cleotides of wild-type HNF-4α-binding site and NF-κB-binding site demonstrated the specificity of the binding as exemplified in Fig. 8A. In addition, the complex in RFB culture was retarded further with anti-HNF-4α antibodies (shown as SS) (Fig. 8A, lanes 7 and 8).

To characterize the nature of protein-HNF-4α-binding site complex in the nuclear extracts from RFB culture treated with rifampicin, we performed a long-time electrophoresis in the EMSA. As shown in Fig. 8B, lanes 5 and 6, we found a retarded mobility of the protein-HNF-4α-binding site complex present in rifampicin-treated RFB culture (shown as SLC), which was hardly recognized in the standard condition of polyacrylamide gel electrophoresis (Fig. 8B, lanes 1-4). Such a super-shifted band was not observed in the monolayer culture in the presence of rifampicin (Fig. 8B, lane 3). Thus, it seemed possible that rifampicin induced formation of nuclear protein-DNA complex with higher molecular weight in the RFB culture.

Discussion

We have established 7 human HCC cell lines, and 5 of these can be incubated in serum-free medium. Analysis of CYP isoforms is being conducted in 3 cell lines. In FLC-4, FLC-5, and FLC-7, we have confirmed the expression of CYP3A as well as CYP1A1, CYP1B1, and CYP2E1, which are important phase-1 reaction enzymes, responsible for approximately 50% of therapeutic drug-metabolizing activity in the liver.[14,16] The present study investigated enzyme induction in FLC-5 cells cultured in the RFB, focusing on CYP3A4.

First, we confirmed the induction of CYP3A4 by rifampicin in FLC-5 cells, as reflected by the increase in the CYP3A4 mRNA and protein. Thus, an enzyme-inducing system has been established for human CYP3A4. It remains to be clarified as to why rifampicin caused more intense CYP3A4 induction in the 3-dimensional perfusion culture. It is possible that expression of CYP3A4 at the transcription level requires maintenance of the 3-dimensional form of the cells, the shear stress caused by perfusion, and the development of intercellular adhesion structures, such as junctional complexes. It was recently suggested that PXR/RXRα could be transcriptional factors related to CYP3A4 induction due to binding at the ER6 site. It would be of interest to clarify whether or not these transcriptional factors are also involved in FLC-5 cells. One of the recognized interspecies differences is that, whereas pregnenolone induces CYP3A4 in rat hepatocytes, it does not do so in human hepatocytes.

We assumed that enhancement of CYP3A4 expression during culture in the RFB is attributable to either increased expression of PXR/RXRα or to enhancement of their DNA-binding activity. Based on this assumption, we measured the mRNA levels of PXR/RXRα by quantitative PCR and evaluated their DNA binding potentials by EMSA.

RXRα mRNA expression in the RFB culture did not differ from that in monolayer culture, but the expression of PXR showed an approximately 2-fold increase; when the culture was treated with rifampicin, the increase was 22-fold (Fig. 6). When evaluated by EMSA, culture in the RFB caused retardation of the electrophoretic activity of ER6 and the PXR/RXRα complex. Retardation in the EMSA assay of the electrophoretic activity of ER6 and PXR/RXRα following 3-dimensional culture or rifampicin treatment has not been previously reported. The result obtained in the present study is probably attributable to the increase in molecular weight of the DNA-protein complex because of further binding of cofactors to the PXR/RXRα heterodimer (Fig. 7). This higher molecular weight complex, which is presumably accompanied by its conformational change, may bind to the ER6 motif with higher affinity and up-regulate its transcriptional activity. We still cannot rule out the possibility that the PXR/RXRα-independent pathway involves in increasing CYP3A4 expression. These findings suggest that 3-dimensional culture in the RFB results in the formation of a transcriptional control complex with a greater capacity to induce transcription.

It is known that HNF-4α may be involved in the control of PXR expression and that HNF-4α knockout mice are completely deficient in PXR.[17] In our study, the level of HNF-4α mRNA expression was elevated only slightly (by about 20%) in RFB culture, but the mobility of HNF-4α and its DNA-binding site were markedly retarded following culture in the RFB. Also, retardation of the electrophoretic activity of HNF-4α in EMSA following 3-dimensional culture or treatment with rifampicin has not been reported previously. However, because both complexes were found to be competitive in the competition assay, it seems likely that HNF-4α formed a complex with other factors during 3-dimensional culture in the RFB. The formation of this complex probably led to enhancement of the transcription control capability. During 3-dimensional culture, rifampicin evidently enhanced the induction of CYP3A4, and this correlated well with PXR induction. Furthermore, during 3-dimensional culture with RFB, rifampicin treatment retarded mobility of the complex of PXR/RXRα and CYP3A4 ER6 and that of HNF-4α and their respective binding sites. Interestingly, subsequent analysis revealed that prolonged EMSA resulted in the formation of a complex having a much larger molecular weight (Figs. 7 and 8). Although the relationship between the larger complex formation including

PXR or HNF-4α and the induction of CYP3A4 is still not clear, this suggests that during 3-dimensional culture in the RFB, rifampicin, which serves as a PXR ligand, induces the formation of a complex that allows more efficient transcription, leading to a more than 100-fold increase in CYP3A4 induction. From these results, we may say that 3-dimensional culture in the RFB enhances the transcriptional control capability of HNF-4α, and, with the induction of transcription of PXR in the downstream region, the expression of CYP3A4 eventually increases. Our results indicated that the induction of CYP3A4 in FLC-5 cells incubated in the RFB is akin to that seen in normal human livers.

In the experiment conducted to study actual drug metabolism in the bioreactor, induction of CYP3A by rifampicin resulted in degradation of testosterone to 6β-hydroxy testosterone. It was shown at the cell level that induction of CYP3A was associated with enhancement of the drug-metabolizing activity. As shown in Fig. 1, this system can therefore be utilized for the screening of unknown drugs that can induce CYP3A, *i.e.*, for examining drug interactions mediated by CYP3A. If induction of CYP3A4 expression by various drugs were studied using the RFB system and FLC-5 cells, it would become possible to establish a simulation of drug metabolism in a setting closely resembling that prevailing in the human body. Drug metabolism in the liver is determined not only by CYP expression but also by many other factors, such as the affinity of the drugs for binding proteins and membranous components. Drugs that can be nonselectively taken up by the liver can affect drug metabolism mediated by CYP, and they are usually deemed as being dangerous for clinical use. The bioartificial liver is therefore useful for conducting studies of drug metabolism in the liver.

In the present study, we confirmed that the expression of CYP3A4, specific to human liver, is enhanced in an HCC cell line derived from human liver, at the mRNA level, protein level, and at the level of functional activity in cultured cells by incubation in the RFB. PXR/RXRα regulates the inductivity of CYP3A4 in human HCC cells under 3-dimensional perfusion culture. The bioartificial liver composed of the human functional HCC cell line was useful in studying drug interactions during induction of human CYP3A4.

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PHARMACOGENETICS AND GENOMICS

Population differences in *S*-warfarin metabolism between *CYP2C9* genotype–matched Caucasian and Japanese patients

Objective: Our objective was to investigate population differences in the metabolic activity of cytochrome P450 (CYP) 2C9 between genotypically matched Caucasian and Japanese patients by using the unbound oral clearance of *S*-warfarin as an in vivo phenotypic trait measure.

Methods: Ninety Japanese and 47 Caucasian patients receiving maintenance warfarin therapy were studied. Steady-state plasma unbound concentrations of *S*-warfarin were measured by a chiral HPLC method coupled with an ultrafiltration technique, and unbound oral clearance for *S*-warfarin was estimated. By combining plasma unbound concentrations of *S*-warfarin with the urinary excretion rates of *S*-7-hydroxywarfarin, the formation clearance of *S*-7-hydroxywarfarin was also determined. Genotyping of CYP2C9 was performed for 6 distinct alleles (*CYP2C9*1*, *CYP2C9*2*, *CYP2C9*3*, *CYP2C9*4*, *CYP2C9*5*, and a *T/C* transition in intron 2).

Results: The frequency distribution of unbound oral clearance for *S*-warfarin obtained from Japanese patients was shifted toward higher values as compared with that in Caucasian patients. Japanese patients had lower allelic frequencies for the 5 variants than Caucasian patients. When interpopulation comparisons of CYP2C9 activity were made for genotype-matched subjects, Japanese patients with the homozygous *CYP2C9*1* (wild-type) genotype (n = 85) had significantly ($P < .01$) greater median values for unbound oral clearance and formation clearance than Caucasian patients with the corresponding genotype (n = 26), 10.4 mL × min^{-1} × kg^{-1} versus 4.25 mL × min^{-1} × kg^{-1} and 0.015 mL × min^{-1} × kg^{-1} versus 0.010 mL × min^{-1} × kg^{-1}, respectively. In addition, Japanese patients heterozygous for the *CYP2C9*3* genotype (n = 4) showed a significantly ($P < .05$) reduced unbound oral clearance for *S*-warfarin, by 63%, as compared with Japanese patients possessing the homozygous *CYP2C9*1* genotype. By contrast, in Caucasian patients, no significant differences were observed in this parameter between *CYP2C9*1* homozygous subjects and those with heterozygous *CYP2C9*2* or *CYP2C9*3* genotypes.

Conclusions: These findings indicate that population differences in the frequencies of known variant *CYP2C9* alleles account only in part for the variability observed in in vivo CYP2C9 activity in different populations. In addition, a gene-dose effect of defective *CYP2C9* alleles on the in vivo CYP2C9 activity is evident in Japanese patients but not in Caucasian patients. Further studies are required to identify currently unknown factor(s) (eg, transcriptional regulation) responsible for the large intrapopulation and interpopulation variability in CYP2C9 activity. (Clin Pharmacol Ther 2003;73:253-63.)

