

Identification of Cytochrome P450 Oxidoreductase Gene Variants That Are Significantly Associated with the Interindividual Variations in Warfarin Maintenance Dose^[S]

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ABSTRACT:

Cytochrome P450 oxidoreductase (POR) is required for drug metabolism by all microsomal cytochrome P450 enzymes. The aim of this study was to investigate whether any of the common single nucleotide polymorphisms (SNPs) in the *POR* gene and its flanking intergenic sequences correlate with interindividual variations in the warfarin maintenance dose (which is determined partly by rates of warfarin metabolism) in patients undergoing anticoagulation therapy. Warfarin dose and patients' demographic and clinical information were collected from 124 patients, who had attained a stable warfarin dose while receiving treatment at the Stratton VA Medical Center. Genomic DNAs were isolated from blood samples and were genotyped for 15 SNPs (including 10 SNPs on the *POR* gene). Association analysis was performed on 122 male patients by linear regression. Simple regression analysis revealed that vitamin

K epoxide reductase complex subunit 1 (*VKORC1*) –1639A>G, *CYP2C9*2*, *CYP2C9*3*, age, and chronic aspirin therapy were significantly associated with warfarin dose. In contrast, multiple regression analysis revealed that, in addition to several known factors contributing to the variations in warfarin maintenance dose (*VKORC1* –1639A>G, *CYP2C9*2*, *CYP2C9*3*, *CYP4F2* rs2108622, and chronic aspirin therapy), three common *POR* SNPs (–173C>A, –208C>T, and rs2868177) were also significantly associated with variations in warfarin maintenance dose. These results indicate, for the first time, that three common SNPs in the *POR* gene may contribute to the interindividual variability in warfarin maintenance dose. Further studies on functional characterization of the *POR* SNPs identified, including their impact on the in vivo metabolism of additional drugs, are needed.

Introduction

Cytochrome P450 oxidoreductase (POR) is the obligate electron donor to all microsomal cytochrome P450 (P450) enzymes, which play major roles in the metabolism of most drugs (Porter and Coon, 1991; Guengerich, 2008). Interindividual variations in POR activity (at least 4- to 5-fold differences) have been found in human liver microsomes (Kaminsky et al., 1984; Hart et al., 2008). The notion that variations in POR expression or activity influence the rates of P450-mediated drug metabolism in patients is supported by several lines of data, including the impact of *Por* gene knockout on drug clearance in mouse models (Gu et al., 2003; Henderson et al., 2003; Zhang et al., 2009), positive correlations between POR activity and P450-mediated

drug metabolism activities in human liver microsomes (Kaminsky et al., 1984; Hart et al., 2008), and the impact of many *POR* single nucleotide polymorphisms (SNPs) on P450 activities toward drugs and other xenobiotics in reconstituted enzyme systems (Agrawal et al., 2010; Flück et al., 2010; Marohnic et al., 2010; Nicolo et al., 2010; Sandee et al., 2010; Miller et al., 2011).

At present, more than 800 *POR* SNPs (Hart et al., 2008; Huang et al., 2008; Gomes et al., 2008; NCBI dbSNP database, <http://www.ncbi.nlm.nih.gov/snp>) and 41 *POR* alleles (Sim et al., 2009; <http://www.cypalleles.ki.se/por.htm>) have been identified. Rare *POR* coding region mutations, identified in patients with Antley-Bixler syndrome and congenital adrenal hyperplasia, cause dramatic decreases in POR activity and the activities of microsomal steroidogenic P450 enzymes (Arlt et al., 2004; Flück et al., 2004; Huang et al., 2005). The impact of the rare *POR* mutant A287P on drug metabolism in a patient (Tomalik-Scharte et al., 2010), the effects of various *POR* SNPs on human liver microsomal drug metabolism activity (Hart et al., 2008; Gomes et al., 2009), and the potential role of the common A503V variation (*POR*28*) in interindividual differences in midazolam metabolism (Oneda et al., 2009) have been reported. However, few studies have examined the impact of common *POR* SNPs on drug metabolism in a clinical setting.

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ABBREVIATIONS: POR, cytochrome P450 oxidoreductase; P450, cytochrome P450; SNP, single nucleotide polymorphism; NCBI, National Center for Biotechnology Information; *VKORC1*, vitamin K epoxide reductase complex subunit 1; INR, international normalized ratio; PCR, polymerase chain reaction; ASPE, allele-specific primer extension; GWAS, genome-wide association study.

In this study, we aimed to investigate whether any of the common SNPs in the *POR* gene and its flanking intergenic sequences correlate with interindividual variations in the warfarin maintenance dose (which is determined partly by rates of warfarin metabolism) in patients undergoing anticoagulation therapy. Our hypothesis was that *POR* SNPs influence *POR* activity or expression, thereby influencing warfarin metabolism, through P450 pathways, and contributing to the interindividual variations in warfarin dose requirement. Warfarin is a widely prescribed and effective oral anticoagulant for the treatment and prevention of thromboembolic diseases. However, warfarin has a very narrow therapeutic range and a large potential to produce hemorrhage and thrombotic complications. Interindividual variability in the warfarin maintenance dose is very high (estimated to be >10 fold) (Rettie and Tai, 2006). The safe achievement of a maintenance dose requires intensive monitoring of patients to ensure their safety.

