Warfarin Dose Model for the Prediction of Stable Maintenance Dose in Indian Patients

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Abstract

The main aim of this study was to screen various genetic and nongenetic factors that are known to alter warfarin response and to generate a model to predict stable warfarin maintenance dose for Indian patients. The study comprised of 300 warfarin-treated patients. Followed by extensive literature review, 10 single-nucleotide polymorphisms, that is, *VKORC1*-1639 G>A (rs9923231), *CYP2C9**2 (rs1799853), *CYP2C9**3 (rs1057910), *FVII* R353Q (rs6046), *GGCX* 12970 C>G (rs11676382), *CALU* c.*4A>G (rs1043550), *EPHX1* c.337T>C (rs1051740), *GGCX*: c.214+597G>A (rs12714145), *GGCX*: 8016G>A (rs699664), and *CYP4F2* V433M (rs2108622), and 5 nongenetic factors, that is, age, gender, smoking, alcoholism, and diet, were selected to find their association with warfarin response. The univariate analysis was carried out for 15 variables (10 genetic and 5 nongenetic). Five variables, that is, *VKORC1*-1639 G>A, *CYP2C9**2, *CYP2C9**3, age, and diet, were found to be significantly associated with warfarin response in univariate analysis. These 5 variables were entered in stepwise and multiple regression analysis to generate a prediction model for stable warfarin maintenance dose. The generated model scored R^2 of .67, which indicates that this model can explain 67% of warfarin dose variability. The generated model will help in prescribing more accurate warfarin maintenance dosing in Indian patients and will also help in minimizing warfarin-induced adverse drug reactions and a better quality of life in these patients.

Keywords

warfarin, dosing algorithm, multiple regression analysis, India

Introduction

The goal of pharmacogenetics is to predict patient's response to a specific drug, based on the genetic variations, in order to deliver the best possible medical treatment. Prediction of the drug response of an individual can increase the success of therapies and reduce the incidence of adverse side effects. Till date, numerous single-nucleotide polymorphisms (SNPs) are identified in genes coding for liver enzymes responsible for drug deposition and pharmacokinetics. Warfarin (coumarin) therapy is one of the many clinical situations in which knowledge of pharmacogenomics can deliver safe personalized dosing.

Several evidences indicate that major genetic variables responsible for individual pharmacological response to warfarin are polymorphisms in 2 genes, namely, vitamin K epoxide reductase complex subunit 1 (*VKORC1*) and cytochrome P450-2C9 (*CYP2C9*).¹⁻⁵ Genetic variations in cytochrome P450-4F2 (*CYP4F2*), γ -glutamyl carboxylase (*GGCX*), epoxide hydrolase 1 (*EPHX1*), calumenin (*Calu*), factor IX (*F9*), and factor VII (*F7*) gene also have shown subsidiary association with warfarin response.⁶⁻¹² Other nongenetic factors including ethnicity, age, body mass index, weight, and gender

are also known to have a minor role in warfarin sensitivity.¹³⁻¹⁹ Based on pharmacogenetics, clinical, and demographic factors, population-based warfarin pharmacogenomic algorithms have been generated for various ethnic groups.²⁰⁻²²

In this study, we aim to generate a prediction model for stable warfarin maintenance dose in Indian patients by comprehensive analysis of genetic and nongenetic factors using multiple linear regression model. The outcome of such study will help in prescribing more accurate warfarin dosing and will

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improve management of this challenging therapy by minimizing warfarin-induced adverse drug reactions in Indian patients.

