Original Article

Effect of Vitamin K Epoxide Reductase Complex 1 Polymorphism on Warfarin Dose Requirement among Patients in Tertiary Care Hospital

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Abstract

Background: Warfarin, anticoagulant is used for thromboembolic disorders. Inter-individual variation in clinical response to warfarin is due to various factors, including polymorphism of Vitamin K epoxide reductase complex 1 (VKORC1)-1639G>A. The aim of our study was to evaluate the effect of *VKORC1* polymorphism on the maintenance dose of warfarin. **Materials and Methods:** Cross-sectional study conducted by the departments of Pharmacology, Cell Biology and Molecular Genetics on patients attending cardiology clinic, receiving warfarin for at least 2 months. Genomic deoxyribonucleic acid was extracted and genotyping was done by Polymerase Chain Reaction - Restriction Fragment Length Polymorphism. The correlation between VKORC1 gene polymorphism and warfarin maintenance dose was analyzed. **Results:** A total of 102 patients with a mean age of 47.72 ± 10.31 years, of which 58 (56.86%) were male. The frequency of *VKORC1* G>A for GG, GA, and AA genotypes was 74.51%, 19.61%, and 5.88%, respectively. Variant allele AA was less frequent than the wild type. Mean weekly warfarin dose was 23.12 ± 8.08, 22.93 ± 8.21, and 15.6 ± 5.35 mg in patients with GG, GA, and AA genotypes, respectively. Patients with GG genotype required therapeutic dose compared to variant type (P = 0.001). Multiple stepwise regression model showed 26.3% variability in warfarin dose was due to *VKORC1* genotype (R = 0.513, R^2 = 0.263, adjusted R^2 = 0.256, P = 0.0001). **Conclusion:** *VKORC1* polymorphism alone influence 26.3% variability in warfarin dose and AA genotype patients required lower dose.

Keywords: *Oral anticoagulant, Vitamin K epoxide reductase complex 1 polymorphism, warfarin*

Introduction

Anticoagulants interfere with the function of clotting factors thereby interrupt the coagulation cascade and prevent the formation of a stable fibrin meshwork. Warfarin, an oral anticoagulant acts by inhibiting the enzyme Vitamin K Epoxide Reductase (VKOR) resulting in reduced production of Vitamin K-dependent clotting factors II, VII, IX, and X. It is used for the prevention and treatment of thromboembolic conditions.[1] Warfarin to be effective and safe necessitate using the correct dose to achieve the therapeutic concentration because low dose can cause therapeutic failure and increased dose can result in fatal bleeding.[2] Hence, warfarin dose has to be monitored by estimating prothrombin time which is expressed as the international normalized ratio (INR). In addition, various factors such as age of the patient, lifestyle, body mass index (BMI), body surface area, environmental and genetic factors play a role in clinical response to the prescribed dose of warfarin.[3,4]

Warfarin exhibits more than ten-fold interindividual variability in dose requirement to attain therapeutic concentration.[3] Interindividual variability in warfarin dose requirement is influenced by two genes, *CYP2C9* which encodes for the enzyme cytochrome P450, family 2, subfamily C, polypeptide 9 (CYP2C9) involved in warfarin metabolism and VKOR complex subunit 1 (*VKORC1*) which encodes for VKOR, the target enzyme for warfarin. Genetic variations in *CYP2C9* gene alters the rate of metabolism of warfarin.[5] *VKORC1*-1639G >A in the promoter region of the gene which influences the VKOR enzyme activity is the most widely studied single nucleotide polymorphism.[6] *VKORC1*-1639 genotypes are GG (wild), AA (homomutant), and GA (heteromutant).[7] The identification of polymorphisms affecting these genes, oral anticoagulation therapy can be tailored to

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the patient requirement. These genetic polymorphisms are widely distributed and may vary by region and ethnicity.[3,5]

Genetic polymorphism involving *VKORC1* is associated with approximately 25% of the variation in the maintenance dose requirement of warfarin.[8,9] There is limited information available from Karnataka on *VKORC1* polymorphism and its influence on warfarin dose requirement. Hence, this study was undertaken to determine the relationship between *VKORC1* polymorphism and warfarin dose requirement and INR.

