Relationship of S/R warfarin ratio with CYP2C9 genotypes in Pakistani population

Aisha Qayyum[1*], Muzammil Hasan Najmi[2], Syed Irfan Raza[3], Tausif Ahmad Rajput[4] and Abdul Khaliq Naveed[5]

[1]Department of Pharmacology, Fazaia Medical College, Air University, Islamabad, Pakistan
[2]Department of Pharmacology, Foundation University Medical College, Islamabad, Pakistan
[3]Department of Biosciences, University of Wah, Wah, Pakistan
[4]Shifa College of Pharmaceutical Sciences, Shifa Tameer-e-Millat University, Islamabad, Pakistan
[5]Department of Biochemistry, Islamic International Medical College, Riphah International University, Rawalpindi, Pakistan

Abstract: Warfarin is administered as a racemic preparation of R- and S-enantiomers. S-warfarin is more potent than R-warfarin, so changes in blood levels of S-warfarin affect the anticoagulant response. This study was carried out to determine the effect of CYP2C9*2 and CYP2C9*3 polymorphisms on S/R warfarin ratio. A single blood sample was collected 12-16 hours after drug administration from 170 stable patients fulfilling the criteria. Genotyping of the CYP2C9 polymorphisms was done by polymerase chain reaction-restriction fragment length polymorphism assay. S- and R-warfarin enantiomers extraction from plasma was accomplished by a validated HPLC method. The concentration of S-warfarin was significantly different among CYP2C9 genotypes (p =0.018) whereas there was no effect on R-warfarin (p =0.134). There was statistically significant effect of different CYP2C9 genotypes on S/R warfarin ratio (p=0.000). It is concluded that CYP2C9 polymorphisms influence CYP2C9 enzymatic activity in turn affecting S-warfarin levels but not R-warfarin, thus leading to different S/R warfarin enantiomers ratio among different CYP2C9 genotypes.

Keywords: S-Warfarin Enantiomer, R-Warfarin Enantiomer, CYP2C9 Genotypes, HPLC, PCR-RFLP.

INTRODUCTION

Warfarin has been the most commonly used oral anticoagulant for treatment and prophylaxis of various arterial and venous thromboembolic diseases. Warfarin is administered as a racemic preparation of R- and S-enantiomers. Two enantiomers not only differ in potency but also in their half-lives and metabolism. R-warfarin has a half-life of 20-60 hours whereas it is 18-35 hours for S-enantiomer because of its faster rate of clearance than R-enantiomer. The concentrations of two enantiomers differ in plasma due to stereo-selective metabolism and difference in their half-lives (Ghoneim and Tawfik, 2004; Locatelli *et al.*, 2005; Wittkowsky, 2005; Malakova *et al.*, 2009; Firriolo and Hupp, 2012; Jensen *et al.*, 2012).

Metabolism of warfarin occurs mainly by hydroxylation and also involves oxidation and reduction. Both enantiomers are metabolized by different cytochrome P450 (CYP450) enzymes and are mainly converted to inactive 6-, 7-, 8- and 10-hydroxy metabolites. R-warfarin is mainly metabolized by CYP1A2, CYP3A4, CYP2C19 and CYP1A1 whereas S-warfarin mainly by CYP2C9. S-warfarin exists at only half the concentration of R-warfarin at steady state because of rapid rate of metabolism than that of R-warfarin (Chan *et al.*, 1994; Kaminsky and Zhang, 1997; Zhang *et al.*, 2001; Locatelli *et al.*, 2005; Clapauch and Benchimol-Barbosa, 2012; Firriolo and Hupp, 2012; Maddison *et al.*, 2013; Shao and Jia, 2013). As S-warfarin is more potent than R-warfarin, so changes in blood levels of S-warfarin affects the anticoagulant response significantly (Kaminsky and Zhang, 1997; Zhang *et al.*, 2001; Clapauch and Benchimol-Barbosa, 2012; Jensen *et al.*, 2012).

