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Original Article

The effect of *CYP2C9*2, CYP2C9*3,* and *VKORC1-1639 G>A* polymorphism in patients under warfarin therapy in city of Kermanshah

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Abstract

Polymorphism in the genes encoding *CYP2C9* enzyme and *VKORC1* reductase significantly influence warfarin dose requirement since patients with *CYP2C9*2, CYP2C9*3* and *VKORC1* mutant alleles require lower warfarin maintenance doses. Studies have reported the ethnic variations in the frequency of these genes within the various populations in Iran and other parts of the world. However, no such study has been done yet on Kurdish population in Kermanshah. From Kurdish population of Kermanshah province in Iran, a total of 110 patients who had heart surgery and taking warfarin, were genotyped for polymorphisms of *VKORC1-1639 G>A*, *CYP2C9*2*, and *CYP2C9*3*. Polymorphism genotyping was performed by sequencing as well as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using restriction enzymes of *MspI*, *AVAII* and *KpnI*, respectively. The frequencies of *VKORC1*-1639 GG, GA, and AA genotypes were 42%, 36%, and 22%, respectively and for *CYP2C9* 1*/1*, 1*/2*, 2*/2*, 1*/3*, 3*/3*, 2*/3* were 71%, 17%, 5.4%, 1.8%, 4.5%, and 0%, respectively. The frequency of *VKORC1*-1639A allele was 42.3% and the frequencies of *CYP2C9*2* and *3 alleles were 14% and 5.4%, respectively. It was indicated that low warfarin dose requirements are strongly associated with the presence of *CYP2C9* and *VKORC1-1639* variant alleles. Our results confirmed the supply to understand the distribution of genomic biomarkers related to the drugs metabolism for future planning health programs.

Keywords: Cytochrome P-450 CYP2C9; International normalized ratio; Polymorphism; Vitamin K1 epoxide reductase; Warfarin.

INTRODUCTION

Warfarin is an anti-coagulation which is derived from coumarin and widely used in treating thrombosis by reducing the risk of blood clotting (1-3), (Fig. 1). The required warfarin dose among different individuals is influenced both by non-genetic factors including age, sex, weight, smoking, drug use, and genetic factors such as cytochrome P-450 family 2, subfamily C, polypeptide 9 (*CYP2C9*) and vitamin K epoxide reductase complex, and subunit 1 (*VKORC1*) genes (1,4). Clinical factors and variations in two genes considerably affect the already mentioned requirements for warfarin in various patients (5-7). The Food and Drug Administration (FDA) recommended an initial range of warfarin dose requirement for individuals based on their *CYP2C9* and *VKORC1* genotypes presented in Table 1.

CYP2C9 is an essential enzyme expressed in the liver and transcribed by a gene located on the 10q22 chromosome. The gene is about 55 kb and contains 9 exons (8) having alleles among which *CYP2C9*2* (single nucleotide polymorphisms (SNP) ID: rs1799853) (arginine- to -cysteine change at codon 144) and *CYP2C9*3* (SNP ID: rs1057910) (isoleucine- to -leucine change at codon 359) can reduce the activity of *CYP2C9* enzyme in comparison to *CYP2C9*1*, the wild type allele, due to the lower catalytic activity of the two allelic variants (9).

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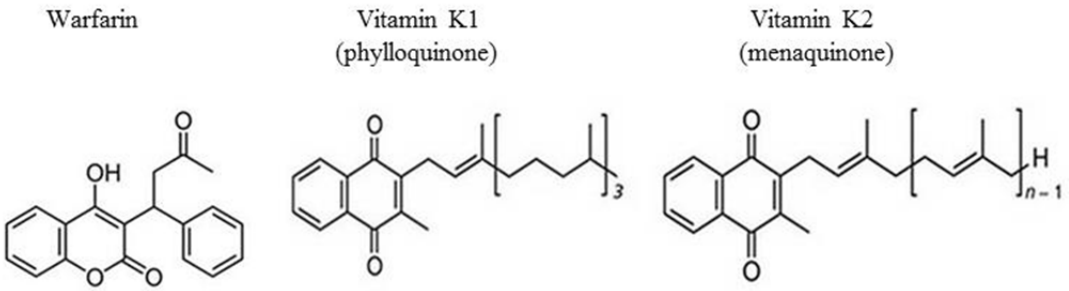

Fig. 1. Chemical structure of warfarin, and two naturally forms of vitamin K1 (phylloquinone) and vitamin K2 (menaquinone) (32).

