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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

Rina Kimura [a], Kotaro Miyashita [b], Yoshihiro Kokubo [c], Yasuhisa Akaiwa [b], Ryoichi Otsubo [b], Kazuyuki Nagatsuka [b], Toshiho Otsuki [b], Akira Okayama [c], Kazuo Minematsu [b], Hiroaki Naritomi [b], Shigenori Honda [a], Hitonobu Tomoike [c], Toshiyuki Miyata [a,*]

[a] *Research Institute, Japan*
[b] *Cerebrovascular Division, Department of Medicine, Japan*
[c] *Department of Preventive Cardiology, National Cardiovascular Center, Osaka, Japan*

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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*

* Corresponding author. Tel.: +81 6 6833 5012x2512; fax: +81 6 6835 1176.
E-mail address: miyata@ri.ncvc.go.jp (T. Miyata).

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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 (R325Q)	GG	48	3.25±1.19	
		GA	39	2.63±0.77	0.022
		AA	6	2.79±1.07	
CYP2C9	42613 A>C (CYP2C9*3) (I359L)	AA	83	3.06±1.05	
		AC	9	2.17±0.84	0.015
		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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REGULAR ARTICLE

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

Shoko Sugiyama [a,b], Hisao Hirota [a,✠], Rina Kimura [b], Yoshihiro Kokubo [c], Tomio Kawasaki [d,*], Etsuji Suehisa [e], Akira Okayama [c], Hitonobu Tomoike [c], Tokio Hayashi [f], Kazuhiro Nishigami [f], Ichiro Kawase [g], Toshiyuki Miyata [b]

[a] *Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita City, Osaka 565-0871, Japan*
[b] *Research Institute, National Cardiovascular Center, Japan*
[c] *Department of Preventive Cardiology, National Cardiovascular Center, Japan*
[d] *Cardiovascular Surgery, Osaka University Graduate School of Medicine, Japan*
[e] *Department of Laboratory Medicine, Osaka University Hospital, Japan*
[f] *Department of Cardiology, National Cardiovascular Center, Japan*
[g] *Respiratory Medicine and Rheumatic Diseases, Osaka University Graduate School of Medicine, Japan*

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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.

* Corresponding author. Tel.: +81 6 6879 3251; fax: +81 6 6879 3259.
E-mail address: kawasaki@sug2.med.osaka-u.ac.jp (T. Kawasaki).
✠ Deceased.

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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.

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 ± 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. $>5 \times 10^5$ 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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REVIEW ARTICLE

Warfarin dose and the pharmacogenomics of *CYP2C9* and *VKORC1* — Rationale and perspectives ☆

Tong Yin [1], Toshiyuki Miyata *

National Cardiovascular Center Research Institute, Suita, Osaka, Japan

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Abstract Warfarin is the most widely prescribed oral anticoagulant, but there is greater than 10-fold interindividual variability in the dose required to attain a therapeutic response. Information from pharmacogenomics, the study of the interaction of an individual's genotype and drug response, can help optimize drug efficacy while minimizing adverse drug reactions. Pharmacogenetic analysis of two genes, the warfarin metabolic enzyme *CYP2C9* and warfarin target enzyme, vitamin K epoxide reductase complex 1 *VKORC1*, confirmed their influence on warfarin maintenance dose. Possession of *CYP2C9*2* or *CYP2C9*3* variant alleles, which result in decreased enzyme activity, is associated with a significant decrease in the mean warfarin dose. Several single nucleotide polymorphisms (SNPs) in *VKORC1* are associated with warfarin dose across the normal dose range. Haplotypes based on these SNPs explain a large fraction of the interindividual variation in warfarin dose, and *VKORC1* has an approximately three-fold greater effect than *CYP2C9*. Algorithms incorporating genetic (*CYP2C9* and *VKORC1*), demographic, and clinical factors to estimate the warfarin dosage, could potentially minimize the risk of over dose during warfarin induction.

Contents

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* Corresponding author. Department of Etiology and Pathogenesis, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 5658565, Japan. Tel.: +81 66833 5012; fax: +81 66835 1176.
E-mail address: miyata@ri.ncvc.go.jp (T. Miyata).
[1] Recipient of Takada Foundation, from Institute of Geriatric Cardiology, General Hospital of People's Liberation Army, Beijing, China.

