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Association between Cytochrome P450 2 C9 and Vitamin K Epoxide Reductase Complex Subunit 1 Polymorphisms with Warfarin Dose among Iranian Patients

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Abstract

Background: Warfarin is a common anticoagulant drug that has a narrow therapeutic index; higher dose causes excessive bleeding and lower dose leads to cerebrovascular clotting and stroke in patients. Genetic factors that have been associated with warfarin response are the genes of cytochrome P450 2C9 (CYP2C9), which metabolize the more active S-enantiomer of warfarin, and vitamin K epoxide reductase (VKOR), the target site for warfarin. The present study was conducted to investigate the association between CYP2C9*2, CYP2C9*3 and VKORC1 (-1639 G>A) polymorphisms with warfarin daily dose on 118 Iranian patients under warfarin treatment.

Materials and Methods: This study is comprised of 118 Iranian patients on warfarin treatment who attended the PT Clinic. Genotyping of CYP2C9*2, CYP2C9*3 and VKORC1 (-1639 G>A) was performed by PCR-RFLP method. Multiple regression model was performed for statistical analyses and $P<0.05$ was considered as significance level.

Results: The allelic frequencies of CYP2C9*2 and CYP2C9*3 were 19% and 7%, respectively. Patients with ≥1 CYP2C9 variant allele had a significantly lower mean warfarin daily dose compared with patients with the wild-type genotype. The allelic frequencies of VKORC1 were 14.4%, 57.6% and 27.9% for GG, GA, and AA genotypes, respectively. The mean (SD) warfarin daily dose in patients with the VKORC1 (–1639) GG genotype was significantly higher than GA and AA patients.

Conclusion: CYP2C9*2, CYP2C9*3 and VKORC1 (-1639 G>A) polymorphisms had significant association with warfarin daily dose. Furthermore, the daily warfarin dose was not influenced by age, height, weight and sex.

Keywords: CYP2C9*2; CYP2C9*3; VKORC1; Warfarin; RFLP

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Introduction

Warfarin is a common drug used for the prevention of coagulation in patients with heart disease. Warfarin is a potent inhibitor of the vitamin K epoxide reductase (VKOR) encoded by the vitamin K epoxide reductase complex subunit 1 (VKORC1) gene (1, 2). Warfarin exerts its anticoagulant effects by inhibiting the ability of VKOR enzyme to recycle vitamin K epoxide to vitamin K hydroquinone (KH2) (3). Thus, warfarin inhibits coagulation pathway by preventing the vitamin K cycle (4). The International normalized ratio (INR) is a standard index for monitoring of coagulation. An INR value between 2

and 3 is regarded as the therapeutic range for an individual (5). Warfarin has a narrow therapeutic rang. This narrow window range means that patients may suffer from bleeding when INR be upper than therapeutic range and thrombosis when INR be under therapeutic range (6, 7). There is substantial inter-individual variability in the warfarin dose needed to achieve optimal anticoagulation. Age, weight, height, vitamin K intake, and the use of concomitant medications that affect warfarin metabolism and disposition are a number of nongenetic factors known to influence warfarin dose requirements (8, 9).

Genetic factors have been associated with warfarin response are the genes for cytochrome P450 2C9 (CYP2C9), which metabolize the more active S-enantiomer of warfarin, and vitamin K epoxide reductase (VKOR), the target site for warfarin. Specifically, the CYP2C9*2 (R144C; rs1799853) allele in exon 3 and *3 (I359L; rs1057910) allele in exon 7 result in reduced CYP2C9 enzyme activity and lower warfarin dose requirements compared to the CYP2C9*1/*1 genotype (10, 11). The −1639G>A (rs9923231) polymorphism located in the VKOR complex-1 (VKORC1) gene promoter region has been correlated with 2-fold lower level of VKORC1 mRNA and lower warfarin dose requirements in predominately European Caucasian and Asian populations (12).