Table 1. Food and Drug Administration (FDA) recommended initial doses (mg/day) requirements of warfarin for individuals based on their CYP2C9 and VKORC1 genotypes.

VKORC1	***CYP2C9***	**Dose**
GG	*1/*3, *2/*2, *2/*3	
AG	*1/*2, *1/*3, *2/*2	3-4 mg
AA	*1/*1, *1/*2	
GG	*1/*1, *1/*2	
AG	*1/*1	5-7 mg
GG	*3/*3	
AG	*2/*3, *3/*3	0.5-2 mg
AA	*1/*2, *2/*2, *2/*3, *3/*3	

The *VKORC1* gene contains multiple polymorphisms associated with the variety of responses to warfarin. This gene is located in the promoter region and its polymorphism changes the expression of vitamin K epoxide reductase enzyme (10). The presence of a G nucleotide position 1639 instead of an A in *VKORC1* gene (*-1639 G>A*) (SNP ID: rs9923231), increases the activity of the enzyme; therefore, promoter activity and then mRNA expression are reduced (11). *VKORC1* is located on 16p11 chromosome and contains 3 exons and 5125 base pairs (8). The presence of *CYP2C9*2, CYP2C9*3,* and *VKORC1-1639 G>A* in individuals reduces the dose requirements for warfarin (12). Accordingly, it may be stated that *CYP2C9*2, CYP2C9*3,* and *VKORC1-1639 G>A* polymorphisms affect warfarin dose requirements to maintain a target international normalized ratio (INR = (prothrombin time (PT) measured ISI (international sensitivity index)/PT normal)) in warfarin users (13), (Table 1). The aim of the present study was to determine the prevalence of *CYP2C9*2, CYP2C0*3*, and *VKORC1-1639 G>A* polymorphisms and also their correlation to warfarin dose requirement in patients taking warfarin living in the city of Kermanshah, I.R. Iran.

MATERIALS AND METHODS

A total of 110 patients under warfarin therapy (59 males and 51 females) (warfarin tablet, 5 mg, Orion Corporation, Espoo, Finland) were enrolled in the present study. The patients had undergone heart surgery in the Imam Ali Hospital (Kermanshah, I.R. Iran). The research protocol was approved by Ethics Committee of the Kermanshah University of Medical Sciences in accordance with international agreements (ID number: 2260) (14). An informed consent form was voluntarily signed by all participants.

Blood samples were taken from patients to measure INR value and extract DNA. Genomic DNA was extracted from peripheral blood leukocytes by phenol-chloroform extraction method (15). The extracted DNA was stored at -20 °C until it was used. The *CYP2C9*2, CYP2C9*3,* and *VKORC1-1639 G>A* polymorphisms were genotyped using

polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The used primers were as follows: *CYP2C9*2* forward: 5´-GTA TTT TGG CCT GAA ACC C-3´, *CYP2C9*2* reverse: 5´ -GGC CTT GGT TTT TCT CAA CTC-3´ (16); *CYP2C9*3* forward: 5´-TGC ACG AGG TCC AGA GGT AC-3´, *CYP2C9*3* reverse: 5´-ACA AAC TTA CCT TGG GAA TGA GA-3´ (17); *VKORC1-1639 G>A* forward: 5´-GCC AGC AGG AGA GGG AAA TA-3´, *VKORC1-1639 G>A* reverse: 5´-AGT TTG GAC TAC GGT GCC T-3´ (18). The PCR reaction consisted of buffer 10× (100 mM tris-HCl, 500 mM KCl, 50 mM $MgCl_2$, pH 8.3), 0.2 mM dNTPs, 200 ng template DNA, and 1U AmpliTaq DNA polymerase. PCR thermal cycling conditions were as follows: for CYP2C9*2, one cycle of initial denaturation at 95 °C for 10 min, then 35 cycle of initial denaturation at 94 °C for 45 sec followed by annealing at 54 °C for 45 sec, and final extension at 72 °C for 10 min; for CYP2C9*3, one cycle of initial denaturation at 95 °C for 10 min, then 35 cycle of initial denaturation at 94 °C for 45 sec followed by annealing at 53 °C for 45 sec, and final extension at 72 °C for 10 min; for VKORC1-1639 G>A, one cycle of initial denaturation at 95 °C for 10 min, then 35 cycle of initial denaturation at 94 °C for 45 sec followed by annealing at 58 °C for 45 sec, and final extension at 72 °C for 10 min. PCR products were examined on 2% agarose gel with Gel Red DNA stain under ultraviolet light. The used restriction enzymes (Fermentas) were AVA II for *CYP2C9*2*, KpnI for *CYP2C9*3*, and MSP I for *VKORC1* (9,19). For restriction enzyme digestion of RCR products, 12 µL of PCR products was mixed with 1 µL restriction enzyme and 2 µL buffer, and incubated for 16 h at 37 °C. For genotypes sequencing, the samples were analyzed by DNA Sequencer apparatus (Macrogen, South Korea).

Data analysis

Deviation from Hardy-Weinberg Equilibrium (HWE) was tested using the calculator. The data was also analyzed by t-test using statistical package for social sciences (SPSS) version 18 and presented as the medians with 95% confidence intervals (20).

RESULTS

The results of the allele variants of *CYP2C9*2*, *CYP2C9*3* and *VKORC1-1639 G>A* obtained from PCR-RFLP are shown in Figs. 2-4. The estimated genotype and allele frequencies of *CYP2C9*2*, *CYP2C9*3*, and *VKORC1-1639 G>A* and 0.054 (12 alleles), respectively and the *VKORC1-1639 A* allele frequency was 0.423.

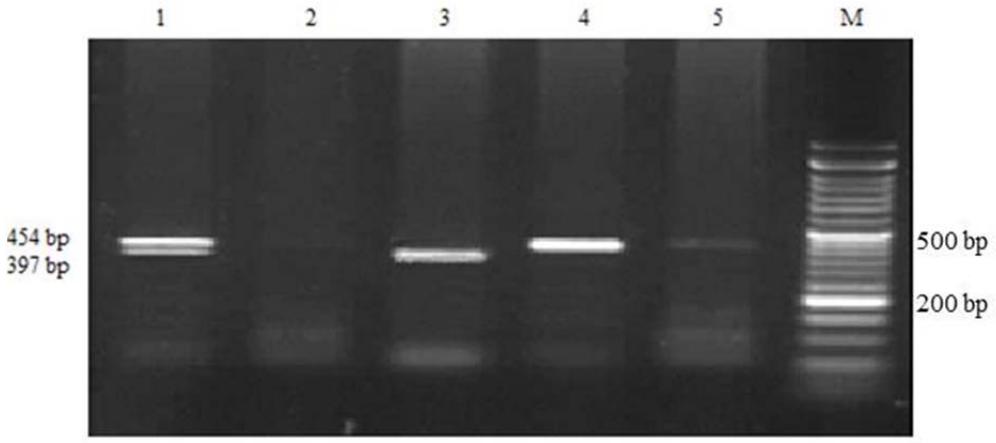

Fig. 2. Agarose gel electrophoresis for *CYP2C9*2* polymorphism detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and restriction enzyme *AVA II*. M, marker; lane 1, CT heterozygous; lane 2, negative control; lane 3, CC homozygous wild type; lanes 4 and 5, TT homozygous mutant type.