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Interindividual differences in the disposition of drugs are well recognized and considered an important factor in bridging therapeutic doses and safety profiles of drugs between populations, especially where ethnic background may be different. For example, studies have demonstrated that there are substantial population differences in the activities of several cytochrome P450 (CYP) isoforms (eg, CYP2D6,[1] CYP2C19,[2] and CYP2E1[3]), phase II enzymes (eg, N-acetyltransferase[4] and glucuronosyltransferase[5]), and drug transporters (eg, *MDR1*[6]) between populations of different origin (ie, Caucasian, African American, and Southeast Asian). Recent advances in molecular biology have revealed single-nucleotide polymorphisms (SNPs) in the genes encoding these proteins and have demonstrated that there are pronounced differences in the frequencies of the functionally defective alleles among different populations.[7] These findings may explain, at least in part, differences in the therapeutic doses and frequency of adverse reactions in different patient groups for drugs whose metabolism predominantly involves such a polymorphic enzyme. In addition, it has become evident that the distributions of CYP2D6 and CYP2C19 activities determined in Caucasian patients, phenotyped as extensive metabolizers of the respective CYP isoforms, are significantly shifted relative to the corresponding values measured in Southeast Asian extensive metabolizers so that the population means are different.[1,2]

CYP2C9 accounts for approximately 20% of total CYP content constitutively expressed in human liver.[8] This CYP isoform is importantly involved in the metabolism of many clinically relevant drugs with narrow therapeutic ranges (eg, warfarin, acenocoumarol, tolbutamide, glipizide, and phenytoin).[9] Although the actual mechanism(s) remains unclear, therapeutic maintenance doses of warfarin for Caucasian patients appear to be twice as large as those for Japanese patients,[10,11] implying differences in either the pharmacokinetics or the pharmacodynamics of this CYP2C9 substrate. At present, 6 distinct alleles in the open reading frame of CYP2C9 (ie, *CYP2C9*1* [wild type], *CYP2C9*2*,[12] *CYP2C9*3*,[13,14] *CYP2C9*4*,[15] *CYP2C9*5*,[16] and CYP2C9*6[17]), a *T/C* transition in intron 2,[18] and 7 SNPs in the 5′-noncoding region[19] have been reported. The allelic frequencies of the common variants *CYP2C9*2* and *CYP2C9*3* have been shown to be markedly different across populations; for example, Caucasian patients possess higher frequencies of *CYP2C9*2* and *CYP2C9*3* alleles than Southeast Asian patients.[9,20] On the other hand, *CYP2C9*4*[15] and *CYP2C9*5*[16] appear to be rare. Previous studies[12-17] have demonstrated that these variant alleles are associated with reduced catalytic activity of CYP2C9 in in vitro expression systems and that subjects genotyped as *CYP2C9*2/*3* or *CYP2C9*3/*3* have substantially reduced metabolic activities for representative CYP2C9 substrates (ie, tolbutamide,[13] *S*-warfarin,[10,11,21] acenocoumarol,[22] and phenytoin[23]), thereby requiring reduced maintenance doses for these drugs.[10,11,21-29] Collectively, the extant data suggest that differences in the genetic variability of CYP2C9 in the open reading frame may explain, in part, the variability in the in vivo metabolic activity of CYP2C9 in different populations. On the other hand, increasing evidence indicates that 5′-promoter region SNPs may be an additional contributory factor so that a phenotype may reflect several different genetic mechanisms. In this case, enzyme activity measured in vivo would differ, for example, between subjects matched for genotype based only on coding region SNPs. Accordingly, in this study we tested the hypothesis that differences in in vivo CYP2C9 activity between populations of different racial origin may not be explained solely by known differences in the frequencies of open reading frame variant alleles, with *S*-warfarin used as a specific probe for in vivo CYP2C9 activity.

METHODS

Formulations of warfarin

The warfarin formulations used were Coumadin (Dupont Pharmaceuticals, Wilmington, Del) and Warfarin (Eisai Co, Ltd, Tokyo, Japan) for Caucasian and Japanese patients, respectively. The rates and extent of dissolution for each formulation (1-mg tablet) were measured under 2 separate conditions (1000 mL of diluted hydrochloric acid solution [pH 1.2] and 0.2-mol/L phosphate buffer [pH 6.8] at 37°C) by the Japanese Pharmacopeia (JP) XIV paddle method.[30] The dissolved warfarin enantiomers in each solution were analyzed at 1, 2, 5, 10, 20, and 30 minutes by a chiral HPLC-ultraviolet method,[31] and the time required to reach 50% dissolution and the amounts dissolved at 30 minutes were compared between the 2 formulations.

Patients

The demographic data of patients who participated in this study and their daily warfarin doses are summarized in Table I. Ninety Japanese patients (50 men and 40 women; mean age, 61 years [range, 26-75 years]; mean weight, 56.3 kg [range, 36.5-82.0 kg]) and 47 Caucasian patients (19 men and 28 women; mean age, 63 years [range, 33-86 years]; mean weight, 80.2 kg [range, 40.0-168.0 kg]) were recruited from patients at

Table I. Demographic data of Japanese and Caucasian patients and warfarin regimens

Characteristic	*Japanese patients (n = 90)*	*Caucasian patients (n = 47)*
Gender (No.)		
Men	50	19
Women	40	28
Age (y, mean ± SD and range)	60.5 ± 9.2 (26-75)	62.9 ± 14.2 (33-86)
Body weight (kg, mean ± SD and range)	56.3 ± 10.9 (36.5-82)	80.2 ± 22.2 (40-168)*
Dose of racemic warfarin (mg/d, mean ± SD)	3.4 ± 1.7	4.9 ± 3.0*
Dose normalized to body weight ($mg \cdot d^{-1} \cdot kg^{-1}$, mean ± SD)	0.060 ± 0.027	0.062 ± 0.039

*$P < .01$ between Japanese and Caucasian groups.

the International Medical Center of Japan, Tokyo, Japan; Hadassah University Hospital, Jerusalem, Israel; and Vanderbilt University Hospital, Nashville, Tenn. Clinical indications for anticoagulation therapy with warfarin were prevention or treatment of thromboembolic disease for various clinical conditions (eg, prosthetic valve replacements, atrial fibrillation, and deep venous thrombosis with or without pulmonary embolism). Concurrent medications with the potential to affect *S*-warfarin metabolism were amiodarone (n = 4), nonsteroidal anti-inflammatory drugs (n = 3), cimetidine (n = 2), thyroid hormone (n = 6), and carbamazepine (n = 1). In each patient the once-daily oral dose of racemic warfarin had been clinically adjusted based on anticoagulation response, as measured by the international normalized ratio (INR). When blood samples for this study were obtained from the patients, all had received a constant maintenance dose of racemic warfarin for at least 1 month before collection. Biochemical and hematologic tests performed before the study revealed no evidence of hepatic impairment but indicated renal impairment in 3 patients (ie, creatinine clearance, 12.4-22.7 mL/min) and congestive heart failure in 21 patients. Informed consent was obtained from each patient after the purpose of the study was thoroughly explained before the study began. The study protocol was approved by institutional review boards at the respective medical institutions.

Study protocol

Blood and urine samples were obtained from most patients during a routine outpatient visit. Urine was collected over a period of about 2 hours after complete voiding, and its volume was measured. In addition, an aliquot of 24-hour urine samples was obtained from 25 hospitalized patients. Blood samples (5-10 mL) were obtained at approximately the midpoint of the urine collection period, corresponding to approximately 15 hours after oral administration of the last dose of warfarin. Blood was centrifuged, separated plasma and urine samples were stored at −70°C until analyzed, and the buffy coat of the blood was stored at 4°C until extraction of deoxyribonucleic acid (DNA) was undertaken.

Plasma concentrations and protein binding of S-warfarin and urine concentrations of S-7-hydroxywarfarin

Analytic methods were used as previously described for determining plasma concentrations of *S*-warfarin by a chiral HPLC-ultraviolet method, urinary concentrations of *S*-7-hydroxywarfarin by a chiral HPLC-fluorescence method, and plasma unbound fractions of *S*-warfarin by an ultrafiltration technique.[31]

Pharmacokinetic analysis for S-warfarin

Previous studies have demonstrated that the *S*-enantiomer of warfarin is 3 to 5 times more potent than its optical congener in anticoagulant effect,[32] thereby accounting for most of the anticoagulant responses elicited by the administration of racemic warfarin. Thus differences in the disposition of warfarin with respect to that of pharmacologically more active *S*-warfarin were investigated in this study. Because the plasma elimination half-life of *S*-warfarin is, on average, approximately 25 hours,[32-35] the plasma concentration and plasma unbound concentration obtained from the patients with the described protocol were considered to approximate the average steady-state concentration of *S*-warfarin, as verified in our previous studies.[10,11] The oral clearance (CL_{oral}) and unbound oral clearance ($CL_{oral,u}$) for *S*-warfarin and the formation clearance for *S*-7-hydroxywarfarin (CL_m) were calculated according to the following equations:

$$CL_{oral}(S) = (D/2\tau)/Cp_{ss}(S) \quad (1)$$

$$CL_{oral,u}(S) = (D/2\tau)/Cu_{ss}(S) \quad (2)$$

$$CL_m = (dAe/dt)/Cp_{ss}(S) \quad (3)$$

Table II. Pharmacokinetic parameters for *S*-warfarin and anticoagulation response in Japanese and Caucasian patients

Parameter	*Japanese patients (n = 90)*	*Caucasian patients (n = 47)*
Plasma unbound fraction (%)	0.90 ± 0.18	0.91 ± 0.31
Plasma unbound concentration (ng/mL)	2.05 ± 0.92	4.75 ± 2.49*
$CL_{oral,u}$ (mL/min)	617 ± 297	412 ± 271*
Body weight–normalized $CL_{oral,u}$ ($mL \cdot min^{-1} \cdot kg^{-1}$)	11.2 ± 5.43	5.13 ± 3.10*
CL_m to *S*-7-hydroxywarfarin (mL/min)	1.67 ± 2.45	0.84 ± 0.71*
Body weight-normalized CL_m ($mL \cdot min^{-1} \cdot kg^{-1}$)	0.030 ± 0.040	0.010 ± 0.009*
INR	1.58 ± 0.43 (0.96-2.85)	2.82 ± 1.21 (1.00-6.60)*

Data are mean ± SD (range).
$CL_{oral,u}$, Unbound plasma clearance; CL_m, formation clearance; INR, international normalized ratio of prothrombin time.
*$P < .01$, Japanese versus Caucasian patients.

in which D is the daily dose of racemic warfarin (thus D/2 is the daily dose of *S*-warfarin); τ is the dosing interval (ie, 24 hours); $Cp_{ss}(S)$ and $Cu_{ss}(S)$ are the average total (bound plus unbound) and unbound concentrations of *S*-warfarin at steady state, respectively; and dAe/dt is the urinary excretion rate of *S*-7-hydroxywarfarin expressed as warfarin equivalent during the urine collection period. $Cu_{ss}(S)$ was calculated as the product of $Cp_{ss}(S)$ and plasma unbound fraction.[10]

CYP2C9 genotyping

DNA was extracted from the buffy coat of blood with the sodium iodide method through use of a commercially available kit (Wako, Tokyo, Japan). Genotyping was performed for *CYP2C9*1* (wild type), *CYP2C9*2*, *CYP2C9*3*, *CYP2C9*4*, and a *T/C* transition in intron 2 by amplifying genomic DNA by a polymerase chain reaction method, followed by restriction fragment length polymorphism methods.[10,11,15,18] The *CYP2C9*5* allele was determined according to the method of Dickmann et al[16] with use of a single-strand conformational polymorphism of the polymerase chain reaction products.

Statistical analysis

Parametric comparisons between the mean values for the pharmacokinetic and clinical data obtained from Caucasian and Japanese patients were performed by unpaired *t* tests. Comparisons of age, body weight-normalized $CL_{oral,u}$ for *S*-warfarin, and CL_m between the 2 groups were made by the Wilcoxon rank sum test, because the distributions of these parameters appeared skewed by visual inspection. Because the distributions of warfarin dose, $CL_{oral,u}$, and CL_m obtained from the participants with different CYP2C9 genotypes (ie, *CYP2C9*1/*1*, **1/*3*, **1/*2*, and **2/*2*) appeared not to be normally distributed, a nonparametric test for multiple comparisons (Steel-Dwass test) was used for the statistical analysis of these parameters.

The distributions of the body weight–normalized $CL_{oral,u}$ for *S*-warfarin obtained from Japanese and Caucasian patients were compared graphically by histograms and log-normal probit plots. Allelic frequencies of CYP2C9 variants were compared by unpaired *z* tests. Data are presented as mean ± SD or median and range where appropriate. A *P* value of less than .05 was considered statistically significant for all analyses.

