

Different contributions of polymorphisms in *VKORC1* and *CYP2C9* to intra- and inter-population differences in maintenance dose of warfarin in Japanese, Caucasians and African-Americans

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Objective To investigate pharmacokinetic and pharmacodynamic factors associated with population differences in warfarin doses needed to achieve anticoagulation, in particular the possible involvement of genetic variability in vitamin K epoxide reductase (*VKOR*) and *CYP2C9*.

Methods Warfarin maintenance dose, unbound plasma S-warfarin concentration [Cu(S)] and INR were determined in 157 Caucasians, 172 Japanese, and 36 African-Americans stably anticoagulated patients. In a subset ($n=166$), fully carboxylated plasma normal prothrombin levels (NPT) were also measured. Genotyping for seven *CYP2C9* (*CYP2C9*1* through 6 and *11) and seven *VKORC1* variants were performed in 115 Caucasians and 64 Japanese patients and 66 healthy African-Americans. Multivariate analysis was performed to identify covariates associated with warfarin requirement.

Results The relationship between NPT and Cu(S) indicated Japanese are more susceptible to inhibition of NPT production by S-warfarin than the other two populations. *VKORC1* 1173 C>T had a greater frequency in Japanese (89.1%) than Caucasians (42.2%) and African-Americans (8.6%). *CYP2C9* variants with reduced metabolizing ability were less frequent in Japanese compared to the other two populations. The median warfarin dose was significantly higher in Caucasians than Japanese patients (5.5 versus 3.5 mg/day), however, when matched for *CYP2C9*1* homozygosity, no difference in dose was observed between *VKORC1* genotype-matched groups. Furthermore, *VKORC1* 1173C>T and *CYP2C9* (*2/*3/*11) genotypes, age and weight were identified as independent covariates contributing to interpatient variability in warfarin dosage.

Introduction

Warfarin is the mainstay of anticoagulation therapy, worldwide. Its clinical use, however, is complicated by the fact that it has a narrow therapeutic index with associated adverse effects that are potentially serious, i.e., bleeding, and the dosage requirement to produce a

Conclusions Both *VKORC1* and *CYP2C9* polymorphisms contribute to inter-population difference in warfarin doses among the three populations, but their contribution to intra-population variability may differ within each population. *Pharmacogenetics and Genomics* 16:101–110 © 2006 Lippincott Williams & Wilkins.

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required degree of anticoagulation varies widely between patients. The reason for the latter is multifactorial and includes determinants such as age [1–3], diet [4], and race [5–10]. Additionally, genetic factors determining the activity of *CYP2C9* have been recently demonstrated to be important. This cytochrome P450 is largely

responsible for the metabolism of S-warfarin, which is the enantiomer predominantly responsible for the drug's anticoagulant activity [11] – warfarin is administered as a racemate. In particular, two structural variants, *CYP2C9.2* and *CYP2C9.3*, have greatly reduced catalytic activity compared to the wild-type enzyme, *CYP2C9.1* [9,12], and retrospective studies have shown associations between the various genotypes and warfarin dose requirement and adverse effects [1–3,9,12–14]. It is apparent, however, that other factors, also possibly genetic, are important because, even when matched according to *CYP2C9* genotype, the dosing requirements for a similar degree of anticoagulation varies across populations and appear to be related to racial ancestry. For example, patients of Asian descent (Chinese [5,8,10], Japanese [9] and Malay [8,10]) require a lower maintenance dose of warfarin than Caucasians and Indians; by contrast, a higher dose is needed in African-Americans [6,7].

Warfarin's anticoagulant activity results from inhibition of hepatic vitamin K epoxide reductase (*VKOR*) that affects the synthesis of various coagulation factors. Recently, variants of the vitamin K epoxide reductase complex subunit 1 gene (*VKORC1*) have been described to have potentially functional consequences [15–21]. For instance, Rieder *et al.* [21] identified five major haplotypes (H1, H2, H7, H8 and H9) based upon 10 common single nucleotide polymorphisms (SNPs) of *VKORC1* in Caucasian and Asian populations and found that those having either H1 or H2 haplotypes required significantly lower dose of warfarin than those having H7, H8 or H9. In addition, these *VKORC1* haplotypes were correlated with the level of expression of mRNA of *VKORC1* in human liver.

Collectively, genetic polymorphisms involved in both pharmacokinetic (*CYP2C9*) and pharmacodynamic (*VKORC1*) factors, therefore, appear to interplay in the overall interindividual variability of warfarin doses; moreover, the contribution of each factor may differ among different ethnic populations. In this context, we initially studied the pharmacokinetics and pharmacodynamics of warfarin separately in a large number of patients having different ethnic backgrounds to assess population difference in the pharmacokinetic and pharmacodynamic phenotypes of warfarin among Caucasians, Japanese and African-Americans. We then examined the contribution of genetic polymorphisms of *CYP2C9* and *VKORC1* in smaller subsets of patients in order to study whether differences in the frequencies of *CYP2C9* and *VKORC1* variants would provide a possible explanation for the difference in warfarin requirements between these populations after taking other clinical covariates (e.g., demographics) into account.

Methods

Patients

Three hundred and sixty-five patients (157 Caucasians, 172 Japanese and 36 African-Americans) participated in

the present study. The majority of them (140 Caucasians and 90 Japanese) had been previously investigated with regard to S-warfarin metabolism [9,12]. Further analysis was performed in 179 patients in whom genetic information was available for both *CYP2C9* and *VKORC1*. Each patient received warfarin orally once daily for at least one month with the dose being titrated to an international normalized ratio (INR) target value of 2.0 to 3.0 for Caucasian and African-Americans [22] and 1.5 to 2.5 for Japanese patients [23]. Clinical indications for anticoagulant therapy were prevention or treatment of thromboembolic disease (e.g., atrial fibrillation, deep vein thrombosis, or prosthetic valve replacement). Standard clinical laboratory tests indicated that all of the patients had normal liver function but three had impaired renal function (creatinine clearance ranging from 12 to 23 ml/min). Concurrent medications with potential to affect S-warfarin's metabolism included amiodarone ($n = 4$), NSAIDs ($n = 3$), cimetidine ($n = 2$), thyroid hormone ($n = 6$) and carbamazepine ($n = 1$).