The clearance of drugs that has been eliminated by CYP2C9 has become increased by the treatment with rifampicin. In healthy subjects or patients who have been treated with rifampicin, the clearance of losartan, phenytoin, tolbutamide and S-warfarin is almost doubled (13).

Amiodarone, fluconazole and sulphaphenazole can inhibit CYP2C9. When an inhibitor is added to a therapeutic regime that includes drugs like warfarin, tolbutamide and phenytoin, then we face with drug-drug interaction (14). For example, There are different studies that indicated drug interaction between warfarin and amiodarone (15, 16).

Materials and methods

Study population and data collection

Enrolled subjects were Iranian patients on warfarin treatment who attended the PT Clinic at Shahid Rajaei cardiovascular and research center for regular INR monitoring from July to October 2012. After obtaining informed consent, these candidates were asked to fill out a questionnaire which contained demographic characteristics inclusive of sex, age, height, weight, ethnicity, use of any concomitant drugs, cause of warfarin therapy, daily warfarin dose requirement and all INR measurements during the three subsequent weeks. All patients had to be aged ≥18 years (Our patients were in the range of 18 – 87 years old). The recruited subjects had to achieve a stable INR in the target range of 2 to 3.5 at least during three consecutive months.

Patients in the study were excluded if: they had received any concurrent therapy known to interfere the anticoagulant effect of warfarin clinical evidence, showed hyperthyroidism or hypothyroidism, hepatic disease or dysfunction, or renal disease or dysfunction (17).

Genotyping

Genotyping of CYP2C9*2, CYP2C9*3 and VKORC1 (-1639 G>A) were performed by PCR-restriction fragment length polymorphism. The CYP2C9*3 allele was analyzed by amplification of a fragment including an NsiI restriction site present in the wild type allele but absent in the CYP2C9*3 allele because of the I359L mutation. The CYP2C9*2 allele was resistant to cleavage by AvaII and remained at 454bp. The wild type allele, cleaved into two fragments of 400 bp and 54 bp. For VKORC1 the amplified products (290 bp) were digested with a MspI restriction enzyme which produced a 124-and a 166- bp fragment in the absence of mutant allele. Primers and PCR product sizes for VKORC1 and CYP2C9 are shown in Table 5.

Table 5. Primers sequence and fragments length

Polymorphism	Primers sequence	Fragments length
CYP2C9*2	gTATTTTggCCTgAAACCCATA ggCCTTggTTTTTCTCAACTC	454 bp
CYP2C9*3	TGTGGTGCACGAGGTCCAGAGAT ACCCGGTGATGGTAGAGGTT	188 bp
VKORC1	gCCAgCAggAgAgggAAATA AgTTTggACTACAggTgCCT	290 bp

For CYP2C9*2 and CYP2C9*3, PCR was performed with an initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of denaturation (94 °C for 1 minute), annealing (57 °C for 1 minute), elongation (72 °C for 1 minute), and final extension (72 °C for 5 minutes). For VKORC1 the PCR condition was

similar, but annealing was performed at 60 °C for 1 minute.

Results

Hardy Weinberg equilibrium test for CYP2C9 * 2, CYP2C9 * 3 and VKORC1 polymorphisms was performed and according to the p-values of 0.651, 0.068 and 0.058 respectively, the Population was in Hardy Weinberg equilibrium. The relationship between warfarin dose and CYP2C9 polymorphisms was tested using the Kruskal-Wallis test. All tests were performed using SPSS, (version 16.0). $P < 0.05$ was considered significant.

The allelic frequencies of CYP2C9*2 and CYP2C9*3 were 19% and 7% respectively. Two groups of CYP2C9 genotypes were considered: CYP2C9*1/*1 (wild type) and CYP2C9X/Y (patients with ≥1 variant allele). Patients with ≥1 variant allele (n = 79) had a significantly lower mean warfarin daily dose compared with patients with the wild-type genotype.