RESULTS

The warfarin tablets taken by Caucasian patients (Coumadin) and those taken by Japanese patients (Warfarin) exhibited similar in vitro dissolution curves under both studied conditions (ie, pH 1.2 and 6.8) (data not shown). The mean times required to reach 50% dissolution for *S*-warfarin were 7.9 ± 2.3 minutes and 4.6 ± 1.3 minutes ($P = .10$) at pH 6.8 for Coumadin and warfarin, respectively, and no significant differences were observed between the two formulations in the amount of *S*-warfarin dissolved within 30 minutes (90.7% ± 3.7% versus 84.9% ± 1.5% at pH 1.2 and 91.3% ± 2.8% versus 87.7% ± 3.3% at pH 6.8). These data indicate that both formulations essentially had similar dissolution characteristics.

The Caucasian and Japanese patients did not differ with regard to age (Table I) or its distribution: median, 64 years versus 62 years, respectively, with the 10th and 90th percentile values being 39.8 and 79.6 years versus 49.7 and 71.3 years, respectively. However, the body weight for the Japanese patients was approximately 30% lower ($P < .01$) than that of the Caucasian patients (Table I). Furthermore, the mean oral dose of racemic warfarin in Japanese patients was 30% smaller ($P < .01$) than that administered to Caucasian patients

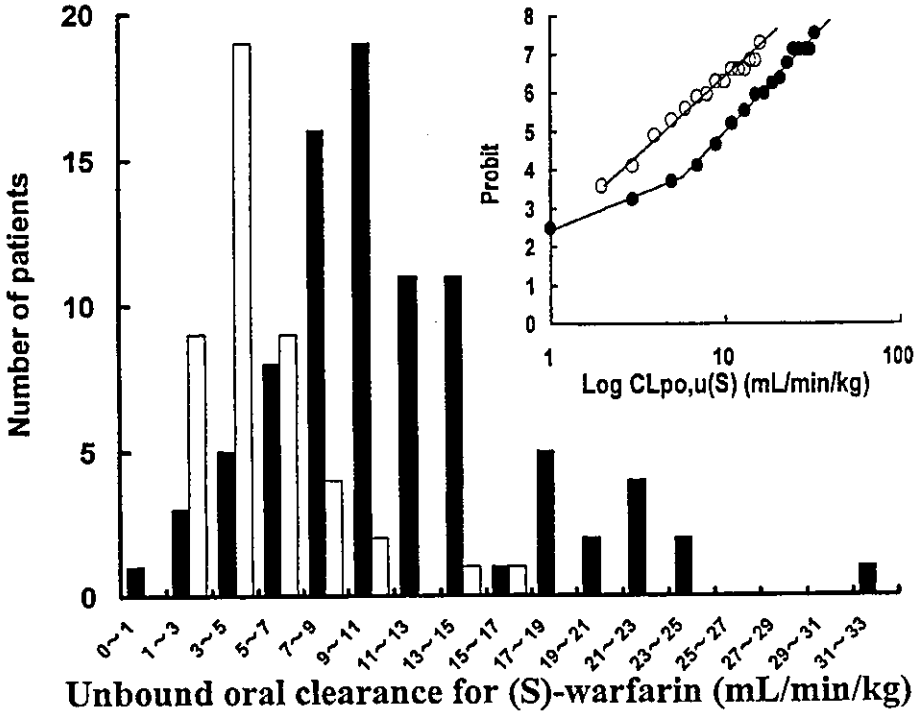

Fig 1. Distribution of body weight–normalized unbound oral clearances for *S*-warfarin [CLpo,u(S)] among 90 Japanese (*filled columns* and *circles*) and 47 Caucasian (*open columns* and *circles*) patients. Data are presented as histograms and log-normal probit plots *(inset). Lines in inset* represent regression lines for data obtained from each population. Note that distribution of Japanese patients appears shifted toward higher values as compared with Caucasian patients. In addition, note that log-normal probit plots for Japanese patients appear to have an inflection around body weight–normalized unbound oral clearance ($CL_{oral,u}$) of 4.0 mL × min^{-1} × kg^{-1}.

(3.4 ± 1.7 mg/d versus 4.9 ± 3.0 mg/d, respectively). Accordingly, the body weight–normalized warfarin daily dose was similar in both populations (0.060 ± 0.027 mg × d^{-1} × kg^{-1} versus 0.062 ± 0.039 mg × d^{-1} × kg^{-1}).

The mean total (bound plus unbound) plasma clearance for *S*-warfarin obtained from the Caucasian patients studied herein (0.042 ± 0.023 mL × min^{-1} × kg^{-1}) was comparable with those values reported in previous studies (0.052-0.075 mL × min^{-1} × kg^{-1}).[33-35] No significant differences were found in the plasma binding of *S*-warfarin between Japanese and Caucasian patients (0.90% ± 0.18% unbound versus 0.91% ± 0.31% unbound, respectively). However, in the Caucasian patients, *S*-warfarin's observed plasma unbound concentration was 2.3 times higher ($P < .01$) than that in the Japanese patients. As a result, the mean $CL_{oral,u}$ estimated in Japanese patients was 1.5-fold higher than that in Caucasian patients ($P < .01$), and this difference was even greater (2.2-fold) after this parameter was normalized on the basis of body weight (Table II). Differences in the mean CL_m by 7-hydroxylation of *S*-warfarin (2-fold) and its body weight–normalized value (3-fold) between the Japanese and Caucasian patients were also present ($P < .01$). Because low-intensity warfarin therapy (INR, 1.5-2.5) was reported to be associated with lower frequencies of bleeding complications than the conventional warfarin therapy (INR, 2.0-3.0) in Japanese patients,[36] the mean INR value obtained from the Japanese patients was significantly lower than that obtained from the Caucasian patients (1.6 ± 0.4 versus 2.8 ± 1.2) ($P < .01$). This finding may be associated with the fact that the Caucasian patients had significantly greater plasma unbound concentration values of *S*-warfarin, as described ($P < .01$).

The distribution histograms of body weight–normalized $CL_{oral,u}$ for *S*-warfarin obtained from the 2

Table III. Allelic frequencies of wild-type and 5 distinct CYP2C9 variants in Japanese and Caucasian patients

	Japanese patients (n = 90)	*Caucasian patients (n = 47)*
*CYP2C9*1* (wild type)	0.967	0.723†
*CYP2C9*2* (exon 3)	0	0.223†
*CYP2C9*3* (exon 7)	0.033	0.053
*CYP2C9*4* (exon 7)	0	0
*CYP2C9*5* (exon 7)	0	0
T/C transition (intron 2)	0.006	0.175†

According to the recommendation of the Human Cytochrome P450 Allele Nomenclature Committee,[14] we refer *CYP2C9*1*, *CYP2C9*2*, *CYP2C9*3*, *CYP2C9*4*, and *CYP2C9*5* to the Arg144/Ile359, Cys144/Ile359, Arg144/Leu359, Arg144/Thr359, and Asp/Glu360 alleles, respectively. The *T/C* transition allele is located 73 base pairs downstream from exon 2.

†$P < .01$, Japanese versus Caucasian patients.

study populations appeared unimodal, but the mean of the distribution among the Caucasian patients was shifted toward lower values, as compared with the Japanese patients (Fig 1), which is consistent with the finding that the group measure of in vivo CYP2C9 activity in Caucasian patients is lower than that in Japanese patients (Table II). Probit plots not only confirmed such a population difference but also indicated that the distribution of log-normal transformed values of body weight–normalized $CL_{oral,u}$ among the Japanese patients demonstrated an inflection around 4.0 mL × min^{-1} × kg^{-1}. These data suggested that in the group of Japanese patients studied a small number of patients having reduced $CL_{oral,u}$ or CYP2C9 activity may have been present.

Next, CYP2C9 genotyping of both populations was performed to determine whether the common genetic polymorphisms of CYP2C9 may account for the observed interpopulation or intrapopulation (or both) variability of $CL_{oral,u}$ for *S*-warfarin between the 2 study groups. As a result, there were significant population differences in the allelic frequency for *CYP2C9*1*, *CYP2C9*2*, and a *T/C* transition in intron 2 (Table III). The observed allelic frequencies of *CYP2C9*2* and *CYP2C9*3* were comparable with those reported by us and others in healthy subjects of each population.[10-13] Because the Caucasian patients had greater allelic frequencies for these CYP2C9 variants than the Japanese patients, the former had a much lower allelic frequency of *CYP2C9*1* than the latter (0.723 versus 0.967, $P < .01$). None of the subjects in either population carried the *CYP2C9*4* or *CYP2C9*5* allele.

Table IV shows body weight–normalized doses of warfarin and pharmacokinetic parameters of *S*-warfarin in the Caucasian and Japanese patients according to genotype. The median maintenance doses of racemic warfarin in Japanese patients who were heterozygotes and homozygotes for the *CYP2C9*3* allele (ie, 0.031 and 0.007 mg × d^{-1} × kg^{-1}, respectively) were 48% ($P < .01$) and 88% smaller than the dose in *CYP2C9*1* homozygotes (ie, 0.060 mg × d^{-1} × kg^{-1}) in the same population. By contrast, no significant differences were observed for the median maintenance doses of warfarin among Caucasian patients with *CYP2C9*1/*1*, *CYP2C9*1/*3*, and *CYP2C9*1/*2* genotypes (ie, 0.06, 0.05, and 0.04 mg × d^{-1} × kg^{-1}, respectively). A Caucasian patient who was a combined heterozygote of *CYP2C9*2* and *CYP2C9*3* (ie, *CYP2C9*2/*3*) received a maintenance warfarin dose of approximately 77% less than that in Caucasian patients who were genotyped as homozygotes of *CYP2C9*1* (wild type).

The median $CL_{oral,u}$ values for *S*-warfarin obtained from the homozygotes and heterozygotes of *CYP2C9*3* (ie, 1.1 and 3.9 mL × min^{-1} × kg^{-1}, respectively) were reduced by 90% and 63% ($P < .01$), respectively, as compared with that obtained in homozygotes of *CYP2C9*1* (ie, 10.4 mL × min^{-1} × kg^{-1}) in the Japanese patients (Fig 2, *A*; Table IV). In contrast, multiple comparisons for the median $CL_{oral,u}$ for *S*-warfarin in Caucasian patients possessing different CYP2C9 genotypes (ie, 4.25, 4.27, 4.26, and 6.84 mL × min^{-1} × kg^{-1} for *CYP2C9*1/*1*, *CYP2C9*1/*3*, *CYP2C9*1/*2*, and *CYP2C9*2/*2*, respectively) showed no significant differences (Fig 2, *B*; Table IV). Although there was only one Caucasian patient with the *CYP2C9*2/*3* genotype, the $CL_{oral,u}$ for *S*-warfarin in this patient appeared to be approximately 70% lower than the median value obtained from the Caucasian patients with the *CYP2C9*1/*1* (wild-type) genotype.

