Association Between CYP2C9 Genetic Variants and Anticoagulation-Related Outcomes During Warfarin Therapy

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Context Warfarin is a commonly used anticoagulant that requires careful clinical management to balance the risks of overanticoagulation and bleeding with those of underanticoagulation and clotting. The principal enzyme involved in warfarin metabolism is CYP2C9, and 2 relatively common variant forms with reduced activity have been identified, *CYP2C9*2* and *CYP2C9*3*. Patients with these genetic variants have been shown to require lower maintenance doses of warfarin, but a direct association between CYP2C9 genotype and anticoagulation status or bleeding risk has not been established.

Objective To determine if *CYP2C9*2* and *CYP2C9*3* variants are associated with overanticoagulation and bleeding events during warfarin therapy.

Design and Setting Retrospective cohort study conducted at 2 anticoagulation clinics based in Seattle, Wash.

Participants Two hundred patients receiving long-term warfarin therapy for various indications during April 3, 1990, to May 31, 2001. Only patients with a complete history of warfarin exposure were included.

Main Outcome Measures Anticoagulation status, measured by time to therapeutic international normalized ratio (INR), rate of above-range INRs, and time to stable warfarin dosing; and time to serious or life-threatening bleeding events.

Results Among 185 patients with analyzable data, 58 (31.4%) had at least 1 variant CYP2C9 allele and 127 (68.6%) had the wild-type (**1/*1*) genotype. Mean maintenance dose varied significantly among the 6 genotype groups (**1/*1* [n=127], **1/*2* [n=28], **1/*3* [n=18], **2/*2* [n=4], **2/*3* [n=3], **3/*3* [n=5]) (by Kruskall-Wallis test, χ^2_5=37.348; $P<.001$). Compared with patients with the wild-type genotype, patients with at least 1 variant allele had an increased risk of above-range INRs (hazard ratio [HR], 1.40; 95% confidence interval [CI], 1.03-1.90). The variant group also required more time to achieve stable dosing (HR, 0.65; 95% CI, 0.45-0.94), with a median difference of 95 days (P=.004). In addition, although numbers were small for some genotypes, representing potentially unstable estimates, patients with a variant genotype had a significantly increased risk of a serious or life-threatening bleeding event (HR, 2.39; 95% CI, 1.18-4.86).

Conclusions The results of our study suggest that the *CYP2C9*2* and *CYP2C9*3* polymorphisms are associated with an increased risk of overanticoagulation and of bleeding events among patients in a warfarin anticoagulation clinic setting, although small numbers in some cases would suggest the need for caution in interpretation. Screening for CYP2C9 variants may allow clinicians to develop dosing protocols and surveillance techniques to reduce the risk of adverse drug reactions in patients receiving warfarin.

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Warfarin is an anticoagulant agent used for the prevention of thromboembolic events in patients with chronic conditions such as atrial fibrillation, and is prescribed to more than 1 million patients in the United States annually.[1] Because warfarin has a narrow therapeutic range and may increase the risk of bleeding events, therapy is individualized by monitoring the prothrombin time international normalized ratio (INR), a measure of anticoagulation status. The management of warfarin therapy is challenging because of variability in patient response due to a multitude of factors including drug, diet, and disease-state interactions.[1] In addition, genetic variation of the hepatic microsomal enzyme CYP2C9, the activity of which constitutes the primary pathway for the metabolism of S-warfarin, may lead to significant differences in patient response to warfarin.

Two common variant alleles (polymorphisms) of CYP2C9 have been identified.[2-10] The **2* allele (*R144C*) and the **3* allele (*I359L*) cause decreased enzymatic activity of 30% and 80%, respectively.[11,12] The frequencies of the **2* and **3* alleles have been estimated at 11% and 7%, respectively.[13]

Several studies have evaluated the association of these polymorphisms with

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clinical phenotypes in patients treated with warfarin. Aithal et al[14] compared individuals having at least 1 variant CYP2C9 allele with individuals having the wild-type (*1/*1) genotype. They reported significant associations between variant CYP2C9 genotype and low-dose requirements for warfarin, and between low-dose warfarin requirements (but not genotype) and major but not minor bleeding events. Taube et al[15] also found a significantly lower maintenance dose in patients with a variant allele vs patients having the wild-type genotype, but did not find evidence of an association between genotype and anticoagulation status (as assessed via INR). Loebstein et al,[16] using multiple regression analysis, reported that CYP2C9 genotype was independently associated with warfarin maintenance doses. Thus, although the *2 and *3 polymorphisms have been associated with lower warfarin dose requirements, a direct association with anticoagulation status or bleeding events has not been reported.

The current study differs from prior studies in that it was designed to evaluate the association between variant CYP2C9 alleles and clinical outcomes such as anticoagulation status and bleeding events. We conducted a retrospective cohort study of patients managed in a university hospital–based anticoagulation clinic. In the study, strict inclusion criteria and direct genetic sequencing were used, and adjustments made for potential confounders. Although medical management practices are crucial in determining anticoagulation state, we hypothesized that variant CYP2C9 alleles would also play a role and result in a longer time to therapeutic INR, a higher rate of out-of-range INRs, a longer time to achieve stable dosing, and a higher risk of serious or life-threatening bleeding events.