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Introduction

Genetic polymorphisms can affect an individual's response to pharmacologic agents, and the study of these interactions is pharmacogenomics. Pharmacogenomic information may allow predictions about effective drug dose and therapeutic and toxic effects to be made prior to drug administration [1]. Most current pharmacogenomic information is based on association studies examining polymorphisms in genes encoding drug-metabolizing enzymes, transporters, receptors, and proteins involved in drug-signaling pathways. In current clinical practice, pharmacogenomic testing is performed for only a few drugs, and an important potential candidate is warfarin.

Warfarin, a derivative of coumarin, is a commonly prescribed oral anticoagulant for the treatment and prevention of thrombotic diseases, including myocardial infarction, ischemic stroke, venous thrombosis, and following heart valve replacement and atrial fibrillation [2]. Recently, oral anticoagulation therapy was confirmed to be superior to clopidogrel plus aspirin for prevention of vascular events in patients with atrial fibrillation at high risk of stroke [3]. However, warfarin has a narrow therapeutic range and a given dose has a large interindividual variation. An insufficient dose may fail to prevent thromboembolism, while an overdose increases the risk of bleeding. The degree of anticoagulation achieved in each patient is followed by obtaining the prothrombin time expressed as the international normalized ratio (PT–INR).

Warfarin therapy management is challenging for several reasons including the need to determine a safe and effective maintenance dose during the early phase of therapy and the fact that maintenance doses must be adjusted to compensate for changes in patients' weight, diet, disease state, concomitant use of other medications, and genetic factors. Traditional warfarin induction algorithms rely on trial-and-error dosing after an initial warfarin dose of 5 mg or 10 mg in Caucasians and 3.5 mg in Asian, rather than being tailored to individual genetic and clinical factors [4–7]. It usually takes not less than several weeks to obtain the stable warfarin control. The alternative to these algorithms incorporates pharmacogenomic, demographic, and clinical factors to more accurately estimate the warfarin dose a priori, potentially decreasing the risk of over dose during therapy induction and minimizing the warfarin induction period [8]. In particular, increasing evidence suggests that genetic variation in *CYP2C9* and *VKORC1* greatly influences effective warfarin dose. In this review, we discuss the implications of variability in *CYP2C9* and *VKORC1* with respect to warfarin dose and its clinical efficacy. Additionally, we describe novel algorithms incorporating genetic and clinical factors to predict effective warfarin doses and the risk of side effects.

Mechanisms of warfarin anticoagulation

Warfarin is a specific inhibitor of the vitamin K epoxide reductase (VKOR) encoded by the vitamin K epoxide reductase complex subunit 1 (*VKORC1*) gene [9,10]. Warfarin exerts its anticoagulant effects by preventing the ability of *VKORC1* to regenerate reduced vitamin K from its epoxide form [11]. Reduced vitamin K is an essential cofactor for γ-glutamylcarboxylase (GGCX), the enzyme catalyzing the post-translational γ-glutamyl carboxylation

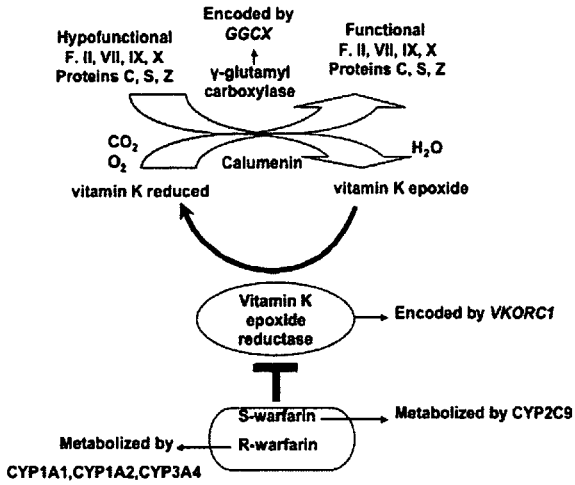

Figure 1 Pathway of warfarin metabolism.