When CL_m values for *S*-warfarin 7-hydroxylation estimated in Japanese and Caucasian patients having different CYP2C9 genotypes were compared, the median CL_m obtained from the Japanese patients having a *CYP2C9*1/*3* genotype (ie, 0.003 mL × min^{-1} × kg^{-1}) was reduced by approximately 80% ($P < .01$), as compared with that obtained from those with the *CYP2C9*1/*1* genotype (Table IV). Again, no significant differences were observed for the median CL_m among the Caucasian patients with *CYP2C9*1/*1*, *CYP2C9*1/*3*, *CYP2C9*1/*2*, and *CYP2C9*2/*2* genotypes. However, CL_m obtained from the Caucasian patient with the *CYP2C9*2/*3* genotype was less than 10% of the corresponding value obtained from those with the *CYP2C9*1/*1* genotype.

A comparison of the differences between genotype-matched Japanese and Caucasian patients showed that

Table IV. Body weight-normalized doses of warfarin, unbound oral clearance of *S*-warfarin, and formation clearance for *S*-warfarin 7-hydroxylation in Japanese and Caucasian patients with different *CYP2C9* genotypes

		Dose (mg/kg)		$CL_{oral,u}$ ($mL \cdot min^{-1} \cdot kg^{-1}$)		CL_m ($mL \cdot min^{-1} \cdot kg^{-1}$)	
Genotype	*No. of patients*	*Median*	*Range*	*Median*	*Range*	*Median*	*Range*
Japanese							
**1/*1*	85 (94.4%)	0.060	0.016-0.167	10.4	4.47-32.3	0.015	0.002-0.239
**1/*3*	4 (4.4%)	0.031	0.018-0.038	3.91†	2.97-4.41	0.003†	0.001-0.014
**3/*3*	1 (1.1%)	0.007	—	1.12	—	ND	ND
**1/*2*	0 (0%)	—	—	—	—	—	—
**2/*2*	0 (0%)	—	—	—	—	—	—
**2/*3*	0 (0%)	—	—	—	—	—	—
Caucasian							
**1/*1*‡	26 (55.3%)	0.060	0.019-0.231	4.25†	1.94-13.7	0.010†	0.001-0.034
**1/*3*‡	4 (8.5%)	0.051	0.037-0.069	4.27	3.70-10.9	0.010	0.001-0.012
**3/*3*	0 (0%)	—	—	—	—	—	—
**1/*2*‡	12 (25.5%)	0.044	0.027-0.123	4.26	2.10-10.4	0.011	0.001-0.037
**2/*2*‡	4 (8.5%)	0.091	0.029-0.095	6.84	1.61-16.0	0.004	0.003-0.005
**2/*3*	1 (2.1%)	0.014	—	1.23	—	0.001	—

ND, Not detected because of lack of urine samples.
†$P < .01$, as compared with corresponding values obtained from Japanese patients genotyped as homozygous *CYP2C9*1* (**1/*1*).
‡No significant differences were observed in any pairs of median values for warfarin doses, $CL_{oral,u}$, and CL_m by multiple comparisons between Caucasian patients with different CYP2C9 genotypes (ie, **1/*1*, **1/*3*, **1/*2*, and **2/*2*).

the median values for $CL_{oral,u}$ and CL_m in *CYP2C9*1/*1* homozygotes were significantly ($P < .01$) greater (10.4 and 0.015 mL × min^{-1} × kg^{-1}, respectively) in Japanese compared with Caucasian patients with the corresponding genotype (4.25 and 0.010 mL × min^{-1} × kg^{-1}, respectively) (Fig 3, *A*; Table IV). In contrast, the Japanese *CYP2C9*1/*3* heterozygous patients had $CL_{oral,u}$ values for *S*-warfarin and CL_m (3.91 and 0.003 mL × min^{-1} × kg^{-1}, respectively) that were essentially comparable to those in Caucasian patients with the same genotype (4.27 and 0.010 mL × min^{-1} × kg^{-1}, respectively) (Fig 3, *B*; Table IV). No significant age differences were observed between Japanese and Caucasian patients carrying the *CYP2C9*1/*1* and *CYP2C9*1/*3* genotypes, respectively (60.6 ± 9.1 years versus 65.2 ± 14.1 years for patients with the *CYP2C9*1/*1* genotype and 62.5 ± 10.8 years versus 57.0 ± 10.7 years for patients with the *CYP2C9*1/*3* genotype).

DISCUSSION

International clinical studies performed in different countries present challenges that have a potential to generate confounding factors. Several efforts were made to minimize these factors. For example, warfarin formulations used in Japan and the United States differed from each other; however, on the basis of the data obtained from the dissolution tests, the formulations used in this study appeared to be equivalent. In addition, the plasma clearance of warfarin decreases with age[21]; thus Japanese and Caucasian patients of similar ages were selected for this study, and the distribution of age among the 2 populations was comparable. To recruit a large number of subjects, the study was mostly performed in an outpatient setting, and the plasma concentration of *S*-warfarin obtained 10 to 20 hours after the last dose under steady-state conditions was used as a measure of the true average plasma concentration during the dosage interval. Previously, we have shown that the plasma *S*-warfarin concentrations measured under such a protocol differ from the theoretic average plasma drug concentrations by only 4% to −22%[10] and, therefore, did not consider that this approximation affects the study's findings.

Few studies have investigated possible population differences in the in vivo disposition of drugs predominantly metabolized by CYP2C9. Phenytoin is eliminated by an extensive hepatic metabolism in which CYP2C9 is mainly involved (ie, 80%-90%).[9] Grasela et al[37] and Chan et al[38] reported that Asian patients have lower mean Michaelis-Menten constant (K_m) values than Caucasian patients but have similar or even greater mean maximum rates of metabolism by an enzyme-mediated reaction (V_{max}) on the basis of the analysis of the steady-state plasma concentrations of phenytoin. Because V_{max}/K_m can be considered as an index of the intrinsic clearance for phenytoin, these data suggest that Asian patients may have a greater in vivo CYP2C9

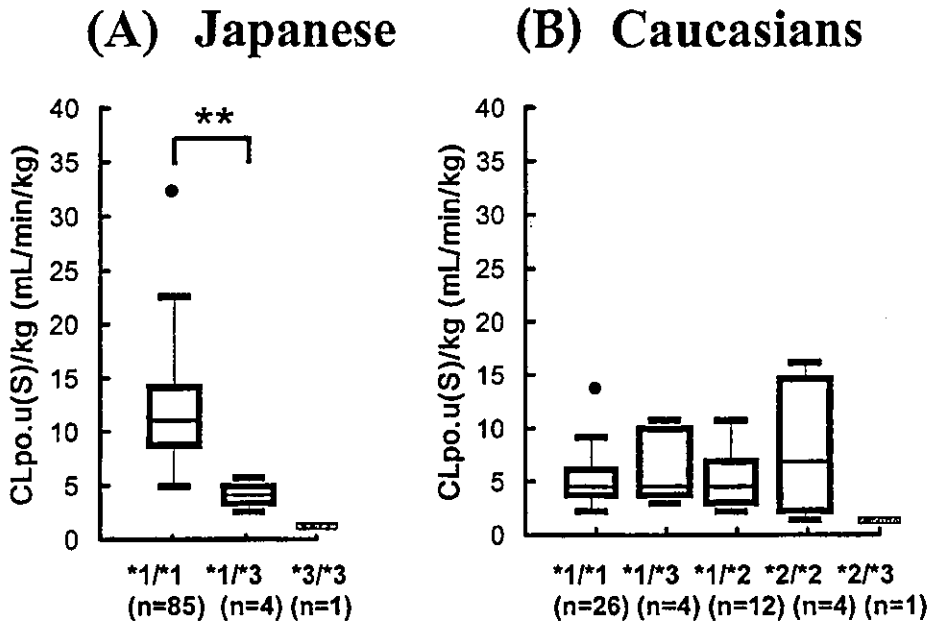

Fig 2. Box-and-whisker plots comparing body weight–normalized unbound oral clearance [CLpo.u(S)] for *S*-warfarin in Japanese (A) and Caucasian (B) patients with different CYP2C9 genotypes. *Subdivisions of boxes* and *top and bottom lines* on *boxes* represent median values and upper and lower quartiles, respectively. *Closed circles* are outlying values beyond maximum length in terms of interquartile range. Numbers of patients in each genotype group are shown in *parentheses*. *Two asterisks*, $P < .01$, for comparison between genotype groups of *CYP2C9*1/*1* and *CYP2C9*1/*3* in Japanese patients. No significant differences were observed between any pairs of genotype groups in Caucasian patients.

activity than Caucasian patients. In addition, Kromann et al[39] reported that in Greenland Eskimos—considered to be of Asian origin—the total (bound plus unbound) plasma clearance of phenytoin after a single oral dose was approximately double that in Danish Caucasian patients. The current findings are consistent with this notion in that, as a group, Japanese patients undergoing anticoagulation therapy with racemic warfarin have significantly greater body weight–normalized $CL_{oral,u}$ for *S*-warfarin and CL_m of *S*-warfarin 7-hydroxylation than Caucasian patients (Fig 1; Table II).

Although the number of patients recruited in this study to determine allelic frequencies was small (<100) for Japanese and Caucasian groups, our results were consistent with previously reported findings[9,20] that there are substantial differences in the allelic frequencies of *CYP2C9*2* and *CYP2C9*3* between Asian and Caucasian patients: no *CYP2C9*2* allele has been detected in Asian populations thus far, and the allelic frequency of *CYP2C9*3* in Asian populations (1.7%-5%) tended to be less than that in Caucasian populations (5.3%-10%).[9,20] The *CYP2C9*2* and *CYP2C9*3* alleles were considered functionally impaired on the basis of in vitro studies.[13,40] This provides an explanation for why the Japanese patient genotyped as *CYP2C9*3/*3* and a *CYP2C9*2/*3* heterozygous Caucasian patient (ie, compound heterozygote) exhibited substantially lower $CL_{oral,u}$ for *S*-warfarin and CL_m for 7-hydroxylation of *S*-warfarin than *CYP2C9*1/*1* individuals in each population (Fig 2; Table IV). On the basis of these findings, it is reasonable to suggest that the reason why Japanese patients as a group have a greater in vivo CYP2C9 activity than Caucasian patients may be accounted for by the fact that Caucasian patients have higher allelic frequencies of *CYP2C9*2* and *CYP2C9*3* alleles than Japanese patients. However, comparisons of body weight–normalized $CL_{oral,u}$ and CL_m between genotypically matched (ie, *CYP2C9*1/*1*) Japanese and Caucasian patients indicated an unexpected difference in that $CL_{oral,u}$ and CL_m values were higher in Japanese patients than Caucasian patients (Fig 3). These data indicate that known genetic polymorphisms in CYP2C9 do not adequately account for the observed differences in CYP2C9 activity in