The allelic frequencies of VKORC1 were 14.4% (17/118), 57.6% (68/118), and 27.9% (33/118) for GG, GA, and AA genotypes, respectively. The mean (SD) warfarin daily dose was 6.02 mg in patients with the VKORC1 (–1639) GG genotype. This was significantly higher than the weekly dose among those with the GA genotype (5.13 mg) or the AAgenotype (4.06 mg). Regression analysis models were designed for patients who had all demographic variables and allelic frequencies of the three enzymes available.

Table 1. Characteristics of studied patients.

Variables	values	Standard Derivation
Sex, no		
Men	42	
Women	76	
Age, year		
Mean	57.8	14.03
Range	18-87	
Weight, kg		
Mean	68.6	14.12
Range	40-105	
Height, cm		
Mean	164.4	10.31
Range	142-193	
Daily warfarin dose, mg		
Range	4.98	1.91
	1.25-10.89	

Multiple linear regression analysis found that sex (P = 0.717), height (P = 0.925), weight (P= 0.769), ethnicity (P= 0.270) and age (P = 0.188) had no significant influence on the maintenance dose of warfarin whereas CYP2C9 and VKORC1 polymorphisms had the greatest effects on the warfarin maintenance dose requirement. Characteristics of studied patients are mentioned in Table 1 and the distribution of CYP2C9 and VKORC1 genotypes in patients receiving warfarin are shown in Table 2.

Discussion

Warfarin is a well-accepted drug for many clinical conditions. However, it has a narrow therapeutic index; higher dose is the cause of excessive bleeding and lower dose leads to cerebrovascular clotting and stroke in patients. Many reports demonstrated some relationships between warfarin dose and warfarin sensitivity genotype (6, 11, 18). The two genes showing the greatest interaction with variation in warfarin dose are CYP2C9 and VKORC1. Combined, polymorphisms in these two alleles plus age, sex, and weight account for 56% to 64% of warfarin dose variation (19).

There are substantial differences in the frequencies of CYP2C9*2 and CYP2C9*3 allelic variants among different populations (20, 21). Our results indicated that most (66.9%) of Iranian participants had wild-type CYP2C9, 19% carried CYP2C9*2 allele and 7% had CYP2C9*3.

Previous published study introduced CYP2C9*2 variant as the most common mutant allele among Caucasians (10–13%), the present study found a slightly higher frequency (19%); this finding is different from previous study that reported the

frequency of 10–13% in other Caucasians, 0% in Japanese and 2% in Africans (19, 22).

Table 2. Distribution of CYP2C9 and VKORC1 genotypes in patients receiving warfarin

Genotype	Frequency	Percent	Mean warfarin dose (mg/day)
CYP2C9*1/*1	79	0.669	5.37
CYP2C9*1/*2	21	0.178	4.68
CYP2C9*1/*3	14	0.119	3.81
CYP2C9*2/*2	1	0.008	3.75
CYP2C9*2/*3	2	0.016	2.76
CYP2C9*3/*3	1	0.008	1.78
VKORC1 GG	17	0.144	6.02
VKORC1 GA	68	0.576	5.13
VKORC1 AA	33	0.279	4.06

The observed frequency for CYP2C9*3 was 7% in the present study. This finding was similar to the other Caucasian frequency (6–9%) but different from the frequency found in Japanese (2.3%) and Africans (1%) (21, 23). A comparison between CYP2C9 allele and genotype frequencies of different populations are summarized in Table 3.

Table 3. A summary of CYP2C9*2 and CYP2C9*3 allelic variant* frequencies of different population.