Materials and Methods

A cross-sectional study was conducted by the Department of Pharmacology on patients attending Cardiology Centre in association with the Department of Cell Biology and Molecular Genetics from January 2015 to June 2016. The protocol was approved by Institutional Ethics Committee and written informed consent was obtained from all the patients. Patients of either gender aged between 18 and 65 years with BMI of 18–29 kg/m^2 and who were on oral warfarin therapy for at least 2 months were included in the study. Patients with specific systemic disorders such as renal or hepatic insufficiency, receiving concomitant medications such as phenytoin, rifampicin, isoniazid, tetracycline, erythromycin, metronidazole, cephalosporins, barbiturates, and oral contraceptive pills, those addicted to smoking and alcohol, pregnant, and lactating women were excluded. A proforma was given to all participants to capture the demographic details such as age, gender, occupation, BMI, daily warfarin dose, change in warfarin dose, INR values, diet history, and any bleeding complications.

The experiments were carried out in the Central Research Laboratory of the institution by the principal investigator and the entire work was supervised by a molecular biologist who is a coinvestigator. Three milliliters of venous blood were collected from each participant under strict aseptic precautions. Deoxyribonucleic acid was extracted using standard salt extraction method and was stored at −40°C until further analysis. Genotyping of *VKORC1* was performed using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). Genotyping of *VKORC1* was performed as described by Sconce *et al*.[10] Sequences for the forward and reverse primers are 5'-GCCAGCAGGAGAGGGAAATA-3' and 5'-AGTTTGGACTACAGGTGCCT-3', respectively.

The PCR was performed using BIORAD C1000 touch thermal cycles at 25 cycles of 1 min at each of the following temperatures, 94°C, 61°C, and 72°C. The PCR product (10 µL) was digested with ten units of restriction enzyme Msp1 in a final volume of 20 µL in the appropriate digestion buffer at 37°C for 16 h. The digested product was visualized on 2% agarose gel stained with ethidium bromide. PCR-RFLP pattern of *VKORC1*-1639 for GG is 168-bp (base pairs), GA 122-bp, and AA 290-bp. To validate the PCR - RFLP tests, positive controls were used for each batch of sample testing. 10% of the samples were repeated to confirm and the results were 100% concordant.

Statistical methods

A precision of 4%, desired confidence interval of 80%, to have an expected proportion of polymorphism of 0.86, the sample size was calculated to be 92. Demographic data were analyzed using descriptive statistics and expressed as mean ± standard deviation. The regression tool was used to study the association of independent variables on warfarin dose. P <0.05 was considered statistically significant.

Results

A total of 125 patients were screened for the study, among them 102 patients were recruited, and genotyping was performed using PCR-RFLP method. There were 58 (56.86%) male and 44 (43.13%) female patients with mean age of 47.72 ± 10.31 years and 42.95 ± 11.98 years, respectively. The characteristics of patients with GG, GA, and AA genotypes are represented in Table 1.

Majority of the patients in wild GG genotype were male (56.57%), prosthetic/mechanical heart valves following valve replacement surgeries, and valvular heart diseases were the most common indications for warfarin use and digoxin being the most commonly used concomitant medication. Majority of the patients (84.31%) had INR in the therapeutic range. INR in subtherapeutic

Table 1: Demographic profile of patients

Patient characteristics	*VKORC1* genotype		
	GG (*n*=76)	GA (*n*=20)	AA (*n*=6)
Age (years)*	40.03±10.78	42.70±12.71	50.83±11.56
Gender, *n* (%)			
Male	43 (56.57)	12 (60)	3 (50)
Female	33 (43.42)	8 (40)	3 (50)
BMI (kg/m^2)*	23.05±3.42	22.20±2.60	24.27±2.77
Indications for warfarin therapy, *n* (%)			
Prosthetic valves	37 (48.68)	7 (35)	2 (33.33)
Valvular heart disease	27 (35.52)	10 (50)	1 (16.66)
Atrial fibrillation	8 (10.52)	2 (10)	1 (16.66)
Venous thrombosis	4 (5.23)	1 (5)	2 (33.33)
Concomitant medications, *n*			
Digoxin	22	8	3
Furosemide	18	11	4
Aldactone	12	8	1
Metoprolol	9	5	2
Atenolol	4	0	2
Mean INR value*	2.5±0.55	2.37±0.50	2.52±1.66

*Values in mean±SD, percentage in parentheses. SD: Standard deviation; BMI: Body mass index; INR: International normalized ratio; *VKORC*: Vitamin K epoxide reductase complex 1

range was observed in 16 (15.7%) patients, among these 10, 4, and 2 belonged to GG, GA, and AA genotypes, respectively [Table 1].

The major type allele G is digested to two fragments of 168-bp and 122-bp, and the minor allele A being resistant to digestion by restriction enzyme is represented as a single band of 290-bp. The presence of all three bands indicates GA (variant heteromutant), two bands indicate GG (wild) and one band indicates AA (variant homomutant) genotype as shown in Figure 1.