Study protocol

Blood (5–10 ml) was obtained 12 to 16 h after administration of the last dose of warfarin, during a routine clinic visit. Separated plasma was stored at -70°C until analyzed whereas the buffy coat was maintained at 4°C until extracted for DNA. The study protocol was approved by the IRBs of the respective institutions and written informed consent was obtained from each patient.

Pharmacokinetics and pharmacodynamics of warfarin

The plasma concentrations of warfarin's enantiomers were determined by a chiral high-pressure liquid chromatography-based method as previously described [24]. The extent of plasma protein binding was measured using ultrafiltration [24], which permitted estimation of the steady-state unbound plasma concentration [Cu(S)] and unbound oral clearance of S-warfarin [CL_{po,u}(S)] [9,25].

In addition to the INR value, warfarin's anticoagulant effect was also assessed in 166 patients (54 Caucasians, 91 Japanese and 21 African-Americans) through measurement of the plasma concentration of fully carboxylated or normal prothrombin (NPT) by the carinactivase-1 method [26]. A 'warfarin sensitivity index' [INR/Cu(S)] was also estimated for all patients.

VKORC1 and *CYP2C9* genotyping

DNA was extracted from the buffy coat of blood using a commercially available kit (Qiagen, Tokyo, Japan). Genotyping for variants in all coding regions and intron/exon boundaries of *VKORC1* (GenBank accession number AY587020) was performed by PCR and direct sequencing

using described primers to identify *VKORC1* 129C > T, 497T > G, 1173C > T, 1196G > A, 1331G > A, 3462C > T and 3730G > A [15,16,21]. In the present study, the position of a nucleotide was numbered according to a previously described system [16]: the A of the ATG initiation codon of AY587020 being denoted as position 1. Thus, the positions of 381, 3673, 6484, 6853 and 7566 of the reference sequence (AY587020) correspond to -4931, -1639, 1173, 1542 and 2255, respectively. Allelic variants of *CYP2C9* (*CYP2C9*1* through *CYP2C9*6*, and *CYP2C9*11*) were determined by either RFLP analysis or direct sequencing [9,27].

Genotypes for both *VKORC1* and *CYP2C9* were available for 179 patients (115 Caucasians and 64 Japanese). Because no DNA samples were available from African-American patients on warfarin, blood was commercially obtained from 64 healthy African-American subjects (ProMedDx, LLC, Norton, Massachusetts, USA) for analysis of the frequencies of the two gene's allelic variants. The patient haplotypes and their frequencies were estimated by PowerMarker (Ver. 3.23) and a haplotype association test was performed according to the method of Rieder *et al.* [21], which allowed classification of each patient into either Group A (comprising either H1 or H2 haplotypes) or Group B (comprising either H7, H8 or H9 haplotypes). Because the nucleotide at position 861 according to the Rieder's system was not examined, patients with the H7 haplotype were not distinguishable from those with an H8 haplotype. However, this did not affect classification of such individuals into Group B. A log-transformed maintenance dose adjusted for age, sex, body weight and *CYP2C9* genotype and warfarin sensitivity index [INR/Cu(S)] were compared between the patient groups with different haplotypes.

Statistics

Multiple comparisons between the mean values for the pharmacokinetic, pharmacodynamic and demographic data obtained from three populations were performed by ANOVA followed by the Tukey–Kramer test. Relationship between Cu(S) and INR in patients with different *VKORC1* (1173C > T) genotypes was examined by the Pearson's correlation test. Genetic data for deviation from the Hardy–Weinberg proportions were tested using the chi-square test. Multiple comparisons for allelic frequencies of *VKORC1* and *CYP2C9* variants between Caucasian, Japanese and African-American patients were performed by the chi-square test followed by the Tukey–Kramer test. Spearman's rank correlation test followed by the stepwise multiple regression analysis were performed to assess the contribution of patients' covariates [i.e., age, sex, body weight, racial ancestry (Caucasian versus Japanese) and genotypes (wild-type versus heterozygote versus homo- or the combined homozygote) of *VKORC1* and *CYP2C9*] to the overall variability of maintenance doses of warfarin. Squares of the adjusted correlation coefficient (r^2) and Akaike's Information Criterion (AIC) were employed to evaluate the goodness of model fitting. Data are presented as means \pm SD or medians and the upper and lower quartile ranges (25 and 75 percentiles) where appropriate. A *P*-value of less than 0.05 was considered statistically significant for all analyses.