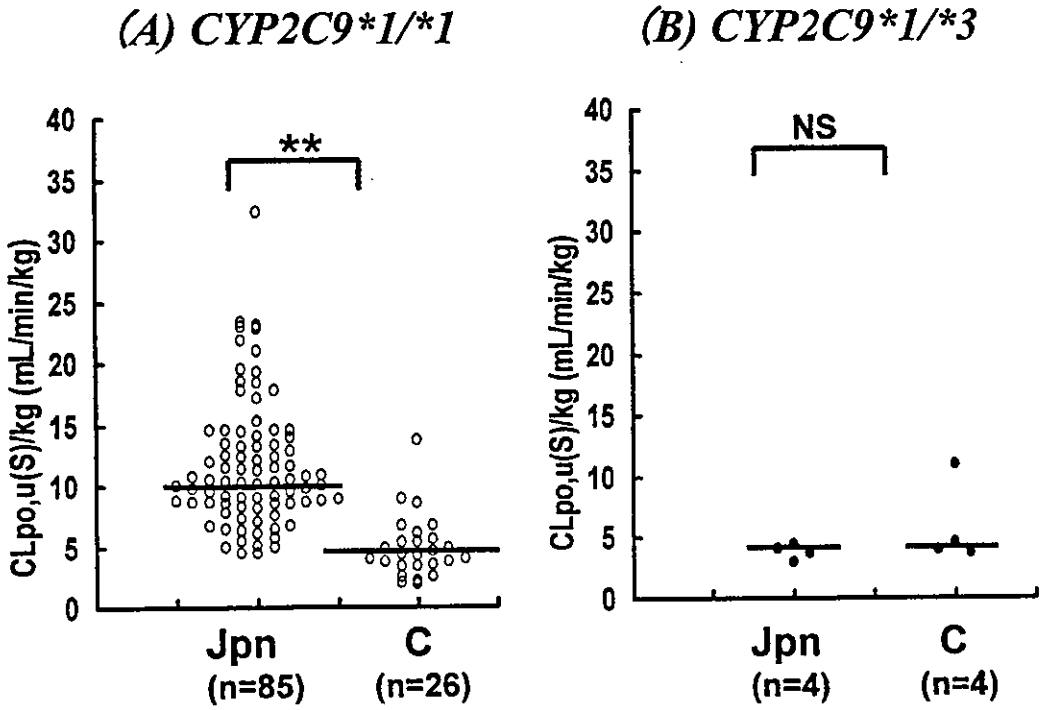

Fig 3. Interethnic comparisons of body weight–normalized unbound oral clearance [CLpo,u(S)] for *S*-warfarin in genotype-matched Japanese (Jpn) and Caucasian (C) patients. A, Data obtained from patients with homozygous *CYP2C9*1* genotype *(open circles)*. B, Data obtained from patients with heterozygous *CYP2C9*3* genotype *(filled circles)*. *Horizontal bars* represent median values. *Two asterisks*, $P < .01$, for comparison between the 2 ethnic groups. NS, Not significant.

vivo. It, therefore, appears that unidentified factors, such as environmental, dietary, and other factors (eg, concomitant drugs), other than the previously reported open reading frame SNPs of CYP2C9 are involved in the differences in the pharmacokinetic parameters of *S*-warfarin and possibly in vivo CYP2C9 activity between Japanese and Caucasian patients. One possibility is that additional unidentified defective alleles exist in the open reading frame of *CYP2C9* or, alternatively, that unknown genetic polymorphism(s) in the 5′-flanking region of the *CYP2C9* gene may be involved in its transcriptional regulation. Although Leung et al[41] reported 4 new SNPs in exon 4 in Chinese patients, these data may be flawed by inappropriate primer design based on the comparisons of their primers with those previously reported for amplifying comparable portions of exon 4.[13] Recently, Shintani et al[19] reported 7 distinct SNPs in the 5′-flanking region of *CYP2C9* in Japanese patients, and we have discovered 4 additional SNPs in this region in Caucasian patients (unpublished data). Accordingly, we speculate that one of these SNPs in the transcriptional regulatory region or a combination of these may account for, at least to some extent, the interethnic differences in the in vivo CYP2C9 activity in Caucasian patients and Southeast Asian patients with the *CYP2C9*1/*1* genotype.

We previously reported that the *CYP2C9*3* allele was associated with a reduction in in vivo CYP2C9 activity assessed by $CL_{oral,u}$ for *S*-warfarin and CL_m for 7-hydroxylation of *S*-warfarin in a gene-dose manner.[10,11] $CL_{oral,u}$ and CL_m obtained from the Japanese patients with the *CYP2C9*1/*3* and *CYP2C9*3/*3* genotypes were 62% and 80% lower than those obtained from patients with the *CYP2C9*1/*1* genotype, respectively. Surprisingly, however, this study showed that the Caucasian patients with either the *CYP2C9*1/*2* or *CYP2C9*1/*3* genotype and those with the *CYP2C9*2/*2* genotype showed no discernible differences in the body weight–normalized $CL_{oral,u}$ for *S*-warfarin (Fig 2). The maintenance doses of racemic warfarin obtained from Caucasian patients with *CYP2C9*2* or *CYP2C9*3* alleles as heterozygote forms

were slightly smaller (15% and 27%, respectively) than the dose obtained from those with the *CYP2C9*1/*1* genotype, but differences were marginal, except for the *CYP2C9*2/*3* genotype (Fig 2; Table IV). Previous reports investigating the contribution of the genetic polymorphisms of CYP2C9 to the interindividual variability in the daily doses of racemic warfarin in Caucasian patients[21,24-27,42,43] found that patients carrying *CYP2C9*2* or *CYP2C9*3* alleles received significantly smaller doses of racemic warfarin (14%-30% and 21%-49%, respectively[21,24-27,42,43]) than those without the variant alleles. However, these investigations did not assess in vivo CYP2C9 activity as we did by more specific parameters such as $CL_{oral,u}$ for *S*-warfarin or CL_m for 7-hydroxylation of *S*-warfarin. Although several investigators[24,25,42] have indicated that patients carrying either the *CYP2C9*2* or *CYP2C9*3* allele had greater risks of bleeding complications during anticoagulation therapy with warfarin than those carrying none of these alleles, this finding has not been confirmed by others.[26,27] Collectively, further studies with a large number of Caucasian subjects will be required to determine the relative contribution of these CYP2C9 open reading frame variants to $CL_{oral,u}$ for *S*-warfarin taking all other genetic, clinical, and environmental variables including 5′-promoter region SNPs, age, sex, clinical indications, interacting drugs and foods, and the like into account.[21]

In conclusion, this study strongly indicates that there are substantial interpopulation differences in the in vivo CYP2C9 activity between Japanese and Caucasian patients and that the differences cannot be fully accounted for by previously known genetic polymorphisms in the open reading frame of CYP2C9. Accordingly, further studies are necessary to identify other unknown factor(s). Furthermore, possible interpopulation difference in the pharmacodynamics of warfarin between Caucasian and Southeast Asian patients may also be important and contribute, along with the demonstrated differences in the pharmacokinetics of *S*-warfarin, to the overall dose-response relationship between the two populations.

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Pharmacogenetics of *CYP2C9* and interindividual variability in anticoagulant response to warfarin

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INTRODUCTION

A large (>10-fold) interindividual variation in the dose requirement of warfarin to achieve optimal therapeutic anticoagulation responses has often posed difficulties for clinicians to individualize warfarin doses to their patients. In addition, therapeutic targets measured by international normalized ratio (INR) of prothrombin time appear to differ between populations: INR of 2–3 for most indications in Caucasian patients[1] and 1.5–2.5 for Asian patients.[2,3] In this context, there is a strong demand to clarify concealed factors associated with a substantial interindividual variability of anticoagulation responses to warfarin. It is widely recognized that pharmacokinetic and pharmacodynamic factors contribute to the intra- and interindividual variabilities in the dosage requirement of warfarin. Clinically available warfarin is a racemic mixture of (*R*)- and (*S*)-warfarin and the anticoagulation effect of (*S*)-warfarin is three to five times more potent than (*R*)-warfarin.[4] Therefore, the interindividual variability in the disposition of (*S*)-warfarin would be the most important pharmacokinetic parameter associated with variable anticoagulation responses to warfarin. (*S*)-warfarin is metabolized almost exclusively by CYP2C9 to its major metabolite, (*S*)-7-hydroxywarfarin in humans.[5] According to the physiological model,[6] the steady-state unbound plasma concentrations (C_u) of an orally administered drug being eliminated mainly by hepatic metabolism depend solely on the hepatic intrinsic clearance ($CL_{int,h}$) for the drug. This pharmacokinetic parameter represents the hepatic enzyme activity involved in the metabolism of the drug. Collectively, the hepatic CYP2C9 activity would be one of the most critical determinants of the interindividual variability in the anticoagulation response to warfarin.

Genetic polymorphisms of *CYP2C9* have been extensively studied in the connection with the alterations in enzyme activity. Among the 11 variant alleles of *CYP2C9* so far reported,[7] *CYP2C9*2* and *CYP2C9*3* would be most important in the light of their influence on *in vitro* and *in vivo* metabolic activities and allelic frequencies in different ethnic populations.[8–15] *CYP2C9*4*[16] has been detected so far in only one Japanese patient whose phenytoin clearance was shown to be substantially reduced as compared with those possessing *CYP2C9*1/*1*. The allelic frequency of this variant should be very low in Japanese and no information is available in Caucasian and African-American populations. *CYP2C9*5* was detected in five of 110 African-American[17] and three of 183 Tanzanians,[18] but none in Caucasian or Asians. Although no information is available for the *in vivo* enzyme activity of *CYP2C9*5* at present, recombinant proteins of *CYP2C9*5* and *CYP2C9*4* exhibited markedly reduced enzyme activities as compared with that of the wild-type *CYP2C9 in vitro*.[17,19] While the allelic frequency of *CYP2C9*6*,[20] a null allele of *CYP2C9*, has been shown to be 0.6% in African-American, none of the Caucasians or Asians have been shown to possess this variant allele. The finding that a homozygote of *CYP2C9*6* developed severe phenytoin toxicity by an oral phenytoin dosing of 300 mg/day indicates that this allele would be associated with a substantially reduced *in vivo* enzyme activity. Among the six other newly discovered *CYP2C9* variant alleles (ie, from *CYP2C9*7* to *CYP2C9*12*),[21] *CYP2C9*11* (R335W) showed markedly defective metabolic activity in *in vitro* systems. Nonetheless, there is a paucity of information regarding the *in vivo* activities of these variants. Recently, different groups of investigators have reported the relation between *CYP2C9* polymorphisms (ie, *CYP2C9*2* and *CYP2C9*3*) and the daily dose requirement of warfarin and risks of bleeding complications during anticoagulation therapy.[22–32] These studies support an idea that the genetic polymorphisms of *CYP2C9* would contribute to the interindividual variability of the anticoagulation responses to warfarin along with the previously known clinical and environmental variables (eg, age, concomitantly administered drugs, foods and clinical indications).[33,34]

Our knowledge about pharmacodynamic factors associated with the variability in the anticoagulation of warfarin is relatively limited. Hereditary resistance to warfarin and to other analogous compounds has been reported in humans[35] and in a few strains of rodents.[36] The mechanism(s) of warfarin resistance in humans and rodents has been considered to have orthologous genetic underpinnings in the same enzyme complex (presumably a vitamin K_1 2,3,-epoxide

reductase, VKOR) or related systems.[37] Recently, Wallin *et al*[38] demonstrated that VKOR partially purified from hereditary warfarin resistant rats showed no significant differences in the affinity constant (K_m), specific activity and sensitivity to warfarin as compared with that purified from normal rats. However, they found that the mRNA of calumenin was overexpressed in the liver of warfarin-resistant rats compared with normal rats.[38] Calumenin belongs to the CREC family of Ca^{2+}-binding proteins found in the various secretory pathways of mammalian cells and has chaperone functions in the endoplasmic reticulum. Wallin *et al*[38] suggested that calumenin may interfere with VKOR-supported γ-carboxylase activity. This study may open a new avenue to elucidate genetic mechanism(s) associated with warfarin resistance and interindividual variability of warfarin sensitivity in humans.