METHODS

Study Setting and Design

This study was approved by the Human Subjects Review Committee at the University of Washington. The study was conducted at the pharmacist-run anticoagulation clinics affiliated with the University of Washington Medical Center (UWMC), Seattle. Five hundred twenty-six patients attend these clinics at regular intervals of 2 to 6 weeks. The mean age of patients in the clinic is 60 years, 54% are male, and the distribution of ancestry is 91.0% European, 4% Asian, 3% African, and 2% Hispanic.

Patient care is managed via a physician-approved prescriptive authority protocol that provides a standardized approach to dosing adjustments based on INR results, management of overanticoagulation and underanticoagulation, and frequency of follow-up. For patients in whom warfarin therapy was initiated after enrollment in clinic, dosing was adjusted based on a protocol developed by Harrison and colleagues.[17] Dosing adjustments during maintenance therapy were made according to previously published protocols.[18] Once receiving maintenance therapy, patients requiring dosage adjustments were reevaluated within a maximum of 2 weeks. Routine follow-up of medically stable and reliable patients occurred every 4 to 6 weeks.

Retrospective clinical data were abstracted from medical charts, anticoagulation records, and prescription records. Genotype data were collected from patients currently attending the anticoagulation clinics. The investigators were blinded to genotype information, as DNA analysis was not conducted until data abstraction was completed.

Power calculations were performed using Egret Sample Size Module v1.01.03 (Statistics and Epidemiology Research Corp, Seattle, Wash). Analysis of the UWMC clinic data revealed an above-range INR rate of 0.32 per patient-year and mean follow-up time of 2 years per patient. A study size of 200 patients was estimated to have 80% power to detect an above-range INR hazard of 2.0 at an α of .05 (2-tailed).

Eligibility

Eligibility criteria included patients with a confirmed index date of first warfarin exposure, patients currently undergoing anticoagulation therapy, and patients older than 18 years. Our goal was to determine the genotype of patients with accurate warfarin initiation information in order to include the effect of CYP2C9 genotype following the post-initiation period when the risk of overanticoagulation is highest and the subsequent bleeding risk is also highest.[19,20] Patients attending the clinic at some point during April 3, 1990, to May 31, 2001, were eligible for the study.

Exclusion criteria were (1) patients of Asian or African descent (n=36), (2) patients managed via telephone rather than in person (n=185), (3) inability to obtain verbal and written consent (non–English-speaking) (n=5), (4) inability to draw blood (n=3), and (5) inability to ascertain the index date (n=11). We excluded patients of known Asian and African descent because CYP2C9 allele frequencies are associated with ethnicity and the UWMC anticoagulation clinic population, being predominantly (91.0%) white, could not provide a sample of sufficient size to control for potential confounding in a regression model.[3,5,21-23] The study base was restricted to patients currently attending the local anticoagulation clinics because a blood sample is required for the genotyping portion of the analysis. The study base was further restricted to patients who were at least 18 years of age at the index date due to age criteria specified by the Human Subjects Review Committee. There was no restriction on the amount of follow-up time required for each patient. Patients were screened to determine their eligibility and enrolled if informed consent was obtained. Two hundred eighty-six patients (54.4%) were determined eligible to participate.

Clinical Data Collection

Data collection consisted of a review of inpatient and outpatient medical records and a venous blood sample from consenting participants. Two trained abstractors collected data using standardized abstract forms.

We used the UWMC anticoagulation database to obtain information on INR measurements, warfarin daily dose, prescription drugs, over-the-counter drugs, and vitamin use. This database

has been used since 1994 by all UWMC-affiliated clinics to maintain records for patients receiving long-term warfarin therapy. Data for patients before 1994 (n=4) were obtained from the paper chart.

We used the electronic medical records database MINDscape (University of Washington, Seattle) to obtain information on bleeding events, comorbid conditions, and demographic variables. The MINDscape database has been used by the UWMC and affiliated clinics to record medical records electronically since 1994. Information obtained from this database was supplemented and confirmed by reviewing the paper medical chart. For bleeding events, the concordance between these 2 data sources was 94%.

Genotyping

Blood (5 mL) was drawn in a sodium citrate vacutainer during a regularly scheduled blood draw. Genomic DNA isolated from whole blood using the Qiagen blood kits (Qiagen Inc, Chatsworth, Calif) was amplified using intron-specific primers for exon 3 and exon 7 of the gene *CYP2C9*, yielding 490-base pair (bp) and 284-bp fragments, respectively (exon 3 [*2] sense: 5′-GCTGCATGGATATGAAGCA-3′, antisense: 5′-CCAAGAATGTCAGTAGAGAAGATAG-3′; exon 7 (*3) sense: 5′-CTCCTTTTCCATCAGTTTTTACT-3′, antisense: 5′-GATACTATGAATTTGGGACTTC-3′). Both exons were amplified by polymerase chain reaction (PCR) and subsequently sequenced using the ABI Prism 377 and BigDye dye terminator cycle sequencing (Applied Biosystems, Foster City, Calif). The PCR for the Arg144 to Cys144 variant in exon 3 was performed in 10× PCR buffer (Qiagen, Inc), 3.0 mM of $MgCl_2$, 0.4 mM of each primer, 0.2 mM of dNTPs, 0.15 U of HotStarTaq DNA polymerase (Qiagen Inc), and approximately 100 ng of genomic DNA. The PCR for the Ile359 to Leu359 variant in exon 7 was performed in 10× PCR buffer (Life Technologies, Rockville, Md), 1.6 mM of $MgCl_2$, 0.24 mM of each primer, 0.2 mM of dNTPs, 0.02 U of Taq polymerase (Life Technologies), and approximately 100 ng of genomic DNA. The BigDye-based reactions were performed according to the manufacturer's protocol.