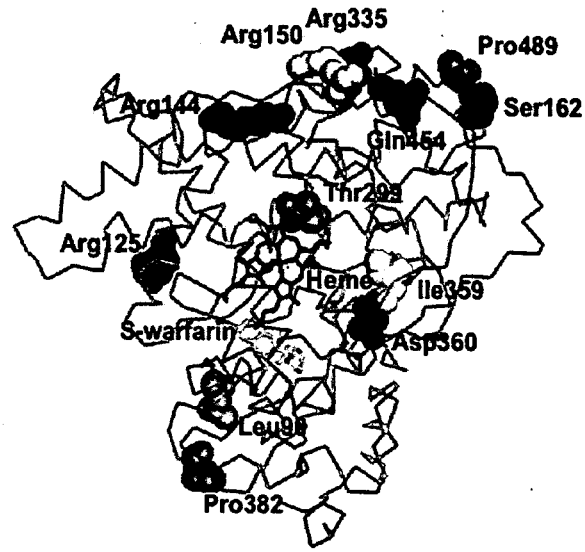

Figure 2 Missense mutations with functional effects mapped in the crystal structure of human *CYP2C9* protein bound with warfarin (PDB: 10G5). *S*-warfarin and heme are shown in the skeleton model with pink and red, respectively. Amino acid residues are shown in the sphere mode with colors.

of the vitamin K-dependent clotting factors, II (prothrombin), VII, IX and X (Fig. 1). Thus, warfarin prevents the functional maturation of vitamin K-dependent clotting factors, leading to reduced coagulation [12,13]. Patients with congenital deficiencies in *GGCX* and *VKORC1* have disordered hemostasis, and these conditions are known as combined deficiency of vitamin K-dependent clotting factors type 1 and 2, respectively [9,14]. Functional abnormalities in *VKORC1* also confer resistance to coumarin-type anticoagulant drugs (warfarin resistance) [9].

Table 1 Nonsynonymous mutations in *CYP2C9* with functional effects

Alleles	Nucleotide change in cDNA	Amino acid change	Enzymatic activity	References
*CYP2C9*2*	430C>T	Arg144Cys	Decrease: an approximately 50% decrease of the maximum rate of metabolism (*V*max) and 30–50% lower turnover (kcat) of *S*-warfarin	[22]
*CYP2C9*3*	1075A>C	Ile359Leu	Decrease: a markedly higher *K*m and lower intrinsic clearance with an approximately 90% decrease of *S*-warfarin	[23]
*CYP2C9*4*	1076T>C	Ile359Thr	Decrease: 72–81% reduction of intrinsic clearance of diclofenac	[28,29]
*CYP2C9*5*	1080C>G	Asp360Glu	Decrease: intrinsic clearance of warfarin approximately 10% of wild type	[30]
*CYP2C9*6*	del818A	Frame shift	Null	[31]
*CYP2C9*8*	449G>A	Arg150His	Increase: more than two-fold increase in the intrinsic clearance of tolbutamide	[32]
*CYP2C9*11*	1003C>T	Arg335Trp	Decrease: a three-fold increase in the *K*m and more than a two-fold decrease in the intrinsic clearance of tolbutamide	[32,33]
*CYP2C9*12*	1465C>T	Pro489Ser	Decrease: a modest decrease in the *V*max and the intrinsic clearance of tolbutamide	[32]
*CYP2C9*13*	269T>C	Leu90Pro	Decrease: decreased activity toward all studied CYP2C9 substrates	[34–36]
*CYP2C9*14*	374G>A	Arg125His	Decrease: 80–90% lower catalytic activity toward tolbutamide	[37,38]
*CYP2C9*15*	485C>A	Ser162X	Null	[37,38]
*CYP2C9*16*	895A>G	Thr299Ala	Decrease: 80–90% lower catalytic activity toward tolbutamide	[37,38]
*CYP2C9*17*	1144C>T	Pro382Ser	Decrease: modest 30 to 40% decreases in caltalytic activity toward tolbutamide	[37,38]
*CYP2C9*19*	1362G>C	Gln454His	Decrease: modest 30 to 40% decreases in caltalytic activity toward tolbutamide	[37,38]

Nonsynonymous mutations with functional activity are listed. Those that functional activity has not been examined were not listed.

