in the present study population.

Influence of Polymorphisms on Serum MCP-1 Level

Next, we examined the influence of polymorphisms of CCL2 on residuals of the MCP-1 level after adjusting for the above-mentioned confounding factors (Adj-MCP1) (Table 7). Two polymorphisms, A(-2138)T and G(-928)C, tended to affect Adj-MCP1. The A(-2138)T and G(-928)C polymorphisms were in tight LD (R-square=0.97084) in this study population (Table 4). Interestingly, the influence of these polymorphisms on Adj-MCP1 seemed to be exaggerated in subjects 65 years and older whose MCP-1 levels were significantly higher than those of younger subjects.

Association Study Between CCL2 Polymorphisms and MI

No significant difference was found in the frequencies of any of the polymorphisms between the cases and controls (Table 8). Multiple logistic analyses including age and BMI indicated that none of the polymorphisms contributed to MI. Moreover, none of them affected IMT after adjusting for sex, age, SBP, and BMI (Table 9).

Haplotype Analysis

We constructed haplotypes based on the G(-2581)A, A(-2138)T, A(-1811)T, G(-928)C, and C7320112G polymorphisms and identified 5 common haplotypes that accounted for 99.7% of all haplotypes. The C(-972)G polymorphism was not included because of its low frequency. No significant difference was observed in haplotype fre-

quencies between subjects with and without MI (Table 8).

The haplotype frequencies reported in the Framingham study¹¹ were significantly different from those in the present study population (Table 10). Although H2 and H3, which accounted for 20.3% and 18.6%, respectively, in the Framingham study, were very rare in this study population, Haplo4 and 5, which were rare in the Framingham study, were common.

Discussion

This report describes a comprehensive analysis of the common polymorphisms of *CCL2* in both a large community-based population and subjects with MI. No significant differences in the frequencies of any of the polymorphisms were found between cases and controls. Moreover, none of the polymorphisms of *CCL2* affected carotid atherosclerosis as assessed by IMT. However, the A(-2136)T and G(-928)C polymorphisms tended to affect the serum MCP-1 level. Although genetic variations in *CCL2* may have some influence on MCP-1 production, their does not seem to contribute appreciably to atherosclerosis in Japanese subjects. Thus, our findings do not support the recently published result from the Framingham Heart Study¹¹ that genetic variations in *CCL2* significantly influence serum MCP-1 levels and the incidence of MI.

There may be several reasons for this discrepancy. The MCP-1 levels in the Framingham Heart Study were approximately 1.4-fold higher than those in the present study population. Genetic variation might well have an influence under a stimulated state. MCP-1 levels are influenced by various factors, as described in Table 5. It is conceivable that subjects in the Framingham Heart Study may have had higher MCP-1 levels because of stimulation by atherogenic factors that may be more prevalent in Caucasians. Indeed, the influence of genetic variations was more evident in the present study population when the analysis was limited to older subjects who had higher MCP-1 levels (Table 7).

In the Framingham Heart Study, the haplotype H2 was reported to contribute to higher MCP-1 levels, and the frequency of this haplotype was 20.3%.¹¹ It is defined by the (-2138)T and (77320112)G genotypes, and although the A(-2138)T and G(7320112)C polymorphisms were observed in the present study population, the H2 haplotype was not ($p < 0.01\%$). This difference in the haplotype structure between Caucasians and Japanese might also contribute to the discrepancy between the 2 studies.

The reported positive association between the A(-2581)T polymorphism and MI in the Framingham Heart Study was based on 1,797 study subjects, including just 107 MI subjects,¹¹ which was insufficient statistical power ($p < 0.50$) to conclude that there was a positive association between the genotype and MI. Moreover, although the H2 haplotype was reported to be associated with the serum MCP-1 level, the H1 haplotype but not the H2 haplotype, was reported to be associated with MI. This inconsistency might also indicate that the Framingham study had insufficient statistical power.

Although the serum MCP-1 level is an excellent indicator of atherosclerosis,⁷⁻¹⁰ MCP-1 itself appears to make only a slight contribution to atherosclerosis (Table 6). Thus, it is unlikely that genetic polymorphisms that may only slightly influence the serum MCP-1 level will contribute significantly to the occurrence of MI and atherosclerosis. Our present findings suggest that, although genetic variations in *CCL2* may have some influence on MCP-1 production, their influ-

ence on the incidence of MI is not appreciable in Japanese. The present study also indicates the importance of clarifying the haplotype structure for comparing genetic association studies involving different ethnic backgrounds.

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Original Article

Genetic Variations of *HSD11B2* in Hypertensive Patients and in the General Population, Six Rare Missense/Frameshift Mutations

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Mutations in the gene encoding 11 β -hydroxysteroid dehydrogenase type 2, *HSD11B2*, cause a rare monogenic juvenile hypertensive syndrome called apparent mineralocorticoid excess (AME). In AME, defective *HSD11B2* enzyme activity results in overstimulation of the mineralocorticoid receptor (MR) by cortisol, causing sodium retention, hypokalemia, and salt-dependent hypertension. Here, we have studied whether genetic variations in *HSD11B2* are implicated in essential hypertension in Japanese hypertensives and the general population. By sequencing the entire coding region and the promoter region of *HSD11B2* in 953 Japanese hypertensives, we identified five missense mutations in 11 patients (L14F, $n=5$; R74H, $n=1$; R147H, $n=3$; T156I, $n=1$; R335H, $n=1$) and one novel frameshift mutation (4884Gdel, $n=1$) in a heterozygous state, in addition to 19 genetic variations. All genetic variations identified were rare, with minor allele frequencies less than 0.005. Four of 12 patients with the missense/frameshift mutations showed renal failure. Four missense mutations, L14F, R74H, R147H, and R335H, were successfully genotyped in the general population, with a sample size of 3,655 individuals (2,175 normotensives and 1,480 hypertensives). Mutations L14F, R74H, R147H, and R335H were identified in hypertensives ($n=6, 8, 3,$ and 0 , respectively) and normotensives ($n=8, 12, 5,$ and 0 , respectively) with a similar frequency, suggesting that these missense mutations may not strongly affect the etiology of essential hypertension. Since the allele frequency of all of the genetic variations identified in this study was rare, an association study was not conducted. Taken together, our results indicate that missense mutations in *HSD11B2* do not substantially contribute to essential hypertension in Japanese. (*Hypertens Res* 2006; 29: 243–252)