All *CYP2C9**2* and *CYP2C9**3* homozygotes detected during primary sequencing were confirmed by sequencing the reverse complementary strand using a second PCR reaction. In addition, randomly selected heterozygote variants (n=19) and wild-type homozygotes (n=34) were confirmed using the same method.

Outcomes

The primary end point of this study was anticoagulation status, as measured by INR in 3 ways: time to therapeutic INR, rate of above-range INRs, and time to stable warfarin dosing (defined as 3 consecutive clinic visits for which INR measurements were within therapeutic range for the same mean daily dose).[14] Once a patient achieved stable dosing, we recorded the maintenance dose in order to compare mean maintenance doses among the various CYP2C9 genotypes. Therapeutic INR was defined as the first INR measured within the optimal therapeutic range for a given indication. If the target therapeutic range was 2.0 to 3.0,[1] then INRs between 2.0 and 3.0 were defined as within range. Above-range INRs were defined as measurements of 4.0 or greater.[14] Twelve patients receiving warfarin therapy for prosthetic valve replacement were anticoagulated at a higher target range (2.5-3.5).[1] Consequently, these patients required an above-range INR definition of 4.5 or greater to account for their higher baseline level of anticoagulation.[24] This approach was used to try to ensure that the 12 patients did not contribute a disproportionate number of events to the analysis.

Although INR values between 3.0 and 4.0 (3.5-4.5 for patients with prosthetic valves) were not defined as "above-range," this does not mean that clinicians necessarily consider these levels of anticoagulation "normal." However, in given instances, these levels could be regarded as acceptable, factoring in perceived thromboembolic risk, diet or drug changes, or compliance.

We recorded every INR value for all patient visits. In this data set, there were 5415 INR measurements obtained for patients with a 2.0 to 3.0 target range. For these patients, there were 676 observations (12.5%) occurring between 3.01 and 3.99. For the 12 patients with prosthetic valves, there were 850 INR measurements obtained. For these patients, there were 180 observations (21.2%) between 3.51 and 4.49.

The minimum cutoff for above-range INR was considered to be 4.0 because INR measurements above 4.0 are less likely to be misclassified as above-range when compared with measurements below 4.0.[25] Also, INR values between 3.0 and 4.0 are not considered to be strong predictors of bleeding risk.[19,26] Levels of 4.0 or greater have been previously used[14] to define "above range."

The above-range INR model was modified to include recurrent events. All 185 patients (see below) contributed exposure time to this analysis. The sample size and exposure time would not change regardless of how above-range INR is defined; it would only change the number of above-range events contributed by each patient. A higher cutoff would result in fewer events contributed by each patient and less power to detect an association. For this study, an INR of 4.0 was considered to be the minimum clinically relevant event (see above).

The secondary end point was time until first serious or life-threatening bleeding event. We used the criteria of Fihn et al[20] to classify bleeding episodes as serious (requiring treatment or medical evaluation) or as life threatening. Examples of serious bleeding included overt gastrointestinal bleeding, occult gastrointestinal bleeding if endoscopic or radiographic studies were performed, gross hematuria that prompted cystoscopy or intravenous urography or lasted more than 2 days, and hemoptysis. Episodes involving blood transfusions of 2 units or more were classified as serious bleeding events. We defined life-threatening bleeding events as those

leading to cardiopulmonary arrest, surgical or angiographic intervention, or irreversible sequelae such as myocardial infarction, neurologic deficit consequent to intracerebral hemorrhage, or massive hemothorax. Bleeding was also considered to be life threatening if it resulted in 2 of the following consequences: (1) loss of 3 or more units of blood, (2) systolic hypotension (<90 mm Hg), or (3) critical anemia (hematocrit of 20% or less).

Statistical Analysis

The associations between CYP2C9 genotype and the primary and secondary end points were evaluated using survival analysis techniques. Patients were divided into 2 groups based on genotype: wild type (*CYP2C9*1/*1* homozygotes) and variant (1 or more mutant alleles). For each analysis, a hazard ratio (HR) and 95% CI comparing variant and wild-type genotype groups were computed. We used Cox proportional hazards models to adjust for the potential confounding effect of sex, age, warfarin indication, comorbid conditions, prescription medications, and over-the-counter products (TABLE 1) and to increase the precision of the model.[27,28] Any prescription medicine metabolized by CYP2C9 and used by at least 5% of this clinic population was considered in the analysis. Covariates were added to the model 1 at a time to assess potential confounding effects on the variant genotype HR. A covariate was defined to have an important effect on the HR if the HR changed by more than 5% upon inclusion of the covariate in the model. Duration of warfarin therapy was measured in days. Patients were followed up from the index date of first warfarin exposure until either the date of an event observation or the end-of-study date (May 31, 2001), when all data were subject to administrative right-censoring. Because this study required a blood sample at enrollment followed by a retrospective chart review, no patients were withdrawn or lost to follow-up.