Genetic polymorphisms in *CYP2C9* relevant to warfarin metabolism

Warfarin metabolism by cytochrome *P450*, CYPs

Warfarin is a racemic mixture of *R*- and *S*-enantiomers [2], and these differ both in their potency and metabolism. *S*-warfarin is a five-fold more potent vitamin K antagonist than *R*-warfarin [2]. Under steady state conditions, *S*-warfarin accounts for 60–70% of the anticoagulation response, with the *R*-enantiomer accounting for 30–40% [15]. *S*-warfarin is metabolized primarily by *CYP2C9*, but *R*-warfarin is metabolized by *CYP3A4*, *1A2* and *1A1* [16]. Genetic variations in *CYP2C9*, *3A4*, *1A2* and *1A1* can potentially lead to the interindividual variation in effective warfarin dose [17,18], and the most extensively studied isomer among the four is *CYP2C9*. To date, more than 50 variants in *CYP2C9* have been described, and two variants, *CYP2C9*2* and *CYP2C9*3*, have been examined with respect to warfarin dosing.

Metabolic activity of *CYP2C9*2* and *CYP2C9*3* proteins

The human *CYP2C9* gene is approximately 55-kb long and located on chromosome 10q24.2 [19,20]. The most common allele is designated *CYP2C9*1*, and it is considered the wild-type genotype. Approximately 24 nonsynonymous variations in *CYP2C9* have been identified [21], and the functional consequences of *CYP2C9*2* (Arg144Cys) and *CYP2C9*3* (Ile359Leu) are well defined. The maximum rate of metabolism (*V*max) of the *CYP2C9*2* protein is approximately 50% that of the wild-type protein, and the turnover (kcat) is reduced by 30 to 50%. The *CYP2C9*3* protein has a markedly higher *K*m and lower intrinsic clearance leading to an approximately 90% decrease in *S*-warfarin 7-hydroxylation [22–24].

CYP2C9 genotype and adverse bleeding events

Most clinical studies examining warfarin pharmacogenomics assessed differences in the mean daily warfarin dose and susceptibility to bleeding. A direct association between *CYP2C9* genotype and anticoagulation status or bleeding was first reported by Higashi et al. [25]. Subsequently, a systematic meta-analysis showed that patients with either the *CYP2C9*2* or *CYP2C9*3* variant required a lower warfarin maintenance dose, and this was especially pronounced for patients with *CYP2C9*3* (a 30% dose reduction) [26]. However, the risk of bleeding for patients with the *CYP2C9*2* and/or *CYP2C9*3* alleles is approximately doubled. Patients with *CYP2C9*2* and/or *CYP2C9*3* metabolize warfarin more slowly than wild-type patients, and a traditional warfarin dose would more likely lead to overdose and bleeding in these individuals [8]. Patients with the *CYP2C9* variants, particularly the *CYP2C9*3* allele or a combination of *CYP2C9*2* and *CYP2C9*3*, may have elevated PT–INRs, require longer to achieve a stable warfarin dose, and have a higher risk of serious or life threatening bleeding events during the induction or dose-titration period of warfarin therapy. However, there was no association between these variants and either PT–INR stability or risk of excessive anticoagulation during long-term treatment [27].

Potential relevance of deleterious mutations in *CYP2C9* to warfarin

Rare missense mutations in *CYP2C9* may affect enzyme function and warfarin clearance [28–38], and these mutations are summarized in Table 1. Missense mutations with functional effects were mapped in the crystal structure of human *CYP2C9* bound with warfarin (Fig. 2) [39]. The population frequencies of these *CYP2C9* variants have not been studied thoroughly. The *CYP2C9*4* allele has only been found at very low frequencies in Asian individuals [28]. The *CYP2C9*5* and *CYP2C9*6* alleles have been identified in approximately no more than 1% of black individuals, and they are virtually absent in Caucasian and Asian populations [30,31,40,41]. The presences of other recently identified *CYP2C9* alleles need to be confirmed in different ethnic populations.

Genetic polymorphisms in *VKORC1* relevant to warfarin

Genetic mutations in *VKORC1* as combined deficiency of vitamin K-dependent clotting factors type 2

As mentioned above, VKOR is the target enzyme of warfarin. VKOR was first identified in 1974, but the gene encoding VKOR, *VKORC1*, was not identified until 2004 [9,10]. *VKORC1* is found on chromosome 16p11.2, and it is approximately 4-kb long. Congenital deficiency of *VKORC1* leads to a bleeding phenotype, named combined deficiency of vitamin K-dependent clotting factors type 2, and a missense mutation, Arg98Trp, has been identified in this patient [9]. Other *VKORC1* missense mutations, Val45Ala, Arg58Gly, and Leu128Arg, have also been identified in patients with warfarin resistance

[9,42,43]. These missense mutations could affect *VKORC1* enzyme function, leading to a global decrease in all vitamin K coagulation factors. Alternatively, these mutations could lead to warfarin non-responsiveness. However, several more common SNPs in *VKORC1* significantly affect warfarin maintenance dose, as described below.