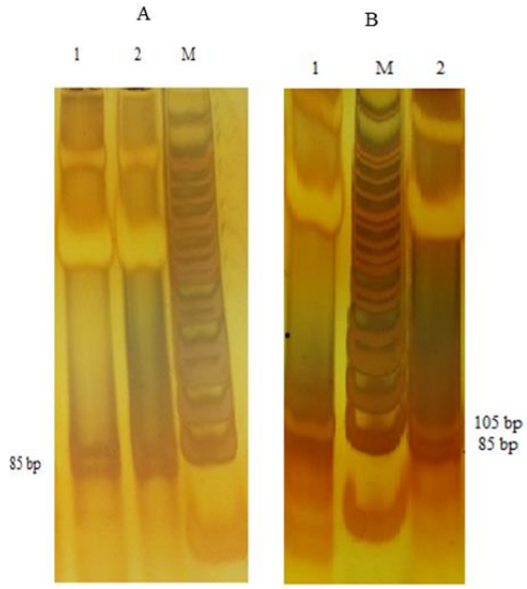

Fig. 3. Agarose gel electrophoresis for *CYP2C9**3 polymorphism detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and restriction enzyme *KpnI*. (A) M, marker; lane 2, CC homozygous mutant type. (B) M, marker; lane 1, AC heterozygous; lane 2, AA homozygous wild type.

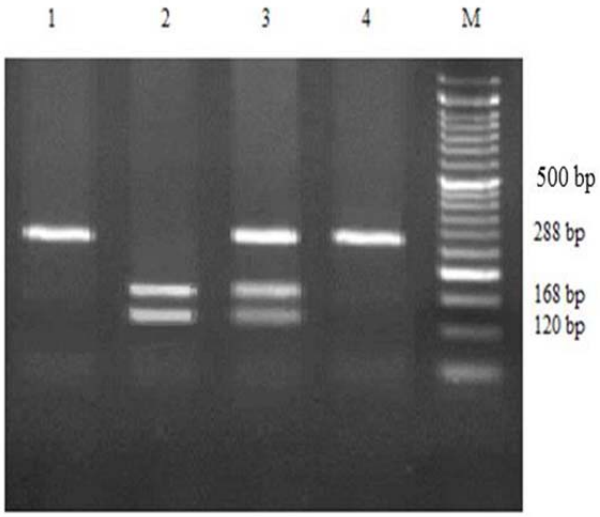

Fig. 4. Agarose gel electrophoresis for *VKORC1* G>A polymorphism detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and restriction enzyme *MSP I*. M, marker; lane 1 and 4, AA homozygous mutant type; lane 2, AC heterozygous; lane 3, GG homozygous wild type.

Table 2. Genotype and allele frequencies of the *CYP2C9*2, CYP2C9*3* alleles, and *VKORC1-1639 G>A* polymorphism in Kurdish population of Kermanshah.

Sex	**N (%)**	**95% Confidence interval**
Female	51 (46.36)	
Male	59 (53.64)	
***CYP2C9* genotype**		
1*1*	78 (71)	68-76
1*2*	19 (17.2)	15-19.3
1*3*	2 (1.8)	1-3.1
2*2*	6 (5.4)	2.4-6.8
3*3*	5 (4.5)	3.6-7.2
***CYP2C9* allele**		
CYP2C9*1	177 (80.4)	
CYP2C9*2	31 (14)	
CYP2C9*3	12 (5.45)	
***VKORC1* genotype**		
GG	46 (42)	40.67-43
GA	35 (32)	28-36.1
AA	29 (26)	23-28.4
***VKORC1* allele**		
G	127 (57.7)	
A	93 (42.3)	

There were 78 subjects with *CYP2C9*1/*1* genotype (0.71), 19 subjects with *CYP2C9*1/*2* genotype (0.17), 6 subjects with *CYP2C9*2/2* genotype (0.054), 2 subjects with *CYP2C9*1/*3* genotype (0.018), 5 subjects with *CYP2C9*3/3* genotype (0.045), and none carried *CYP2C9*2/*3* genotype. For *VKORC1-1639 G>A* polymorphism, there were 46 subjects with *VKORC1-1639 GG* genotype (0.42), 35 with *VKORC1-1639 G>A* genotype (0.32), and 29 with *VKORC1-1639 AA* genotype (0.26) (Table 2). The results of direct DNA sequencing from the patients with wild genotype and mutant genotype for each gene are shown in Fig. 5.