Key Words: HSD11B2, missense mutation, genetic variation, essential hypertension, salt-sensitivity

Introduction

In mineralocorticoid target organs, the 11 β -hydroxysteroid dehydrogenase (*HSD11B*) catalyzes the interconversion of

the endogenous cortisol and cortisone in humans. Two distinct forms, *HSD11B1* and *HSD11B2*, of *HSD11B* have been characterized and cloned (1–3). *HSD11B1* is expressed in most tissues. In contrast, *HSD11B2* has been identified in a limited range of tissues, such as the distal tubules of the kid-

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ney (2, 4, 5). In mineralocorticoid-responsive cells, HSD11B2 converts cortisol to cortisone, which is not a ligand for the mineralocorticoid receptor, permitting aldosterone to occupy the receptor.

Apparent mineralocorticoid excess syndrome (AME) is an autosomal recessive disorder that results in severe low-renin hypertension and other characteristic clinical features (6–8). Typical patients present with severe hypertension, hypokalemia, and undetectable aldosterone. Most patients also have low birth weight, polyuria and polydipsia, failure to thrive, and nephrocalcinosis. The syndrome has been associated with sudden fatality. The HSD11B2 deficiency has been demonstrated in patients with AME and explains the pathogenesis of the disease, which results from excess cortisol binding to the mineralocorticoid receptor due to a failure to convert cortisol to cortisone (9–11). Over the last two decades, various genetic mutations in the *HSD11B2* gene have been reported (12–17). In Japanese patients with AME, two missense mutations (S180F, R208H) and a deletion of 3 nucleotides resulting in R337H and delta Y338 have been identified (14, 18).

In 1998, a mild form of this disease characterized by P227L mutation in the *HSD11B2* gene was reported (19). In contrast to the patients with AME, this patient had low-renin hypertension and hypoaldosteronism but no other phenotypic features that would lead to the diagnosis of AME. Afterwards, it was reported that the defective allele frequency in a cohort of Mennonites was 1.7% (20). The genetic mutation in the *HSD11B2* gene, which results in a mild HSD11B2 deficiency, may represent an important cause of low-renin hypertension, the diagnostic basis of which is mostly unknown. Together, these findings suggest that, because 40% of patients with essential hypertension have low renin, these patients may have a mild form of AME.

In the *HSD11B2* gene, the 535G>A polymorphism (synonymous mutation at E178) in exon 3, which can be distinguished by *Alu* I cleavage and the polymorphic microsatellite marker (21), have been reported. The minor allele frequency of the 535G>A polymorphism was 0.086 in a healthy Caucasian population and 0.180 in a group of renal transplant patients ($n=61$), indicating association of this polymorphism with end-stage renal disease. This polymorphism was not associated with essential hypertension (22). As for the microsatellite marker, a total of 12 alleles were detected. The urinary ratio of cortisol to cortisone metabolites was higher in subjects homozygous for the A7 microsatellite allele than in the corresponding control subjects. Thus, the association of a polymorphic microsatellite marker of the *HSD11B2* gene with reduced HSD11B2 activity suggests that variants of the *HSD11B2* gene contribute to enhanced blood pressure response to salt in humans (23). The study demonstrated that a salt-induced blood pressure increase is associated with impaired HSD11B2 activity, as measured by the urinary excretion ratio of cortisol to cortisone metabolites in young Caucasian salt-sensitive men.

The present study was undertaken 1) to identify the genetic

Table 1. General Characteristics of Patients with Hypertension

Number	953
Age (years)	65.1±10.5
Gender (M/F)	522/431
Body mass index (kg/m ²)	24.2±3.3
SBP (mmHg)	145.5±19.2
DBP (mmHg)	84.8±13.4
Essential hypertension	880
Secondary hypertension	73
Renal hypertension	36
Renovascular hypertension	23
Primary aldosteronism	11
Hypothyroid-induced hypertension	2
Renal impairment/failure*	110
Ischemic heart disease	102
Stroke	145

Values are expressed as mean±SD. *Serum creatinine ≥ 1.4 mg/dl. M, male; F, female; SBP, systolic blood pressure; DBP, diastolic blood pressure.

variants in the *HSD11B2* gene in Japanese hypertensives, 2) to address whether individuals with heterozygous missense/frameshift mutations show hypertension or renal impairment, and 3) to explain the genetic contribution to a mild form of hypertension including low-renin hypertension and hypoaldosteronism. We sequenced the promoter and exon regions of *HSD11B2* in Japanese hypertensives and genotyped the rare missense/frameshift mutations in the general population. We assessed the role of these genetic variations in hypertension and clarified their contribution to hypertension in Japanese.

Methods

Hypertensive Patients

A total of 953 hypertensive patients (522 men and 431 woman; average age: 65.0±10.5 years) were recruited from the Division of Hypertension and Nephrology at the National Cardiovascular Center as reported previously (24–27). Briefly, 92% of study subjects (880 subjects) were diagnosed with essential hypertension, and the rest had secondary hypertension (Table 1). Hypertension was defined as systolic blood pressure (SBP) of ≥ 140 mmHg, and/or diastolic blood pressure (DBP) of ≥ 90 mmHg, or current use of antihypertensive medication. Hyperlipidemia was defined by total cholesterol ≥ 220 mg/dl or current use of antihyperlipidemia medication. Diabetes mellitus was defined by fasting plasma glucose ≥ 126 mg/dl or HbA1c $\geq 6.5\%$ or current use of anti-diabetic medication. Study subjects had routine laboratory tests including electrolytes, renal function, blood glucose, HbA1c, plasma renin activity and plasma aldosterone concentration.