To account for changes in the prescribed dose of warfarin, we programmed mean daily dose as a time-varying covariate. When there was a change in warfarin dose or a new INR value, we updated the regression model's covariates accordingly. Consequently, the regression model always used patients' most recent mean dose to adjust the HR for genotype.

Individuals could contribute more than 1 event to the above-range INR outcome. We stratified the analysis on patients' history of above-range INR values so that patients who experienced a recurrent above-range INR were always compared with patients who had experienced the same number of prior above-range INRs. In doing so, we also produced valid CIs for recurrent events that do not violate the independence assumption.

In order to assess potential confounding, we fit each covariate (Table 1) to the model to determine changes to the exposure coefficient. We also fit interaction terms to assess potential effect modification of the genotype exposure by each covariate. We then conducted model diagnostics and identified potentially influential cases, ie, the 12 patients having prosthetic valves with a higher target INR range (2.5-3.5). An analysis was performed in which they were excluded to determine their effect on the exposure HR. Statistical testing of the Schoenfeld residuals and graphical assessment revealed no significant departure from the proportional hazards assumption.

Testing for deviation of genotype frequencies from Hardy-Weinberg equilibrium was calculated by applying the Hardy-Weinberg model to our data.[29] For comparing expected vs actual preva-

Table 1. Comparison of Subject Characteristics for Patients With Variant vs Wild-Type CYP2C9 Genotypes*

Variable	Wild-Type	Variant	All Patients
Demographics			
Subjects, No. (%)	127 (68.6)	58 (31.4)	185
Men, No. (%)	83 (65.4)	35 (60.3)	118 (63.8)
White, No. (%)	121 (95.3)	57 (98.3)	178 (96.2)
Age, mean (SD), y	58.4 (16.5)	61.6 (14.1)	59.9 (15.7)
Indication for warfarin, No. (%)			
Atrial fibrillation	67 (53.2)	28 (48.3)	95 (51.4)
Dilated cardiomyopathy	27 (21.3)	11 (19.0)	38 (20.5)
DVT/PE	27 (21.3)	13 (22.4)	40 (21.6)
Valve replacement	6 (4.7)	6 (10.3)	12 (6.5)
Follow-up, d			
Mean	823	807	818
Median	617	422	543
Range	14-2670	23-4032	14-4032
Comorbid conditions, No. (%)			
Arrhythmia	54 (42.5)	28 (48.3)	82 (44.3)
Congestive heart failure	52 (40.9)	22 (37.9)	74 (40.0)
Type 1 diabetes mellitus	5 (3.9)	1 (1.7)	6 (3.2)
Type 2 diabetes mellitus†	27 (21.3)	5 (8.6)	32 (17.3)
Malignancy	15 (11.8)	11 (19.0)	26 (14.1)
Tobacco dependence	20 (15.7)	7 (12.1)	27 (14.6)
Hypertension	59 (46.5)	22 (37.9)	81 (43.8)
Prescription medication, No. (%)			
Amiodarone	17 (13.4)	7 (12.1)	24 (13.0)
Losartan	15 (11.8)	3 (5.2)	18 (9.7)
Torsemide	7 (5.5)	2 (3.4)	9 (4.9)
Over-the-counter products, No. (%)			
Acetaminophen	34 (26.8)	17 (29.3)	51 (27.6)
Vitamin C	15 (11.8)	12 (20.7)	27 (14.6)
Vitamin E†	13 (10.2)	12 (20.7)	25 (13.5)

*DVT indicates deep vein thrombosis; PE, pulmonary embolus.
†Variant and wild-type groups differed significantly ($P<.05$) using the χ^2 test.

lence of each CYP2C9 genotype, because of small samples for some genotypes, the χ^2 goodness-of-fit test could not be used. Thus, the likelihood ratio test was used, which is appropriate for use with small sample sizes when testing for Hardy-Weinberg equilibrium with rare allele frequencies.[30,31] The likelihood ratio test statistic has an approximate χ^2 distribution with 3 *df*. With the general model, there are 6 parameters (6 different genotype frequencies) and 1 constraint, giving 5 *df*. With the Hardy-Weinberg model, there are 3 parameters (3 allele frequencies) and 1 constraint, giving 2 *df*. Thus, the *df* for the χ^2 test statistic is equal to the dimensions of the null hypothesis of Hardy-Weinberg equilibrium subtracted from the dimensions of the general model (5−2=3).

We calculated the HR for bleeding events during the first 3 months of treatment and during the entire follow-up period. We did not calculate the HR for only the maintenance phase because patients became "at risk" for a bleeding event on the index date of first warfarin administration (time zero). Using survival analysis techniques, we were able to examine the risk conferred by genotype during initiation, and during initiation and maintenance phases combined. However, we could not assess the risk during the maintenance phase exclusively because this would involve recoding time zero at a later point, which could introduce bias if persons with variant genotypes have more first bleeding events during initiation and are thus censored, and would not allow us to define the time until first bleeding event following warfarin administration. An unadjusted incidence rate ratio was also calculated for bleeding events by taking the ratio of the unadjusted bleeding rates in the wild-type and variant genotype groups.