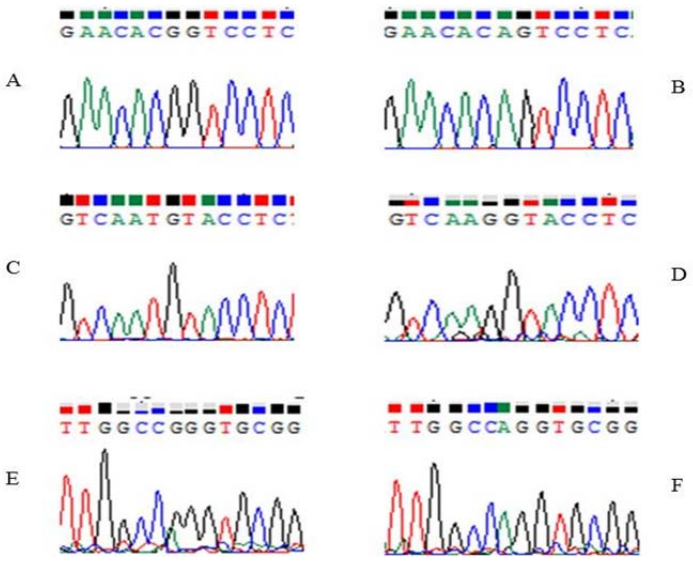

Fig. 5. Sequence analysis of DNA samples. (A) Sequence of *CYP2C9*1*1* genotype GG. (B) Sequence of *CYP2C9**2*2 genotype AG. (C) Sequence of *CYP2C9*1*1* genotype TG. (D) Sequence of *CYP2C9**3*3 genotype GG. (E) Sequence of *VKORC1-1639* GG. (F) Sequence of *VKORC1-1639* AG**.**

Table 3. The warfarin dose requirement for the patients in our study to reach international normalized ratio (INR) goal of 2-3 according to the genotype (mg/day).

Genotype	***VKORC1GG***	***VKORC1AG***	***VKORC1AA***
CYP2C9*1*1	6.4	5.2	4
CYP2C9*1*2	5	3.9	2.6
CYP2C9*1*3	4.3	3.5	2.2
CYP2C9*3*3	2	1.8	1
CYP2C9*2*2	4.1	3	2

Table 4. The calculated *P* values for each SNP.

VKORC1	*CYP2C9**2	*CYP2C9**3
0.005	0.001	0.003

Warfarin dosing is typically adjusted to maintain the INR at 2.5 ± 0.5 and at 3.0 ± 0.5 for higher risk patients, including those with certain mechanical heart valves (21). Considering the achieved data including the range of INR and calculating the genotype frequency, it was observed that warfarin dose requirements for reaching the target INR had a significant relationship with individuals´ genotype. Thereby, people with a mutation in *CYP2C9*2*, *CYP2C9*3*, and *VKORC1-1639 G>A* genes require less warfarin dose daily. Table 3 presents the relationship between warfarin dose requirements and individuals' genotype. The calculated *P* values for each SNP are also shown in Table 4.

DISCUSSION

Drug metabolism varies from one patient to another due to genetic differences. Accordingly, the required warfarin dose depends upon various factors including age, diet, sex, and last but not least, the genetics. Each society has its own specific gene polymorphism considered a significant determinant in drug metabolism. Patients with *VKORC1*, *CYP2C9*2*, and *CYP2C9*3* mutant alleles require lower warfarin maintenance doses (1,4). Studies showed that the mutations on these genes may lead to an abnormal three-dimensional conformation of the molecule, which would have a remarkable impact on the

protein structure. Furthermore, these mutations can alter the amino acid sequence of the proteins which result in altered enzymatic activity (22).

The basic method used in this study was PCR-RFLP which is an appropriate method in SNP genotyping and mutation detection. Also, our sequencing results confirmed the results derived from RFLP-PCR. The frequencies of *CYP2C9*2* and *CYP2C9*3* alleles are different in various populations, as they are very common in some countries while being rare in others (9). Since individuals with *CYP2C9*2* and *CYP2C9*3* alleles have lower *CYP2C9* enzyme activity, they are prone to bleeding following warfarin therapy. Individuals with *CYP2C9*2* and *CYP2C9*3* genotypes have 12% and 5% lower enzyme activity, respectively than the wild type. Therefore, studying the frequencies of *CYP2C9*2* and *CYP2C9*3* genes in populations with high frequency is extremely important prior to warfarin therapy (23). In this study, the frequency of *VKORC1-1639 G>A* SNP genotypes as well as the frequencies of *CYP2C9*2* and *CYP2C9*3* alleles were evaluated.