Results

The Caucasian patients were slightly older than the other two populations and there were also differences in body weight between the groups (Table 1). The daily maintenance dose of warfarin and its associated unbound concentration of the S-enantiomer were higher in African-Americans than in Caucasians who, in turn, had larger values than the Japanese; the reverse ranking was present

Table 1 Demographic characteristics of study patients

Parameter	African-American	Caucasian	Japanese
Number of patients studied			
Dose-Cu(S)-INR relationship	36	157	172
Plasma normal prothrombin	21	54	91
Genotyping of <i>CYP2C9</i> and <i>VKORC1</i>	(64)*	115	64
Gender (M/F)	12/24	87/70	101/71
Age (years)	61 \pm 11	65 \pm 13	61 \pm 10 [†]
Body weight (kg)	89.5 \pm 26.4 [§]	73.7 \pm 17.1	56.5 \pm 10.9 ^{†,‡}
Dose of racemic warfarin (mg/day)	5.3 \pm 2.6	4.7 \pm 2.4	3.5 \pm 1.6 ^{†,‡}
Cu(S) (ng/ml)	6.76 \pm 2.97 [§]	4.09 \pm 2.08	2.19 \pm 1.25 ^{†,‡}
CL _{po,u} (S) (ml/min)	314.7 \pm 163.1 [§]	469.4 \pm 294.4	654.3 \pm 376.8 ^{†,‡}
INR	2.67 \pm 0.81	2.50 \pm 0.89	1.84 \pm 0.59 ^{†,‡}
INR/Cu(S) (ml/ng)	0.46 \pm 0.21 [§]	0.75 \pm 0.45	1.05 \pm 0.58 ^{†,‡}
Normal prothrombin level (μ g/ml)	54.6 \pm 23.2	60.3 \pm 36.1	52.5 \pm 26.1

Abbreviations: Cu(S), plasma unbound concentration of S-warfarin; CL_{po,u}(S), unbound oral clearance of S-warfarin; INR, international normalized ratio of prothrombin time.

Data are mean values \pm SD.

*DNA samples were obtained from healthy subjects.

[†]*P* < 0.01 between the Caucasian and Japanese groups.

[‡]*P* < 0.01 between the Japanese and African-American groups.

[§]*P* < 0.05 between the Caucasian and African-American groups.

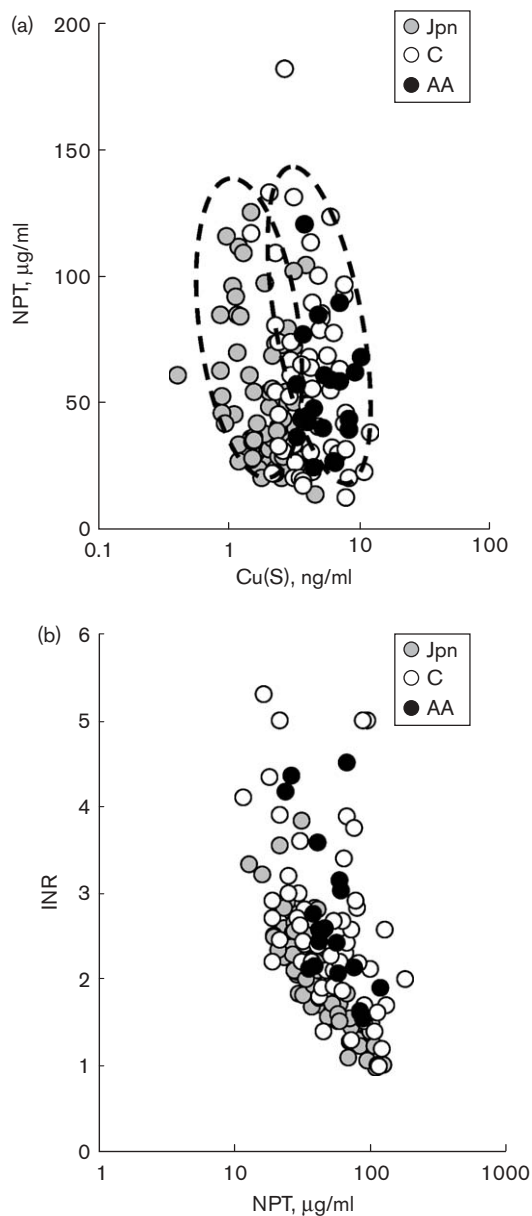
in the oral clearance of unbound S-warfarin (Table 1). No apparent differences in unbound S-warfarin's oral clearance were observed between patients who were given either amiodarone (458 ± 98 ml/min, $n = 4$) or thyroid hormone (330 ± 119 ml/min, $n = 6$) with warfarin and those were given warfarin alone. There was a significant ($P < 0.0001$) correlation between the oral clearances of S-warfarin and R-warfarin ($r = 0.706$).

Population differences were also apparent in the associated measures of anticoagulation (Table 1) with INR values in the Japanese patients being lower than in either of the other two populations. However, the 'warfarin sensitivity index' – a measure of the degree of anticoagulation normalized for the unbound S-warfarin plasma concentration – was higher in Japanese compared to Caucasians or African-Americans. No significant differences were present in the NPT concentrations between the populations, however, the distribution of NPT levels in the Japanese patients relative to the unbound plasma concentration of S-warfarin was shifted to the left compared to that in the Caucasian and African-American populations (Fig. 1a). On the other hand, the relationships between the NPT level and INR value in the three populations overlapped each other (Fig. 1b).

Seven allelic variants in the *VKORC1* gene were identified and these all exhibited differences in frequency between the populations studied (Table 2). With the exception of the 1173C > T transition in Japanese, Hardy-Weinberg equilibrium was present. A synonymous 3462C > T transition (Leu120Leu) in exon 3 was selectively present in African-Americans and two heterozygous cases of an exon 2 substitution (1331G > A, Val66Met) were also found in this population. In contrast, the transitions at 129C > T in exon 1, 497T > G in intron 1 and 1196G > A in intron 1 appeared to be present in Caucasians at a low frequency and the allelic frequencies of the transition at 3730G > A in the 3'-downstream region was significantly higher in African-American and Caucasians compared with Japanese. The most common allelic variant with a significant difference in frequency in all three populations was an 1173C > T polymorphism in intron 1 which was found in 8.6% of African-Americans, 42.2% of Caucasians and 89.1% of Japanese. Population differences in the allelic frequencies of the various *CYP2C9* variants were also found (Table 2); *CYP2C9* variants with reduced metabolizing ability were present at higher frequencies in Caucasians and African-Americans compared with Japanese.