Materials and Methods

This study comprised of 300 patients who were prescribed warfarin for various clinical conditions. Patients were recruited from King Edward Memorial Hospital, Mumbai, BYL Nair Charitable Hospital, Mumbai, as well as clinics located in Mumbai and other parts of India. The study was approved by the institutional ethics committee review board for Research on Human Subjects of National Institute of Immunohaematology (ICMR). Patients on any other medication known to interact with warfarin and patients with abnormal renal or liver function test were excluded from the study. A clinical pro forma was designed to include all the relevant patient information such as international normalized ratio (INR) values, warfarin daily dose, changes in warfarin dose, gender, body weight, height, smoking, diet, race, bleeding complications, new thrombotic episodes, surgical procedures, or any other major health complications. Warfarin initiation and maintenance dose adjustment was blinded to genotype results. Initially, INR was tested every 2 to 4 days. After 2 consecutive therapeutic INR, testing interval was increased to 2 weeks. After 4 consecutive therapeutic INR, this was increased to 4 weeks and continued with the same monitoring interval.

The minimum follow-up period was 6 months and the maximum period was 15 months. None of the patients were on any vitamin K supplements.

Operational Definitions

Overanticoagulation: A patient was said to be overanticoagulated if patient's INR was > 4.0.

Bleeding episodes: Patients were considered to have "bleeding episodes" when it required medical attention or attendance at hospital.

Alcoholism: We categorized weekly alcohol intake as light (1-6 drinks), moderate (7-13 drinks), and chronic (≥ 14 drinks).

Diet: Patients who have nonvegetarian diet 2 or more times in a week were included in nonvegetarian category, whereas patients who avoid all food derived from animals or those who occasionally eat nonvegetarian diet were included in vegetarian/occasionally nonvegetarian category.

Selected Variables

Followed by extensive literature review, 10 SNPs, that is, *VKORC1*-1639 G>A (rs9923231), *CYP2C9**2 (rs1799853), *CYP2C9**3 (rs1057910), *FVII* R353Q (rs6046), *GGCX* 12970 C>G (rs11676382), *CALU* c.*4A>G (rs1043550), *EPHX1* c.337T>C (rs1051740), *GGCX*: c.214+597G>A (rs12714145), *GGCX*: 8016G>A (rs699664), and *CYP4F2* V433M (rs2108622), and 5 nongenetic factors, that is, age, gender, smoking, alcoholism, and diet, were selected to see their

association with warfarin dose requirement and risk of overanticoagulation.

Blood Collection and Processing

After obtaining informed consent, 10 mL of venous blood sample was collected in 3.13% sodium citrate (1:9 anticoagulant to blood) from each patient. The samples were spun at 4000 rpm for 15 minutes at 4°C. The platelet poor plasma was used for Prothrombin time-International normalized ratio (PT-INR) test. Usually, the test is performed within 1 hour of collection of blood. The cell pellet was preserved at -20°C for DNA analysis. DNA was extracted by standard phenol-chloroform method.

Genotyping

The polymerase chain reaction (PCR) amplification was done using oligonucleotide primers (Sigma Chemicals, Bangalore, India) in a 96-well thermal cycler (Applied Biosystems, Carlsbad, California). Genotyping for 10 SNPs was carried out by restriction fragment length polymorphism (RFLP), allele-specific PCR, and direct sequencing method.

Restriction fragment length polymorphism. *VKORC1*-1639 G>A (rs9923231), *CYP2C9**2 (rs1799853), *CYP2C9**3 (rs1057910), and *FVII* R353Q (rs6046) polymorphisms were genotyped using RFLP technique. Briefly, the protocol for DNA amplification was as follows: the PCR mix consisted of a final volume of 25 μ L, containing 1.25 μ L of 10 pmol each primers, 0.2 μ L 25 mM deoxynucleotides triphosphate, 1.5 μ L 25 mM MgCl₂, 2.5 μ L 10 $\times$ complete buffer (Bioron, Ludwigshafen, Germany), 0.15 μ L DNA Free Sensitive (DFS) Taq DNA polymerase (Bioron, Ludwigshafen, Germany), and 2 μ L DNA sample. Ten microliters of amplified product was used for RFLP analysis using 5 units of *Hpa* II, *Ava* II, *Nsi* I, and *Msp* I restriction enzyme (New England Biolabs, Hertfordshire, United Kingdom) for *VKORC1*-1639 G/A, *CYP2C9**2, *CYP2C9**3, and *FVII* R353Q genotypes, respectively. The PCR program and the primer sequences for genotyping are given in Table S1 (Online Resource 1).

