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REGULAR ARTICLE

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

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

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

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

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

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

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

Materials and methods

Subjects

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

DNA analyses

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

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

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

Statistical analysis

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

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

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

Results

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

Table 1 Characteristics of patients

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

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

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

Gene	SNP	Genotype	*n*	Mean±SD (mg/day)	*P*
VKORC1	−1639 G>A*	AA	79	2.83±1.00	
		GA	14	3.70±1.11	0.004
		GG	0	–	
VKORC1	1173 C>T*	TT	79	2.83±1.00	
		CT	14	3.70±1.11	0.004
		CC	0	–	
VKORC1	3730 G>A*	GG	79	2.84±1.00	
		GA	14	3.68±1.12	0.006
		AA	0	–	
GGCX	8016 G>A (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.

Acknowledgments

This study was supported by the Program for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), a Grant-in-Aid from the Ministry of Health, Labor, and Welfare of Japan, and the Ministry of Education, Culture, Sports, Science, and Technology of Japan. We thank Ms. Junko Ishikawa for her technical assistance.

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Age- and gender-related differences of plasma prothrombin activity levels

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Dear Sir,

Advancing age is an important risk factor for venous or arterial thrombosis in both sexes (1–3). Moreover, gender is associated with differences in the prothrombotic state and in the progression of atherosclerosis that occurs with aging (4, 5). Prothrombin is one of the dominant factors influencing thrombin generation (6), and the prothrombin G20210A mutation accompanied by an increased level of prothrombin poses a risk factor for venous or arterial thrombosis (7, 8). However, gender differences in age-related changes in plasma prothrombin activity have not been investigated until now. In the present study, we measured prothrombin activity in 742 individuals derived from a general Japanese population which was supposed to be free of prothrombin G20210A mutation (9).

The study population was composed of samples randomly selected from the residents of Suita, a city located in the second largest urban area in Japan (the Suita Study) (4). All subjects had been visiting the National Cardiovascular Center every two years since 1989 for regular health checkups. Only subjects who provided written informed consent to have a blood examination were enrolled in this study. We excluded subjects treated with oral anticoagulant therapy. Finally, 742 subjects, aged 36 to 85 years (mean age: 64 years), were included in this study. Spearman correlation analysis was used to assess the association between aging and the level of prothrombin activity within a given gender. For comparison between the two gender groups, the Mann-Whitney U test was used. Differences with a value of $p<0.01$ for the Spearman correlation analysis and $p<0.05$ for the Mann-Whitney U test were considered to be significant. Statistical calculations were performed using SPSS version 12.0 (SPSS Inc, Chicago, IL, USA). Prothrombin activity was measured according to a published method (10) with a modification. Briefly, 200 µl of 20 mM Tris-HCl, 0.14 M NaCl, pH 7.5 buffer containing 1 mg/ml of bovine serum albumin (TBSA) was added to 50 µl of plasma anticoagulated with 0.13% sodium citrate. Then, diluted plasma was incubated for 150 seconds at 37°C, and we detected ΔA/min at 405 nm after adding 50 µl of the reagent containing 6 mM $CaCl_2$, 0.5 mM Boc-Val-Pro-Arg-pNA as a thrombin substrate, 500 pM carinactivase-1 as a thrombin activator, and TBSA. Calibration was performed with a standard-human-plasma (Dade Behring GmbH, Marburg, Germany). The coefficient of intra-assay variation for prothrombin activity assay was 2.0%.

The mean ± SD of prothrombin activity level in men and women was 110.2 ± 17.0 (range: 54.5–158.5%) and 120.4 ± 17.4 (range: 57.5–194.4%), respectively. Figure 1 shows the age-related distribution (36–85 years) of prothrombin activity in 348 men (Fig. 1A) and 394 women (Fig. 1B). As a whole, a linear decrease of prothrombin activity level with age was observed in

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men (r=-0.34, p<0.0001), but not in women (r=-0.04, p=0.47). When prothrombin activity level was analyzed in 10-year age groups, significant decreases were observed in the men aged 46–55 years and 56–65 years (p<0.0001), aged 56–65 years and 76–85 years (p<0.05), and in the women aged 66–75 years and 76–85 years (p<0.0001). Levels of prothrombin activity were decreased in both sexes in the oldest age group (aged 76–85 years). With regards to gender-related change, the prothrombin activity level in the age group of 56–65 years, 66–75 years, and 76–85 years was significantly lower in men than in women.