Low but statistically significant ($P < 0.05$) correlations were present between the INR value and the unbound plasma concentrations of S-warfarin in *VKORC1* 1173 C > T heterozygotes and variant homozygotes but not homozygote wild-type in the collective results from all patients (Fig. 2). For any given genotype, the data from

Fig. 1



Relationships between plasma unbound concentrations of S-warfarin [Cu(S)] and plasma concentrations of fully carboxylated normal prothrombin (NPT) (a) and those between plasma concentrations of NPT and INR (b) in Caucasian (open circles), Japanese (grey or halftone circles) and African-American (closed circles) patients.

the Caucasians and Japanese patients overlapped. Additionally, the slopes of the relationships were steeper in the heterozygous and homozygous variant groups (0.163 and 0.183 ml/ng, respectively) than in the wild-type population (0.021 ml/ng). Regarding the novel *VKORC1* 1196 G > A transition, all four such Caucasian patients had an INR value greater than 2.5 at an unbound plasma concentration of S-warfarin < 5 ng/ml (i.e., they had increased warfarin sensitivity). Three of them also carried

Table 2 Allelic frequencies of *VKORC1* and *CYP2C9* variants

	African-American (n=64)	Caucasian (n=115)	Japanese (n=64)
<i>VKORC1</i> 129 C>T (Cys43Cys, exon 1)	0	0.009	0
<i>VKORC1</i> 497T>G (intron 1)	0.039 [§]	0.288	0 [†]
<i>VKORC1</i> 1173C>T (intron 1)	0.086 [§]	0.422	0.891 ^{†,‡}
<i>VKORC1</i> 1196G>A* (intron 1)	0	0.017	0
<i>VKORC1</i> 1331G>A (Val66Met, exon 2)	0.016	0	0
<i>VKORC1</i> 3462C>T (Leu120Leu, exon 3)	0.227 [§]	0.004	0 [‡]
<i>VKORC1</i> 3730G>A 3'-downstream)	0.523 [§]	0.374	0.167 ^{†,‡}
<i>CYP2C9</i> *1 (wild-type) (Arg ₁₄₄ / Arg ₃₃₅ /Ile ₃₅₉)	0.953 [§]	0.743	0.984 [†]
<i>CYP2C9</i> *2 (exon 3) (Arg/Cys ₁₄₄)	0 [§]	0.143	0 [†]
<i>CYP2C9</i> *3 (exon 7) (Ile/Leu ₃₅₉)	0.008 [§]	0.109	0.016 [†]
<i>CYP2C9</i> *4 (exon 7) (Ile/Thr ₃₅₉)	0	0	0
<i>CYP2C9</i> *5 (exon 7) (Asp/Glu ₃₆₀)	0.008	0	0
<i>CYP2C9</i> *6 (exon 5) (818delA)	0.008	0	0
<i>CYP2C9</i> *11 (exon 7) (Arg/Trp ₃₃₅)	0.023	0.004	0

African-American DNA samples were obtained from healthy subjects.

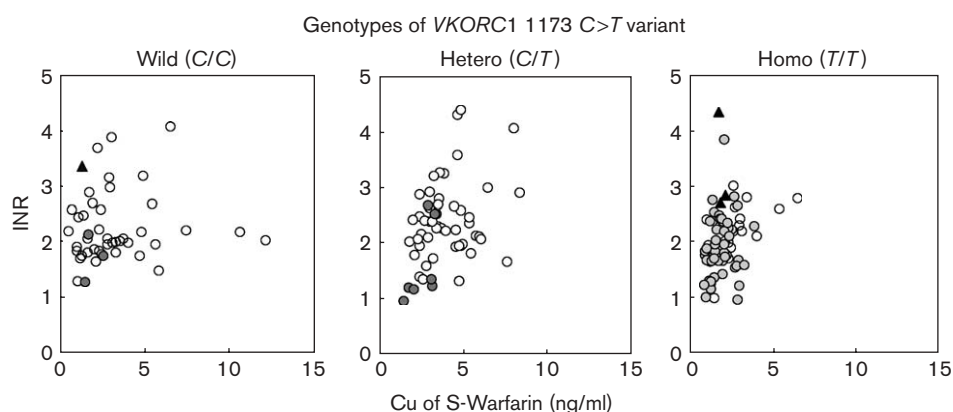
* a novel polymorphism.

[†] $P < 0.01$ between the Caucasian and Japanese groups.

[‡] $P < 0.01$ between the Japanese and African-American groups.

[§] $P < 0.05$ between the Caucasian and African-American groups.

Fig. 2



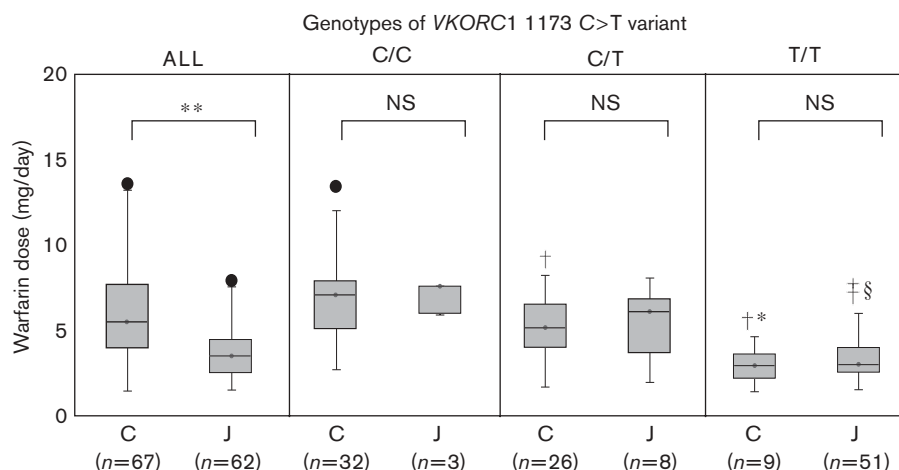
Relationships between plasma unbound concentrations (Cu) of S-warfarin and INR in Caucasian (open circles) and Japanese (grey or halftone circles) patients with three different genotypes of *VKORC1* 1173C>T: those with the wild-type (C/C), heterozygote (C/T) and homozygote (T/T) are shown separately. Four Caucasian patients carrying *VKORC1* 1196G>A are presented by black triangles. Significant ($P < 0.05$) and apparently steeper correlations between the two parameters were observed in the C/T ($r = 0.35$) and T/T genotypes ($r = 0.36$), respectively.