Recent pharmacogenomics studies have demonstrated that the polymorphisms in genes that are involved in warfarin metabolism or action, such as *CYP2C9*, *CYP4F2*, and vitamin K epoxide reductase complex subunit 1 (*VKORC1*), contribute to the interindividual variability in response to warfarin (Marsh and McLeod, 2006; Wadelius and Pirmohamed, 2007; Yin and Miyata, 2007; Caldwell et al., 2008; Limdi and Veenstra, 2008; Takeuchi et al., 2009). These findings indicated that personalized dosing, based on genotyping data, may be a solution for safe warfarin therapy. However, the currently identified genetic factors, combined with nongenetic factors, can only explain approximately 33 to 60% of the interindividual variations in warfarin dose required to achieve therapeutic effects, leaving the factors influencing the remaining 40 to 67% unknown.

In the current study, DNA samples were genotyped for the common SNPs (with $\geq 5\%$ variant allele frequency) in the *POR* gene, using newly developed rapid, high-throughput genotyping assays based on Luminex xTAG technology. The genotypes were then correlated with levels of the warfarin maintenance dose for the individual donor patients. Statistically significant correlations indicate association of the genotypes with the warfarin dose requirements of the individual patients and further imply that the relevant *POR* SNPs can lead to changes in *POR* expression or activity. The identification of "relevant" genetic polymorphisms in the *POR* gene and of other genetic or nongenetic interacting factors will not only contribute to the efforts to develop accurate warfarin dosing algorithms for predicting the required warfarin maintenance dose for each patient but also provide the basis for further determination of the impact of the relevant *POR* SNPs on the in vivo metabolism of numerous other clinical drugs.

Materials and Methods

Participating Patients. The study was approved by the institutional review boards of the Stratton VA Medical Center and the New York State Department of Health. The study subjects were patients who were receiving warfarin anticoagulation therapy in the Stratton VA Medical Center during 2008 and 2009 and who gave written informed consent. The inclusion criteria were 1) target therapeutic international normalized ratio (INR) range of 2 to 3 (INR is standard unit used to report the result of a prothrombin time test) and 2) attainment of a stable warfarin maintenance dose as reflected in the medical records. The most recent three or more consecutive INR measurements that are in the target range (2–3) were identified, for calculation of the average weekly dose (median dose), which was taken to represent the stable warfarin maintenance dose. In a few cases, the INR range was extended to 1.8 or 3.2, because a difference of 0.2 in INR values would not cause dose modification (Aquilante et al., 2006). A total of 124 patients (including both inpatients and outpatients) met the inclusion criteria, of which only 2 were female. Thus, only males were included in the study. The demographic and clinical information for these patients is summarized in Table 1. The age of patients ranged from 42 to 93 years at the time their medical records were reviewed. Of the patients, 115

TABLE 1

Summary of demographics and clinical variables

A total of 122 male patients were studied. Data are *n* (%) unless indicated otherwise.

	Values
Demographic variables	
Age	
Mean \pm S.D.	72 \pm 10
40–49 yr	2 (2)
50–59 yr	11 (9)
60–69 yr	37 (30)
70–79 yr	41 (34)
80–89 yr	29 (24)
>89 yr	2 (2)
Weight (lb)	
Mean \pm S.D.	209 \pm 48
Race	
White	115 (94)
African American	6 (5)
Other	1 (1)
Clinical variables	
Warfarin dose (mg/wk), median (minimum, maximum)	33 (8, 71)
Target INR (range)	2–3
Clinical indications for warfarin treatment	
Atrial fibrillation or flutter	79 (65)
Deep venous thrombosis	22 (18)
Cerebrovascular accident	3 (2)
Pulmonary embolism	2 (2)
Other diseases	16 (13)
Other medication	
Aspirin	26 (21)
Amlodipine	14 (11)
Medicines containing statin	86 (70)
Multivitamin	22 (18)
Insulin	11 (9)

patients were white, six were black, and one was Hispanic. The indications for treatment included atrial fibrillation or flutter, deep venous thrombosis, cerebrovascular accident, pulmonary embolism, and other diseases.

Data Collection. Weekly warfarin dose (milligrams per week); INRs; aspirin (dose range: 0, 81, or 325 mg/day); use of other concomitant medications (yes or no), including statins (all statin-containing medicines), multivitamins, insulin, and amlodipine; and patients' demographic information including age (years), gender, race (white or nonwhite), and weight (pounds) were obtained by reviewing the patient's medical records. All of the extracted information was deidentified and linked to the corresponding blood samples by coding. Peripheral blood samples (~5 ml) were taken during the patient's blood draw for INR measurement and stored at -30°C until isolation of genomic DNA.

Genotyping. Genomic DNA was isolated from whole blood using a QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA). Genotyping assays were performed for the following four groups of SNPs:

1. Five SNPs, four of which are known to be associated with varied warfarin dose requirement: *VKORC1* -1639A>G (rs9923231), *CYP2C9**2 (rs1799853), *CYP2C9**3 (rs1057910), and *CYP4F2* V33M; the SNP rs4889606 in *STX1B* intron 4 (located at ~90 kilobases downstream of *VKORC1* and significantly associated with varying levels of *VKORC1* mRNA expression) (Schadt et al., 2008) was also included (to our knowledge, there has been no report on the relationship between SNP rs4889606 in *STX1B* with varied warfarin dose requirement).
2. Two *POR* SNPs known to be associated with *POR* functional consequences: *POR* A503V (rs1057868) (causes ~30% decrease in *POR* activity) (Huang et al., 2008) and *POR* rs41301472 (within intron 12, G>A change, correlated with decreased *POR* activity in liver microsomes) (Hart et al., 2008).
3. Six tag SNPs in *POR* and its flanking intergenic sequences (chr7: 75356180-75454513), which were identified in the CEU population from HapMap (Data Rel 27, PhaseII+III, Feb09, on NCBI B36

assembly, dbSNP b126), using the Annotate tag SNP Picker (Tagger Pairwise, R^2 cutoff: 0.8, MAF cutoff: 0.05): rs10280802, rs28737229, rs1057870, rs17148944, rs2868177, and rs17685 (<http://www.hapmap.org/>).