In the present study, we showed the age-related decrease in the plasma prothrombin activity of men and gender-related change in the plasma prothrombin activity. These results contribute to the understanding of age-related hypercoagulability and to the practical institution of anticoagulant therapy in older patients. It has been established that thrombin generation increases with age in both sexes, evidenced by plasma prothrombin fragment F1+2 levels produced by the cleavage of prothrombin by factor Xa (11, 12). Age-related hypercoagulability does not likely stem from the prothrombin activity, because the prothrombin activity of men showed the age-related decrease, but it may result from some other mechanisms including decreased levels of anticoagulant proteins such as protein C and S (11, 13). We presented here the gender-related change of significantly lower prothrombin activity levels in men in the age of 56–85 years than in women. Men tend to develop thrombotic events including recurrent venous thrombosis (14), but this tendency was not related to the plasma level of prothrombin activity. Our work sheds further light on the point that, when considering relative hypercoagulability, gender-adjustment is necessary for the comparison of prothrombin activity levels.

With regards to anticoagulant therapy, the plasma levels of vitamin K-dependent coagulation factors decrease with increasing intensity of anticoagulation therapy (15). At the same time, the risks of major haemorrhage increase according to the intensity of anticoagulation therapy, especially in patients older than 80 years (16). Given our current study results, the markedly decreased prothrombin level in the age group of 76–85 years, especially in men, provides a potential mechanistic explanation for the increased rate of major haemorrhage observed in elderly patients receiving anticoagulant therapy.

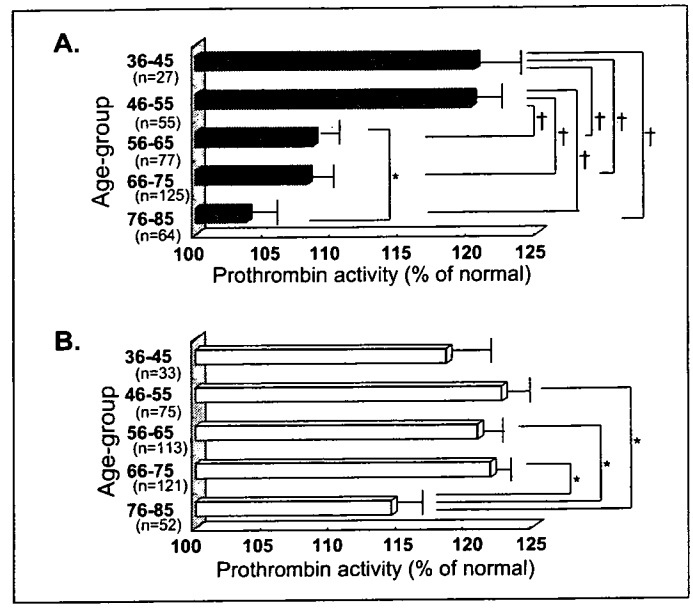

Figure 1: Age-related changes of plasma prothrombin activity levels according to gender (A: men, B: women). Populations aged from 36 to 85 years old were divided into five age groups by gender. Data are expressed as the mean ± SEM. *: P<0.05, †: P<0.0001, compared between two age groups of the same gender.

In conclusion, there are significant age- and gender-related differences in plasma prothrombin activity levels. In particular, the prothrombin activity level in men in the age group of 76–85 years was lower than that of any other age group in either gender.