the *VKORC1* 1173 homozygous mutant allele (T/T), but one had the 1173 wild-type genotype. No differences in metabolizing ability, as measured by the oral clearance of unbound S-warfarin, were observed between the three *VKORC1* 1173 C > T genotype groups in Caucasians and Japanese. However, reduced maintenance doses of warfarin in patients carrying *CYP2C9**2 and/or *CYP2C9**3 were observed in the Caucasians and Japanese patients (5.5 ± 2.6 , 4.0 ± 1.8 , 3.2 ± 1.5 , 2.0 ± 1.3 mg/day in Caucasians with *CYP2C9**1/*1, *1/*2, *1/*3 versus *2/*3 or versus *2/*2 or versus *3/*3, respectively, and 3.6 ± 1.7 and 1.8 ± 0.5 mg/day in Japanese with *CYP2C9**1/*1 and *1/*3 genotypes, respectively). In order to perform further genotype: phenotype analysis (Fig. 3), patients homozygous for the wild-type *CYP2C9* gene (67 Caucasian and

62 Japanese patients) were selected to exclude the influence of population differences in the frequencies of defective *CYP2C9**2 and *CYP2C9**3 alleles on the maintenance doses.

The median daily warfarin dose in Caucasians was significantly greater ($P < 0.01$) than that in Japanese (5.5 versus 3.5 mg/day, respectively), when the two such populations were compared irrespective of *VKORC1* genotype (ALL in Fig. 3). There was a significant ($P < 0.05$) *VKORC1* 1173C > T gene-dose effect present in each population, e.g., a lower dose was observed in patients carrying homozygous mutations (T/T) compared with those with wild-type (C/C) and heterozygous mutations (C/T) except for Japanese patients with C/C

Fig. 3



Comparisons of the median maintenance doses of warfarin between Caucasian (C) and Japanese (J) patients carrying the wild-type *CYP2C9* genotype. Comparisons were made irrespective of *VKORC1* 1173C>T genotypes (ALL) and with regard to the *VKORC1* 1173C>T genotype (C/C, C/T and T/T, respectively) between Caucasian and Japanese patients. Data are shown by box-and-whisker plots. Subdivisions of the boxes and the top and bottom lines on the boxes represent median values and the upper and lower quartiles, respectively. The closed circles (●) are outlying values beyond the maximum length in terms of the interquartile range. Numbers of patients in each group are shown in the parentheses. There was a significant difference in warfarin doses between Caucasian and Japanese patients when compared irrespective of *VKORC1* genotype (ALL). There were also significant differences in warfarin doses between Caucasian patients having different *VKORC1* genotypes and between Japanese patients having 1173 C/C and T/T genotypes and between patients with 1173 C/T and T/T genotypes. ** $P < 0.01$ between the Caucasian and Japanese groups; † $P < 0.01$ between Caucasian patients with 1173 C/C and those with C/T or T/T genotypes; * $P < 0.05$ between Caucasian patients with 1173 C/T and those with T/T genotypes; ‡ $P < 0.01$ between Japanese patients with 1173 C/C and those with T/T genotypes; § $P < 0.01$ between Japanese patients with 1173 C/T and those with T/T genotypes.

and C/T genotypes: the mean maintenance doses obtained from Caucasian patients carrying C/C, C/T and T/T genotypes were 6.9 versus 5.2 versus 3.0 mg/day, respectively, and the corresponding values obtained from Japanese patients were 7.0 versus 5.4 versus 3.3 mg/day. In contrast, no significant differences were observed between these two populations in the daily dose within each 1173C > T genotype (Fig. 3).

Haplotype frequencies were 0.156 and 0.847 for H1, 0.256 and 0 for H2, 0.363 and 0.109 for H7/H8 and 0.200 and 0 for H9 in Caucasian and Japanese patients, respectively. Haplotype analysis revealed no significant differences in warfarin doses adjusted for age, sex, body weight and *CYP2C9* genotype and 'warfarin sensitivity index' for S-warfarin between patients in Group A, i.e., with the H1 versus H2 haplotype (3.4 versus 3.5 mg/day, and 1.0 versus 1.0 ml/ng, respectively). No significant differences were observed in the corresponding values in Group B patients with the H7/H8 haplotype and those with the H9 haplotype (5.8 versus 5.2 mg/day, and 0.66 versus 0.58 ml/ng). Haplotype groups of A/A, A/B and B/B completely corresponded to the genotype groups of *VKORC1* 1173 T/T, T/C and C/C.