- Two SNPs located in the *POR* proximal promoter region: *POR* -173C>A (a newly identified SNP at the time of the study, now also included in dbSNP; rs72553971) and *POR* -208C>T (rs12537282) (Huang et al., 2008); the transcription start site (first nucleotide of *POR* mRNA sequence) was designated as +1.

Genotyping assays for the 15 SNPs above were developed on the basis of Luminex xTAG technology, which uses a proprietary universal tag system. Multiplex PCR primers and allele-specific primer extension (ASPE) primers were designed according to the NCBI GenBank sequences [*CYP2C9*: NT_030059.12 (GI: 51467897); *VKORC1*: NT_010393.15 (GI: 51472974); *POR*: NT_007933.14 (GI: 51493052); *CYP4F2*: NT_011295.10 (GI: 29801560); and *STX1B*: NT_010393.15 (GI: 51472974)]. All the primers were synthesized at Integrated DNA Technologies, Inc. (Coralville, IA), and the ASPE primers were high-performance liquid chromatography-purified. Details of PCR and ASPE primers are listed in Supplemental Tables S1 and S2, respectively.

The 15 SNPs above were detected in four multiplex genotyping assays: assay 1 for 6 SNPs (*CYP2C9**2, *CYP2C9**3, *VKORC1* -1639G>A, *STX1B* rs4889606, *CYP4F2* rs2108622, and *POR* A503V); assay 2 for 3 SNPs (*POR* rs10280802, *POR* rs28737229, and *POR* rs41301427); assay 3 for 3 SNPs (*POR* rs1057870, *POR* rs17148944, and *POR* rs2868177); and assay 4 for 3 SNPs (*POR* -173C>A, *POR* -208C>T, and *POR* rs17685). The experimental procedures of the four assays differed only at the multiplex PCR step. The genotyping assays consisted of the following five steps.

Step 1. Multiplex PCR. DNA fragments covering the SNPs studied for each genotyping assay were amplified in one reaction on a model 9800 Fast Thermal Cycler (Applied Biosystems, Foster City, CA). For assays 1 to 3, after an initial denaturation at 95°C for 15 min, the amplification reaction was performed for 35 cycles, with each cycle consisting of a denaturation at 95°C for 30 s, an annealing at 55°C for 30 s, and an extension at 72°C for 40 s, followed by a final extension at 72°C for 10 min. The reaction mixtures, in a total volume of 25 μ l, contained 1 \times PCR buffer (20 mM Tris-HCl, pH 8.4, and 50 mM KCl), 0.2 mM concentrations each of dNTPs, 0.2 μ M concentrations each of the PCR primers, 0.625 unit of HotStarTaq DNA Polymerase (Qiagen, Valencia, CA), and 40 ng of genomic DNA. For assay 4, the TaKaRa LA Taq system (TaKaRa Bio, Inc., Madison, WI) was used, and after initial denaturation at 94°C for 2 min, 35 PCR cycles were performed, with each consisting of a denaturation at 94°C for 30 s, and an annealing and extension at 64°C for 100 s, followed by a final extension at 68°C for 10 min; the reaction mixture contained, in a total volume of 25 μ l, 1 \times LA PCR buffer II (Mg²⁺ PLUS), 1.25 mM concentrations each of dNTPs, 0.2 μ M concentrations each of the 12 PCR primers, 1.25 units of TaKaRa LA Taq Polymerase, and 40 ng of genomic DNA. An H₂O blank (no template) control was routinely used for detecting potential contamination of reagents; the results were also used later for fluorescence background correction at step 5. Identity of each PCR product was confirmed by direct DNA sequence analysis.

Step 2. Shrimp alkaline phosphatase and exonuclease I treatment. To remove unincorporated deoxynucleotide triphosphates and primers from the last PCR amplification step, the PCR products were treated with shrimp alkaline phosphatase and exonuclease I (ExoSAP-IT; USB, Cleveland, OH). A mixture of ExoSAP-IT (2 μ l) and the PCR product (5 μ l) was incubated at 37°C for 30 min, followed by an incubation at 80°C for 15 min (to inactivate the enzymes), in the model 9800 Fast Thermal Cycler. The treated PCR products were kept at 4°C until use.

Step 3. Multiplex ASPE. Multiplex ASPE reactions were performed in the model 9800 Fast Thermal Cycler, with 5 μ l of ExoSAP-IT-treated PCR product, in a total volume of 20 μ l. Each reaction mixture consisted of 1 \times PCR buffer, 1.25 mM MgCl₂, 25 nM concentrations each of Tag-ASPE primers, 5 μ M concentrations each of dGTP, dATP, and dTTP (Invitrogen, Carlsbad, CA), 5 μ M biotin-14-dCTP (Invitrogen), and 0.75 unit of Platinum Genotype *Tsp*DNA Polymerase (Invitrogen). The reaction was started with preincubation at 96°C for 2 min (to denature DNA) and then followed by 30 cycles of PCR, with each consisting of 94°C for 30 s, 55°C for 1 min, and 74°C for 2 min. The ASPE products were kept at 4°C until use.