Based on our findings, allele frequency of *CYP2C9*2* in Kermanshah population (14%) was similar to the results from German (14%) (24), French (14%) (25), and Caucasian (14.3%) (25) populations. In case of frequency of *CYP2C9*3* allele, similar results were also obtained in our study (5.4%) compared to the report from German population (5%) (24) (Table 5). However, the estimated frequency of *CYP2C9*2* and **2* alleles in our study compared with the reported corresponding results from Japanese, Caucasian, and African-American populations (26) showed a considerable difference (Table 5). Moreover, the frequency of *VKORC1* in our study (42.3%) was similar to the results from the German, French, and Caucasian populations and different from the Japanese (89%) and African-American (8.6%) populations. In addition, our results were different from the findings obtained from the studies on other populations in Iran. For example, Azarpira, *et al*. in Shiraz determined the allele frequency of *CYP2C9*2*, *CYP2C9**3* and *VKORC1-1639 G>A* with the frequencies of 25.3%, 9.8%, and 55.6%, respectively (27). In another study by Namazi, *et al.* in Shiraz (28), the allele frequency for *CYP2C9*2*, *CYP2C9**3*, and *VKORC1-1639 G>A* was obtained as 27%, 9%, and 18%, respectively (Table 5). In contrast to Kermanshah population, most of the subjects in Birjand showed more variant allele frequency of *CYP2C9*2*, *CYP2C9*3*, and *VKORC1-1639 G>A* as 9.1, 10, and 31.9%, respectively (29). Furthermore, Dalily and Ramazani in 2012 (30) and Kameli, *et al.* in 2016 (31) in two separate studies in Iran reported different genotype frequencies for *CYP2C9*2*, *CYP2C9*3* and/or *VKORC1* compared to the obtained results in our study (Table 5).

Table 5. Comparative allele frequency (%) of three single nucleotide polymorphisms (SNPs) (*CYP2C9**2*, *CYP2C9**3* and *VKORC1*-1639 A) in Iran and the different world populations.

	N	***CYP2C9*2***	***CYP2C9*3***	***VKORC1-1639 A***
Iran (Kermanshah)	110	14	5.4	42.3
German (Burian)[24]	118	14	5	ND
German (Geisen)[33]	200	ND[1]	8.6	42
French (Bodin)[25]	222	14	20.9	42
Caucasian (Takahashi)[26]	157	14.3	0.8	42.2
African-American[26]	36	0	1.6	8.6
Japanese[26]	172	0	10	89
Iran (Birjand)[29]	120	9.1	9	31.9
Iran (Shiraz)[28]	100	27	9.8	18
Iran (Shiraz) [27]	150	25.3	7	55.6
Iran[30]	118	19	10	57.6
Iran[34]	400	26	ND	ND

[1] ND: not determined.

Collectively, it can be concluded that the frequencies of *CYP2C9*2*, *CYP2C9*3*, and *VKORC1* genes are different in various populations. Regarding the use of warfarin dose and individuals' genotypes, it was observed that people having mutation in these genes require less warfarin doses to reach the target INR in comparison to people with no gene mutation (32). Therefore, it can be stated that warfarin dose requirement for reaching the target INR is influenced by individuals, genetics.

CONCLUSION

The role of *VKORC1* and *CYP2C9* polymorphisms has been long studied regarding warfarin therapy and to help clinicians with the new field of pharmacogenetics in order to determine the proper warfarin dose in patients treatment strategy. Our study confirmed the previous reports about the significance of pharmacogenetic testing in predicting a high risk of bleeding before initiation of anticoagulation with warfarin in the patients carrying either the *VKORC1* variant (1639A) or the *CYP2C92*, CYP2C9*3 alleles. According to the association between drug dosage and genotype, conducting regional studies based on races and geographical conditions is considered essential.

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