Table 2. Expected vs Actual Prevalence of CYP2C9 Genotypes Based on Hardy-Weinberg Equilibrium*

Genotype	Expected Prevalence, %	Actual Prevalence, No. (%)
*1/*1	65.7	127 (68.6)
*1/*2	17.1	28 (15.1)
*1/*3	13.6	18 (9.7)
*2/*2	1.1	4 (2.2)
*2/*3	1.8	3 (1.6)
*3/*3	0.7	5 (2.7)

*Hardy-Weinberg $\chi^2_5 = 10.454$; $P = .02$. Because of small samples for some genotypes, the χ^2 goodness-of-fit test could not be used. Thus, the likelihood ratio test was used, which is appropriate for use with small sample sizes when testing for Hardy-Weinberg equilibrium with rare allele frequencies (see text).

RESULTS

Of the 286 patients eligible for participation, 213 were randomly approached regarding participation in this study. Thirteen patients declined to participate, resulting in 200 enrolled patients. Two patients subsequently were excluded due to African ancestry, 2 were excluded due to warfarin therapy prior to their clinic initiation date, and 1 was excluded due to a liver transplant. Ten samples were excluded because of difficulties in generating PCR products for either exon 3 or exon 7. There were 185 patients available for analysis—127 with the wild-type genotype, and 58 with a variant genotype.

Table 1 summarizes the main characteristics of the cohort. The mean age of all patients at the start of therapy was 59.9 years, and 63.8% of the patients were men. White patients comprised 100% of the sample with 3.8% of those classified as Hispanic ethnicity. The majority of patients (51%) were receiving warfarin for atrial fibrillation. The mean follow-up time was 818 days (2.24 years). Patients were seen a median of 23 times over the follow-up period and for a median of 543 days. With the exception of type 2 diabetes mellitus and use of vitamin E, there were no significant differences in characteristics between patients with the wild-type genotype (*1/*1) and patients with at least 1 variant allele.

TABLE 2 summarizes the prevalence of each genotype within the cohort. The allelic frequencies were similar to those from a large study from Sweden.[13] However, the prevalence of the *CYP2C9*3/*3* genotype was 4 times greater than expected based on Hardy-Weinberg calculations.[29] In addition, we discovered that 2 of the *CYP2C9*1/*1* patients had a novel polymorphism, a C/T at nucleotide 1003 (GenBank Accession NM 00771) resulting in an Arg→Trp substitution at position 335 of exon 7. The mean daily dose of warfarin for these 2 patients was 5.00 and 5.18 mg.

TABLE 3 summarizes the mean maintenance dose of warfarin stratified by genotype. Maintenance dose was significantly related to genotype. Furthermore, a possible gene-dose relationship is suggested when comparing the *1/*1, *1/*2, and *1/*3 genotypes, with mean maintenance doses of 5.63, 4.88, and 3.32 mg, respectively (Table 3).

The unadjusted incidence rate of bleeding complications (serious and life threatening combined [n=32]) was 7.72 per 100 patient-years (TABLE 4). For patients with a variant allele, the rate of se-

Table 3. Prescribed Daily Dose of Warfarin in Relation to CYP2C9 Genotype*

	Genotype					
	*1/*1	*1/*2	*1/*3	*2/*2	*2/*3	*3/*3
No.	127	28	18	4	3	5
Daily maintenance warfarin dose, mg						
Mean (SD)	5.63 (2.56)	4.88 (2.57)	3.32 (0.94)	4.07 (1.48)†	2.34 (0.35)	1.60 (0.81)
Median (IQR)	5.27 (3.93-7.14)	4.64 (3.61-5.29)	2.92 (2.50-3.93)	3.86 (2.50-4.00)	2.32 (2.00-2.70)	1.61 (1.14-1.96)

*Kruskal-Wallis $\chi^2_5 = 37.348$; $P<.001$. IQR indicates interquartile range.
†Mean doses (mg) for the 4 patients were 2.50, 3.71, 4.00, and 6.07. The mean of these is 4.07; however, the 75th percentile is 4.00. The patient with the mean daily dose of 6.07 had a prosthetic valve and experienced a serious bleeding event. This reflects the potential skewness that data from patients with prosthetic valves can introduce to small samples and reflects the range of maintenance doses that can occur in a clinical setting. An analysis was performed in which 12 patients having prosthetic valves with a higher target international normalized ratio range (2.5-3.5) were excluded; the effect on hazard ratio estimates was trivial (see text).

Table 4. Person-Years of Follow-up and First Bleeding Events Stratified by CYP2C9 Genotype

Characteristic	Genotype						Total (N = 185)
	*1/*1 (n = 127)	*1/*2 (n = 28)	*1/*3 (n = 18)	*2/*2 (n = 4)	*2/*3 (n = 3)	*3/*3 (n = 5)	
Follow-up, person-years	286.29	63.93	39.51	8.84	1.02	14.91	414.49
Bleeding events							
Serious	14	6	4	2	1	1	28
Life-threatening	2	0	1	0	0	1	4

rious bleeding events (n=14) was 10.92 per 100 patient-years, and the rate of life-threatening bleeding events (n=2) was 1.56 per 100 patient-years. For patients with the wild-type genotype, the rate of serious (n=14) and life-threatening (n=2) bleeding was 4.89 and 0.70 per 100 patient-years, respectively. Patients with the variant genotype experienced a significantly higher bleeding rate. For variant vs wild-type genotype, the unadjusted incidence rate ratio for serious and life-threatening bleeding combined was 2.23 (95% CI, 1.05-4.77).

