Warfarin maintenance dose associated with genetic polymorphisms of CYP2C9

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Abstract

Background: Cytochrome P450 2C9 (*CYP2C9*) gene polymorphisms alters the required warfarin dose in patients, due to pharmacogenetic events. This study aimed to identify the frequency of the allele *CYP2C9* polymorphic variants *2 and *3, and the association of these allelic variants with warfarin dosage in the population of the west Azerbaijan province in Iran.
Methods: One hundred and seventy patients receiving warfarin were examined to evaluate the genotype frequency of common *CYP2C9* polymorphisms. Genotype analysis for *CYP2C9**2 and *CYP2C9**3 polymorphisms was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. To assess if the dosage is different between genotypes we used one-way analysis of variance (ANOVA).
Results: Frequency of the two variants in studied subjects was 12 % for *CYP2C9**2 and 25.8 % for *CYP2C9**3. Comparison of the warfarin daily maintenance dose between genotype groups showed that the daily mean dose of warfarin in patients who have homozygous wild-type genotype for *CYP2C9* (*1/*1) was 5.26 ± 2.32 mg, which was significantly higher compared to *1/*2, *1/*3 (3.57 ± 2.25 mg, $p < 0.001$) and *2/*2, *2/*3 and *3/*3 patients (3.76 ± 2.4 mg, $p = 0.024$). Further analysis revealed that the allelic frequency of *CYP2C9* polymorphisms in the study population was similar to that of the Turkish population.
Conclusions: Due to the relatively high frequency of *CYP2C9* polymorphisms in the study population, the clinicians should become aware of these results to reduce the risk of hemorrhage when prescribing warfarin. HIPPOKRATIA 2017, 21(2): 93-96.

Keywords: *CYP2C9* polymorphisms, warfarin, pharmacogenetic, polymerase chain reaction-restriction fragment length polymorphism assay, PCR-RFLP

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Introduction

Warfarin is administered for various therapeutic purposes including prevention of stroke in patients with chronic atrial fibrillation, embolism in patients with rheumatic heart disease, prevention and treatment of venous thrombosis, pulmonary embolism, and transient ischemic attack[1]. Warfarin dose requirement is estimated by measurements of the prothrombin time (PT) and international normalized ratio (INR) blood tests. Therapy usually starts with a fixed dose of warfarin, followed by regular measurements of the coagulation rate. The weekly dose for each patient is adjusted to ensure that the patient's INR is in targeted range[2].

Despite the effectiveness of warfarin in treated patients, it may be associated with toxic effects in some, especially in the initial stage of its administration[3]. Clinical potency and safety of warfarin depend on maintaining a therapeutic INR range and this is achieved by personalizing the drug dose for each patient[4]. If the drug level in the blood is lower than targeted, therapeutic goals are not achieved, and there is a risk of blood clot formation while if it is too high, there is an increased potential risk of bleeding. This is particular-

ly important due to the wide variation among individuals regarding their response to warfarin doses[5]. Genotyping of the single nucleotide polymorphisms (SNPs) in genes regulating warfarin metabolism or its target enzymes could enable doctors to prescribe each patient with the appropriate therapeutic dose of warfarin[6]. The *CYP2C9* gene is responsible for catalyzing warfarin metabolism[5], and several common variant alleles have been reported for it, but of these allelic variants *CYP2C9**2 (rs 1799853, c. 430 C>T, p. Arg144Cys) and *CYP2C9**3 (rs 1057910, c. 1075 A>C, p. Ile359Leu), are the most common functionally significant coding SNPs that affect enzyme activities and cause impaired metabolic capacity of the CYP2C9 enzyme by amino acid substitution. Patients with one or two of these variants require a lower dose of warfarin and encounter a higher risk of bleeding compared with patients carrying the wild-type (*1) allele[7].

In the present study, we assessed the alleles and genotypes of *CYP2C9* and established minor allele frequencies in the heart valve replacement cohort of the west Azerbaijan province in Iran. We also tested how

polymorphic variants within *CYP2C9* could affect the required dose of warfarin.

Materials and Methods
Patients and Criteria

In this prospective study conducted between February 2016 and March 2017, 170 consecutive heart valve replacement patients, from Seyyedal shohada Hospital in Urmia, Northwest of Iran who required warfarin therapy were enrolled and had their INR checked. The targeted INR range for the studied cohort was calculated at 2.5-3.5. Their mean daily warfarin dose was 4.23 ± 2.43 (range 0.28-12.10 mg). Patient characteristics are presented in Table 1. The study was approved by the Scientific and Ethical Committee of Urmia University of Medical Sciences (Ir.umsu.rec. 1394.282 /05.02.2016).