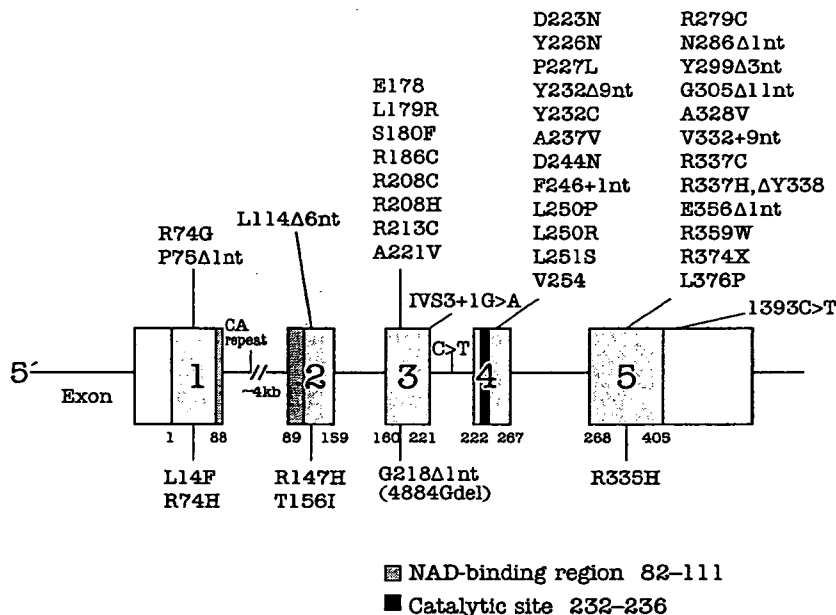


Fig. 1. Summary of the reported genetic polymorphisms in HSD11B2. All polymorphisms in the upper section were reported previously, and the six polymorphisms in the lower section were identified in present study.

Sequencing of the HSD11B2 Gene

We sequenced all exons and the promoter region of HSD11B2 in 953 Japanese hypertensive patients. Blood samples were obtained from hypertensive patients and genomic DNA was isolated from peripheral blood leukocytes. All exons with their flanking sequences and about 1.6 kb of the upstream region were directly sequenced with an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, USA) using seven sets of primers, as described previously (28). Information on the primers and polymerase chain reaction (PCR) conditions is available on request. The obtained sequences were examined for the presence of variations using Sequencher software (Gene Codes Corporation, Ann Arbor, USA), followed by visual inspection. The A of the ATG of the initiator Met codon is denoted as nucleotide +1. The nucleotide sequence (GenBank Accession ID: NT_010498) was used as a reference sequence.

General Population (the Suita Study)

The sample selection and study design of the Suita Study have been described previously (29, 30). Briefly, the subjects visited the National Cardiovascular Center every 2 years for general health checkups. In addition to performing a routine blood examination that included lipid profiles, glucose levels, blood pressure, anthropometric measurements, a physician or

nurse administered questionnaires covering personal history of cardiovascular diseases, including angina pectoris, myocardial infarction, and/or stroke. Blood pressure was measured after at least 10 min of rest in a sitting position. SBP and DBP were means of two measurements performed by well-trained doctors using a mercury sphygmomanometer (with a 3-min interval). The subjects were classified as current drinkers if they drank at least 30 ml ethanol per day, nondrinkers if they had never drunk, and past drinkers if they previously had drunk above 30 ml ethanol per day.

Genotyping of Genetic Variations in the General Population

Genotyping was attempted for six rare missense/frameshift mutations using the TaqMan-PCR method (31). The sequences of PCR primers and probes for the TaqMan-PCR method are available on request. Genotyping for two of the six rare mutations—4582C>T (encoded T156I) and 4884Gdel (a frameshift mutation)—failed. Thus, four genetic variations were successfully genotyped in 3,655 participants (1,709 men and 1,946 women) of the large cohort known as the Suita Study. All of the participants for genetic analysis in the present study gave their written informed consent. All clinical data and sequencing and genotyping results were anonymous. The study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center.

Table 2. Sequence Variations in the Promoter Region and All Exons in *HSD11B2* Identified in Approximately 953 Japanese Patients with Hypertension and/or Renal Failure

SNP name	Region	Amino acid substitution	Allele 1 freq.	Allele 2 freq.	Flanking sequence	Genotyping
-879C>T	promoter		0.999	0.001	TCCTCTGACA[C/T]CCCACCCTCC	
-687C>A	promoter		0.999	0.001	CAGGGGTGAG[C/A]GCGCCTTAGG	
-596 to -595 CGGCAGins	promoter		0.999	0.001	GCAGCGGCAG[CGGCAG]CGGAGACCGG	
-562G>T	promoter		0.999	0.001	TGGTTCCTCG[G/T]GGTGTTCCTG	
-74C>G	promoter		0.999	0.001	ACTCCGCGCC[C/G]CGGCCTAGAA	
40C>T	exon 1	L14F	0.997	0.003	CGCCTGGCTG[C/T]TCGTGGCTGC	done
42C>A	exon 1	L14L	0.999	0.001	CCTGGCTGCT[C/A]GTGGCTGCC	
82C>T	exon 1	L28L	0.999	0.001	GCGCTCAGAC[C/T]TGCCTCTGGG	
221G>A	exon 1	R74H	0.999	0.001	CGCCTGGCGC[G/A]CCCAGCGC	done
4554G>A	exon 2	R147H	0.999	0.001	GACATTAGCC[G/A]CGTGCTAGAG	done
4582C>T	exon 2	T156I	1.000	0.000	AAGGCCACA[C/T]CACCAGCACC	failed
4681G>A	intron 2		1.000	0.000	GCTGACCTAA[G/A]GCTTCCCTCC	
4884Gdel	exon 3	frame shift	1.000	0.000	TGACTGTGGG[G]AGCCCAGCGG	failed
4910C>G	intron 3		0.995	0.005	TGCCCCCCCC[C/G]ACTGGAGCAA	
4902insC(8-10)	intron 3		0.998	0.002	GCCCCCCCC[C]ACTGGAGCAA	
4964C>G	intron 3		0.999	0.001	GAGCCCCTTG[C/G]CAAAGCTGAG	
5017G>A	exon 4	P227P	0.997	0.003	TGCCATATCC[G/A]TGCTTGGGGG	
5205G>A	intron 4		0.999	0.001	TATGGGGGCA[G/A]GTCAGGTTTG	
5267G>A	intron 4		0.999	0.001	CAGACCTGGC[G/A]CGGGTAAAC	
5334C>T	intron 4		0.999	0.001	GCCACTCCTT[C/T]CCCAGAGTCA	
5422C>T	exon 5	Y295Y	1.000	0.000	TGCAGGCCTA[C/T]JGGCAAGGACT	
5541G>A	exon 5	R335H	1.000	0.000	GCTCGGCCCC[G/A]CCGCCGCTAT	done
5698G>A	exon 5	Q387Q	1.000	0.000	CCCCACCACA[G/A]GACGCAGCCC	
5759A>G	3'-UTR		1.000	0.000	TCGGTGAGCC[A/G]TGTGCACCTA	
5784C>T	3'-UTR		0.996	0.004	CCAGCCACTG[C/T]AGCACAGGAG	