Acknowledgments

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REVIEW ARTICLE

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

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Polymorphism

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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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

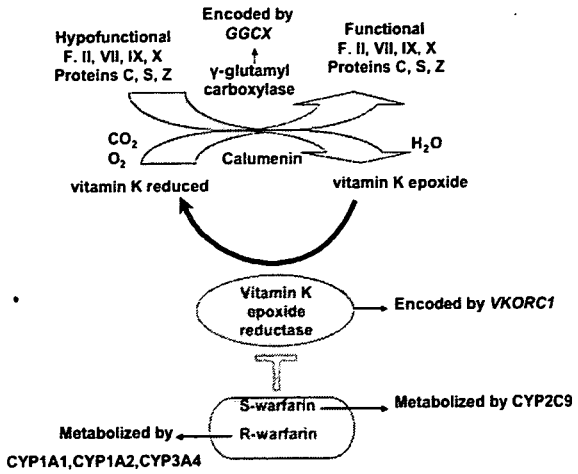

Figure 1 Pathway of warfarin metabolism.

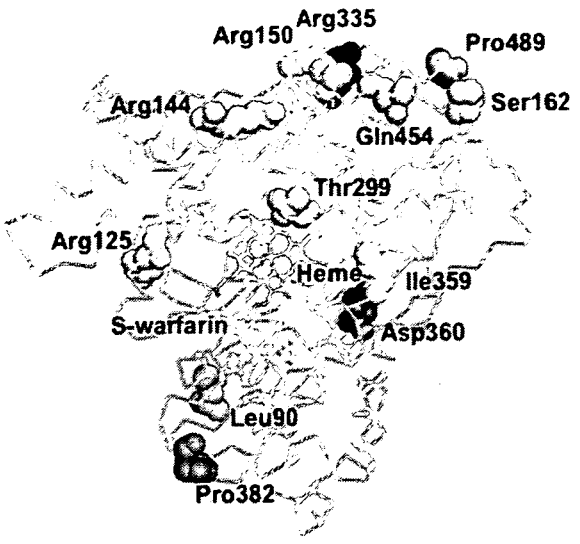

Figure 2 Missense mutations with functional effects mapped in the crystal structure of human *CYP2C9* protein bound with warfarin (PDB: 10G5). S-warfarin and heme are shown in the skeleton model with pink and red, respectively. Amino acid residues are shown in the sphere mode with colors.

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

Table 2 Estimated contribution of various factors for interindividual variation of warfarin dose

Variable	Estimated contribution[a]		Reference
VKORC1	14%		D'Andrea et al. [44]
CYP2C9	22%		
VKORC1	21%		Rieder et al. [45]
CYP2C9	6%		
VKORC1	37%[b]	30%[c]	Bodin et al. [46]
CYP2C9	14%[b]		
Body weight, *VKORC1*, *CYP2C9*	54%[b]	40%[c]	
Age	17%		Sconce et al. [47]
VKORC1	15%		
CYP2C9	18%		
Age, *VKORC1*, *CYP2C9*, height	54%		
VKORC1	30%		Wadelius et al. [48]
CYP2C9	12%		
Age, *VKORC1*, *CYP2C9*, *GGCX*, body weight, interacting drugs, indication for treatment	56%		
Age	21.5%		Veenstra et al. [49]
Gender	0.4%		
VKORC1	31.0%		
CYP2C9	7.9%		
Age, gender, *VKORC1*, *CYP2C9*	60.8%		
Age	1.7%		Kimura et al. [50]
Gender	8.1%		
Body Weight	7.8%		
VKORC1	5.9%		
CYP2C9	4.6%		
GGCX	5.2%		
Age, gender, body weight, *VKORC1*, *CYP2C9*, *GGCX*	33.3%		

[a] Estimated contribution of variables is denoted as R^2 (coefficient of determination), calculated from multivariate linear regression models.
[b] Decrease in factor VII in healthy individuals.
[c] PT–INR change in healthy individuals.

maintenance dose in Asian patients was approximately 30–40% less than that of Caucasian patients [37,50,51,56], and these differences are, in part, attributable to genetic differences in *CYP2C9* and *VKORC1*.