Allele-specific PCR method. Following polymorphisms were genotyped by allele-specific PCR standardized in the laboratory: *CALU* c.*4A>G (rs1043550), *CYP4F2* V433M (rs2108622), *EPHX1* c.337T>C (rs1051740), *GGCX* c.214+597G>A (rs12714145), and *GGCX* 8016G>A (rs699664). Two primers were designed, one specific for the wild type and the other for variant, to specifically detect desired genotypes. The PCR conditions were standardized with known wild-type, heterozygous, and homozygous samples. The accuracy of standardized allele-specific PCR method was validated with direct sequencing method, and the data were in agreement.

Direct sequencing. *GGCX* 12970 C>G (rs11676382) polymorphism was genotyped by direct sequencing method. The DNA samples were amplified using oligonucleotide primers in a

96-well thermal cycler (Applied Biosystems Veriti, Carlsbad, California). Briefly, the protocol for DNA amplification was as follows: in a final volume of 25 μ L, containing 1.25 μ L of 10 pmol each primers, 0.2 μ L 25 mM deoxynucleotides triphosphate, 0.5 μ L 25 mM $MgCl_2$, 2.5 μ L 10 $\times$ complete buffer (Bioron), 0.10 μ L DFS Taq DNA polymerase (Bioron), and 2 μ L DNA sample. Primer sequences and PCR program for the same are given in Table S2 (Online Resource 1). Amplified PCR products were purified using commercial kits (Invitrogen, Germany) followed by cycle sequencing using BigDye Terminator v3.1 cycle sequencing kit. Sequencing was performed on an ABI Prism Genetic analyzer.

Statistical Analysis

Hardy-Weinberg equilibrium was estimated for all the analyzed polymorphisms using an Excel-based application (Microsoft Office 2007).

Fisher exact test was used to calculate the *P* value. Data were shown to be significant if *P* value was $\leq .05$. Statistical analysis for Fisher exact was performed with the MedCalc software version 12.3.0 (http://www.medcalc.org/calc/relative_risk.php). In most cases, the mutant homozygotes and heterozygotes were combined and compared to the wild type. For *CYP2C9*, each mutant genotype was compared to the wild type. Warfarin dose was square rooted before analysis to approximately normalize the data and to equalize the variance. Univariate analysis was done using 1-way analysis of variance or *t* tests (2 groups) on the square root of dose.

Multiple regression analysis. Stepwise multiple regression analysis was carried out using statistical program “R” (version 3.1.3).

Results

A total of 300 patients fitting into the inclusion criteria were screened for 15 variables. The demographic and clinical data are shown in Table 1.

Fifty-nine percent were males and age range was from 19 to 80 years. The mean daily stable warfarin dose was 5.05 (± 1.84 mg). Cerebral venous thrombosis and lower limb deep vein thrombosis were the commonest types of thrombosis.

Univariate Analysis

Univariate analysis was carried out to evaluate the association of genetic and nongenetic variables with warfarin response.

Genetic factors. The genotype frequencies of all the polymorphisms and their effect on warfarin dose requirement and risk of overanticoagulation are shown in Table 2. Among the 10 polymorphisms analyzed, *VKORC1*-1639 G>A polymorphism showed significant association with reduced dose requirement and risk of overanticoagulation ($P < .001$). The “A” allele carriers were found to require low warfarin dose and were

Table 1. Demographic and Clinical Data.