Sampling and DNA extraction

Peripheral blood samples were obtained from each patient and transferred to ethylenediamine tetraacetic acid (EDTA) containing tubes in order to prevent clotting. Genomic DNA was extracted using a DNA Extraction mini kit (YTA Company, Tehran, Iran) according to the manufacturer's instructions. The purity and concentration were checked using gel electrophoresis and NanoDrop™ spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Table 1: The demographic characteristics of the 170 patients included in the present study.

Variable		
Age (years)		54.8 ± 14.0
Weight (kg)		72.67 ± 14.8
Height (cm)		162.83 ± 9.98
Daily warfarin dose (mg)		4.23 (0.28-12.10)
Sex	Male	66 (38.8 %)
	Female	104 (61.2 %)
Race	Turk	152 (89.4 %)
	Kurd	16 (9.41 %)
	Fars	2 (1.17 %)

Values are presented as means ± standard deviation (age, weigh, height), mean and range in brackets (daily dose), BMI: body mass index.
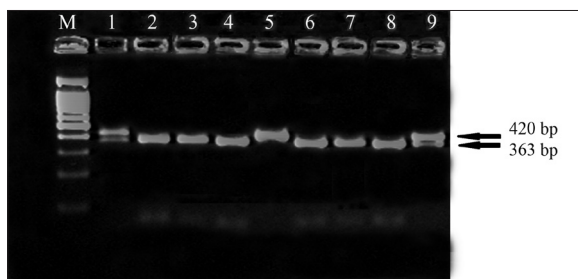

Figure 1: *AvaII* digestion products. Lane M, 100-bp ladder; lanes 1 and 9 two CT heterozygotes with fragments of 420-bp, 363-bp; lane 5, mutant homozygote *AA* with fragment of 420-bp; and lanes 2, 3, 4, 6, 7, and 8, wild homozygote CC with fragment of 363-bp.

Genotyping of CYP2C9 allele

Genotyping for the *CYP2C9*2* and *CYP2C9*3* polymorphisms was performed with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay using the primers (5'-GGATATGAAGCAGTGAA-GGAA-3' and 5'-GGCCTTGGTTTTTCTCAACTC-3) to amplify a 420-bp *CYP2C9*2* amplicons and (5'-TG-CACGAGGTCCAGAGATGC-3' and 5'- AAACATG-GAGTTGCAGTGTA-3') to amplify a 131-bp *CYP2C9*3* amplicons. The PCR reactions were performed as described by Daly et al in 2006[8]. Ten microliters of PCR products were incubated with one unit of the restriction endonuclease *AvaII* and *NsiI* (New England Biolabs, Ipswich, MA, USA) restriction enzymes for *CYP2C9*2* and *3* respectively at 37 °C overnight. The digestion products were analyzed on 2 % agarose gel. For *CYP2C9*2* a 420 bp DNA fragment was amplified by PCR. After RFLP analysis, the presence of the mutant allele T was indicated by loss of the single *AvaII* site and a single band at 420 bp while products containing C allele were digested to two fragments of 363 bp and 57 bp (Figure 1). For *CYP2C9*3* a 131 bp DNA fragment was amplified. The presence of the *CYP2C9*3* allele was indicated by the loss of the single *NsiI* site created by the PCR primer. So amplified products containing mutant allele C were visualized as 131 bp bands, whereas those containing the wild allele T produced bands of 110 bp and 21 bp (Figure 2).

Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistics (IBM SPSS, IBM Corp., Armonk, NY, USA), version 23.0. The significant differences in the daily maintenance dose of warfarin between the different genotype groups were evaluated by ANOVA and Tukey post-hoc test. Box plot analysis was performed to graphically display the dispersion and skewness of genotyping data. The statistical level of significance was set at $p = 0.05$.