Step 4. Hybridization to Luminex MicroPlex-xTAG microspheres and acquisition of data. The hybridization of ASPE products to the MicroPlex-xTAG microspheres was performed in a PerkinElmer thermal cycler 9600 instrument (Applied Biosystems). Data collection was performed on a Luminex 100 IS System (Luminex Corporation, Austin, TX). All procedures of this step were performed in the dark. Microsphere suspension was prepared by gentle vortexing, followed by sonication, in an Ultrasonic cleaner (model 08849-00; Cole-Parmer Instrument Co., Vernon Hills, IL), for ~20 s. Each hybridization reaction was performed with ~2500 each of the populations of carboxylated fluorescent microspheres that are precoupled with the xTAG oligonucleotide sequences (anti-tags) (Luminex Corporation).

Equal amounts of the microsphere sets were combined, concentrated, and then resuspended to 100 beads each/ μ l, in 2 \times Tm hybridization buffer (0.2 M Tris-HCl, pH 8.0, 0.4 M NaCl, and 0.16% Triton X-100). The resuspended beads (25 μ l) were combined with 5 μ l of the ASPE products from step 3 and H₂O to make a total volume of 50 μ l; the mixture was heated at 96°C for 90 s to denature DNA, followed immediately by incubation at 37°C for 60 min. After this hybridization step, the reaction mixtures were transferred to filter plates (Millipore Corporation, Billerica, MA) and prewetted in 1 \times Tm hybridization buffer, and the supernatant was removed under vacuum, as described in the manufacturer's instruction manual. After two washes with 100 μ l of 1 \times Tm hybridization buffer, the beads were resuspended in 100 μ l of 1 \times Tm hybridization buffer containing 2 μ g/ μ l streptavidin-*R*-phycoerythrin (Invitrogen) and incubated at 37°C for 15 min. A 80- μ l portion of the mixture was then analyzed at 35°C on a Luminex 100 analyzer, according to the system manual. For each sample, the instrument was set to read a minimum of 100 events for each type of beads, with the sample timeout setting being 90 s and the doublet discriminator gate setting being 8000 to 13,500. The bead sets were sorted, and the intensity of *R*-phycoerythrin fluorescence on each of the bead sets, corresponding to the alleles analyzed in the assay, was measured.

Step 5. Genotype determination. Genotypes of a given DNA sample for a specific SNP were determined on the basis of the calculated median fluorescence intensity ratios between variant allele and the total (variant + reference), after subtraction of background fluorescence intensity. The threshold ratio values for each genotype were empirically determined: a ratio of <0.25 was found to indicate a homozygous reference call, a ratio between 0.25 and 0.75 was found to indicate a heterozygous call, and a ratio of >0.75 was found to indicate a homozygous variant call, with exceptions for *CYP2C9**3 (<0.13, 0.13–0.35, and >0.35), *VKORC1* (<0.25, 0.25–0.77, and >0.77), and *POR* -173C>A (<0.19, 0.19–0.36, and >0.36). The relative fluorescence intensities of the specific beads used to label each pair of primers (reference versus variant) differed for each SNP, which led to the occasional SNP-specific differences in threshold values. The accuracy of the genotyping assays was validated for each SNP during method development using >30 samples with known genotypes and by additional genotype analysis of two to three randomly selected DNA samples using direct DNA sequencing; 100% concordance was obtained between the genotype calls assessed by direct sequencing and the results of Luminex assays. In addition, in rare cases when a DNA sample had a median fluorescence intensity ratio close to the threshold value, the sample was also resequenced.

Data Analysis. The χ^2 test for goodness of fit was used first to examine whether the genotype distribution of the SNPs studied in the patient population deviated from Hardy-Weinberg equilibrium. Spearman correlation analysis between every two SNPs was performed to see whether they were linked with each other. If the correlation coefficient (r) between the two SNPs was greater than 0.85 (arbitrary), we would choose only one of the two SNPs in the multivariate analysis.

Before the analysis of potential associations between the genotype (SNPs) and the phenotype (varying warfarin dose), the SNP genotype data were converted into quantitative codes: code = 2 if a SNP was a homozygous variant genotype, code = 1 if a SNP was a heterozygous variant genotype, and code = 0 if a SNP was a homozygous reference genotype. The stable warfarin maintenance dose (milligrams per week), which was treated as the dependent variable for linear regression analysis, was square root-transformed to meet the normal distribution requirement. Simple and multiple linear regression analyses were performed to determine whether the SNPs and the nongenetic factors were significantly associated with variations in warfarin dose requirement (using $P < 0.05$ as the criterion for significance). The square of the