The A of the ATG of the initiator Met codon is denoted nucleotide +1, as recommended by the Nomenclature Working Group (37). The nucleotide sequence (GenBank Accession ID: NT_010498) was used as a reference sequence. UTR, untranslated region; freq., frequency. Missense mutations were genotyped for general population except two mutations of which genotypes were not determined.

Results

Identification of Genetic Variations in *HSD11B2* in a Japanese Hypertensive Population

We sequenced the promoter and exon regions of *HSD11B2* in 953 hypertensives. As a result, we did not identify the reported common genetic variations in Caucasians and causative genetic variations of AME in the *HSD11B2* gene. Instead, we identified five novel missense mutations and one frameshift mutation in *HSD11B2* (Fig. 1, Table 2). Five patients had a C-to-T substitution at nucleotide 40 in exon 1, which led to an amino acid substitution from L to F at position 14 (L14F). One patient had a G-to-A substitution at nucleotide 221 in exon 1, resulting in an amino acid substitution from R to H at position 74 (R74H). Three patients had a G-to-A substitution at nucleotide 4554 in exon 2, leading to an amino acid substitution from R to H at position 147 (R147H). One patient had a C-to-T substitution at nucleotide 4582 in

exon 2, leading to an amino acid substitution from T to I at position 156 (T156I). One patient had a G-to-A substitution at nucleotide 5541 in exon 5, resulting in an amino acid substitution from R to H at position 335 (R335H). We also found one patient with a frameshift mutation that resulted from a guanine deletion at position 4884 in exon 3 (4884Gdel). These missense/frameshift mutations were all found in the heterozygous form.

We also identified five synonymous polymorphisms, which encoded for L14 (42C>A in exon 1) with a minor allele frequency of 0.001%, L28 (82C>T in exon 1) with a minor allele frequency of 0.001%, P227 (5017G>A in exon 4) with a minor allele frequency of 0.003%, Y295 (5422C>T in exon 5) with a minor allele frequency of 0.0003% and Q387 (5698G>A in exon 5) with a minor allele frequency of 0.0003%. Fourteen additional genetic variations in the promoter, intronic, and 3'-untranslated regions were also identified. All of the genetic variations were rare, with minor allele frequencies less than 0.005 (Table 2).

Table 3. Clinical Profiles of Twelve Hypertensive Patients with Missense/Frameshift Mutations in HSD11B2 Gene

	Case											
	1	2	3	4	5	6	7	8	9	10	11	12
Polymorphism	L14F	L14F	L14F	L14F	L14F	R74H	R147H	R147H	R147H	T156I	4884Gdel	R335H
Age (years old)	73	71	64	51	59	70	76	69	85	78	75	67
Sex	male	female	male	female	male	male	male	male	male	male	female	female
BMI (kg/m ²)	21.39	20.45	20.20	24.09	30.30	27.92	24.03	22.12	26.17	21.69	29.97	21.50
Diagnosis	EHT, HL, HU, CRF, NIDDM, hypothyroidism	Renal HT, HL, CGN	EHT	ETH, HL	EHT, HL, obesity	EHT, HL, obesity	EHT, HU, OCI	EHT, HU, OCI	EHT, AF, AAA, obesity	EHT	RVHT, NIDDM, HL, obesity	EHT, HL
HT duration (years)	24	21	24	<1	9	15	19	20	21	8	30	41
HT initial onset age (years old)	49	50	40	—	50	55	57	49	64	70	45	26
HT family history	none	none	none	father	none	father, brother	mother, brother	none	none	mother	none	father, mother, brother
SBP (mmHg)	138	136	152	140	130	140	134	138	154	134	170	148
DBP (mmHg)	70	80	88	68	80	86	72	70	84	68	90	80
Antihypertensive drugs	CCB, ARB	CCB, ACEI	CCB, BB, diuretics	CCB, AB	CCB, ARB, BB	CCB, BB	CCB, AB	CCB, ACEI, AB	CCB	CCB, ACEI, AB	CCB, ACEI	CCB, BB
Na ⁺ (mEq/l)	141	141	140	142	140	141	141	140	143	143	140	139
K ⁺ (mEq/l)	4.4	5.2	4.1	4.2	4.2	3.6	4.2	5.2	4.5	4.2	4.6	5.0
Cl ⁻ (mEq/l)	110	109	104	107	102	107	106	108	104	111	104	103
Creatinine (mg/dl)	2.7	0.8	0.6	0.5	0.6	1.1	1.3	2.9	1.2	0.8	0.6	0.8
Overt proteinuria	+	+	-	-	+	+	+	-	-	-	-	-
PRA (ng/ml/h)	3.8	0.9	6.3	0.1	0.5	2.9	1.9	no data	3.4	13.2	19.8	3.2
PAC (ng/dl)	8.8	8.5	no data	27.6	12.4	18.9	43.5	no data	7.7	14.6	7.0	14.1
FBS (mg/dl)	128	92	105	89	113	105	95	91	95	96	137	101
HbA1c (%)	6.0	5.6	5.4	5.2	5.6	6.0	5.1	5.2	5.1	5.0	8.7	5.7

BMI, body mass index; EHT, essential hypertension; HL, hyperlipidemia; HU, hyperuricemia; CRF, chronic renal failure; NIDDM, non-insulin dependent diabetes mellitus; HT, hypertension; CGN, chronic glomerulonephritis; OCI, old cerebral infarction; OCH, old cerebral hemorrhage; AF, atrial fibrillation; AAA, abdominal aortic aneurysm; RVHT, renovascular hypertension; SBP, systolic blood pressure; DBP, diastolic blood pressure; CCB, calcium channel blocker; ARB, angiotensin II receptor blocker; ACEI, angiotensin converting enzyme inhibitor; BB, β-adrenergic blocker; AB, α-adrenergic blocker; PRA, plasma renin activity; PAC, plasma aldosterone concentration; FBS, fasting blood sugar. Normal values in our institute: Na⁺, 136–146 mEq/l; K⁺, 3.6–4.9 mEq/l; Cl⁻, 99–109 mEq/l; creatinine, 0.6–1.1 mg/dl; PRA, 0.2–2.7 ng/ml/h; PAC, 2–13 ng/dl.