Relationship of genetic polymorphisms in *VKORC1* and warfarin dose

Several genetic polymorphisms in *VKORC1* are associated with warfarin dose across the normal dose range [44–54]. Two common polymorphisms, 1173C>T in intron 1 and 3730G>A in the 3′-untranslated region (defined by the nucleotide position from the translation start site), affect the interindividual variability of warfarin dose [44]. Regardless of the presence of confounding variables, the mean warfarin dose was higher (6.2 mg/day) in patients with the *VKORC1* 1173CC genotype than those patients with the CT (4.8 mg/day; $p=0.002$) or TT genotype (3.5 mg/day; $p<0.001$).

Subsequent haplotype analysis established a significant contribution of *VKORC1* to interindividual variability of warfarin dose [45]. The 10 most common SNPs were used to construct five major haplotypes, and the relationship of these haplotypes to warfarin dose was examined in Caucasian patients. A low-dose haplotype group (A) and a high-dose haplotype group (B) were identified. The mean (± SE) warfarin maintenance dose differed significantly between the three combinations of haplotype group, with a dose of 2.7±0.2 mg/day for group A/A, 4.9±0.2 mg/day for group A/B, and 6.2± 0.3 mg/day for group B/B. Thus, *VKORC1* haplotype explained a large degree of the interindividual variations of warfarin dose.

Estimated contribution of *CYP2C9* and *VKORC1* genotypes in interindividual variability of warfarin dose

Since the cloning of *VKORC1*, several pharmacogenomic studies have examined the contribution of *VKORC1* genetic polymorphisms in the interindividual variability of warfarin responsiveness [44–51]. These studies suggest that variations in *CYP2C9* and *VKORC1* can potentially account for 5–22% and 6–37% of the interindividual variability of warfarin dose, respectively (Table 2). Taken together, these data indicate that the interindividual variability of warfarin dose can be partly explained by genetic polymorphisms in *VKORC1* and *CYP2C9*. Thus, when pharmacogenomic knowledge of *CYP2C9* and *VKORC1* is considered together with clinical factors, such as age, gender, body weight, height, concurrent medications, and indication for treatment, more than 33% of the variability in the warfarin dose can be predicted.

Function of *VKORC1* polymorphisms

A component of one of the examined haplotypes is the −1639G>A polymorphism in the *VKORC1* promoter. This polymorphism occurs in the second nucleotide of an E-box (CANNTG) and is predicted to alter the E-box consensus sequence with potential changes in the *VKORC1* promoter activity. When this was examined using a luciferase reporter assay, one study found that the promoter activity of the G allele variant was 44% higher compared with the A allele [52], but another group did not identify any differences in *VKORC1* promoter activity between these variants [46]. When *VKORC1* mRNA levels were examined in human liver tissue, *VKORC1* mRNA expression significantly correlated with haplotype group with expression in the B/B (high-dose) group about three times higher than the A/A (low-dose) group [45]. Thus, despite inconclusive *in vitro* data, *VKORC1* haplotype is associated with variable mRNA levels that can contribute to interindividual variability in warfarin dose.

VKORC1 genotype and adverse bleeding events

Genetic polymorphisms in *VKORC1* can clearly affect warfarin dose, but can polymorphisms affect the occurrence of adverse bleeding events? To address this question, a case-control study examined 110 patients with episodes of severe bleeding during warfarin therapy and 220 control patients without bleeding undergoing the same therapy. They specifically examined the *VKORC1* 1173C>T polymorphism, and carriers of at least one T allele had an increased risk of bleeding (crude odds ratio=1.7, 95% CI: 1.1–2.5) compared to individuals with the CC genotype [55]. In this study, phenprocoumon and acenocoumarol were used for anticoagulation. When analyzed separately, phenprocoumon seems to more strongly modify the bleeding risk of patients with the 1173C>T genotype (crude odds ratio=2.6, 95% CI: 1.2–5.7 for T-allele carriers), whereas genotype did not affect acenocoumarol users (crude odds ratio=1.2, 95% CI: 0.6–2.3).

Ethnicity and interindividual variation in warfarin dose

Ethnicity is an important factor contributing to the warfarin maintenance dose. The warfarin