TABLE 2
Genotype and allele frequency data for the 15 studied SNPs

SNPs Examined	Homozygous Variant Allele	Heterozygous Variant Allele	Homozygous Reference Allele	No. Samples Examined	Variant Allele Frequency
<i>CYP2C9</i> *2	1	25	96	122	0.111
<i>CYP2C9</i> *3	0	11	111	122	0.045
<i>VKORC1</i> -1639G>A	23	53	46	122	0.406
<i>STX1B</i> rs4889606	23	54	45	122	0.410
<i>CYP4F2</i> rs2108622	10	55	57	122	0.307
<i>POR</i> A503V	8	42	72	122	0.238
<i>POR</i> rs10280802	8	42	72	122	0.238
<i>POR</i> rs28737229	25	61	36	122	0.455
<i>POR</i> rs41301427	0	15	107	122	0.061
<i>POR</i> rs1057870	24	58	40	122	0.434
<i>POR</i> rs17148944	0	17	105	122	0.070
<i>POR</i> rs2868177	12	57	53	122	0.332
<i>POR</i> -173C>A	0	17	105	122	0.070
<i>POR</i> -208C>T	3	20	99	122	0.107
<i>POR</i> rs17685	7	41	74	122	0.225

correlation coefficient (R^2) was calculated, which shows the proportion of variations of the dependent variable (square root of the warfarin dose) that can be accounted for by variations of the independent variable(s), such as the SNPs studied. All analyses were performed using SAS 9.2 (SAS Institute, Cary, NC).

Results

The genotype data and the variant allele frequency data for the 15 selected SNPs are shown in Table 2. Among the 122 male patients, frequencies of variant alleles ranged from 4.5 to 46%. Genotype distribution for none of the 15 SNPs deviated from Hardy-Weinberg equilibrium, on the basis of the results from the χ^2 test for goodness of fit, at the $P < 0.05$ criterion. Correlation analysis performed on each SNP pair among the 15 SNPs identified three pairs that are highly correlated with each other: *POR* rs41301427 and *POR* rs17148944 ($r = 0.858$, $P < 0.0001$); *POR* rs17685 and *POR* A503V ($r = 0.969$, $P < 0.0001$); and *STX1B* rs4889606 and *VKORC1* -1639G>A ($r = 0.935$, $P < 0.0001$). Therefore, rs41301427, rs17685, and rs4889606 were not included in subsequent multiple linear regression analysis.

Association analysis of genetic and nongenetic factors with stable warfarin maintenance dose (square root-transformed) was performed through linear regression. Simple regression analysis of all the variables [15 SNPs, age, race, weight, and concomitant medications (aspirin, statins, multivitamin, insulin, and amlodipine)] revealed that age, *CYP2C9**2, *CYP2C9**3, *VKORC1* -1639G>A, *STX1B* rs4889606, and aspirin are significantly associated with the stable warfarin dose. The contribution (R^2) to dose variation was ~3.2% by age, ~12.2% by *CYP2C9**2, ~5.2% by *CYP2C9**3, ~21.4% by *VKORC1* -1639G>A, ~18.6% by *STX1B* rs4889606, and ~5.0% by aspirin (Table 3). However, note that the apparent contribution by *STX1B* probably reflects the contribution by *VKORC1*, given the close linkage between the two genes. None of the *POR* SNPs or the *CYP4F2* SNP showed a significant correlation with warfarin dose in simple regression analysis.

TABLE 3

Significant factors in the simple linear regression analysis ($P < 0.05$)

Variables	Parameter Estimate	Variable Significance in Model	R^2	Adjusted R^2
Age (yr)	-0.02065	$P = 0.0489$	0.0319	0.0239
<i>CYP2C9</i> *2	-0.97160	$P < 0.0001$	0.1224	0.1151
<i>CYP2C9</i> *3	-0.96394	$P = 0.0112$	0.0524	0.0445
<i>VKORC1</i> -1639G>A	-0.76681	$P < 0.0001$	0.2141	0.2076
<i>STX1B</i> rs4889606	-0.71861	$P < 0.0001$	0.1862	0.1794
Aspirin	-0.00426	$P = 0.0095$	0.0499	0.0420

Multiple regression analysis was performed on the variables listed above with rs41301427, rs17685, and rs4889606 excluded from the analysis. Table 4 shows the analysis results from multiple linear regression (backward: P value must be < 0.05 for a variable to stay in the model). The SNPs *CYP2C9**2, *CYP2C9**3, *VKORC1* -1639G>A, *CYP4F2* rs2108622, *POR* rs2868177, *POR* -173C>A, and *POR* -208C>T, weight, and aspirin were significantly associated with stable warfarin dose. The direction of association was negative for *CYP2C9**2, *CYP2C9**3, *VKORC1* -1639G>A, *POR* -173C>A, *POR* -208C>T, and aspirin, meaning that a patient with the indicated variant allele(s) or with a higher aspirin dose required lower doses of warfarin to achieve effective anticoagulation compared with a patient with the reference alleles or with a lower aspirin dose. In contrast, the direction of association was positive for *CYP4F2* rs2108622, *POR* rs2868177, and weight, meaning that a patient with the indicated variant allele(s) or higher body weight range required a higher dose of warfarin to achieve effective anticoagulation compared with a patient with the reference alleles or with lower body weight.

Various regression models were generated for estimation of the relative contributions of the variables studied to the variations in warfarin maintenance dose in the study population. The variables were categorized into four groups: 1) patient's demographic information, including age, weight, and race; 2) SNPs that are known to affect warfarin dose, including *CYP2C9**2, *CYP2C9**3, *VKORC1* -1639A>G, and *CYP4F2* rs2108622; 3) SNPs in the *POR* gene, including A503V, rs10280802, rs28737229, rs1057870, rs17148944, rs2868177, -173C>A, and -208C>T; and 4) other medications, including aspirin, amlodipine, multivitamin, insulin, and medicines containing statin. As shown in Table 5, when all of the variables studied were included at the first step of regression (model 1), nine

TABLE 4

Significant factors in the multiple linear regression analysis

Backward: $P < 0.05$ for variables to stay in the model.