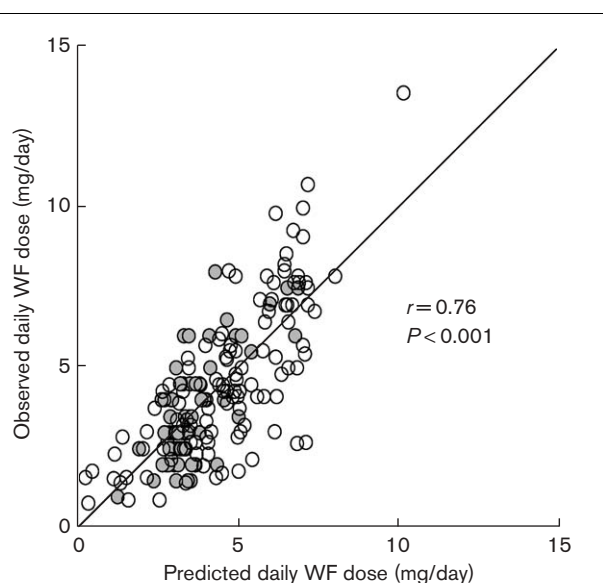
Univariate analysis to identify patient covariates associated with the interindividual variability in daily warfarin dose showed that age ($r = -0.22$), body weight ($r = 0.29$), *CYP2C9* variant ($r = -0.32$), *VKORC1*

1173C > T ($r = -0.58$) and Japanese ancestry ($r = -0.20$) were all significantly ($r < 0.05$) correlated. Further multivariate analysis with these covariates in 115 Caucasian and 64 Japanese patients revealed that *CYP2C9* and *VKORC1* genotypes, age and body weight had independent and statistically significant contributions to the overall variability in warfarin dose (Table 3). The final regression equation for estimating maintenance doses (MD) of warfarin was as follows: for patients with homozygous wild-type genotype for both *CYP2C9* and *VKORC1*: MD (mg) = $6.6 - 0.035 \times (\text{age, years}) + 0.031 \times (\text{body weight, kg})$; for those with either heterozygous or homozygous variant of *CYP2C9*, the MD was reduced by 1.7 and 2.8 mg, respectively, and for those with either heterozygous or homozygous variant of *VKORC1* 1173C > T, the MD 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 inter-patient variability in warfarin requirements. Collectively, the identified covariates accounted for 57% of the overall variability in the daily dose of warfarin. Also, a significant correlation ($r = 0.76$, $P < 0.001$) without systematic bias was observed between the actual maintenance doses taken by the Caucasian and Japanese patients and those predicted from the multiple regression model (Fig. 4).

Table 3 Multivariate analysis for patients' covariates that are associated with interindividual variability of warfarin doses

Covariates	Partial regression coefficient \pm SE	Standardized partial regression coefficient	P-value
Constant	6.656 \pm 0.973		
Age (years)	-0.035 \pm 0.010	-0.252	0.000808
Body weight (kg)	0.031 \pm 0.007	0.298	0.000059
<i>CYP2C9</i> *2/*3/*11 (Heterozygous)	-1.706 \pm 0.290	-0.408	<0.0000005
(Homozygous variant)	-2.815 \pm 0.473	-0.413	<0.0000005
<i>VKORC1</i> 1173 C>T (Heterozygous)	-1.316 \pm 0.309	-0.310	0.000034
(Homozygous variant)	-2.941 \pm 0.310	-0.590	<0.0000005

SE, standard error of mean.

Fig. 4

Relationship between maintenance doses of warfarin predicted from the multiple regression model and those actually observed in the 115 Caucasian (○) and 64 Japanese (●) patients. There is a significant correlation between the predicted and observed doses ($y = x + 0.0008$, $r = 0.76$, $P < 0.001$). The solid line represents the line of identity.

Caucasian and Japanese patients who carried *CYP2C9* variants possessed a lower unbound oral clearance for S-warfarin (decreased metabolic activity), thereby required a smaller daily dose of the drug (Fig. 5a). In addition, those carrying the *VKORC1* 1173C/C wild-type allele needed higher unbound concentrations of S-warfarin to achieve a therapeutic anticoagulation response (reduced sensitivity), and a greater daily dose was required regardless of race (Fig. 5b). Forty-seven percent of Caucasian patients possessed one of the *CYP2C9* variant alleles (*CYP2C9**2, *CYP2C9**3 or *CYP2C9**11) and 48% the *VKORC1* 1173 C/C wild-type allele, respectively. The corresponding values for African-Americans were 11% and 83%, and those for Japanese were 3% and 17%, respectively. These genetic polymorphisms in *CYP2C9* and *VKORC1* were independent to each other and allelic frequencies of