The allele and genotype frequency of *VKORC1* are shown in Table 2. Among 102 patients, 76 had wild genotype, 20 were heterozygous, and 06 homozygous having both the mutant alleles. The VKORC1-1639G >A minor allele frequency was found to be 15.68%.

Mean weekly warfarin dose was 23.12 ± 8.08, 22.93 ± 8.21, and 15.6 ± 5.35 mg in patients with GG, GA, and AA genotypes, respectively. Mean weekly warfarin dose was compared between the wild and the variant genotypes of *VKORC1* and a significant difference in dose requirement was found between *VKORC1* wild and mutant genotypes. The weekly warfarin dose requirement of patients with wild type was significantly higher compared to AA genotype [Figure 2]. Patients with GG and GA genotypes required therapeutic dose of warfarin whereas AA genotype required subtherapeutic dose.

Table 2: Vitamin K epoxide reductase complex 1−1639G >A genotype and allele frequency

Genotype	Number of subjects			Frequency
	Male	Female	Total	
GG	43	33	76	74.51
GA	12	8	20	19.61
AA	3	3	6	5.88
Allele	**Number of alleles**			**Frequency**
G	76×2+20=172			84.32
A	6×2+20=32			

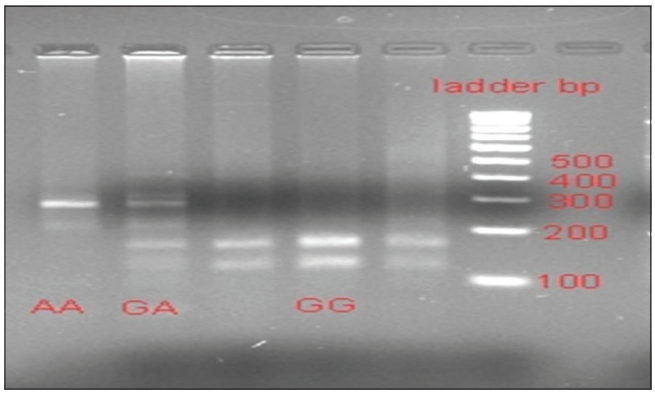

Figure 1: Representative gel electrophoresis of Polymerase Chain Reaction-Restriction Fragment Length Polymorphism for Vitamin K epoxide reductase complex 1 G > A of three patient samples showing wild type GG, variant heterozygous type GA, and variant homozygous type as AA

Multiple linear regression analysis was carried out for factors which are known to influence the warfarin dose requirement. Age, BMI, gender, duration of therapy, and *VKORC1* polymorphism were the independent variables considered to analyze the association with mean warfarin maintenance dose requirement. *VKORC1* genotype was found to be significantly contributing to 26.3% variability in the warfarin dose requirement (P = 0.0001) [Table 3], and there was no significant association between age, gender, BMI, language spoken, duration of therapy, and warfarin dose.

Discussion

Warfarin is the most commonly used oral anticoagulant for thromboembolic conditions. The drug exhibits narrow therapeutic window, with wide variability in dose requirements among different ethnic groups and individuals. Factors such as age, gender, BMI, diet, race, and concomitant medications may contribute to the variability in warfarin dose along with the genetic factors.[3,4,11] Warfarin when used in therapeutic doses may result in INR either below or above the recommended range predisposing to thrombosis or increased risk of bleeding in certain individuals. Hence, individual dose adjustment is a challenge in clinical practice. At present, in our country warfarin dose adjustment is based mostly on the INR value or by trial and error method.[3] Including genotyping of the patient to decide the dose of warfarin can be considered before starting treatment with warfarin.[12] The prevalence of gene polymorphisms is known to vary across different ethnic-geographical populations.[3] In our study, we have determined the frequency of *VKORC1* polymorphism among patients from Kolar district.

In this cross-sectional study, genotyping for VKORC1-1639G >A was performed on 102 patients who were on the maintenance dose of warfarin. In the present study, majority of the patients were in the third and fourth decade of life [Table 1] which is similar to most other Indian studies.[7,13] The number of male and female patients in our study were almost the same. Mechanical heart valves replacement was the most common indication for warfarin therapy and digoxin followed by furosemide were the most common concomitant medications.