Variable	Parameter Estimate	S.E.	F Value	$P > F$
Intercept	5.55931	0.40179	191.44	<0.0001
Weight	0.00431	0.00176	5.99	0.0159
<i>CYP2C9</i> *2	-0.87096	0.19831	19.29	<0.0001
<i>CYP2C9</i> *3	-1.07031	0.29295	13.35	0.0004
<i>VKORC1</i> -1639G>A	-0.81933	0.11751	48.62	<0.0001
<i>CYP4F2</i> rs2108622	0.37928	0.14053	7.28	0.0080
<i>POR</i> rs2868177	0.35491	0.15440	5.28	0.0234
<i>POR</i> -173C>A	-0.70359	0.26143	7.24	0.0082
<i>POR</i> -208C>T	-0.50989	0.21827	5.46	0.0213
Aspirin	-0.00287	0.00139	4.27	0.0411

TABLE 5

Linear regression models for estimation of relative contributions of the variables studied to the variations in warfarin maintenance dose

Backward, $P < 0.05$ for variables to stay in the model.

Regression Models	Variables Entered at the Beginning of Regression	Final Regression Equation	Model P Value	R^2
1. All variables	Patient's demographic information, ^a SNPs known to affect warfarin dose, ^b SNPs in <i>POR</i> gene, ^c other concomitant medications ^d	SQRT(D) = 5.559 + 0.004 (weight) - 0.871 (<i>CYP2C9*2</i>) - 1.070 (<i>CYP2C9*3</i>) - 0.819 (<i>VKORC1</i> -1639 A>G) + 0.379 (<i>CYP4F2</i> rs2108622) + 0.355 (<i>POR</i> rs2868177) - 0.704 (<i>POR</i> -173C>A) - 0.510 (<i>POR</i> -208C>T) - 0.003 (aspirin)	<0.0001	0.477
2. Without other medications	Patient's demographic information, ^a SNPs known to affect warfarin dose, ^b SNPs in <i>POR</i> gene ^c	SQRT(D) = 5.567 + 0.004 (weight) - 0.941 (<i>CYP2C9*2</i>) - 1.087 (<i>CYP2C9*3</i>) - 0.831 (<i>VKORC1</i> -1639 A>G) + 0.312 (<i>CYP4F2</i> rs2108622) + 0.357 (<i>POR</i> rs2868177) - 0.680 (<i>POR</i> -173C>A) - 0.556 (<i>POR</i> -208C>T)	<0.0001	0.457
3. Without other medications and <i>POR</i> SNPs	Patient's demographic information, ^a SNPs known to affect warfarin dose ^b	SQRT(D) = 5.650 + 0.005 (weight) - 0.869 (<i>CYP2C9*2</i>) - 1.091 (<i>CYP2C9*3</i>) - 0.751 (<i>VKORC1</i> -1639 A>G)	<0.0001	0.395

^a Age, weight, and race.

^b *CYP2C9*2*, *CYP2C9*3*, *VKORC1* -1639A>G, and *CYP4F2* rs2108622.

^c *POR* A503V, rs10280802, rs28737229, rs1057870, rs17148944, rs2868177, -173C>A, and -208C>T.

^d Aspirin, amlodipine, multivitamin, insulin, and medicines containing statin.

significant variables (as listed in Table 4) remained in the regression model, and the nine combined can explain 47.7% (R^2) of warfarin dose variation. After removal of concurrent medications (i.e., aspirin) from the analysis (model 2), eight significant variables remained in the model, and they can explain 45.7% (R^2) of warfarin dose variation. The difference in R^2 values (2%) between the two regression models represents the contribution of aspirin to warfarin dose variation.

After further removal of the three significant *POR* SNPs (rs2868177, -173C>A, and -208C>T) from the analysis (model 3), only four variables (*CYP2C9*2*, *CYP2C9*3*, *VKORC1* -1639G>A, and weight) remained significant in the model, and they can explain only 39.5% (R^2) of the warfarin dose variation. The difference in R^2 values between model 2 and model 3 was 6.2%, which represents the combined contributions of the three *POR* SNPs and the *CYP4F2* SNP to warfarin dose variation. Of note, because of the small sample size, we could not determine the specific contributions by the *POR* SNPs; however, given the previous reports (Caldwell et al., 2008; Takeuchi et al., 2009) indicating contributions by *CYP4F2* to be 1 to 2%, we estimate the actual contributions by the *POR* SNPs to be at least 4%. Further studies on a larger population are warranted to confirm this estimation.

The assumption of linearity in each of the three regression models was confirmed by residual analysis, as visualized on scatter plots of standardized residuals versus predicted values of the square root of warfarin dose (data not shown). In addition, as shown in Fig. 1, in plots of the square root of the actual warfarin dose that maintained the target INR within 2 and 3 versus the square root of the warfarin dose predicted by each of the three linear regression models, the scatter above and below the straight regression line was roughly the same.