TABLE 5 summarizes the main findings of the study. For all 3 Cox models using INR measurements as end points, the time-varying covariate, warfarin daily dose, was included in the final model as a possible confounder. None of the covariates (sex, age, warfarin indication, comorbid conditions, prescription medications, and over-the-counter products) added in the stepwise model-fitting procedure produced remarkable changes (ie, greater than 5%) to the HR estimate of bleeding risk. The exclusion of 12 patients having prosthetic valves and a higher target INR range (2.5-3.5) resulted in only trivial effects on the HR estimates. The Kaplan-Meier curves for time to first therapeutic INR value did not differ significantly between groups as evaluated by the log-rank test ($P=.63$) (FIGURE, A). This finding did not change after adjusting for other covariates with a Cox regression model (Table 5). The curves showing time until first above-range INR did not differ significantly between groups ($P=.10$) (Figure, B). Similarly, the curves for a second event ($P=.27$) and third event ($P=.38$) did not differ significantly (M.K.H., unpublished data, November 2001). However, when we adjusted for warfarin daily dose in a Cox regression model, patients with the variant genotype showed an increased risk of above-range INRs (HR, 1.40; 95% CI, 1.03-1.90). The variant group required more time to achieve stable dosing compared with the wild-type group ($P=.004$) (Figure, C), with a median difference of 95 days. The slower rate of stabilization for the variant group was confirmed in a Cox regression model (HR, 0.65; 95% CI, 0.45-0.94) (Table 5).

Table 5. Unadjusted and Adjusted (for Warfarin Daily Dose) HRs for Clinical Outcomes in Patients Having the CYP2C9 Variant Genotype*

End Point	HR (95% CI)	
	Unadjusted	Adjusted†
Time to therapeutic INR	0.91 (0.65-1.27)	0.95 (0.68-1.34)
Time to above-range INR	1.28 (0.94-1.74)	1.40 (1.03-1.90)
Time to stable dosing	0.60 (0.42-0.85)	0.65 (0.45-0.94)
Time to bleeding event		
Initiation phase (first 3 mo)	3.94 (1.29-12.06)	NA
Entire follow-up period	2.39 (1.18-4.86)	NA

*Excluding 12 patients with prosthetic valves, the hazard ratios (HRs) for time to therapeutic international normalized ratio (INR) were 0.99 (95% confidence interval [CI], 0.71-1.38); time to above-range INR, 1.48 (95% CI, 1.04-2.11); time to stable dosing, 0.59 (95% CI, 0.40-0.85); and time to first serious/life-threatening bleeding event, 2.54 (95% CI, 1.18-5.46). NA indicates not applicable (no covariates were included in the final model because they did not result in a >5% change in HR of bleeding risk [see text]).
†Warfarin daily dose was the only covariate used in this adjustment of the HR.

Patients with the variant genotype experienced a first bleeding event sooner than patients with the wild-type genotype ($P=.01$) (Figure, D). In the Cox model, we examined the effect of variant genotype on bleeding risk during the initiation phase of therapy by censoring the data at 90 days postinitiation. Variant genotype increased the risk of bleeding during the initiation phase (HR, 3.94; 95% CI, 1.29-12.06). When all the follow-up data were considered (initiation and maintenance phase), variant genotype still conferred an increased risk of bleeding (HR, 2.39; 95% CI, 1.18-4.86).