Results

The genotype frequencies of the *CYP2C9*2* variant were 75.9 %, 22.9 %, and 1.2 % for CC, CT, and TT genotypes, respectively. The genotype frequencies of the *CYP2C9*3* variant were 50 %, 48.2 %, and 1.8 % for AA, AC, and CC genotypes, respectively.
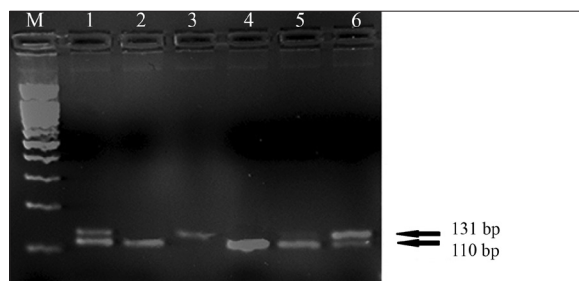

Figure 2: *NsiI* digestion products. Lane M, 100-bp ladder; lanes 1 and 6 two CA heterozygotes with fragments of 131-bp and 110-bp; lane 3, mutant homozygote CC with fragment of 131-bp; lanes 2, 4, and 5, wild homozygote with fragment of 110-bp.

In the studied population, the *CYP2C9*2* mutant allele (allele T) frequency was found to be 12.64 %. Out of 170 patients, 39 were heterozygous (CT; 22.9 %) and two were homozygous (TT; 1.2 %). The *CYP2C9*3* mutant allele frequency (allele C) was found to be 25.8 %. Out of 170 individuals, 82 were heterozygous (AC; 48.2%) and three were homozygous (CC; 1.8%). Genotype frequencies of wild and variant alleles were 64 (37.6 %), 17 (10.0 %), 67 (39.4 %), 2 (1.2 %), 17 (10.0 %), and 3 (1.8%) for *1/*1, *1/*2, *1/*3, *2/*2, *2/*3, and *3/*3, respectively (Table 2).

Comparison of warfarin daily maintenance dose between genotype groups showed that the daily mean dose of warfarin in patients who have homozygous wild-type genotype for *CYP2C9* (*1/*1) was 5.26 ± 2.32 mg, which was significantly higher than that in *1/*2, *1/*3 (3.57 ± 2.25 mg, $p < 0.001$) and *2/*2, *2/*3, and *3/*3 patients (3.76 ± 2.4 mg, $p = 0.024$). The mean daily warfarin dose in the subjects with wild-type *CYP2C9*1 was significantly higher. However, there was no statistically significant difference in the mean daily warfarin dose between heterozygotes and homozygous mutants. Distributions of the dose within the different genotypes are illustrated in Figure 3.

Discussion

Warfarin is an anticoagulant drug metabolized by CYP2C9 and its dose requirement varies between individuals. Optimizing the appropriate dose of warfarin for each treated individual would lead to a reduced incidence of side effects and in turn decreased healthcare costs for patients
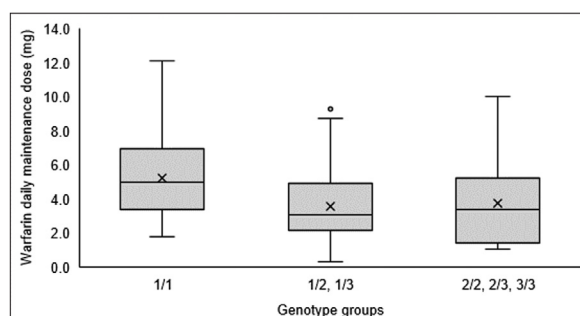

Figure 3: Boxplot showing the different genotype groups of *CYP2C9* against the daily warfarin dosage. Boxes indicate the median and interquartile ranges. Vertical lines above and below boxes indicate the minimum and maximum values. The numbers above whiskers show mean values. An asterisk shows each outlier.

and hospitals[9]. The variability in the individual response to the prescribed daily dose of warfarin is mainly associated with polymorphisms in two genes, *CYP2C9* and *VKORC1*, besides demographic factors such as age and body-surface area[10]. In patients with *CYP2C9*2* and *CYP2C9*3* alleles the half-life of the more bioactive S-warfarin is increased, so the required dose is lower, the probability of bleeding is higher and the time to reach a steady-state may be longer compared with those who carry the wild-type genotype[11].