Ethnic differences in allelic frequencies of *CYP2C9*2* and *CYP2C9*3*

The allelic frequencies of *CYP2C9*2* and *CYP2C9*3* are considerably different between ethnic populations. In Caucasians, the allelic frequencies of *CYP2C9*2* and *CYP2C9*3* are approximately 8% to 20% and 6% to 10%, respectively [40,57–59]. These deleterious variants are less prevalent in Asian and African-American populations. *CYP2C9*2* is not present in Asian populations, and only approximately 2–4% of African-American populations carry the *CYP2C9*2* allele. *CYP2C9*3* is present in 1–4% of Asians and 1–2% of African-Americans [40,60]. The clinical effects of this polymorphism have been widely documented *in vivo* [23,60–63].

Ethnic differences in *VKORC1* variants

The frequencies of different *VKORC1* alleles in Asian, African-American and Caucasian subjects are listed in Table 3. The frequency of the AA genotype of the −1639G>A variant in Japanese (83%) was much higher than that in Caucasians (14%) [53], but it is comparable to Chinese (82%) [52]. The *VKORC1* haplotype group A related to low warfarin dose was highest in Asian populations (89%), while haplotype group B was highest in Caucasian populations (58%) [45]. One studied examined the combination of *CYP2C9*2* and *CYP2C9*3* frequencies and *VKORC1* haplotype in 556 unrelated healthy individuals from different ethnic backgrounds, and the Asian population had the highest frequency (86%) of the "low dose" genotype [64]. African-Americans had the lowest frequency (22%) of the "low dose" phenotype, and these data are consistent with the observations that Asian patients require a lower average maintenance warfarin dose and African-Americans a higher average dose to obtain a therapeutic PT–INR. These results were also confirmed in a Hong Kong Chinese population [49].

Proposed pharmacogenomic algorithms for warfarin dose determination

A dosing algorithm was developed based on the study of 297 Caucasian warfarin-treated patients [47]. The formula predicts that dose = 0.628 − 0.0135 (age) − 0.240 (*CYP2C9*2*) − 0.370 (*CYP2C9*3*) − 0.241 (*VKORC1*) + 0.0162 (height), where age (year), *CYP2C9* (**2 *3*) and *VKORC1* (−1639G>A) genotypes, and height (cm) allow the best estimate of warfarin maintenance dose. This formula accounted for nearly 55% of the variability in warfarin daily dose requirements in Caucasian. In this study, comorbid

Table 3 Common variant alleles and haplotype group frequencies of *VKORC1* in Asian, African and Caucasian individuals

	Frequency (%)		
	African	Asian	Caucasian
−1639G>A	–	82–83	14
1173C>T	9	89	42
1542G>C	25	91	37
3730G>A	49	13	45
Haplotype group A (low dose)	14–23	85–89	37–42
Haplotype group B (high dose)	49	10–14	57–58

Taken from the references of Yuan et al. [52], Mushiroda et al. [53], Rieder et al. [45], Marsh et al. [64], and Veenstra et al. [49].

Sequence number is defined by the nucleotide position from the translational start site ATG.

Haplotype groups A and B are based on classifications from Reider et al. [45] where haplotype A represents individuals at risk for excessive anticoagulation with standard warfarin dosing, and haplotype B represents individuals at risk for subtherapeutic anticoagulation from standard warfarin dosing.

conditions and concurrent medication were exclusion criteria, so that their contributions to warfarin dose could not be determined. This dosing algorithm was validated in an unrelated cohort of patients on warfarin chronic therapy.