Characteristics of Patients with Rare Missense/Frameshift Mutations in the Hypertensive Population

The characteristics of the 12 hypertensive patients who had missense/frameshift mutations (L14F, n=5; R74H, n=1; R147H, n=3; T156I, n=1; 4884Gdel, n=1; R335H, n=1) are shown in Table 3. Five patients out of the twelve had renal impairment including protein urea. Two (cases 1 and 2) of five patients with the L14F mutation had chronic renal failure (CRF) and chronic glomerulonephritis (CGN), and one (case 8) of three patients with the R147H mutation also had CRF. A patient with 4884Gdel (case 11) was diagnosed with renovas-

cular hypertension caused by atherosclerosis with type 2 diabetes, hyperlipidemia and obesity (body mass index [BMI]: 29.97 kg/m²). This patient was 75 years old, female, and had never smoked or drunk alcohol. This patient had microalbuminuria (urinary albumin excretion: 30.8 mg/g creatinine) without renal dysfunction (creatinine clearance: 112.5 ml/min) or cardiac hypertrophy (left ventricular mass index: 126.4 g/m²). The average onset age of hypertension of the 12 patients with these missense mutations was 50.5 years. A patient with the R335H mutation (case 12) showed hypertension at her age of 26. Serum sodium levels of all patients were within normal range. There were no patients with hypokalemia as seen in AME.

Table 4. Basic Characteristics of Subjects in the General Population

	Women (n=1,946)	Men (n=1,709)
Age (years)	63.3±11.0	66.3±11.1*
Systolic blood pressure (mmHg)	128.0±19.7	131.8±19.4*
Diastolic blood pressure (mmHg)	76.5±9.8	79.7±10.7*
Body mass index (kg/m ²)	22.3±3.2	23.3±2.9*
Total cholesterol (mg/dl)	215.6±30.6*	197.9±30.3
HDL-cholesterol (mg/dl)	64.5±15.3*	55.0±14.1
Current smokers (%)	6.3	30.2 [†]
Current drinkers (%)	29.6	67.2 [†]
Present illness (%)		
Hypertension	38.0	47.3 [†]
Hyperlipidemia	54.4 [†]	27.8
Diabetes mellitus	5.2	12.8 [†]

Values are expressed as mean±SD. 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 *t*-test. [†]*p*<0.05 between women and men by χ^2 test. HDL, high-density lipoprotein.

Characteristics of Individuals with Rare Missense/Frameshift Mutations in the General Population

The characteristics of the 3,655 subjects comprising the Japanese general population group (1,709 men, 1,946 women) are summarized in Table 4. Age, SBP, DBP, BMI, percentage of current smokers, percentage of current drinkers, and prevalence of hypertension and diabetes mellitus were significantly higher in men than in women. Total cholesterol, high-density lipoprotein (HDL)-cholesterol, and percentage of hyperlipidemia were significantly higher in women than in men. In this population, 1,480 subjects were diagnosed with hypertension.

We successfully genotyped four genetic variations in the general population, which had a sample size of 3,655 individuals (2,175 normotensives and 1,480 hypertensives), but the genotyping failed for two of the genetic variations, T156I and 4884Gdel. In the general population, a missense mutation, R335H, was not present. The remaining three mutations, L14F, R74H, and R147H, were found in both hypertensive and normotensive subjects (Table 5). We identified 14 individuals with the L14F mutation. Six individuals with the L14F mutation had hypertension and eight were normotensive. We identified 20 individuals with the R74H mutation. Among them, eight showed hypertension and 12 were normotensive. We identified 8 individuals with the R147H mutation. Among them, three showed hypertension and five were

normotensive. There were no statistically significant differences in any clinical characteristics between the subjects with the three missense mutations of *HSD11B2* and the subjects in the general population (Table 5).

Comparison of Missense/Frameshift Mutations in *HSD11B2* between Normotensives and Combined Hypertensives

As seen in Table 6, there was no difference in the prevalence of missense/frameshift mutations of *HSD11B2* between the combined subjects with hypertension and the normotensives.

Discussion

A missense mutation, P227L, in *HSD11B2* was previously identified in a patient with mild low-renin hypertension (32). This patient did not demonstrate the typical features of AME. The authors suggested that patients with mild low-renin hypertension may carry the mutations in the *HSD11B2* gene. In our study, we did not identify the P227L mutation in 953 Japanese hypertensives.

Genetic analyses of *HSD11B2* have been reported in two Japanese AME probands (14, 18). In one family, the proband had a compound heterozygous mutation with a missense mutation, R208H, and a deletion of 3 nucleotides in codons 337–338 resulting in a substitution of Arg337 to His and a deletion of Tyr338 (CGCTAT to CAT: R337H and delta Y338) (18). Their family members, a father, mother, and elderly sister, who carried the heterozygous mutation were all normotensive and normokalemic, and had normal ratios of urinary [THF plus aTHF]/THE (THF, tetrahydrocortisol; aTHF, allotetrahydrocortisol; THE, tetrahydrocortisone). Another Japanese patient with AME had the homozygous missense mutation, S180F. The enzymatic activity of this mutant was 1.8% compared with the wild-type enzyme when cortisol was used as the substrate and 5.7% when corticosterone was used as the substrate (14). Figure 1 summarizes the reported polymorphisms in *HSD11B2*. In our study, none of the three causative genetic defects was identified, indicating that those mutations were not accumulated in the Japanese population.