Total number of patients: 300	
Male, n (%)	177 (59)
Female, n (%)	123 (41)
Age, median (range), years	37 (19-80)
BMI, median (range), kg/m ²	22.10 (17.12-35.42)
Duration of anticoagulant treatment, median (range), months	6 (6-24)
Stable average daily warfarin dose, mean (standard deviation), mg	5.05 (± 1.84)
Indication for anticoagulation with warfarin, n (%)	
Atrial fibrillation	8 (2.7)
Cerebral venous thrombosis	111 (37.0)
Lower limb DVT	123 (41)
Upper limb DVT	10 (3.3)
Heart valve replacement	37 (12.4)
Young stroke	7 (2.3)
Others	4 (1.3)

Abbreviations: BMI, body mass index; DVT, deep vein thrombosis.

posed with a higher risk of overanticoagulation. Among the 2 *CYP2C9* variants, *CYP2C9**3 variant allele carrier patients showed statistically significant association with lower dose requirement as well as risk of overanticoagulation ($P < .001$), while *CYP2C9**2 allele carrier patients showed relatively weak association ($P < .05$) as compared to *CYP2C9**3, but it was statistically significant. *CALUC*.*4A>G (rs1043550), *EPHX1* c.337T>C (rs1051740), *CYP4F2* V433 M (rs2108622), *GGCX*104+597 G>A (rs12714145), *GGCX* 8016G>A (rs699664), and *Factor VII* R353Q (rs6046) polymorphisms showed nonsignificant association with dose requirement and also no statistically significant association was found with risk of overanticoagulation. *GGCX* 12970 C>G (rs11676382) variant allele was found to be absent in the present series.

Nongenetic factors. Five nongenetic factors, that is age, gender, smoking, alcoholism, and diet were selected to study their association with warfarin dose requirement and risk of overanticoagulation (Table 3). Among the nongenetic factors, age was found to have significant association with risk of overanticoagulation (<60 years vs ≥ 60 years, $P < .001$). When the 2 age groups were compared, there was no significant difference in warfarin dose. However, it was significant across all age groups ($P = .013$). With increasing age, the requirement of warfarin dose reduced but the risk of overanticoagulation significantly increased in elderly patients. Gender and smoking were neither found significantly associated with overanticoagulation nor with dose requirement. Although, in the current study, alcoholism has not shown any association with warfarin dose requirement or risk of overanticoagulation, yet the effect of alcoholism on warfarin is unclear in this study, as many chronic alcoholic patients with liver dysfunction and abnormal liver function were excluded from the present study. Vegetarian or occasionally nonvegetarian patients were found to require significantly lower warfarin dose as compared to nonvegetarian patients ($P = .013$). However, no significant association was found with the risk of overanticoagulation.

Table 2. Effect of Genetic Polymorphisms on Warfarin Dose Requirement and Risk of Overanticoagulation.

Polymorphism	Genotype	N = 300, n (%)	Mean Daily Warfarin Dose (mg/d)	P Values for Mean Daily Warfarin Dose ^a	Patients Faced Overanticoagulation (INR > 4), n (%)	P Values for Overanticoagulation (INR > 4)
VKORC1-1639G>A (rs9923231)	GG	223 (74)	5.55	-	86 (39)	-
	GA	73 (25)	3.68	.001	42 (58)	.001
	AA	4 (1)	2.12		4 (100)	
CYP2C9 genotype (rs1799853) (rs1057910)	*1/*1	211 (71)	5.81	-	68 (32)	-
	*1/*2	15 (5)	4.42	.001	8 (53)	.001
	*1/*3	60 (20)	3.18		43 (72)	
	*2/*3	4 (1)	2.68		3 (75)	
	*3/*3	10 (3)	2.2		10 (100)	
CALU c.*4A>G (rs1043550)	AA	182 (61)	5.02	-	77 (43)	-
	GA	97 (32)	5.07	.881	45 (46)	.4602
	GG	21 (7)	5.19		10 (48)	
EPHX1 c.337T>C (rs1051740)	TT	129 (43)	5.09	-	75 (46)	-
	TC	140 (47)	5.03	.97	30 (43)	.4459
	CC	31 (10)	4.97		27 (40)	
GGCX c.214+597 G>A (rs12714145)	GG	163 (54)	5.09	-	75 (46)	-
	GA	70 (24)	4.87	.64	30 (43)	.4459
	AA	67 (22)	5.14		27 (40)	
GGCX 12970 C>G (rs11676382)	CC	300 (100)	5.05	-	132 (44)	-
	CG	-	-		-	NA ^b
	GG	-	-		-	
GGCX 8016G>A (rs699664)	GG	97 (32)	5.07	-	40 (41)	-
	GA	185 (62)	5.04	.953	85 (46)	.5110
	AA	18 (6)	5.01		7 (39)	
CYP4F2 V433M (rs2108622)	GG	93 (31)	4.99	-	41 (44)	-
	GA	178 (59)	5.03	.505	75 (42)	.9839
	AA	29 (10)	5.38		16 (55)	
FVII R353Q 13407G>A (rs6046)	GG	167 (56)	5.15	-	66 (39)	-
	GA	122 (40)	4.86	.213	64 (52)	.0790
	AA	11 (4)	5.63		2 (18)	