While genetic polymorphisms of vitamin K-dependent coagulation factors (F-II, VII, IX, X Protein C, Protein S and γ-glutamyl carboxylase) have also been studied intensively in the light of susceptibility to venous thrombosis,[39] only limited information is available regarding the relation between mutations in these coagulation proteins and the variability in the anticoagulation sensitivity to warfarin in humans. It has been speculated that the impact of genetic polymorphisms of vitamin K-dependent coagulation proteins on the interindividual variability of warfarin sensitivity may be less important than those of CYP2C9,[40] because the allelic frequencies of variants for coagulation proteins such as F-IX reported so far are much lower than those of CYP2C9.[41] In this context, we consider that the pharmacogenetics of *CYP2C9* would be a principal factor involved in the interindividual variability in the anticoagulation response to warfarin. Thus, our goals of the present article are to summarize the clinical data on the relation between the *CYP2C9* polymorphisms and the daily dose requirement of warfarin as well as the susceptibility to bleeding complications and to assess the impact of *CYP2C9* polymorphisms on the pharmacokinetics of (*S*)-warfarin in different populations. In addition, we wish to give a comprehensive pharmacokinetic explanation why the patients carrying genetic polymorphisms associated with reduced CYP2C9 activity would have less stable INR status than those with the wild-type genotype particularly during the initial phase of warfarin therapy.

EFFECTS OF *CYP2C9* GENOTYPES ON THE MAINTENANCE DOSE OF WARFARIN

Caucasians

Furuya *et al*[22] were the first to report that patients with *CYP2C9*2* allele required 20% lower maintenance dose of warfarin than those with the wild-type (*CYP2C9*1*) allele. Recently, Aithal *et al*[23] reported that patients requiring a lower dose of warfarin (ie, <1.5 mg/day) to maintain optimum anticoagulation had a higher incidence (81%) of having one or more *CYP2C9* variant alleles (ie, *CYP2C9*2* and *CYP2C9*3*) than those randomly selected from anticoagulation clinics (38%). Tables 1 and 2 summarize the data obtained from previous studies investigating the relation between the *CYP2C9* genotypes and the maintenance daily dose of warfarin associated with therapeutic anticoagulation. Available data[22–31] agree with the notion that the stable maintenance dose of warfarin would differ significantly among the patients possessing distinct *CYP2C9* genotypes and that the magnitude of impact by *CYP2C9*3* variant on the dose requirement of warfarin would be greater than that by *CYP2C9*2* variant.

The patients with the heterozygous *CYP2C9*2* (*CYP2C9*1/*2*) and *CYP2C9*3* (*CYP2C9*1/*3*) genotypes would require on average 21% (range, 14–27%) and 34% (range, 15–49%) lower daily maintenance dose of warfarin, respectively, as compared with those with the homozygous wild-type genotype (*CYP2C9*1/*1*). In addition, the patients with the homozygous mutation of *CYP2C9*3* or with the combined heterozygous mutation of *CYP2C9*2* and **3* (*CYP2C9*2/*3*) would require 60–75% lower doses of warfarin than those with the homozygous wild-type allele.[27,31]

Asians

As none of the Asians so far studied were carriers of the *CYP2C9*2* variant, it is impossible to assess the impact of this variant on the dose requirement of warfarin in Asians. While most of the previous studies[10–15,22–31] agreed that the patients possessing at least one *CYP2C9*3* variant would require a significantly lower maintenance dose of warfarin than those possessing the homozygous wild-type genotype (*CYP2C9*1/*1*) irrespective of their ethnic backgrounds, there is a discernible difference in the impact of *CYP2C9*3* variant on the warfarin doses between the populations. While Japanese patients with the *CYP2C9*1/*3* and *CYP2C9*3/*3* genotypes have 50 and 90% lower warfarin doses than those with the *CYP2C9*1/*1* genotype, respectively,[12–14] the impact of *CYP2C9*3* on the warfarin dose appeared somewhat attenuated in Caucasians. The Caucasian patients with the *CYP2C9*1/*3* and *CYP2C9*3/*3* genotypes have 20–45% and 75% lower warfarin doses than those with homozygous wild-type *CYP2C9* genotype, respectively.[15,24–31]

Anecdotal observations indicated that the maintenance doses of warfarin obtained from Asians (ie, 3.4 and 3.3 mg/day for Japanese[12–14] and Chinese,[32] respectively) are 20–50% lower than those obtained from Caucasian (ie, 4.1–6.7 mg/day).[10,11,22–31] We will discuss whether or not the assumed population difference in the maintenance dose of warfarin between Caucasians and Asians can be explained by the population difference in the polymorphisms of *CYP2C9* in the separate chapter.

CYP2C9 GENOTYPES AND SUSCEPTIBILITY TO BLEEDING COMPLICATIONS

Induction phase

While the *CYP2C9* polymorphism would influence on the interindividual variability of the maintenance dose of warfarin associated with optimal anticoagulation effect, it may also

Table 1 Effects of the genetic polymorphisms of *CYP2C9* on the daily dose of warfarin and risks of bleeding complications in different ethnic populations

References	*(n)*	*Ethnic background*	*Allelic frequencies of CYP2C9 variants*	*Daily dose of warfarin (mg/day)*	*Bleeding complications*
Furuya *et al*[22]	94	British	0.191 (*2), ND (*3)	4.7 (*1/*1) *vs* 3.8 (*1/*2)	ND
Aithal *et al*[23]	88	British	0.182 (*2), 0.142 (*3)	81% of the low-dose (1.5 mg /day) group had at least one of *3 or *2 alleles *vs* only 38% of control group	↑ Risks of major bleedings in the low-dose group (RR, 3.68; CI, 1.43–9.50) ↑ Likelihood of INR>4.0 at induction (OR, 5.97; CI, 2.26–15.82) in the low-dose group
Aithal *et al*[24]	52	British		4.7 (*1/*1) *vs* 3.7 (*1/*2, *2/*2) *vs* 2.7 (*1/*3)	
Ogg *et al*[25]	233	British	ND (*2), 0.073 (*3)	4.1 (*1/*1) *vs* 2.9 (*1/*3,*3/*3)	↑ Bleedings during initial 3months and at the end of follow-up in patients with *3/*3 *vs* patients with *1/*3 or *1/*1
Taube *et al*[26]	561	British	0.106 (*2), 0.053 (*3)	5.0 (*1/*1) *vs* 4.3 (*1/*2) *vs* 3.0 (*2/*2) *vs* 4.0 (*1/*3) *vs* 4.1 (*2/*3)	No difference in the SD of the mean INR, % of high INR >0.5 INR unit above target, % of time spent in target range
Margaglione *et al*[27]	180	Caucasian 1.8 (*2/*3)	0.178 (*2), 0.089 (*3)	6.7 (*1/*1) *vs* 3.8 (*1/*3) *vs* 5.2 (*1/*2, *2/*2) *vs*	↑ Bleedings in patients with *2 and/or *3 *vs* *1 (OR, 2.57; CI, 1.16–5.73)
Tabrizi *et al*[28-30]	153	Caucasian (*n*=120) African-American (*n*=33)	0.082 (*2),0.078(*3)	5.7 (*1/*1) *vs* 4.4 (*2/*3) *vs* 4.0 (*2) *vs* 4.0 (*3)	ND
Higashi *et al*[31]	185	European (91%) Asian (4%) African-American (3%) Hispanic (2%)	0.105 (*2), 0.084(*3)	5.6 (*1/*1) *vs* 4.9 (*1/*2) 3.3 (*1/*3) *vs* 4.1 (*2/*2) 2.3 (*2/*3) *vs* 1.6 (*3/*3)	↑ Risks of above-range INR (HR, 1.4; CI, 1.03–1.90), ↑ time to achieve stable dosing (HR, 0.65; CI, 0.45–0.95) ↑ Risks of serious bleedings (HR, 2.39; CI, 1.18–4.86), ↑ bleedings during initiation (HR, 3.94; CI, 1.29–12.06) in patients with at least 1 variant allele
Leung *et al*[32]	89	Chinese	0.75 (Leu208Val) *vs* 0.20 (Gln192Pro) *vs* 0.10 (His184Pro) *vs* 0.09 (Ile181Leu)	↑ MD in patients with Ile181Leu ↓ MD in patients with Leu208Val	ND

Abbreviations: *1=*CYP2C9*1*, *2=*CYP2C9*2*, *3=*CYP2C9*3*, CI=confidence interval, HR=hazard ratio, INR=international normalized ratio, MD=maintenance dose (mg/day), ND=not determined, OR=odds ratio, RR=rate ratio, SD=standard deviation

influence on the susceptibility to bleeding complications. Aithal *et al*[23] reported that the patients requiring less than 1.5 mg/day of warfarin for attaining optimal INR (ie, the low-dose group) had a significantly greater probability of developing above-range or supratherapeutic INR defined as >4.0 during the induction phase of the warfarin therapy (odds ratio (OR), 5.97; 95% confidence interval (CI), 2.26–15.82) than those randomly selected patients from anticoagulation clinics. As the majority (81%) of patients in the low-dose group possessed at least one or more of either *CYP2C9*2* or *CYP2C9*3* allele, they considered that the patients carrying these *CYP2C9* variants may be more susceptible to bleeding complications than the genotype-unspecified randomly selected population. Ogg *et al*[25] reported a *post hoc* analysis of a prospective large-scale thrombosis prevention trial with low-intensity warfarin therapy performed in 5499 British men at high risk of ischemic heart disease: they found that the two patients with the homozygous *CYP2C9*3* genotype had a significantly greater probability (ie, 100%) of developing bleeding complications than those with the homozygous wild-type *CYP2C9* genotype (ie, 9%) during the initial 3 months of the trial. However, there was no evidence

Table 2 Effects of the genetic polymorphisms of *CYP2C9* on the metabolic activity for (*S*)-warfarin and INR in different ethnic populations