VKORC1 (1173C>T) and *CYP2C9* (**2/*3/*11*) genotypes, age and weight were identified as independent covariates contributing to interindividual variability in warfarin dose in different ethnic groups [51]. In this study, 70% of Caucasian, 83% of African-American and 20% of Japanese patients carried the *CYP2C9* and *VKORC1* genetic factors respectively, resulting in the observed wide interindividual variation in warfarin dose. The final regression equation for estimating maintenance doses of warfarin was as follows: for patients with homozygous wild-type genotype for both *CYP2C9* and *VKORC1*: maintenance dose (mg) = 6.6 − 0.035 × (age, year) + 0.031 × (body weight, kg); for those either heterozygous or homozygous for variants of *CYP2C9*, the maintenance dose was further reduced by 1.3 and 2.9 mg, respectively, from those predicted by the respective equations. Based on the standardized partial regression coefficients, genotypes of *CYP2C9* and *VKORC1* were the principal covariates contributing equally to interindividual variability in warfarin dose requirements. Collectively, the identified covariates accounted for 57% of the overall variability in the daily dose of warfarin.

An alternative warfarin-dosing algorithm was developed by studying 828 Japanese warfarin-treated patients [53]. Patients were classified into three groups according to *CYP2C9* (**1/*3*) and *VKORC1* (intron 1–136T>C, same as 1173T>C) genotype, and this was referred to as the "warfarin-response index" [53]. The median warfarin daily dose varied significantly in the three index groups, with the lowest median dose being 2.0 mg/day for the *CYP2C9*3/*3* and *VKORC1* 1173T/T group, and highest dose of 3.5 mg/day for the *CYP2C9*1/*1* and *VKORC1* 1173C/C group ($p = 4.4 \times 10^{-13}$).

Contribution of other genes to warfarin interindividual variability

Despite our current knowledge of phamacogenomic and clinical factors, the source of more than 40% of the variability in warfarin dose remains unclear. Additional genetic factors, including multidrug resistance 1 (*MDR1*) [65], genes encoding vitamin K-dependent clotting factors [66], *GGCX* encoding γ-glutamyl carboxylase in the vitamin K cycle (Fig. 1) [48,50], the γ-glutamyl carboxylase inhibitory protein calumenin (Fig. 1) [67], apolipoprotein E [68], candidate genes encoding microsomal epoxide hydrolase (*mEH*) [69], and possible genes encoding additional components of the vitamin K epoxide reductase complex [9], might be responsible for the observed interindividual variability in warfarin dose requirements.

Perspective

We have greatly increased our knowledge of the factors contributing to the interindividual variability of warfarin dose. The relationship between genetic variations in *CYP2C9* and *VKORC1* and therapeutic warfarin dose is biologically and statistically compelling. Use of new warfarin-dosing algorithms will not eliminate the need for PT–INR monitoring, but these algorithms may prevent bleeding caused by excessive warfarin initiation. However, current evidence does not indicate widespread genotyping of *CYP2C9* and *VKORC1* for a variety of reasons.

The utility of pre-prescription *CYP2C9* and *VKORC1* genotyping and the proposed pharmacogenomic algorithms have not yet been established in prospective randomized clinical trials. Comparisons between patients treated based on genotype information and patients treated with only conventional empirical therapy are needed before a widespread genotyping should be performed. The hypothesis that pharmacogenomic based dosing will reduce the risk of bleeding during warfarin induction should be tested prospectively.

A cost-benefit analysis of pre-prescription *CYP2C9* and *VKORC1* genotyping during warfarin treatment should be performed. Genotyping large numbers of patients to identify the small minority with a markedly increased risk of adverse effects may not be cost-effective. However, for patients

treated with warfarin, even a small reduction in the risk of major hemorrhage during induction could make genotyping cost-effective because of the devastating clinical and economic consequences of a major bleeding event [8].

In conclusion, a warfarin-dosing regimen using clinical data and pharmacogenomic information of *CYP2C9* and *VKORC1* genotype could benefit patients treated with warfarin, but treatment algorithms incorporating pharmacogenomic data must be evaluated prospectively in a randomized controlled clinical trial before incorporating into routine clinical practice. Additionally, the prospective validation of a pharmacogenomics dosing model would benefit from a platform that could quickly and economically genotype individuals.

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