CYP2C9 enzyme metabolizes more than 16 percent of clinically used drugs including S-warfarin. This enzyme is encoded by CYP2C9 gene (Kaminsky and Zhang, 1997; Miners and Birkett, 1998; Yamazaki *et al.*, 1998; Nakai *et al.*, 2005; Du *et al.*, 2016) which exhibits several genetic polymorphisms, but out of these CYP2C9*3 and CYP2C9*2 have been well studied because of their significant effect on S-warfarin metabolism. The most commonly present allele is CYP2C9*1 which is regarded as wild-type and produces CYP2C9 enzyme with normal activity. The CYP2C9*2 allele results from single nucleotide base substitution from C to T at codon 430 in exon 3 whereas CYP2C9*3 allele results from substitution from A to C at codon 1075 at exon 7. The presence of these SNPs results in decrease in the activity of CYP2C9 enzyme which is more with CYP2C9*3 than CYP2C9*2 (Rettie *et al.*, 1992; Sullivan-klose *et al.*, 1996; Yamazaki *et al.*, 1998; Miners and Birkett, 1998; Yin and Miyata, 2007; Kusama *et al.*, 2009; Shin, 2012; Niinuma *et al.*, 2013; Flora *et al.*, 2016).

**Corresponding author:* e-mail: aisha30102000@yahoo.com

Table 1: Relationship of S/R warfarin ratio with CYP2C9 genotypes

CYP2C9 Genotypes	Number of Subjects n (%)	S/R Warfarin Ratio Mean±SD	*p*-value
CYP2C9*1/*1	103 (60.6)	0.46±0.13	0.000*
CYP2C9*1/*2	4 (2.4)	0.6±0.09	
CYP2C9*1/*3	54 (31.8)	0.65±0.34	
CYP2C9*2/*2	1 (0.6)	1.02	
CYP2C9*2/*3	3 (1.8)	1.02±0.42	
CYP2C9*3/*3	5 (2.9)	2.75±1.59	

*Significant

Table 2: Pair-wise comparison of S/R warfarin ratio among CYP2C9 genotypes

CYP2C9 Genotypes		*p*-value
CYP2C9*1/*1	CYP2C9*1/*2	0.915^{NS}
	CYP2C9*1/*3	0.008*
	CYP2C9*2/*3	0.038*
	CYP2C9*3/*3	0.000*
CYP2C9*1/*2	CYP2C9*1/*1	0.915^{NS}
	CYP2C9*1/*3	0.999^{NS}
	CYP2C9*2/*3	0.484^{NS}
	CYP2C9*3/*3	0.000*
CYP2C9*1/*3	CYP2C9*1/*1	0.008*
	CYP2C9*1/*2	0.999^{NS}
	CYP2C9*2/*3	0.341^{NS}
	CYP2C9*3/*3	0.000*
CYP2C9*2/*3	CYP2C9*1/*1	0.038*
	CYP2C9*1/*2	0.484^{NS}
	CYP2C9*1/*3	0.341^{NS}
	CYP2C9*3/*3	0.000*
CYP2C9*3/*3	CYP2C9*1/*1	0.000*
	CYP2C9*1/*2	0.000*
	CYP2C9*1/*3	0.000*
	CYP2C9*2/*3	0.000*

* Significant
^{NS} Non Significant

The metabolic rate of S-warfarin has been found to be decreased in the presence of polymorphic alleles of CYP2C9 resulting in increased levels of S-warfarin which in turn significantly affects the anticoagulant response of warfarin. In such individuals lower doses of warfarin are required to produce the therapeutic response without any bleeding risk. At the same time, R-warfarin levels remain unaffected by CYP2C9 polymorphism. S-enantiomer exists at only half the concentration that of R-warfarin at steady state. This S/R ratio of 0.5:1 is changed in individuals possessing polymorphic alleles due to decreased rate of metabolism of S-warfarin. The S/R ratio has been observed to increase even upto 4:1. At steady state, the S/R warfarin ratio has been used to assess the activity of CYP2C9 enzyme. In patients carrying CYP2C9 *3/*3 genotype, S-warfarin clearance is decreased even upto 85%. Patients carrying these alleles are at higher risk of bleeding after administration of usual dose of warfarin. So genotyping of CYP2C9 especially for common alleles CYP2C9*3 and CYP2C9*2 before administering warfarin reduces the chances of adverse complication (Steward *et al.,* 1997; Caraco *et al.,* 2008; Kusama *et al.,* 2009; Ageno *et al.,* 2012; Shin, 2012; Lane *et al.,* 2012; Jorgensen *et al.,* 2012; Wells *et al.,* 2016). The present study was carried out to determine the effect of CYP2C9*2 and CYP2C9*3 polymorphisms on S/R warfarin ratio in Pakistani population.