References	*Number of patients*	*Ethnic background*	*Allelic frequencies of CYP2C9 variants *1 vs *2 vs *3*	*Daily dose of warfarin (mg/day) *1 vs *2 vs *3*	*(S)-warfarin metabolism*	*INR*
Steward *et al*[10]	1	Caucasian		0.5 (*3/*3)	↑ 7.8-fold in plasma *S/R* ratio	INR; 9.7 after 2X LD of 10 mg
Loebstein *et al*[11]	156	Jewish	0.84 *vs* 0.10 *vs* 0.06	6.5 (*1/*1) *vs* 5.2 (*1/*2) *vs* 3.3 (*1/*3)	CLpo (ml/min); 2.4 (*1/*1) *vs* 2.2 (*1/*2) *vs* 1.5 (*1/*3, *3/*3)	Mean INR; 2.65 (*1/*1) *vs* 2.59 (*1/*2) *vs* 2.70(*1/*3,*3/*3)
Takahashi *et al*[12-14]	137	Japanese (*n*=90)	0.97 *vs* 0 *vs* 0.03	3.4 (*1/*1) *vs* 1.75 (*1/*3) *vs* 0.4 (*3/*3)	$CL_{po,u}$(*S*) (ml/min/kg); 10.4 (*1/*1) *vs* 3.9 (*1/*3) *vs* 1.1 (*3/*3)	Mean INR; 1.6 (*1/*1) *vs* 1.5 (*1/*3) *vs* 2.2 (*3/*3)
		Caucasian (*n*=47)	0.72 *vs* 0.22 *vs* 0.05	4.8 (*1/*1) *vs* 4.1(*1/*3) 3.5 (*1/*2) *vs* 7.3 (*2/*2) 1.1 (*2/*3)	4.3 (*1/*1) *vs* 4.3 (*1/*3) 4.3 (*1/*2) *vs* 6.8 (*2/*2) 1.2 (*2/*3)	2.9 (*1/*1) *vs* 3.5 (*1/*3) 2.7 (*1/*2) *vs* 2.4 (*2/*2) 2.1 (*2/*3)
Scordo *et al*[15]	93	Italian	0.75 *vs* 0.12 *vs* 0.13	5.6 (*1/*1) *vs* 3.9 (*1/*2) 21.9 (*1/*3) *vs* 3.0 (*2/*2) 2.6 (*2/*3) *vs* 1.3 (*3/*3)	$CL_{po,u}$(*S*) (ml/min) 660 (*1/*1) *vs* 380 (*1/*2) 345 (*1/*3) *vs* 213 (*2/*2) 155 (*2/*3) *vs* 61 (*3/*3)	Mean INR; 2.4 (low-dose) *vs* 2.3 (high-dose) 2.3 (medium-dose) *vs*

Abbreviations: CLpo(*S*)=total (bound+unbound) oral clearance for (*S*)-warfarin, CLpo,u(*S*)=unbound oral clearance for (*S*)-warfarin, INR=international normalized ratio, LD=loading dose, 2X=twice, plasma S/R ratio=plasma (*S*)-warfarin/(*R*)-warfarin ratio.

suggesting that the heterozygous carriers of *CYP2C9*3* would be more susceptible to bleeding complications than those with homozygous for both groups wild-type *CYP2C9* genotype (ie, 10%) during the initiation 3 months as well as through 3–4 years of the entire follow-up period. Recently, Higashi *et al*[31] conducted a retrospective cohort study where they analyzed the influence of the *CYP2C9* genotypes on the anticoagulation responses and bleeding complications during warfarin therapy. They found that the patients with at least one *CYP2C9* variant allele of either *CYP2C9*2* or *CYP2C9*3* took a longer time to achieve stable dosing of warfarin than those with the homozygous wild-type genotype (hazard ratio (HR), 0.65; 95% CI, 0.45–0.94) with the median difference of 95 days. In addition, the patients with at least one variant *CYP2C9* allele had a greater risk of developing bleeding events during the initial 3 months of warfarin therapy (HR, 3.94; 95% CI, 1.29–12.06).

Maintenance phase

Aithal *et al*[23] reported that the patients requiring less than 1.5 mg/day of warfarin for attaining optimal anticoagulation response (the low-dose group) exhibited a higher incidence of serious and life-threatening bleeding episodes than those randomly selected patients from anticoagulation clinics (rate ratio (RR), 3.68; 95% CI, 1.43–9.50). In support of their findings were the subsequent studies conducted by other investigators[27,31] demonstrating that patients with the heterozygous *CYP2C9*2* or *CYP2C9*3* genotype would carry a higher risk of bleeding complications during warfarin therapy: OR, 2.57; 95% CI, 1.16–5.73[27] and HR, 2.39; 95% CI, 1.18–4.86.[31] One of these studies[31] also showed that the carriers of *CYP2C9*2* or *CYP2C9*3* allele more frequently developed above-range or supratherapeutic INRs (>4.0 for most patients and >4.5 for those with prosthetic valve replacement) with the HR of 1.4 (95% CI, 1.03–1.90).

In contrast, Taube *et al*[26] could not confirm the above findings in their study performed with a large number of patients ($n=561$) and a more extended period (the median of 2.38 years) of follow-up. Despite that their patients with the *CYP2C9*2* and **3* variant alleles had a significantly reduced dose requirement as compared with those with the wild-type genotype (Table 1), they observed no significant differences in the incidence of INR of 8 or greater (OR, 1.52; 95% CI, 0.64–3.58) as well as instability indices for anticoagulation responses (ie, standard deviation (SD) of the mean INR, percentage of high INRs (>0.5 unit greater than the target INR of 2.5), and percentage of person-time spent in the therapeutic range during long-term warfarin therapy) between the patients with the *CYP2C9* variants and those with the wild-type geno-

type. Based upon these results, they concluded that the possession of *CYP2C9*2* or *CYP2C9*3* variant alleles does not increase the likelihood of developing severe overcoagulation or instability in the anticoagulation response during the long-term warfarin therapy once a stable anticoagulant effect is established in each patient. The reason(s) why Taube *et al*'s [26] data are contradictory to those of previous studies[23,25,27,31] remains unclear. However, the extremely strict criterion of Taube *et al* for judging overcoagulation (INR > 8) might have prevented them from detecting the influence of *CYP2C9* polymorphism on the variability of anticoagulation response to warfarin.

Since Asians appear to be more sensitive to warfarin than Caucasians[2,3] as far as the maintenance dose is concerned, it is of great interest whether the *CYP2C9* genotypes would have a significant effect on the susceptibility to bleeding complications of warfarin therapy in non-Caucasian populations. As there is a paucity of knowledge regarding this issue, it would be of value to carry out retrospective studies on this issue before embarking on prospective trials. However, prospective, long-term, controlled clinical studies should ultimately be performed to assess clinical implications of the *CYP2C9* genotypes on the anticoagulation response and bleeding complications during the warfarin therapy in Asians and other non-Caucasian populations.

Collectively, it is suggested that patients with *CYP2C9* variants, particularly *CYP2C9*3* allele or a combination of *CYP2C9*2* and *CYP2C9*3* alleles would be vulnerable to above-range INRs, need more time to achieve stable warfarin dosing and longer hospitalization and have a higher risk of serious or life-threatening bleeding events than those with wild-type *CYP2C9* genotype during the induction or dose-titration period of warfarin therapy.

EFFECTS OF *CYP2C9* GENOTYPES ON (*S*)-WARFARIN METABOLISM

Within-population comparisons

While numerous studies have been conducted to clarify the relation between the *CYP2C9* genotypes and maintenance dose of warfarin, few of them have addressed the question whether *CYP2C9* variants would be associated with altered *in vivo* metabolic activity toward any of the CYP2C9 substrate drugs.[10–15] We were the first to report that Japanese patients with the heterozygous and homozygous *CYP2C9*3* allele exhibited a 63 and 89%, reduced unbound oral clearance ($CL_{po,u}$) for (*S*)-warfarin, respectively, as compared with those with the homozygous wild-type *CYP2C9* allele.[12,13] As a significant gene–dose effect in $CL_{po,u}$ for (*S*)-warfarin by *CYP2C9*3* variant was observed, we considered that the *CYP2C9*3* variant may have a clinically significant impact on the anticoagulation response to warfarin even in a heterozygous genotype in Japanese patients.

Subsequently, we studied the effects of the *CYP2C9* polymorphisms on $CL_{po,u}$ for (*S*)-warfarin in Caucasians using the same protocol as we used in the previous studies performed in Japanese patients.[12,13] To our surprise, we observed no significant difference in $CL_{po,u}$ for (*S*)-warfarin between the Caucasian patients with the homozygous wild-type CYP2C9 genotype and those with the heterozygous *CYP2C9*2* or *CYP2C9*3* genotype. Nonetheless, a patient having a combined heterozygote of *CYP2C9*2* and *CYP2C9*3* (ie, *CYP2C9*2/*3*) had $CL_{po,u}$ for (*S*)-warfarin that was 70% lower than the mean value for those with the homozygous wild-type *CYP2C9* genotype.[14]

Loebstein *et al*[11] reported that the Jewish patients ($n=156$) with the *CYP2C9*1/*2* and *CYP2C9*1/*3* genotypes had 8 and 38% lower total (bound + unbound) oral clearance (CL_{po}) for warfarin than those with the *CYP2C9*1/*1* genotype. Recently, Scordo *et al*[15] reported that there was a significant ($P<0.01$) difference in $CL_{po,u}$ for (*S*)-warfarin among the groups of patients with different CYP2C9 genotypes (ie, six possible combinations of *CYP2C9*2* and *CYP2C9*3* alleles) in 93 Italian patients. However, there was a substantial overlap in $CL_{po,u}$ for (*S*)-warfarin among the different genotype groups, so that 72% of the patients with the *CYP2C9*1/*1* genotype showed $CL_{po,u}$ for (*S*)-warfarin in the same range as those carrying either *CYP2C9*2* or *CYP2C9*3* allele. Collectively, the magnitude of reduction in $CL_{po,u}$ for (*S*)-warfarin associated with the *CYP2C9*1/*3* genotype as compared with the respective control values obtained from those with wild-type CYP2C9 genotype in the above two studies[11,15] (ie, 38 and 48%, respectively) appears somewhat attenuated as compared with that we observed in Japanese patients (63%).[12,13] The data obtained from the studies of Loebstein *et al*[11] and Scordo *et al*[15] agreed that the influence of *CYP2C9*2* mutation on the *in vivo* warfarin metabolism assessed by CL_{po} or $CL_{po,u}$ in humans appears less pronounced than that of *CYP2C9*3*.

Population differences in CYP2C9 activity and allelic frequencies of *CYP2C9* variants

There are substantial differences in the allelic frequencies of *CYP2C9* variants, at least for *CYP2C9*2* and *CYP2C9*3* alleles, across different ethnic populations: the respective allelic frequencies of *CYP2C9*2* and *CYP2C9*3* in African-Americans (1–3.6% and 0.5–1.5%, respectively) and Asians (0% and 1.7–5%, respectively) tended to be lower than the corresponding values of Caucasians (8–19.1% and 5.3–10%, respectively).[8,9] As the enzyme activities of *CYP2C9* variant proteins are reduced as compared with the wild-type protein[42] at least in *in vitro* experiments, there is a possibility that the population differences in the allelic frequencies of distinct *CYP2C9* variants may emerge as those in certain phenotypic traits of *CYP2C9* (eg, $CL_{po,u}$). We have recently studied the population differences of hepatic CYP2C9 activity using $CL_{po,u}$ for (*S*)-warfarin as a specific *in vivo* probe for the CYP isoform between Caucasians and Japanese patients and found that the population mean for body weight-normalized $CL_{po,u}$ for (*S*)-warfarin obtained from Japanese patients was significantly greater than that for Caucasian patients.[14] Considering greater allelic frequencies of *CYP2C9*2*

and *CYP2C9*3* in Caucasians than in Japanese patients,[8,9] one may assume that population differences in the allelic frequencies of *CYP2C9*2* and *CYP2C9*3* between Caucasians and Japanese patients may account at least partly for the above-described population difference in the metabolic activity for (*S*)-warfarin. However, comparisons of $CL_{po,u}$ for (*S*)-warfarin between the genotype-matched Japanese patients and Caucasians with the homozygous wild-type *CYP2C9* genotype revealed that Japanese patients still had a significantly greater body weight-normalized $CL_{po,u}$ than the Caucasians.[14] Collectively, previously recognized genetic polymorphisms of *CYP2C9* cannot adequately account for the observed population differences in the *in vivo* CYP2C9 activity at least between Caucasians and Japanese patients.