We identified five novel missense mutations and one frameshift mutation in *HSD11B2* (Fig. 1, Table 2). As shown in Fig. 2A, five of the missense mutations occurred in residues that were highly conserved among the three different species, indicating that these mutations may result in functional changes in *HSD11B2*. However, neither hypertensive patients nor general subjects with these novel missense mutations showed any distinctive clinical characteristics during their health-check-ups.

We identified one hypertensive patient having renal artery stenosis with a frameshift mutation (4884Gdel) in *HSD11B2*. This deletion caused the frameshift at S219 with a premature stop codon at position 270 (Fig. 2B). A recent report indicated

Table 5. Accumulated Clinical Profiles of Subjects with Missense Mutations in *HSD11B2* in the General Population

	L14F	R74H	R147H
Number	14	20	8
Age (years old)	67.7±12.3	64.8±13.3	61.5±12.0
Sex (M/F)	7/7	9/11	5/3
Body mass index (kg/m ²)	23.4±4.0	22.4±2.9	23.9±1.7
Systolic blood pressure (mmHg)	125.4±23.0	128.7±23.4	124.9±19.9
Diastolic blood pressure (mmHg)	75.4±11.0	78.2±10.0	75.8±12.9
Total cholesterol (mg/dl)	213.9±34.0	213.8±37.0	199.3±36.4
HDL-cholesterol (mg/dl)	57.9±12.1	63.1±16.9	52.1±18.5
Triglyceride (mg/dl)	93.8±49.3	120.1±93.4	140.7±90.1
Creatinine (mg/dl)	0.8±0.2	0.7±0.2	0.8±0.2
Over proteinuria (yes/no)	1/13	0/20	0/8
FBS (mg/dl)	100.4±20.9	94.5±10.3	99.6±22.3
HbA1c (%)	5.7±0.8	5.4±0.7	5.6±0.9
Current smoker (yes/no)	2/12	4/16	1/7
Current drinker (yes/no)	5/9	9/11	4/4
Hypertension (yes/no)	6/8	8/12	3/5
Hyperlipidemia (yes/no)	10/4	11/9	6/2
Diabetes mellitus (yes/no)	6/8	2/18	2/6
Antihypertensive treatment (yes/no)	4/10	2/18	2/6

Values were expressed as mean±SD. 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. M, male; F, female; HDL, high-density lipoprotein; FBS, fasting blood sugar.

Table 6. Number of Subjects with Missense/Frameshift Mutations in the Hypertensive and the General Populations

Mutations	Hypertensive population (n=953)	General population	
		Hypertensive subjects (n=1,480)	Normotensive subjects (n=2,175)
L14F	5	6	8
R74H	1	8	12
R147H	3	3	5
T156I	1	n.d.	n.d.
4884Gdel	1	n.d.	n.d.
R335H	1	0	0
Total	12	17	25

n.d., not determined.

that the heterozygous carriers with the defective allele of the *HSD11B2* gene showed essential hypertension (16). It is evident that this frameshift mutation results in the dysfunction of *HSD11B2*. The allele frequency of this mutation was very low (0.052%, 1 allele/1,906 alleles) in the Japanese hypertensive population. However, it is worth noting that this defective allele might be prevalent in other ethnic populations, because the frequency of some genetic mutations varies with ethnicity. Recently, rare genetic mutations collectively contributing to a quantitative trait variation, such as plasma levels

of HDL-cholesterol, have been reported (33). We have performed large-scale sequence analyses of five hypertension candidate genes, *WINK4*, *SCNN1B*, *SCNN1G*, *NR3C2* and *RGS2*, to evaluate this hypothesis and found that a low but significant percentage of the hypertensive subjects had missense/frameshift mutations (24–26, 34). Collectively, these rare mutations may make an at least partial contribution to hypertension.

The deduced NAD-binding sites reside in the conserved region from T82 to A111 (2), and the deduced catalytic site resides in the conserved region from Y232 to K236 (35). So far, more than ten genetic defects in patients with AME, most of whom had a severe deficiency of enzymatic activity confirmed by the expression analysis, have been reported and none of them overlap with the five missense mutations identified in the present study. Therefore, the effects on the *HSD11B2* enzymatic activity of the mutations are not clear. In the future, an *in vitro* expression study should be performed to evaluate the activity of mutants and the ratios of urinary cortisol to cortisone metabolites in carriers of the mutations.

In the Caucasian population, a mutation at E178 that is synonymous with 553G>A which can be distinguished by *Alu* I restriction enzyme digestion, has been identified with a prevalence of 8.6% in the control subjects (21, 23). This polymorphism was associated with end-stage renal disease but not with essential hypertension. We did not identify this polymor-

A

h-HSD	1	MERWPWPSGGAWLLVAARALLQLLRSDLRLGRPLLAALALLAALD	45
m-HSD	1	MERWPWPSGGAWLLVAARALLQLLRSDLRLGRPLLAALALLAALD	45
r-HSD	1	MERWPWPSGGAWLLVAARALIQLLRADLRLGRPLLAALALLAALD	45
*			
h-HSD	46	WLCQRLPPPAALAVLAAAGWIALSRLARPQRLPVATRAVLITGC	90
m-HSD	46	WLCLRLMPPPAALVVLGAGWIALSRLARPPRLPVATRAVLITGC	90
r-HSD	46	WLCQSLPPSAALAVLAAAGWIALSRLARPQRLPVATRAVLITGC	90
*			
h-HSD	136	QMDLTKPGDISRVLEFTKAHTTSTGLWGLVNNAGHNEVVADAELS	180
m-HSD	136	QMDLTKAEDISRVLEITKAHTASTGLWGLVNNAGLNIVVADVGLS	180
r-HSD	136	QMDLTKPADISRALLEFTKAHTTSTGLWGLVNNAGHNDVVADVLS	180
*			
h-HSD	316	SDLTPVDAITDALLAARPRRRYYPGQGLGLMYFIHYLPEGLRR	360
m-HSD	316	PDLSPVDAIIDALLAAQPRSRYPGRGLGLMYFIHYLPEGLRR	360
r-HSD	316	PDLSPVDAITDALLAARPRPRYPGRGLGLMYFIHYLPEGLRR	360