MATERIALS AND METHODS

Study protocol

The study was conducted in accordance with the current Good Clinical Practices (FDA, 1996) and the Declaration of Helsinki (WMA, 2000). The clinical data collection and laboratory investigations were done at Armed Forces Institute of Cardiology (AFIC) Rawalpindi and National Institute of Cardiovascular Diseases (NICVD) Karachi. The analytical procedures were carried out at Centre for Research in Experimental and Applied Medicine (CREAM), Army Medical College Rawalpindi in

collaboration with University of Veterinary and Animal Sciences (UVAS) Lahore and Institute of Biomedical and Genetic Engineering (IBGE) Islamabad. The study protocol was approved by ethical committees of all concerned institutes.

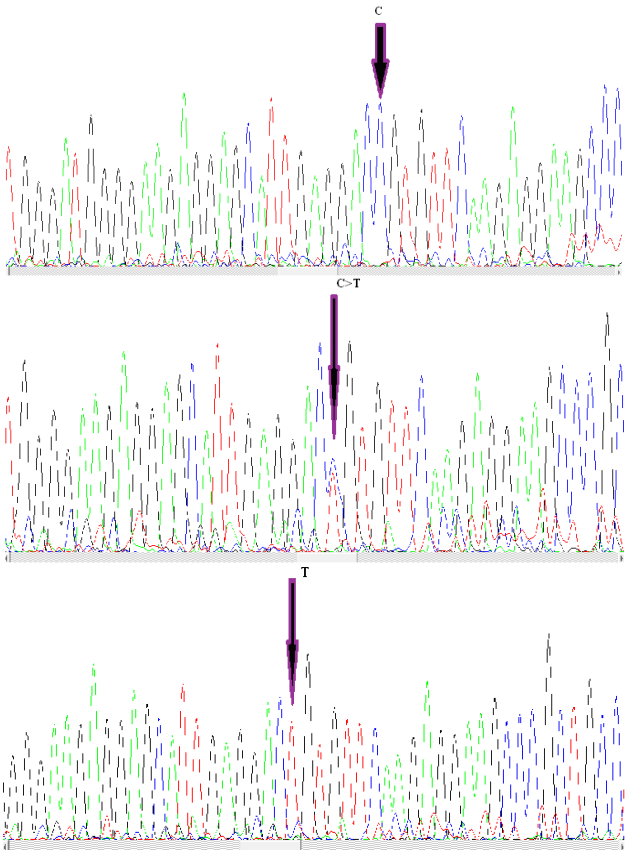

Fig. 1: DNA sequencing electropherogram of CYP2C9*2 polymorphism. The top sequence represents a wild-type sequence (C), the middle sequence a heterozygous (C>T) and the bottom sequence characterizes variant allele (T)

Study subjects

Study subjects were adults of either sex between the age of 18-65 years who were taking warfarin for anticoagulation therapy. One hundred and seventy stable patients fulfilling the criteria participated in the study. A stable patient was defined as the one whose warfarin dose had been constant for at least three previous clinic visits over a minimum period of three months, and had an INR of the prothrombin time (PT) within the range of 1.5-3.5 (Hirsh *et al*., 2001; Miao *et al*., 2007; Wang *et al*., 2008; Yoshizawa *et al*., 2009). The patients suffering from hepatic, renal or any co-morbid disease, taking any medication or diet known to interact with warfarin therapy, were excluded. Subjects were informed of the nature, significance and consequence of the study and the investigational procedures. The written informed consent was obtained.