Abbreviation: INR, International normalized ratio.

^aP values for warfarin dose denote the result of 1-way analysis of variance on the square root of dose.^bVariant allele not present.**Table 3.** Effect of Nongenetic Factors on Warfarin Dose Requirement and Risk of Overanticoagulation.

Characteristic	Parameters	N = 300 (%)	Mean Daily Warfarin Dose (mg/d)	P Values for Mean Daily Warfarin Dose ^a	Patients Who Faced Overanticoagulation (INR > 4), n (%)	P Values for Overanticoagulation (INR > 4)
Age	<60 years	279 (93)	5.07	-	115 (41)	-
	≥60 years	21 (7)	4.74	.53	17 (81)	.001
Gender	Male	177 (59)	4.95		77 (43)	
	Female	123 (41)	5.19	.338	55 (43)	.8348
Smoking	No	268 (89)	5.05	-	121 (45)	-
	Yes	32 (11)	5.01	.686	11 (34)	.2819
Chronic alcoholic	No	260 (87)	5.02	-	119 (46)	-
	Yes	40 (13)	5.24	.888	13 (32)	.1497
Diet	Nonvegetarians	214 (71)	5.23	-	92 (43)	-
	Vegetarian or occasionally nonvegetarian	86 (29)	4.61	.013	40 (47)	.5736

Abbreviation: INR, International normalized ratio.

^aP values for warfarin dose relate to t tests on the square root of the dose.

Table 4. Multiple Linear Regression Analysis.

Coefficients	Estimate	Standard Error	Pr (> t)
(Intercept)	2.609832	0.046119	$<2.0 \times 10^{-16a}$
<i>VKORC1</i> -1639 G>A	-0.406740	0.030869	$<2.0 \times 10^{-16a}$
<i>CYP2C9</i> *1/*2	-0.206816	0.068246	0.00266 ^b
<i>CYP2C9</i> *1/*3	-0.579309	0.037153	$<2.0 \times 10^{-16a}$
<i>CYP2C9</i> *2/*3	-0.862982	0.126904	5.93×10^{-11a}
<i>CYP2C9</i> *3/*3	-0.864116	0.081463	$<2.0 \times 10^{-16a}$
Age	-0.002576	0.001103	0.02023 ^c
Vegetarian	-0.082231	0.032153	0.01105 ^c

Significance codes: ^a0.001, ^b0.01, ^c0.05.

Table 5. Contribution of Factors in Multiple Linear Regression Model.

Predictor	Partial R ² (%)	P Value
<i>VKORC1</i> -1639 G>A	23	.001
<i>CYP2C9</i> *2 and <i>CYP2C9</i> *3 genotype	42	.001
Age	0.80	.01
Diet	0.70	.05
Model R ²	67	.001

Multiple Linear Regression Analysis

Multiple regression analysis was carried out to generate a model to predict stable warfarin maintenance dose. All possible explanatory variables were considered, but only *VKORC1*-1639 G>A, *CYP2C9**2, *CYP2C9**3, age, and diet were found significant in a stepwise multiple linear regression model (Table 4). Based on the multiple regression analysis, the following equation was generated to predict daily stable warfarin maintenance dose in Indian patients:

$$\begin{aligned} \text{Square root of dose(mg)} = & 2.61 - 0.41(\text{VKORC1} - 1639) \\ & - 0.21(*1/*2) - 0.58(*1/*3) \\ & - 0.86(*2/*3) - 0.86(*3/*3) \\ & - 0.002(\text{Age in years}) - 0.08(\text{diet}). \end{aligned}$$