CYP2C9	Present Study (%)	Italy (%)	Sweden (%)	British (%)	UK (%)	Dutch (%)	Japan (%)	African (%)
***1**	61	77.7	81.9	79	84.1	76.6	97.6	9
***2**	19	12.5	10.6	12.5	10.6	14.2	0	2
***3**	7	9.7	7.4	8.5	5.2	9.2	2.3	1
Reference	____	(20)	(23)	(25)	(35)	(38)	(39, 40)	(40)

In the present study, as it was expected, the frequencies of homozygotes for the CYP2C9*2 and CYP2C9*3 variants were low (both 0.8%). The frequencies of the two heterozygous genotypes CYP2C9*1/*2 and CYP2C9*1/*3 were 17.8% and 11.9%, respectively, whereas compound heterozygotes of CYP2C9*2/*3 was 1.6%. Our results are consistent with those of Caucasian population which was studied by Hauge et al. (2008) (24). They evaluated the genotype distributions of CYP2C9*2 and CYP2C9*3 polymorphisms in 212 patients and found that the genotype frequency of the CYP2C9*1/*2 variant was 17.9%, whereas the CYP2C9*1/*3 variant was 9.4%. Homozygotes of the CYP2C9*2 and CYP2C9*3 variant constituted 1.9% and 0.5% of the patients, respectively and CYP2C9 compound heterozygotes (CYP2C9*2/*3) was 0.9%.

In a study in Taiwan (18) 0.4% of 92 white subjects were homozygotes for CYP2C9*3 alleles; in another study in United Kingdom one of 297 patient had CYP2C9*3/*3, wich is consistent with our findings in that this genotype was found in one patient of Iranian subjects (25).

We found that those who had the wild-type variant of CYP2C9 needed a significantly higher mean dose of warfarin (5.37 mg/d) compared with others. The second group belongs to patients who carried one mutation in CYP2C9 gene. In this group, patients with CYP2C9*1/*2 needed higher mean dose of

warfarin (4.76 mg/d) than CYP2C9*1/*3 (3.84 mg/d). The Third group belongs to patients who carried more than one mutation in CYP2C9 gene. This group included patients with CYP2C9*2/*2, CYP2C9*2/*3 and CYP2C9*3/*3 genotype who needed 3.75 mg/d, 2.76 mg/d and 1.87 mg/d, respectively. Previous studies showed that *2 and *3 polymorphisms are associated with reduced metabolism of warfarin; Margaglione et al. (2000) reported similar observations in 180 Italian patients followed up at one specialized clinic from the start of the anticoagulation with warfarin. Required dose of warfarin in patients with CYP2C9*1 haplotype (5.6 mg/d), was more than those carried CYP2C9*2 (4.7 mg/d) and CYP2C9*3 (4.0 mg/d) haplotypes. Patients who had both CYP2C9*2 and CYP2C9*3 haplotypes, with 1.8 mg/d warfarin dose were in therapeutic INR range (26).

The frequencies of different VKORC1 −1639G>A alleles differ among Asian, African-American and Caucasian populations (18). The VKORC1 haplotype group A that leads to low warfarin dose requirement was the highest in Asian populations (89%), whereas haplotype group B was the highest in Caucasian populations (58%) (27).

Our results showed that 14.4% of participants carried a GG genotype, whereas the AA and GA genotypes were seen in 27.9% and 57.6% of participants, respectively. The frequency of the –1639A and –1639G alleles were estimated as 56.7% and 43.2%, respectively; this result agrees with reports from the other Caucasians and Hispanics but is different ($p < 0.05$) from the frequencies seen among Asians and in African American populations. A comparison between VKORC1 allele and genotype frequencies among different ethnics is summarized in Table 4 (28).

Table 4. Comparison between VKORC1 allele and genotype frequencies among different ethnics.

VKORC1 -1639G>A genotype	African-American (n=300)	Asian (n=102)	Caucasian (n=106)	Hispanic (n=101)	Present study (n=118)
GG	80.3	22.5	36.8	30.7	14.4
GA	17.7	21.6	45.3	51.5	57.6
AA	2.0	55.9	17.9	17.8	27.9
VKORC1 -1639G>A Allele					
G	89.5	33.3	59.45	56.45	56.7
A	10.85	66.7	40.55	43.55	43.2

In another study in Mazandaran province of Iran a total of 29 patients taking warfarin were studied. The objective of study was to determine the influence of 3 VKORC1 polymorphisms (-1639G>A, 1173C>T and 3730G>A) on warfarin dose requirement. Significant correlation was obtained only between -1639G>A polymorphism and warfarin dose requirement. In this polymorphism, 25 patients (86.2%) were AA, 2 patients (6.9%) were GA and 2 patients (6.9%) were GG genotype. Mean dose of warfarin in patients with AA genotype was lower but their INR was higher than the other two genotypes (29).