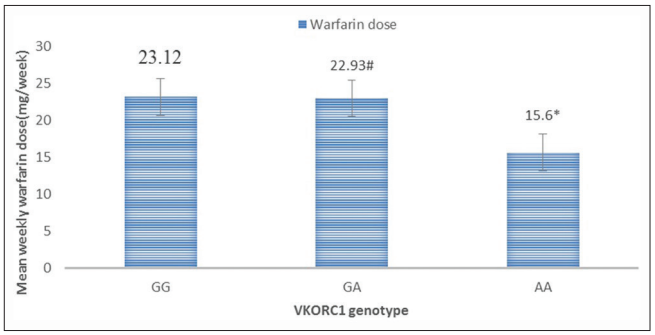

Figure 2: Weekly warfarin dose requirement according to the Vitamin K epoxide reductase complex 1 genotypes. #GA vs GG, *AA vs GG

Table 3: Multiple linear stepwise regression analysis for factors determining warfarin dose

Variables	Unstandardized regression coefficient	Standard error	Standardized regression coefficient	*P*
Constant	31.364			
VKORC1 genotype	−0.576	0.096	−0.513	0.0001

VKORC1: Vitamin K epoxide reductase complex 1

The wild type of polymorphism was the most common type in our study population accounting to about 74.51% and this finding is in concordance with other Indian studies. Madan *et al*.'s study on South Indian population observed frequency of 79.9% and a study on North Indian population showed 76% of wild type polymorphism.[7,13] The VKORC1-1639 G >A minor allele frequency observed in our study was 15.68% and 10.4% in Madan *et al*.'s study which is slightly lower than our study finding.[7] A pilot study conducted on 50 patients from North India found minor allele frequency to be 22% which is higher compared to findings in other South Indian studies and our study.[13] Rathore *et al*. conducted study on 102 North Indian healthy volunteers and showed that minor allele frequency was 14.22%.[14] This implies that region may contribute to variation in gene polymorphism. The VKORC1 minor allele frequency in our study population is smaller compared to Caucasians and rest of the world. Similar other studies conducted in various parts of our country have shown a lesser frequency of the homozygous mutant genotype compared to our study population among whom the variant genotype is slightly higher.[7,12-18]

Individuals with wild genotype required therapeutic dose of warfarin in comparison to the variant genotype individuals who required lesser dose [Figure 2]. We observed a significant association between VKORC1-1639G>A polymorphism and mean weekly warfarin dose which imply that polymorphism in VKORC1 gene is an important determinant of warfarin dose.

VKOR enzyme function in patients with variant homozygous genotype (AA) is least, leading to decreased availability of Vitamin K hydroquinone. This results in reduced action of clotting factors, and hence, they require a lesser dose of warfarin to inhibit the enzyme. In case of variant heterozygous genotype, the enzyme will have intermediate activity and hence require slightly lower than therapeutic dose. Patients with wild genotype whose enzyme is fully functional require the defined therapeutic dose. Thus, carriers of even one variant allele (A) compared to those having major allele (G) were observed to require lower warfarin dose suggesting the requirement for genotyping of patients to be treated with warfarin, to avoid side effects like bleeding due to excess anticoagulation and to administer the correct dose of warfarin to these patients.

In our study, we observed that two patients with mutant homozygous genotype (AA) received the usual adult dose of warfarin and they presented with complications such as malena, epistaxis and bleeding gums. Their INR values were high (INR = 6). Warfarin was withheld, Vitamin K injection was given. Genotyping helped in adjustment of dose of warfarin and on follow-up the INR was within the therapeutic range with no bleeding complications. These events emphasize the need for pharmacogenetic testing at the time of initiation and maintenance of warfarin therapy. Warfarin has been listed by the US Food and Drug Administration under the recommended category of genomic biomarkers for pharmacogenetic testing.[19]

In our study, VKORC1 gene polymorphism has contributed to 26.3% variability in the warfarin dose requirement [Table 3]. A study conducted by Bodin *et al*. has shown that VKORC1 polymorphism alone accounted to 37% variability in the dose requirement of coumarin drugs among the healthy individuals.[20] In our study, we did not find any association between age, gender, BMI, duration of therapy, and warfarin dose. Dang *et al*. observed that even after all confounding factors were adjusted, warfarin maintenance dose varied across ethnic groups suggesting genetic variations to be the cause for interindividual variability.[21] However, Madan *et al*.'s study has found that 36.1% variability was attributed to combined effect of CYP2C9, VKORC1, age, BMI, and duration of the treatment.[7] In our study, we did not assess the influence of other factors such as diet, concomitant medications, and smoking on the patients response to warfarin.[3,4,8] However, all our patients were strictly instructed to restrict intake of green leafy vegetables, cabbage which might have contributed toward reducing diet-related fluctuations in dose.[3,4,22]

Conclusion

VKORC1 polymorphism alone influence variability in warfarin dose, and variant genotype patients required lesser dose. Hence, *VKORC1* genotyping while initiating warfarin therapy can improve patient's response to treatment and limit the bleeding complications.

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Conflicts of interest

There are no conflicts of interest.

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