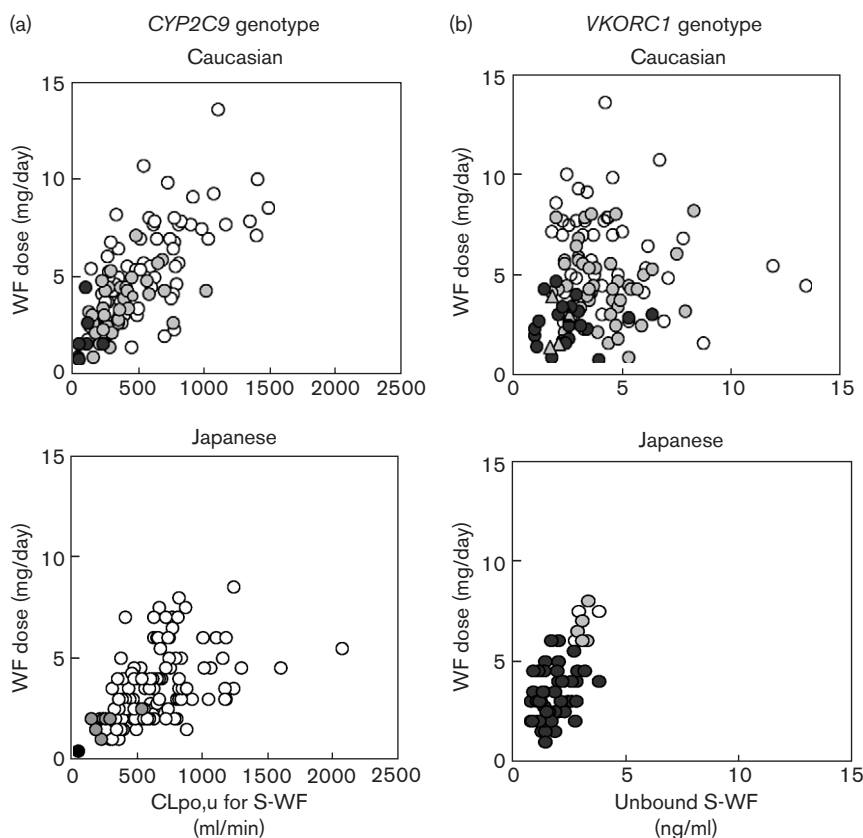
these genetic variants differed among the three populations (Table 2). As a result, 70% of Caucasian, 83% of African-American and 20% of Japanese patients were found to carry pharmacokinetic (*CYP2C9*) and pharmacodynamic (*VKORC1*) genetic factors which are associated with a lower and a higher requirement, respectively, resulting in the wide interindividual variation in warfarin doses.

Discussion

Warfarin therapy is complicated by large interpatient variability in maintenance dose requirement and the associated risk of under- and over-anticoagulation. This is the first study demonstrating that there are population differences not only in pharmacokinetics but also in pharmacodynamics of warfarin based upon the dose-plasma concentration and plasma concentration-INR relationships. The pharmacodynamics of S-warfarin evaluated by its 'warfarin sensitivity index' showed significant differences between African-Americans, Caucasians and Japanese patients, although the number of African-American patients ($n = 36$) participating in the study was smaller than the Caucasians and Japanese groups (Table 1). In addition, the sensitivity of S-warfarin to inhibit normal or fully carboxylated prothrombin (NPT) production was found to differ between populations and this may play a pivotal role in the population differences of warfarin dose requirement.

Readily determinable demographic factors such as age and body weight have been considered as contributing covariates [1–3], and this is confirmed in the present study. The age factor may be related to a reduced ability to metabolize warfarin with aging [1]. A similar mechanistic explanation may also account for the body weight covariate although a pharmacodynamic factor may also be involved, since obese subjects have been found to have elevated plasma levels of fibrinogen and factor VII compared to lean individuals [28]. Nonetheless, such demographic factors only have limited utility for optimizing the warfarin maintenance dose and it has become increasingly appreciated that genetic factors may have an important role. Recent focus has been upon drug metabolizing enzymes involved in warfarin's metabolism that influence its plasma concentration.

Fig. 5



Relationships between unbound oral clearance ($CL_{po,u}$) for S-warfarin and daily doses of warfarin (left column, a) and those between plasma unbound concentration (Cu) for S-warfarin and daily doses of warfarin (right column, b) in Caucasian and Japanese patients with different genotypes of *CYP2C9* (a) and *VKORC1* (b). Symbols (a): *CYP2C9**1/*1 (open circles), *CYP2C9**1/*2 or *1/*3 or *1/*11 (grey circles) and *CYP2C9**2/*2, or *3/*3 or *2/*3 (black circles); symbols in (b): *VKORC1* 1173 C/C and 1196 G/G (open circles), *VKORC1* 1173 C/C and 1196 G/A (open triangle), *VKORC1* 1173 C/T and 1196 G/G (grey circles), *VKORC1* 1173 T/T and 1196 G/G (black circles) and *VKORC1* 1173T/T and 1196 G/A (grey triangles).

Clinically available warfarin is a racemic mixture of R- and S-enantiomers. However, S-warfarin has been shown to be three to five times more potent than R-warfarin based upon the anticoagulation responses elicited after the administration of the respective enantiomers separately in healthy subjects [11]. While plasma concentrations of R-warfarin are, on average, approximately twice those of S-warfarin following oral administration of the racemate, pharmacokinetic-pharmacodynamic analysis concluded that the anticoagulant effect is attributable almost entirely to S-warfarin concentrations [29]. Moreover, as noted in the present study, there was a significant correlation between the oral clearance of unbound S-warfarin and that for R-warfarin ($P < 0.0001$), indicating that demographic factors (e.g., body weight and age), nutritional and certain environmental factors linked with variability in both of these parameters may also be associated. Accordingly, it is likely that interindividual variability in the plasma concentration of S-warfarin is more important than that of R-warfarin when considering

the variability of anticoagulant activity following the administration of racemic warfarin.

CYP2C9 and its allelic variants have been investigated since the encoded enzyme is largely responsible for the metabolism of S-warfarin. Several relatively large retrospective clinical studies in several different populations have now demonstrated associations between warfarin's maintenance dose and adverse events, i.e., increased bleeding complications, and the presence of *CYP2C9* variants leading to markedly reduced catalytic activity of the resulting enzyme such as *CYP2C9.2* and *CYP2C9.3* [1–3,9,12–14]. Collectively, the present data confirm these previous observations that lower doses are required in patients carrying these variant alleles especially *CYP2C9**3. Despite such associations, however, the contribution of such genetic variability to the overall variability in warfarin's maintenance dose is relatively low – less than 20% of the variance [1–3]. The present findings based on the presence of *CYP2C9**2, *CYP2C9**3

and *CYP2C9*11* variants, all of which are associated with reduced enzyme activity, also confirm this small contribution even when variant homozygosity is present. Moreover, the difference in warfarin dosage requirement between Japanese and Caucasians cannot be explained by a greater frequency of *CYP2C9* variants with reduced catalytic activity in Caucasians (Table 2), and the former population have higher unbound oral clearances of S-warfarin than the latter when matched for the wild-type genotype in the 5'-flanking (up to -2 kb) and coding regions of *CYP2C9* [9,27]. Therefore, the present results strongly suggest the involvement of other factors.