COMMENT

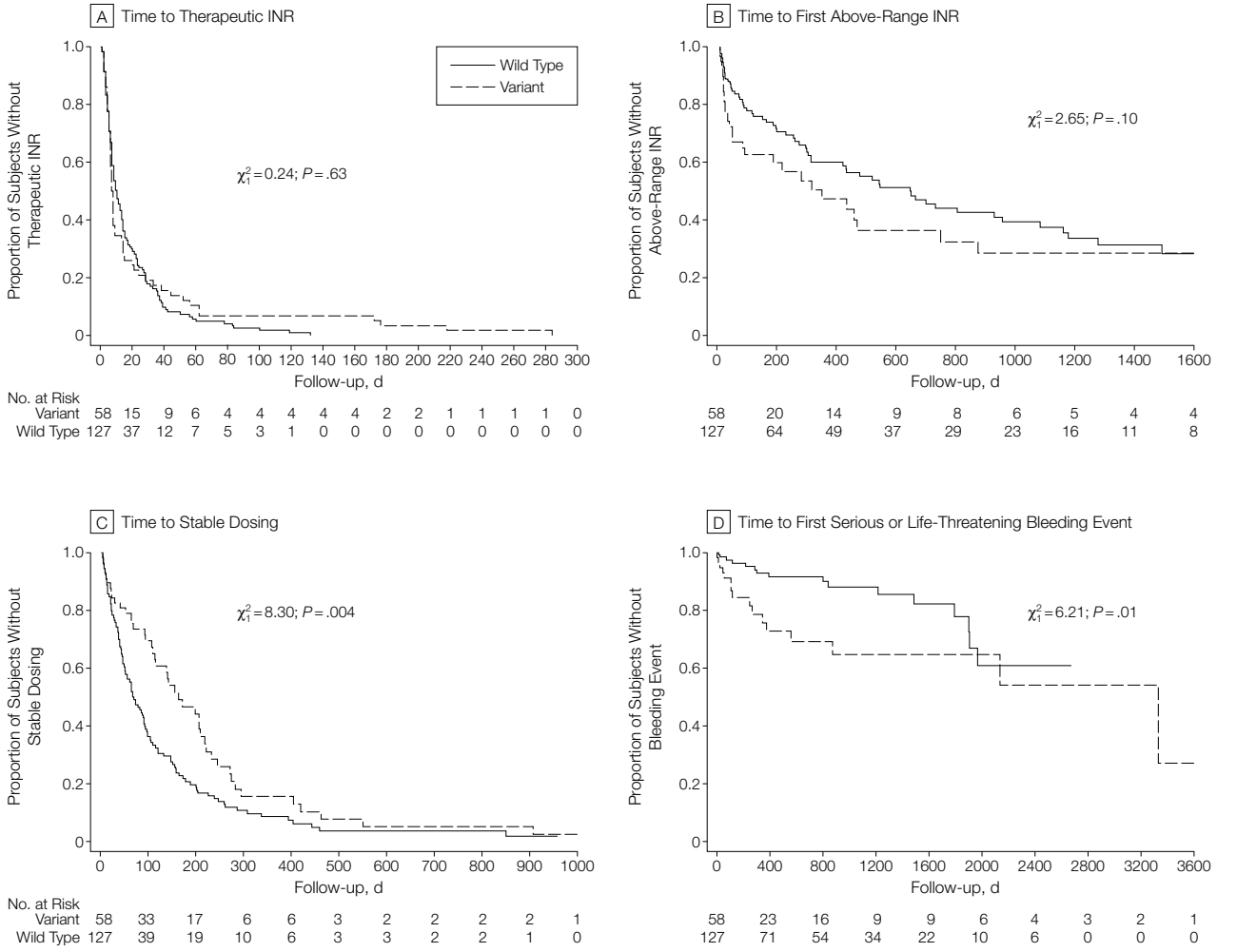
The results of this study suggest that CYP2C9 genotype is associated with (1) warfarin maintenance dose, (2) time to stable warfarin dosing, (3) rate of above-range INRs, and (4) bleeding events in patients taking warfarin. During the initiation period of warfarin therapy, it appears that patients with variant CYP2C9 alleles become overanticoagulated at a faster rate and must undergo additional dose adjustments, thus translating into a longer time until stable dosing is achieved. When these patients do become stable, their daily maintenance dose of warfarin is significantly lower than that of patients without genetic impairment of warfarin metabolism.

Patients with variant CYP2C9 alleles also experience a higher risk of serious and major bleeding events, although numbers were small for some genotypes, representing potentially unstable estimates, suggesting the need for caution in interpretation. Fihn et al[20]

found that recent initiation (first 90 days) of warfarin therapy compared with any time thereafter was an independent predictor of first-episode serious bleeding, with a relative risk of 5.9 (95% CI, 3.8-9.3). We found CYP2C9 genotype to be an independent predictor of a first bleeding event during the initiation phase of therapy (HR, 3.94; 95% CI, 1.29-12.06). This increased risk may be caused by the administration of loading doses that are too high for patients with genetic impairment of CYP2C9. Clinicians make rapid, downward dose adjustments based on above-range INR values,[18] so the increased bleeding risk during initiation should rapidly dissipate. The effect of variant genotype on bleeding holds over the entire course of therapy when comparing rates (unadjusted incidence rate ratio, 2.23; 95% CI, 1.05-4.77) or using a Cox proportional hazards model (HR, 2.39; 95% CI, 1.18-4.86); however, further research is required to determine whether the variant genotype continues to confer a bleeding risk once patients are stabilized with maintenance doses. Serious and life-threatening bleeding episodes were combined as a single end point due to the small number of observed events (n=32). This study is unable to assess the effect of genotype on life-threatening bleeding alone.

The incidences of serious (n=28) and life-threatening (n=4) bleeding events in our study (6.8 per 100 person-years and 1.0 per 100 person-years, respectively) are similar to those found in other studies (eg, 7.5 per 100 person-years and 1.1 per 100 person-years, respectively[20]), suggesting the patients in our study were not at an unusually high risk of bleeding events.[32,33] These re-

Figure. Time to Event for Anticoagulation-Related Outcomes



Curves represent Kaplan-Meier time-to-event analyses. Statistics given in the individual panels represent log-rank test for equality of survivor functions. INR indicates international normalized ratio.

sults also suggest that the patient management practices in our clinic population may be comparable to those of other anticoagulation clinics in the United States, thus increasing the generalizability of our results.