Regarding population differences in the allelic frequencies of *CYP2C9* variants, a recent report of Leung *et al*[32] deserves some comments. They studied the genetic polymorphisms of *CYP2C9* in Chinese patients and found four novel single-nucleotide polymorphisms (SNPs) in *CYP2C9* exon 4. In addition, they found that the patients with one of the SNPs (ie, a nucleotide change of 527A>C corresponding to an amino-acid substitution of 181Ile>Leu) may be associated with greater maintenance dose of warfarin and those with the other SNP (ie, 608T>G or 208Leu>Val) was associated with a lower warfarin dose than those with the wild-type genotype. However, caution must be exercised for interpreting their data, because their data may be flawed by inappropriate primer designs. We critically reappraised their findings using their primers originally reported by Stubbins *et al*[43] and the other primers by Sullivan-Klose *et al*[44] that were validated by us and others.[12–14,16] We found that the nucleotide sequences of the PCR products amplified by the primers of Leung *et al*[32] and Stubbins *et al*[43] differed from those amplified by the latter primers[44] and the authentic DNA sequence of *CYP2C9* exon 4. Using the latter PCR primers we found no SNPs in exon 4 of *CYP2C9* in Japanese patients (unpublished data).

CYP2C9 genotypes and development of enzyme activity

Since many CYP isoforms exhibit substantial ontogenic changes in their enzyme activity particularly during prepubertal period (<12 years), it is of great interest if the genetic polymorphism of *CYP2C9* may influence the developmental changes in the CYP2C9 activity. In this context, we compared $CL_{po,u}$ for (*S*)-warfarin between prepubertal (1–11 years) and adult (37–76 years) Japanese patients with the *CYP2C9*1/*1* or *CYP2C9*1/*3* genotypes.[45] The median $CL_{po,u}$ for (*S*)-warfarin obtained from the adult patients with the wild-type (*CYP2C9*1/*1*) genotype (ie, 667 ml/min) was significantly greater than that obtained from the prepubertal patients with the corresponding genotype (ie, 367 ml/min), indicating a substantial development would take place for the activity of CYP2C9 in these subjects. In contrast, there was no significant difference in the median $CL_{po,u}$ for (*S*)-warfarin between prepubertal and adult patients with the *CYP2C9*1/*3* genotype (ie, 213 and 212 ml/min, respectively), indicating that the development of CYP2C9 activity may be substantially attenuated in carriers of the *CYP2C9*3* allele. However, because the numbers of prepubertal and adult patients with the *CYP2C9*1/*3* allele were rather small ($n=5$ and 4, respectively) and no longitudinal follow-up was made for individual children with *CYP2C9*1/*3* genotype, further studies are required to confirm our findings. At present, to our knowledge, no attempts have been made to study the effect of *CYP2C9* variants on the development of CYP2C9 activity in Caucasians and other ethnic populations.

PHARMACOKINETIC CONSEQUENCES OF *CYP2C9* POLYMORPHISMS

Dose–response relation

We have already discussed the effects of *CYP2C9* polymorphism on the maintenance dose of warfarin and the metabolic activity of CYP2C9. Here, we wish to afford a pharmacokinetic basis for these clinical findings. For many drugs the magnitude of the pharmacological response is a function of plasma unbound drug concentrations, because the unbound drug in plasma is assumed to be equilibrating with that in site(s) of action. Indeed, we previously demonstrated that there is a significant linear relation between Cu for (*S*)-warfarin and INR in Japanese patients undergoing long-term stable anticoagulation therapy with warfarin.[46] Clearance (CL) is a pharmacokinetic parameter that connects the dosing rate of a drug (D/τ) with its steady-state plasma concentration according to the equation $C_{pss}=(D/\tau)/CL$. Thus, assuming that there are no significant differences in the anticoagulation sensitivity to warfarin between patients with different *CYP2C9* genotypes, the maintenance dose of warfarin for those with defective alleles of *CYP2C9* (eg, *CYP2C9*3*) should be reduced largely in parallel with that observed in $CL_{po,u}$ in the respective populations in order to obtain comparable Cu and thereby INR.

Time to reach stable warfarin dosing

When a drug is administered intermittently at regular dosing intervals, the steady-state plasma concentration will be achieved after approximately 4 half-lives ($t_{1/2}$) of the drug, regardless of doses or dosing intervals. Since the mean $t_{1/2}$ of (*S*)-warfarin obtained from randomly selected population (most likely having the *CYP2C9*1/*1* genotype) is approximately 1 day,[47] it would take approximately 4 days to reach the steady-state plasma concentration of (*S*)-warfarin in patients with the *CYP2C9*1/*1* genotype (Figure 1). On the other hand, a patient with a *CYP2C9* variant associated with the reduced enzyme activity would require a longer time to attain the steady state of the warfarin disposition than those with the wild-type genotype. Assuming that the volume of distribution of warfarin is independent of *CYP2C9* genotypes, the elimination $t_{1/2}$ of (*S*)-warfarin in patients with *CYP2C9* variants will be prolonged in proportion to the reciprocal of the systemic

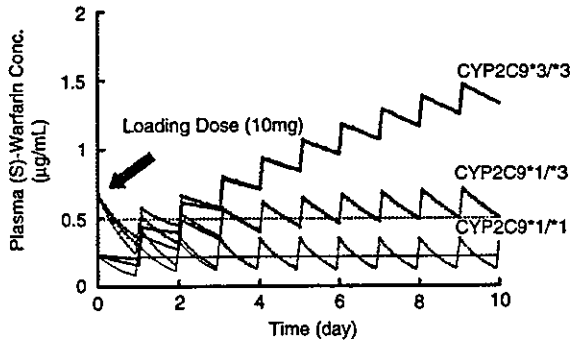

Figure 1 Computer-assisted simulations for the plasma total (bound+unbound) (*S*)-warfarin concentration–time profiles following the intermittent administration of 3.4 mg of racemic warfarin a day in representative Japanese patients with *CYP2C9*1/*1*(thin line), *CYP2C9*1/*3* (medium-bold line) and *CYP2C9*3/*3* (bold line) genotypes following an oral dose of 3.4 mg/day. The symbols ○, △ and ◇ represent plasma concentration–time curves for patients with *CYP2C9*1/*1*, *CYP2C9*1/*3* and *CYP2C9*3/*3* genotypes when a loading dose regimen of 10 mg followed by a maintenance dose of 3.4 mg/day was employed. The simulation was performed assuming that the representative patients with *CYP2C9*1/*1*, *CYP2C9*1/*3* and *CYP2C9*3/*3* genotypes had the total oral clearance for (*S*)-warfarin of 0.34, 0.11 and 0.034 l/h, respectively, 7.4 l for volume of distribution and an oral bioavailability of unity. The horizontal broken line represents the population mean value for the steady-state plasma (*S*)-warfarin concentration with an oral warfarin dose of 6.1 mg/day in Caucasians[53] and the solid line represents that with 3.4 mg/day in Japanese patients.

clearance that largely depends on *CYP2C9* genotypes according to the equation $t_{1/2} = (0.693 \cdot Vd)/CL$.

As Caucasians and Japanese patients with the *CYP2C9*1/*3* genotype possess a 38–48% and 63% lower $CL_{po,u}$ for (*S*)-warfarin than those with the *CYP2C9*1/*1* genotype in the respective populations, their elimination $t_{1/2}$ for (*S*)-warfarin should be 1.6–1.9 and 2.7 times longer than that in those with the *CYP2C9*1/*1* genotype (ie, 1 day). Therefore, Caucasian and Japanese patients with the *CYP2C9*1/*3* genotype would require approximately 6–8 and 11 days, respectively, to reach the steady-state condition. Furthermore, because a Japanese patient having the homozygous *CYP2C9*3* allele has an 89% lower $CL_{po,u}$ for (*S*)-warfarin than those with the homozygous *CYP2C9*1* allele,[12] such a patient would require approximately 36 days to reach the steady-state plasma concentration of (*S*)-warfarin as well as stable INR status. As mentioned above, time required to reach steady state of a drug during intermittent administrations solely depends on its elimination $t_{1/2}$, an adoption of a loading dose warfarin regimen (eg, 10 mg for a loading dose followed by 3.4 mg of a maintenance dose) will not help to shorten the time to achieve the steady-state condition for patients with the *CYP2C9*3* allele. As shown in Figure 1, when an average maintenance dose of warfarin (eg, 3.4 mg) is given to Japanese patients irrespective of their *CYP2C9* genotypes, the resultant steady-state plasma (*S*)-warfarin concentrations for patients with the *CYP2C9*1/*3* and *CYP2C9*3/*3* genotypes would be 2.7 and 9.1 times greater than that obtained from a representative patient with the *CYP2C9*1/*1* genotype.

The above simulation of the plasma (*S*)-warfarin concentrations–time courses in patients with different *CYP2C9* genotypes would give us a further insight into the finding of Higashi *et al*[31]. They found that patients with one or more of *CYP2C9*2* or **3* allele had longer time to achieve stable warfarin dosing with a mean difference of 95 days after the commencement of warfarin therapy than those with the homozygous *CYP2C9* wild-type genotype. In their study, a common dosing protocol of warfarin was employed for their patients irrespective of patients' *CYP2C9* genotypes. Our pharmacokinetic simulation of the plasma concentration–time course of pharmacologically more active (*S*)-warfarin in patients with different *CYP2C9* genotypes predicts that patients with *CYP2C9*3* allele would exhibit a greater plasma concentration of (*S*)-warfarin and thereby exaggerated anticoagulation response to a standard warfarin dosing. In addition, because the patients with *CYP2C9*3* allele have a longer $t_{1/2}$ of (*S*)-warfarin than those with the homozygous wild-type allele, they require longer periods of follow-up and more frequent dosage adjustments before they reach the final steady-state condition for the disposition of (*S*)-warfarin.

Risks of adverse drug reactions

It is commonly believed that patients receiving greater doses of a drug would be at higher risk to develop adverse drug reactions. Contradicting to this notion, lines of evidence indicate that patients receiving lower doses of warfarin tend to have a greater incidence of bleeding complications particularly during the initial phase of warfarin therapy.[23,31] Our discussion about the effects of *CYP2C9* polymorphisms on the *in vivo* pharmacokinetics of (*S*)-warfarin and on the disposition of vitamin K-dependent coagulation factors may explain at least partly an apparently paradoxical relation between the individualized maintenance dose of the drug and incidence of adverse drug reactions.

Loading dose of warfarin is often adopted in order to accelerate to elicit therapeutic anticoagulation response of the drug. However, based upon our simulation of the plasma concentration–time courses of (*S*)-warfarin in patients with different *CYP2C9* genotypes (Figure 1) we assume that the loading dose of warfarin may pose a greater risk of bleeding complications in patients with *CYP2C9* variant alleles particularly around initial two or three doses of the drug as compared with those with the wild-type *CYP2C9* genotype. When a loading dose regimen of warfarin is employed, plasma (*S*)-warfarin concentrations in the patients with the *CYP2C9*1/*3* or *CYP2C9*3/*3* genotype would rise