Different allelic frequencies have been reported for *CYP2C9*2* and *CYP2C9*3* in different racial groups. In the multi-ethnic population of Iran, genetic diversity and different allelic and genotypic prevalence are possible. It is essential to investigate the distribution patterns of genetic variants in different regions of the country to reach a proper dosing algorithm of warfarin and a better understanding of the relationship between *CYP2C9* variants and sensitivity to warfarin. In our study, the majority of patients had allele *1 of *CYP2C9*, with a frequency of 62.2 %, and the variant alleles 2 and 3 with 12 % and 25.8 % frequencies respectively. The allelic frequencies of *CYP2C9*2* and *CYP2C9*3* were compared to those reported in various populations (Iranian, Turkish, Indian, Chinese, Malaysian, and European). For example, in the northeast of Iran, the frequency of *CYP2C9*2* and *CYP2C9*3* variants (9.1 % and 10 %, respectively)[12] was lower compared with our studied population. The region of our studied population is located in the northwest of Iran near to the border with Turkey where the population is comprised of mostly Turkish ethnicities. So we compared our results with those in Turkey and observed similarity to those reported by Yildirim et al (17 % for *2 and 26 % for *3)[13] and Ozer et al (13 % for *2 and 15 % for *3)[14] in a Turkish population. However, the frequencies of *CYP2C9*2* and *CYP2C9*3* in our study were different compared with Gujarati Indians (4.90 % and 3.92 %)[15], Chinese (2.6 % and 4.6 %)[16], and Europeans (12.5 % and 8.5 %)[17].

We also evaluated the effect of *CYP2C9* polymorphisms as a genetic determinant of the daily warfarin dose. Our study showed that patients carrying mutant allele *2 and *3 for *CYP2C9* required a lower dose of warfarin compared with carriers of wild allele *1. Patients with homozygous wild-type *1/*1 genotype required a significantly higher dose, but there was no statistically significant difference between the heterozygous and homozygous mutant patients. The association of *CYP2C9* variants with warfarin

Table 2: Genotype frequency of *CYP2C9* gene in the present study which included 170 patients receiving warfarin therapy.

CYP2C9[b]	All subjects (n: 170)	Males (n: 66)	Females (n: 104)	p value[a]	Odds ratio
*1/*1	64 (37.6)	19 (28.78)	45 (43.26)	0.058	0.541 (0.286-1.024)
*1/*2	17 (10)	6 (9.09)	11 (10.56)	0.947	1.027 (0.462-2.283)
*1/*3	67 (39.4)	29 (43.93)	38 (36.53)	0.459	1.261 (0.682-2.332)
*2/*2	2 (1.2)	1 (1.51)	1 (0.96)	0.331	3.127 (0.278-35.123)
*2/*3	17 (10)	9 (13.63)	8 (7.6)	0.501	1.409 (0.517-3.835)
*3/*3	3 (13.0)	2 (3.03)	1 (0.96)	0.141	4.757 (0.485-46.642)

Values are presented as number of subjects and percentage in brackets, while for Odds ratios 95 % confidence intervals are reported in brackets, CYP2C9: cytochrome P450 2C9 and *1, *2, *3 are the alleles, [a]: p value calculated by χ^2 test.

dose requirement was firstly reported in 1999[18]. To date, a number of studies have confirmed that having at least one of the allelic variants for *CYP2C9* is associated with a lower mean dose requirement for warfarin compared to having wild-type alleles[19]. Clinical trials involving genotype-guided dosing which include genotyping for *CYP2C9* have also be conducted. According to the results of a large pharmacogenomics trial, the Clarification of Optimal Anticoagulation through Genetics (COAG) study by Kimmel et al, genotype-guided dosing of warfarin did not improve the anticoagulation control based on the time within the therapeutic range during the first four weeks of treatment[20]. However, the European Pharmacogenetics of Anticoagulant Therapy (EU-PACT) warfarin trial, indicated that genotype-based dosing on days 1-5 was superior to standard therapy regarding the time spent within the targeted therapeutic range over three months. With genotype-based dosing, a significant reduction in supratherapeutic INRs was detected indicating both safety and efficacy with such a strategy[10]. As discussed by Pirmohamed et al, due to use of a maintenance dose algorithm to determine the initial dose in the COAG study, it is unlikely that steady state was achieved in most patients by day four[21]. While a meta-analysis did not prove the clinical importance of the pharmacogenetic dosing of warfarin on the basis of time within the therapeutic range, evidence of some advantages such as more rapid achievement of stable dosing was obtained[22]. Recently, another study on genotype-guided dosing was published; the Genetic Informatics Trial (GIFT) study, showing a better outcome for patients with genotype-guided dosing compared to controls dosed with a clinically guided algorithm. Factors studied included INR above four, bleeding, and death. The findings appear to be consistent with the EU-PACT[23].

Results of the current study in combination with other reported, suggest that genotype profiling of each patient for *CYP2C9* in the initial warfarin dosing phase might play a critical role. However, to have a better insight, one should consider variants of the other genes, namely *VKORC1*.

Acknowledgement

Conflict of interest

Authors declare no conflict of interest.

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