The lack of a significant difference in the time to first therapeutic INR is likely because the first therapeutic INR is often achieved within a few days of warfarin initiation, may be transient, and is not reflective of stable anticoagulation status. The median difference in time to stable warfarin dosing (95 days) suggests that patients with the variant genotype require more time to achieve stable anticoagulation status.

The frequencies of the *2 and *3 alleles (10.5% and 8.4%, respectively) were similar in our population compared with previously published results. Yasar et al[13] genotyped 430 Swedish volunteers and found *2 and *3 frequencies of 11% and 7%. However, we found 5 *3/*3 homozygotes in our population, or a prevalence of 2.7%. This result was 4 times higher than what we would expect by applying the Hardy-Weinberg derivation to our data (Table 2).[29] Two previous studies in the United Kingdom did not detect any *3/*3 homozygotes in patients receiving warfarin.[14,15] Therefore, the current study is the first to analyze and describe the clinical effect of this genotype in a stable, anticoagulated patient population.

There are several possible reasons why the prevalence of the *3/*3 genotype in our study is higher than expected. First, we used direct sequencing of exons 3 and 7 to detect variant alleles. Prior studies have relied on restriction enzyme analysis using *Nsi*I digestion of the exon 7 product to detect the *2C9*3* allele using a restriction site forced into the forward primer.[13-15] Direct sequencing is a more costly but more accurate approach to the detection of these alleles, and previous studies may have underestimated the prevalence of the *3 allele, resulting in misclassification bias. Second, Aithal et al[14] and Taube et al[15] cited selection bias as a possible explanation for why these UK-based studies failed to detect the *3/*3 homozygote in their warfarin clinic population. The suggestion was that individuals homozygous for this allele have such a low warfarin dose requirement that stabilization is unsuccessful and warfarin treatment is abandoned. Different approaches to maintenance therapy between UK-based and US-based practices could explain a stronger effect of selection bias in the UK-based populations. Finally, the UWMC anticoagulation population may be enriched with patients of the *3/*3 genotype. Patients who are difficult to stabilize (and more likely to carry the *3/*3 genotype) could be preferentially referred to the anticoagulation clinic for maintenance therapy, although physicians may simply refer patients on the basis of general convenience. Review of the clinical records of the 5 *3/*3 homozygotes did not reveal comments or observations to suggest preferential referral but this possibility cannot be excluded.

Adjusting for warfarin dose as a time-varying covariate did not materially change the results of any Cox model except for the above-range INR end point. Although warfarin dose is in the causal pathway of predicting INR values, we controlled for it as a confounder because dose and genotype are associated,[14,15] and because dose influences INR values independent of genotype.[19]

As genomic information becomes more readily available, it is likely that clinicians will need to consider new guidelines for patient management, especially when administering drugs with narrow therapeutic indexes such as warfarin.[34,35] Variant CYP2C9 genotype could be considered a "sensitivity factor" for low-dose requirements when initiating warfarin therapy, and patients with a variant genotype could be considered candidates for increased surveillance for bleeding risk. As oral versions of direct thrombin inhibitors become available,[36] CYP2C9 genotyping could identify patients with impaired warfarin metabolism as potential candidates for these newer alternate therapies.

There are several additional research questions that should be addressed. Our study was conducted in a relatively homogeneous population, and the presence of other functionally important polymorphisms in ethnically diverse groups has not been well studied. For example, the *2 and *3 alleles are relatively rare in African Americans, but approximately 3% carry the recently reported *5 polymorphism (Asp360Glu).[23] A novel and relatively common polymorphism (L208V; homozygous prevalence = 19%) with a reduced warfarin dose requirement has also recently been reported in Chinese patients.[37] Given the ethnically diverse population in the United States, additional studies are needed to evaluate the prevalence and clinical importance of these polymorphisms. Establishing the effectiveness of CYP2C9 genetic testing to reduce adverse bleeding events may require a controlled clinical trial in which some patients are randomized to receive a CYP2C9 genetic test at the time of warfarin initiation. Ideally, this study would be large enough to make meaningful comparisons of the risk-benefit ratio of warfarin therapy in genetic subgroups (eg, *2/*2, *3/*3). The prognostic specificity of CYP2C9 genotype will also be an important factor in its clinical usefulness. Significant additional medical care resources could be consumed unnecessarily by patients with variant genotypes despite low risk of bleeding. It will be necessary to demonstrate the value of the genotype information as being useful in the maintenance phase. For example, it is unclear whether those with variant genotypes would require a different target INR, as there may be substantial overlap in dose requirements with those not having the genetic variant. Finally, the cost-effectiveness of genotyping patients in an anticoagulation clinic must be considered.

Understanding the pharmacogenetics that contribute to variability in the warfarin dose-response relationship may help in tailoring drug therapy to patients in a safe and effective manner. This study confirms the dose-genotype association found in previous studies and is the first to describe the *3/*3 genotype effect in a stable anticoagulated population. We found that patients with a variant genotype experienced a higher rate

of above-range INRs, less stability on maintenance therapy, and a higher risk of serious or life-threatening bleeding events. The use of CYP2C9 testing may be a method to identify high-risk patients who are candidates for lower warfarin doses, more frequent monitoring, or treatment with alternate therapies as they become available.

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