B**Wild type**

ACTGTGGGGAGCCCAGCGGGGACATGCCA
 216 T V G S P A G D M P 225
 TTCAAGACAGAGTCAGTGAGAAACGTGGGT
 265 F K T E S V R N V G 274

4884Gdel allele

ACTGTGGGGAGCCCAGCGGGGACATGCCAT
 216 T V G A Q R G T C H 225
 TCAAGACAGAGTCAGTGAGAAACGTGGGTC
 265 S R Q S Q * * *

Fig. 2. Partial amino acid sequence surrounding the mutations in HSD11B2. **A:** Alignment of partial amino acid sequences of HSD11B2 from two species and human HSD11B2. HSD11B2 sequences are from *Homo sapiens* (h), *Mus musculus* (m), and rabbit (r). Numbers indicate the position of amino acid sequence. The asterisks indicate the positions at which missense mutations occur (L14F, R74H, R147H, T156I, R335H). **B:** Nucleotide and amino acid sequences of wild-type allele and 4884Gdel allele. Numbers indicate the amino acid residues. An asterisk indicates the base deleted in the 4884Gdel allele, which causes a frameshift mutation from S218. This results in a 51-amino-acid extension that is terminated by a stop codon (indicated by three asterisks).

phism in our Japanese population.

In the Caucasian population, an intensive genetic study on the HSD11B2 gene using 587 subjects, including 260 patients with end-stage renal disease, has been conducted, in which one missense mutation, L148V, and three synonymous mutations, T156, E178, and D388, were identified by the combination of single strand conformational polymorphism analysis and DNA sequencing (36). The results showed that allele frequencies did not differ significantly between control subjects and end-stage renal disease patients or between patients with hypertension and patients with end-stage renal disease. We did not identify these mutations in our Japanese population. Our results support their findings that the mutations in the HSD11B2 gene do not affect hypertension.

In summary, we suggest that rare mutations in HSD11B2,

L14F, R74H, R147H, T156I, R335H, and 4884Gdel may not collectively contribute to the pathogenesis of hypertension, although it was not clear whether abnormalities of electrolytes, renin activity, or aldosterone concentration were present, since our hypertensive patients with these missense/frameshift mutations were taking antihypertensive drugs. Further functional analyses of HSD11B2 mutants are necessary to clarify the functional defects caused by these genetic variations in Japanese.

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REGULAR ARTICLE

Haplotype of thrombomodulin gene associated with plasma thrombomodulin level and deep vein thrombosis in the Japanese population

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Abstract

Introduction: Thrombomodulin (TM) is an essential cofactor in protein C activation by thrombin. Here, we evaluated the contribution of genetic variations in the TM gene to soluble TM (sTM) level and deep vein thrombosis (DVT) in Japanese.

Patients and methods: We sequenced the TM putative promoter, exon, and 3'-untranslated region in DVT patients ($n=118$). Among 17 genetic variations we identified, two missense mutations (R385K, D468Y) and three common single nucleotide polymorphisms ($-202G>A$, $2487A>T$, $2729A>C$) were genotyped in a general population of 2247 subjects (1032 men and 1215 women) whose sTM levels were measured. We then compared the frequency of these mutations in DVT patients

Abbreviations: DVT, deep vein thrombosis; TM, thrombomodulin; PC, protein C; APC, activated protein C; PS, protein S; EGF, epidermal growth factor; SNP, single-nucleotide polymorphism; sTM, soluble TM; 5'-UTR, 5'-untranslated region; 3'-UTR, 3'-untranslated region.

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with that in the age, body mass index-adjusted population-based controls.

Results: We identified one neutral mutation (H381) and three missense mutations (R385K; $n=2$, A455V; $n=53$ heterozygous, $n=14$ homozygous, D468Y; $n=2$) of TM in the DVT patients. Age-adjusted mean values of sTM were lower in C-allele carriers of 2729A>C than in noncarriers in the Japanese 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, $p = 0.03$). Additionally, the CC genotype of this mutation was more common in the male DVT patients than in the male individuals of the general population (odds ratio = 2.76, 95% confidence interval = 1.14–6.67; $p = 0.02$). This mutation was in linkage disequilibrium (r -square > 0.9) with A455V mutation.

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

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Introduction

Family-based studies have established that venous thromboembolism is, at least in part, an inherited disease with estimated heritabilities of approximately 60% [1,2]. The mode of inheritance of venous thromboembolism is probably complex [2]. Moreover, family-based and twin studies have established that over 25 plasma hemostasis-related analytes (traits) both correlate with thrombosis and are heritable [3–5]. In Caucasians, the factor V-Leiden mutation and prothrombin G20210A mutation are widely recognized as genetic risk factors for deep vein thrombosis (DVT) [6]. However these mutations are not present in the Japanese [7,8]. Recently, we and others found that the protein S (PS) K196E mutation, known as the PS Tokushima mutation, is a genetic risk for DVT in the Japanese population, indicating large differences in the genetics of DVT among ethnicities [9,10].

Thrombomodulin (TM) is a transmembrane protein that is constitutively expressed on the luminal surface of vascular endothelial cells [11]. The anticoagulant function of TM is mediated by interaction with thrombin and protein C (PC). Endothelial membrane-bound TM forms a high-affinity complex with thrombin via thrombin exosite 1, and inhibits thrombin interaction with fibrinogen and protease-activated receptor-1. In contrast, the thrombin–TM complex is a potent activator of PC, and TM enhances thrombin-dependent PC activation by more than two orders of magnitude. Due to the abundance of TM in the microvasculature, the vast majority of thrombin generated under ambient conditions is sequestered by TM. Constitutive inhibition of the procoagulant function of thrombin and tonic formation of activated PC (APC) comprise an essential anticoagulant mechanism that prevents the amplification of

thrombin generation, via proteolysis of activated coagulation factors Va and VIIIa by APC.