Discussion

The current study investigated whether the common SNPs in the *POR* gene are associated with warfarin dose variation in patients undergoing anticoagulation therapy. Simple regression analysis confirmed that *VKORC1* -1639A>G, *CYP2C9*2*, *CYP2C9*3*, age, and chronic aspirin therapy were significantly associated with warfarin dose variation in our study population, whereas multiple regression analysis confirmed *CYP4F2* rs2108622 as an additional contributing factor and revealed that three common *POR* SNPs (-173C>A, -208C>T, and rs2868177) were also significantly associated with variations in warfarin maintenance dose. Our findings regarding the contributions of *CYP2C9*2*, *CYP2C9*3*, *VKORC1* -1639A>G, and

CYP4F2 rs2108622 to warfarin dose variation are consistent with results reported previously by others (Marsh and McLeod, 2006; Wadelius and Pirmohamed, 2007; Yin and Miyata, 2007; Caldwell et al., 2008; Limdi and Veenstra, 2008; Takeuchi et al., 2009). Our simple regression analysis also showed an association between the *STX1B* SNP and warfarin dose variation, which has not been reported previously; however, the result was not unexpected, given the known association of the *STX1B* SNP with *VKORC1* mRNA expression.

Both simple and multiple regression analyses were used here and in previous studies (see Takeuchi et al., 2009) to examine the relationships between various predictors and warfarin maintenance dose. Simple regression analysis, which estimates the impact of each individual predictor on the response variable without considering the impact of any other predictors, may fail to identify predictors that have a weak impact, as was demonstrated in previous work. Multiple regression analysis considers multiple predictors simultaneously; it yields an overall estimate of the impact of multiple predictors on a response variable, as well as estimates of the impact of individual predictors after controlling for the effects of other predictors. Multiple regression analysis may provide enhanced detection of predictors with weak impact on a response variable, as well as potential interactions among the variables tested; however, given the inclusion of multiple variables, it also requires larger sample sizes.

In our simple regression analysis, none of the *POR* SNPs was identified as being significantly associated with warfarin dose variation. Similar negative results were also found in the forward multiple regression analysis, in which the variables were sequentially added to the model in order of greatest significance to the model, rather than sequentially removed from the model in order of least significance to the model (data not shown). The fact that three *POR* SNPs were identified as being significantly associated with warfarin dose variation in the backward multiple regression analysis reflects the known dependence of P450 function on *POR* function and the reality that neither enzyme alone can metabolize warfarin. Furthermore, the impact of *POR* SNPs on warfarin dose requirement would be maximal in the presence of the P450 SNPs, given the theoretical possibility that a deficiency in P450 function can be either exacerbated or compensated for by variations in *POR* function.

It should be noted that, given the relatively small sample size and the large number of variables tested in the multiple regression models, our results should be viewed as exploratory, and the estimates of

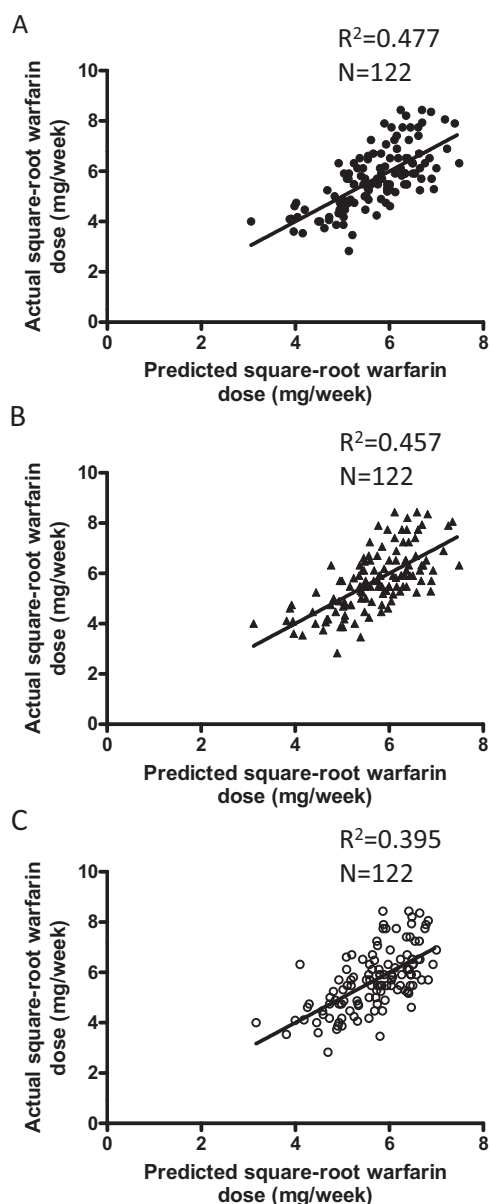


FIG. 1. Correlation between actual and predicted warfarin doses. The square root of the actual warfarin dose that maintained the target INR within 2 and 3 was compared with the square root of the warfarin dose predicted by the model, for different groups of variables used in the linear regression models (as in Table 5). A, all variables (model 1); B, without other medications (model 2); C, without other medications and *POR* SNPs (model 3).

regression will need to be replicated in additional, independent studies. Nonetheless, the positive identification of *CYP4F2*, a previously known minor contributor, in our multiple regression analysis seems to further support the validity of our findings with *POR*. In that connection, the specific contribution by *CYP4F2*, which could not be determined in our study because of the limited sample size, was estimated to be 1 to 2% on the basis of the results from two previous studies on a U.S. population and a northern European (Swedish) population (Caldwell et al., 2008; Takeuchi et al., 2009). The majority of the study subjects in these two latter studies, as in our study, were white. Differing results have also been reported in studies of other populations, with predicted *CYP4F2* contributions ranging from 0 to 11%. We did not consider the results from these other studies, given that they were conducted on populations from regions other than the

United States or northern Europe, such as Italy (Borgiani et al., 2009), Singapore (Singh et al., 2011), and Japan (Harada et al., 2010), and that there are known differences in the extent of genetic polymorphisms in genes relevant to warfarin dose requirement among various populations, even between northern (e.g., Swedish) and southern (e.g., Italian) European populations (Schelleman et al., 2008; Borgiani et al., 2009; Singh et al., 2011).