The molecular target of warfarin is vitamin K epoxide reductase, which is critically involved in the production of functionally active vitamin K-dependent coagulation factors [e.g., factors II (prothrombin), VII, IX and X] through γ -glutamyl carboxylation [30]. Subunit 1 of this lipoprotein complex has recently been shown to exhibit genetic polymorphisms, and several such allelic variants have been shown to have reduced catalytic activity that is associated with 'warfarin-resistance', i.e., require substantially higher doses to achieve satisfactory anticoagulation [15,17]. However, only two such heterozygous *VKORC1* 1331G > A, Val66Met, African-American individuals were found in the present study. Other variants reported to be associated with 'warfarin-resistance' [15] were not detected. A number of other nucleotide transitions including a novel *VKORC1* 1196G > A were, however, identified and appeared to have selective distribution according to racial ancestry, but their rarity made it impossible to assess whether they have functional consequences. On the other hand, a haplotype combination including a *VKORC1* 1173C > T transition, previously reported to be present in 40% of European-Caucasians, was found to be common with higher and lower frequencies in Japanese and African-Americans, respectively [16-21]. This variant was also found to be associated with a gene-dose effect and a lower warfarin maintenance dose [16-21]. The present findings confirm this observation in Caucasians and extend the relationship to Japanese. Interestingly, this *VKORC1* variant appeared to affect the relationship between the unbound concentrations of S-warfarin and the resulting INR value - the slopes of the regression curves of the relationship being steeper in heterozygous and homozygous variant patients than in those homozygous for the wild-type allele. Importantly, the different population frequency of the *VKORC1* 1173T variant allele in Japanese compared to Caucasians, appeared to account for the increased 'warfarin sensitivity' of the former group of patients, matched according to *CYP2C9* genotype, i.e., *CYP2C9*1* homozygous, since no differences in dosage requirement was observed between the populations when stratified according to *VKORC1* genotype. Furthermore, multiple regression analysis showed that the *VKORC1* 1173C > T variant was an

important covariate with respect to the interindividual variability in warfarin dosage. Patients carrying the T allele at the position of 1173 of *VKORC1* gene are classified into the Group A haplotype associated with a lower dose requirement [21]. However, this haplotype system is no more informative than a single segregating SNPs among those at positions 381, 3673, 6484, 6853 and 7566 of the reference sequence (GenBank accession number AY587020) as shown previously by others [16], when the influence of *VKORC1* genotype on the interindividual variability in warfarin doses is considered. Overall, these results also suggest that the higher dose requirements in African-Americans [6,7] may possibly reflect the higher frequency of the *VKORC1* 1173C allele (91%) compared to Japanese (11%) and Caucasians (58%) (Table 2).

The 1173C > T transition in intron 1 of *VKORC1* was recently reported to be in complete disequilibrium with -1639G > A at a putative NF1 binding site [18], -4931T > C, 1542G > C and 2255C > T [21]. While there is a controversy regarding the influence of this *VKORC1* haplotype on the transcriptional activity of this gene [16,18,19], a recent report indicates that this haplotype was associated with lower mRNA levels in human liver [21]. This finding suggests that the 1173C > T variant may be associated with the lower levels of reduced form of vitamin K, thereby making patients with this variant more susceptible to the anticoagulation effect of warfarin. In addition to the conventional measure of anticoagulation, namely, the INR value, the concentration of NPT was also determined in the patients. No population differences could be discerned in the relationship between these two biomarkers, indicating comparable functionality of the involved fully carboxylated vitamin K-dependent factors and fibrinogen. However, Japanese patients appeared to be more sensitive to γ -carboxylation of prothrombin in that a comparable NPT response was achievable at lower plasma concentrations of unbound S-warfarin compared to Caucasians and African-Americans. The reason for this difference is unknown but may involve population differences in NPT's baseline level (preliminary unreported data), and further studies are required to explore this possibility. In addition, the question of whether the *VKORC1* haplotypes may influence the baseline levels of VKOR and NPT remains to be clarified. Regarding functionally related genes, multiple variants in several vitamin K-dependent proteins have been identified including factor II, factor VII and γ -glutamyl carboxylase [20,31]. Moreover, some of these are associated with altered 'warfarin sensitivity' [20,31] and preliminary data (not shown) indicates that their allelic frequencies differ between Caucasian and Japanese populations. Therefore, influences of these polymorphisms on the overall variability in warfarin responses are also to be clarified.

In summary, the present study shows that interindividual variability and population differences in the maintenance dose of warfarin required to achieve anticoagulation involves demographic, pharmacokinetic, and pharmacodynamic factors. Furthermore, genetic variability in CYP2C9-mediated metabolism of S-warfarin and the drug's molecular target, VKOR, are specific determinants. The present study shows that 70% Caucasian and 83% African-American patients carried either *CYP2C9* or/and *VKORC1* genotype(s) which leads to either reduced metabolic activity or attenuated sensitivity of warfarin. In contrast, only 20% of Japanese population possesses these genotypes. Thus, the relative contribution of the *VKORC1* and *CYP2C9* genotypes to the overall inter-patient variability in warfarin doses differs between the three populations according to racial ancestry. Moreover, it should be of note that the identified demographic and genetic covariates of warfarin doses only account for 57% of interindividual variability. Accordingly, other currently unknown determinants remain to be identified, and populations other than those currently studied need to be investigated.

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