Our results suggest that VKORC1 polymorphism may have a greater effect on warfarin dose than CYP2C9 polymorphism, and this is in accordance with the findings of previous studies. RHaug et al. studied 105 Norwegian patients of Caucasian race and the findings confirmed that the VKORC1 polymorphism had a larger impact (25%) compared to CYP2C9 (7%) (24). Another study by Herman et al. which analyzed 165 Slovenians showed that VKORC1 contributed 34% and CYP2C9 18% (22). Based on a review article by Wadelius and Pirmohamed, six studies found that VKORC1. had a greater effect on warfarin dose than CYP2C9 (30-33). In contrast, two studies which were performed by Scon et al. and D'Andrea et al., found the greatest effect of CYP2C9 (34, 35) and one study by Vecsler et al. reported equal contribution of the two polymorphisms (21). Previous studies reported the influence of age on warfarin dose requirement but we failed to find any association between age and dose of warfarin. For example, a study of 297 patients on stable warfarin doses reported that mean warfarin daily dose requirements fell by 0.5 to 0.7 mg per decade between the ages of 20 to 90 years (35). In another study of 115 African American stable patients, it was observed that CYP2C9 genotype, age, and body surface area account for 33% of the warfarin dose variability, whereas VKORC1 seemed to have no effect on warfarin dose (28). Hossam et al. studied 207 Egyptian patients taking warfarin for more than two months (36). VKORC1 genotype ($P < 0.0001$), CYP2C9 gene ($P= 0.0004$) and increasing age ($P < 0.0001$) were associated with warfarin doses. Similar to our observation height and weight were not significant predictors of dose; in our study height and weight didn't have any association with the dose

requirement either. Furthermore, in regard to P= 0.717 and P= 0.270 for sex and ethnicity respectively, these two factors had no influence on warfarin dose. These findings are similar to the findings of a study that was conducted in 2010 in wich sex (P=0.36), weight (P=0.75), body mass index (BMI) (P=0.67) and height (P=0.55) had no influence on warfarin dose (37).

Conclusion

Warfarin has been one of the most important coagulation drugs for a long time. Despite its efficiency,the possibility of bleeding in sensitive patients, makes it difficult to be used. Warfarin sensivity has a multigenic and multifactorial inheritance. However, a combination of genetic polymophisms has the greatest contribution to warfarin sensivity. The correlation between the maintenance dose of warfarin and allelic variants of CYP2C9 and VKORC1 has been demonstrated by many previous studies and is reconfirmed in this report.

The present study found a higher frequency for CYP2C9*2 (19%) than those previously found in other Caucasians. For CYP2C9*3, the frequency of 7% found in the present study was similar to the reports on other Caucasians. The frequency of the –1639A and –1639G alleles were estimated 56.7% and 43.2%, respectively; this result agrees with reports from the other Caucasians. Moreover, our study showed that CYP2C9 and VKORC1 polymorphisms had significant influence on Iranian daily warfarin dose, but the effect of age was not significant. Height and weight did not have a significant correlation with the warfarin maintenance dose. In addition, there was no significant relationship between sex, ethnicity and the maintenance dose of warfarin (the data are not shown).

Understanding the pharmacogenetics involved in warfarin variability, helps physicians to prescribe appropriate dose that prevents bleeding or clotting effects. If such studies improve the outcomes of therapy, then more investigations lead to design algorithms that present a new way to achieve therapeutic INR in a rapid and easy manner.

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