Our results indicate, for the first time, that three common SNPs in the *POR* gene may contribute to the interindividual variability in warfarin maintenance dose. Several reasons may explain why *POR* did not emerge as a significant contributor to warfarin dose in previous genome-wide association studies (GWASs) (Caldwell et al., 2008; Takeuchi et al., 2009). Takeuchi et al. (2009), who determined that *CYP4F2* accounted for ~1.5% of warfarin dose variance by multiple regression analysis, suggested that, although their study probably detected the most common SNPs that contribute >1.5% to warfarin dose variation, it may have failed to detect other common SNPs that have lower effects or rare variants with greater effects than that of *CYP4F2*. For the three *POR* SNPs found to be significant in our study, it is possible that their individual contributions were each <1.5%, thus making it difficult to detect in a GWAS. Furthermore, of the three *POR* SNPs, $-173C>A$ was newly identified and hence was not included in dbSNP or in any previous GWAS. As for the other two *POR* SNPs (*POR* $-208C>T$ and *POR* rs2868177), they were not included on the DMET chip (Caldwell et al., 2008), and it is not clear whether they were included in the published GWASs, given the reported call rates of <95% (Takeuchi et al., 2009). In addition, it is possible that the opposing directions of the influence of the *CYP2C9* and *CYP4F2* variant alleles might have prevented detection of the *POR* effects in the GWASs.

Given the small sample size, we were not able to estimate the relative contributions by each of the three significant *POR* SNPs. However, the fact that the three *POR* SNPs (which are all noncoding) had opposing effects (one positive and two negative) on warfarin dose suggests that the SNPs have a differential impact on *POR* expression, and this is currently being tested experimentally. *POR* $-173C>A$ (genomic position: 75382183) and *POR* $-208C>T$ (genomic position: 75382148) are located just upstream of the *POR* transcription start site and were identified here to be negatively associated with warfarin dose. Database analysis for potential transcription factor binding sites, using TFsearch (<http://www.cbrc.jp/research/db/TFSEARCH.html>) (Heinemeyer et al., 1998), showed that *POR* $-173C>A$ and *POR* $-208C>T$ are both located within potential binding sites for multiple transcription factors, such as the homeo domain factor Nkx-2.5 (Chen and Schwartz, 1995) (identity score 81.4 for *POR* $-173C>A$) and alcohol dehydrogenase gene regulator 1 (Cheng et al., 1994) (identity score 81.5 for *POR* $-208C>T$). There are two possible mechanisms to explain how these two *POR* SNPs might affect warfarin metabolism: one is that these two upstream SNPs may decrease *POR* expression and thus lower metabolism of warfarin through P450 pathways. Another possible mechanism is that the two SNPs are in linkage disequilibrium with other unknown SNPs that affect *POR* expression. In that connection, Tee et al. (2011) reported that neither of the two promoter region SNPs had an impact on the transcription of a 325-base pair *POR* basal promoter in reporter gene assays. Thus, it appears that the second mechanism is more likely; i.e., other unknown SNPs that are in linkage disequilibrium with the SNP at -178 or -208 are critical for *POR* expression. Further studies are needed to clarify these issues. *POR* rs2868177, which showed positive correlation with warfarin dose, is in linkage disequilibrium with rs2868180, rs7804806, and rs2868178; all four SNPs are located centrally in intron 2 of *POR*.

The underlying mechanisms for the involvement of *POR* rs2868177 are unclear.

The common *POR* coding region polymorphism A503V, with an allele frequency of 19 to 36%, causes 30 to 40% reduction in POR activity (Huang et al., 2005, 2008). However, the impact of A503V on P450 activity has been found to be variable, depending on the P450 enzyme involved and the substrate studied (Arlt et al., 2004; Gomes et al., 2008, 2009; Miller et al., 2009, 2011; Oneda et al., 2009; Sandee et al., 2010). The ability of this variant to support P450-mediated warfarin metabolism has not been examined in vitro. In the present study, we did not find a significant correlation between *POR* A503V and warfarin dose in patients. It appears that this SNP may not affect warfarin metabolism through either the CYP2C9 or the CYP4F2 pathway.

In conclusion, three SNPs in the *POR* gene are significantly associated with variable warfarin dose. Further examination of a complete list of common *POR* SNPs, in a larger patient population and molecular studies on the functional impact of the significant SNPs on POR expression/function are needed to better understand the nature and extent of the impact of *POR* SNPs on warfarin dose requirement. In addition, studies on the potential functional impact of the *POR* SNPs on the in vivo metabolism of numerous additional drugs are warranted.

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Authorship Contributions

Participated in research design: Zhang, Ding, and Kaminsky.
Conducted experiments: Zhang and Li.
Performed data analysis: Zhang and Li.
Wrote or contributed to the writing of the manuscript: Zhang, Li, Ding, and Kaminsky.

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