VKORC1-1639: “0” for GG, “1” for GA, “2” for AA; *CYP2C9* *1/*2, *1/*3, *2/*3, and *3/*3, “0” if variant genotype is absent, and “1” if variant genotype is present; “0” if patient is nonvegetarian and “1” if patient is vegetarian or occasionally nonvegetarian.

R² of the multiple regression model was scored to .67, indicating that the prediction model could explain approximately 67% of dose variability. The maximum dose variability was explained by *CYP2C9* (*2 and *3) and *VKORC1* genotypes, that is, 42% and 23%, respectively, followed by age and diet (Table 5).

Discussion

The pharmacogenetic data have been helping the clinicians in improving the patient care by way of reduction in adverse drug

reactions or by decreasing the cost of treatment by minimizing hospital admissions. The aim of our study was to develop a model for prediction of stable warfarin maintenance dose in Indian patients. In the present study, of 15 analyzed markers, only 5 markers, that is, *CYP2C9**2, *CYP2C9**3, *VKORC1*-1639 G>A, age, and diet, were found to be significantly associated with warfarin response in univariate analysis.

Many studies have reported that *VKORC1* and *CYP2C9* polymorphisms are the major predicting factors of warfarin dose requirement, but the present study shows that integrating patient's diet and age can marginally improve the power of dose prediction. It is reported that a vitamin K-rich diet can prevent the action of warfarin (vitamin K antagonist) and may need high warfarin maintenance dose,^{23,24,25} whereas Ford et al in a review of literature concluded that vitamin K supplementation may reduce variation in drug response caused by dietary intake.²⁶ Few other studies have suggested that patients on warfarin need to maintain consistent dietary vitamin K intake to achieve more stable anticoagulation response.^{23,27} In the present study, nonvegetarian patients were found to require higher warfarin dose than the vegetarian patients.

Reports have shown that warfarin dose is inversely proportional to age and also elderly patients (>75 years) are at more risk for bleeding complications ($P < .05$).^{18,28-30} In the present study, significant association of age with decreased warfarin dose requirement was observed and a linear trend across all ages was seen ($P < .05$). The risk of warfarin-induced bleeding was also significant when compared between the 2 age groups (ie, <60 years vs ≥ 60 years, $P < .001$).

The generated prediction model scored R² to .67 indicates that this prediction model could explain nearly 67% dose variability. The prediction power of this study is quite good as compared to other reported prediction models in Indian patients.³¹⁻³⁴ However, the remaining 33% dose variability is still unsolved. It might be due to the genetic and/or nongenetic factors that are yet to be identified. More well-designed and large-scale studies are needed to uncover these unknown factors affecting warfarin response. Although this is a comprehensive analysis of most of the genetic and nongenetic risk factors associated with warfarin sensitivity, yet there is a lack of systematic data on comedications and other comorbidities associated with warfarin response. For instance, although alcohol intake has been considered as one of the factors in the present analysis, chronic alcoholics with liver dysfunction had to be excluded from the present study. Although the generated model can predict warfarin dose modestly and not the exact dosing, it can still help in dividing patients into low-, intermediate-, and high-dose requirement groups, which will help in prescribing more appropriate stable warfarin maintenance dosage than the conventional dosing and will also help in minimizing warfarin-induced adverse drug reactions and a better quality of life in patients who are treated with warfarin.

Authors' Note

SS and KG conceived and designed the experiments. TG performed the experiments. PA, FK, and TG analyzed the data. PA and FK

contributed analysis tools. TG wrote the manuscript. SS contributed to critical revision of manuscript for important intellectual content and final approval.

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The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplementary material for this article is available online.

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