TM encoded by an intron-less gene consists of a large N-terminal extracellular region, a single transmembrane segment, and a short cytoplasmic tail [12]. The extracellular region is comprised of an N-terminal lectin-like domain followed by six tandem repeats of epidermal growth factor (EGF)-like domains, and a glycosylated (chondroitin sulfate) serine/threonine-rich domain. The thrombin-binding region has been localized to the fifth and sixth EGF-like domains, while the fourth EGF-like domain is required for PC binding to the thrombin–TM complex. The serine/threonine-rich spacer region is required for both thrombin binding and TM cofactor activity for membrane-associated TM. The chondroitin sulfate domain may stabilize thrombin binding to TM, possibly by interacting with the thrombin apolar region [13,14].

Animal model data suggest that TM dysfunction or deficiency is associated with a prothrombotic disorder. Knock-in mice with a TM mutant that has a mutation corresponding to human E387P exhibit a prothrombotic disorder [15]. This amino acid change is located between the interdomain loop of the fourth and fifth EGF-like domains and abolishes the ability of soluble TM (sTM) to catalyze in vitro thrombin activation of PC to APC. Mice with TM deficiency limited to the vascular endothelium die shortly after birth as a result of a consumptive coagulopathy that can be prevented by warfarin anticoagulation [16].

Based on the important antithrombotic role of TM, we hypothesized that genetic variations within the TM gene that alter TM expression and/or impair anticoagulant function could predispose to venous thromboembolism. To test this hypothesis, we screened the promoter, exon, and 3'-untranslated regions (3'-UTR) of the TM gene in unrelated patients with idiopathic, objectively confirmed

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 \pm S.D.	52.3 \pm 16.1	Nephrotic syndrome, <i>n</i> (%)	0 (0.0)
Women, <i>n</i> (%)	59 (50.0)	Chronic heart failure, <i>n</i> (%)	17 (14.4)
BMI, kg/m ² , mean \pm S.D.	23.7 \pm 3.2	Diabetes Mellitus, <i>n</i> (%)	47 (39.8)
DVT family history, <i>n</i> (%)	8 (6.8)	Hyperlipidemia, <i>n</i> (%)	48 (40.7)
Previous DVT, <i>n</i> (%)	12 (10.2)	Autoimmune disease, <i>n</i> (%)	11 (9.3)
Pregnancy, <i>n</i> (%)	5 (4.2)	Inflammatory bowel disease, <i>n</i> (%)	2 (1.7)
Stroke, <i>n</i> (%)	1 (1.5)	Estrogen use, <i>n</i> (%)	3 (2.5)
Prolonged immobility, <i>n</i> (%)	14 (11.9)	Steroid use, <i>n</i> (%)	9 (7.6)
Malignancy, <i>n</i> (%)	16 (13.6)	Paralysis, <i>n</i> (%)	5 (4.2)
Major surgery (abd, hip, leg), <i>n</i> (%)	21 (17.8)	Myeloproliferative disease, <i>n</i> (%)	1 (0.8)
Trauma (pelvis, hip, leg), <i>n</i> (%)	3 (2.5)	Reduced plasminogen activity, <i>n</i> (%)	7 (5.9)
Stasis due to compression, <i>n</i> (%)	6 (5.1)	Reduced antithrombin activity, <i>n</i> (%)	7 (5.9)
Central venous catheter, <i>n</i> (%)	0 (0.0)	Reduced protein C activity, <i>n</i> (%)	8 (6.8)
		Reduced protein S antigen, <i>n</i> (%)	10 (8.5)
		Lupus anticoagulant (cardiolipin, ACLb2), <i>n</i> (%)	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 $\geq 6.5\%$ 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. $>5 \times 10^9$ 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 \pm 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 estab-

lished 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	gggcagaggcgc [c/a] tgggttaggcc	
*-754G>C		Promoter		99.1	0.9	caagcgcgctcc [g/c] ctgtttcctga	
*-265C>A		Exon(5'UTR)		99.6	0.4	aatccgagtatg [c/a] ggcacagccct	
-202G>A	A	Exon(5'UTR)		89.2	10.8	ggagggagggcc [g/a] ggcactataaa	
*-58G>C		Exon(5'UTR)		98.3	1.7	ctgctccggcac [g/c] gccctgtcgcag	
*1197C>T		Exon(EGF4)	H381	99.6	0.4	gccattcccca [c/t] gagccgcacagg	
1208G>A		Exon(EGF4)	R385K	99.1	0.9	acgagccgcaca [g/a]gtccagatgtt	
1418C>T	B	Exon(EGF6)	A455V	65.1	34.9	actcggcccttg [c/t] ccgaccattgg	rs1042579
1456G>T		Exon(Ser/Thr-rich)	D468Y	99.1	0.9	tccggcaagggtg [g/t] acggteggcaca	
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]ggcgaggaggg	rs3176134
*2230T>C		Exon(3'UTR)		99.6	0.4	tcttggtgaatt [t/c] tttttcctagc	
*2487A>T		Exon(3'UTR)		93.1	6.9	ttccagagcaa [a/t] ataattttaaac	
2521A>G		Exon(3'UTR)		79.8	20.2	gatgtaaaagt [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	ctcgggtgtgt [-/t] gtctgtcactt	
*3559T>A		3'flanking		99.6	0.4	gccctcattta [t/a] gtcattaatgg	

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 \pm S.D.	64.6 \pm 10.7	67.1 \pm 10.9	<0.0001
Systolic blood pressure, mm Hg \pm S.D.	123.5 \pm 19.8	126.1 \pm 17.9	0.0008
Diastolic blood pressure, mm Hg \pm S.D.	74.3 \pm 10.4	77.2 \pm 10.4	<0.0001
Body mass index, kg/m ² \pm S.D.	22.4 \pm 3.2	23.4 \pm 3.0	<0.0001
Total cholesterol, mg/dl \pm S.D.	215.9 \pm 31.6	198.7 \pm 31.5	<0.0001
HDL-cholesterol, mg/dl \pm S.D.	64.4 \pm 15.1	55.2 \pm 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 \geq 140 mm Hg and/or diastolic blood pressure \geq 90 mm Hg or use of antihypertensive medication; Hyperlipidemia, total cholesterol \geq 220 mg/dl or use of antihyperlipidemia medication; Diabetes mellitus, fasting plasma glucose \geq 126 mg/dl or non-fasting plasma glucose \geq 200 mg/dl or HbA1c \geq 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 χ^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
		<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)
-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