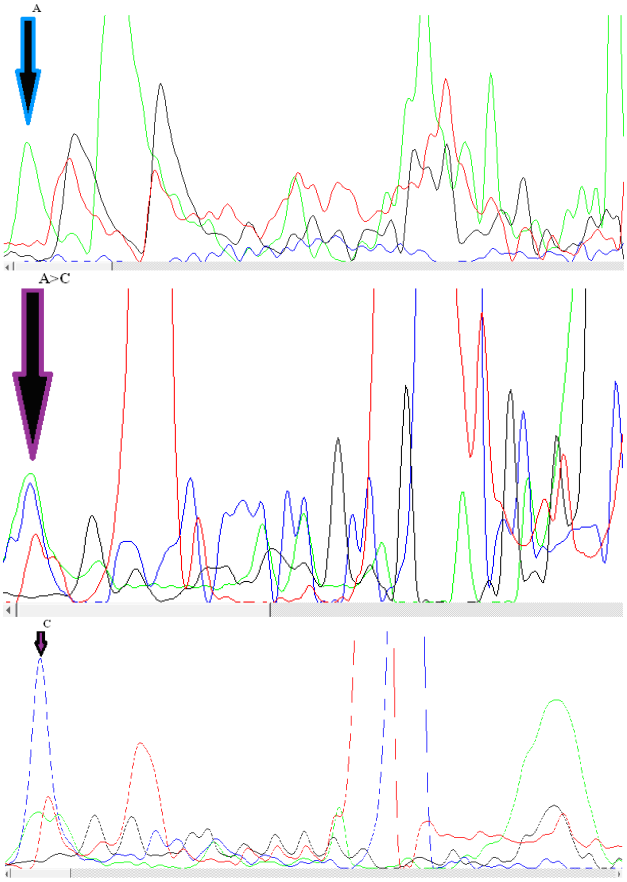

Fig. 2: DNA sequencing electropherogram of CYP2C9*3 polymorphism. The top sequence represents a wild-type sequence (A), the middle sequence a heterozygous (A>C) and the bottom sequence characterizes variant allele (C)

Blood sampling

A blood sample of 10 ml was drawn 12–16 hours after last administered warfarin dose from each recruited subject. Out of this sample, 2 ml of blood was collected in EDTA (ethylene diamine tetraacetic acid) containing tube and stored at 4ºC for genotyping (Miao *et al*., 2007). A blood sample of 5 ml blood was transferred to heparinised tube and centrifuged immediately to separate plasma. Plasma was stored at -80ºC till further analysis on high performance liquid chromatographic (HPLC) system for estimation of S- and R-warfarin levels in plasma (Unge *et al*., 1992; Jensen *et al*., 2012; Takahashi *et al*., 2006). Rest of the blood was distributed in respective tubes for baseline investigations that included complete blood picture (blood CP), ESR, liver function tests (Serum ALT and bilirubin), renal function tests (Serum creatinine and urea), PT and INR.

Genotyping of CYP2C9*2 and CYP2C9*3 polymorphisms

The genomic DNA from all of the samples was isolated mainly by standard organic method which involved two principal organic chemicals chloroform and phenol

(Sambrook and Russell, 2001). The protocol was slightly modified as per requirement of the laboratory working. DNA isolation kit was used (QIAamp DNA Mini, Qiagen Inc, USA) to extract genomic DNA from some of the blood samples which were either less in quantity or somewhat clotted. Genotyping of the CYP2C9*2 and CYP2C9*3 polymorphisms was done by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. The detailed methodology of PCR-RFLP assay has already been reported by Qayyum *et al* (2016). A brief account of method is given here. The optimization of PCR with reproducible results was performed. The forward and reverse primers for CYP2C9*2 and CYP2C9*3 allelic variants being used were reported in a previous study (Moridani *et al.,* 2006). The sequences of pairs of primers were for CYP2C9*2 (forward GGAGGATGGAAAACAGAGACTTA; reverse TGAGCTAACAACCAGGACTCAT) and CYP2C9*3 (forward GCTGTGGTGCACGACGTCCAGAGATGC; reverse ACACACACTGCCAGACACTAGG). The sequences of these primers were confirmed through Primer-BLAST search (http://blast.ncbi.nlm.nih.gov/Blast .cgi). The amplified DNA fragment spanning the SNP CYP2C9*2 was digested with *AVAII* restriction enzyme, whereas for CYP2C9*3 restriction enzyme *Nsi1* was used. In order to validate the PCR-RFLP genotypes; direct sequencing of a number of samples was done through automated capillary sequencing method. Both SNPs were amplified using the same primer as used in PCR. The purified sequencing reaction product was loaded to ABI Genetic Analyzer 3130 (Applied Biosystem®, Life Technologies, USA) for sequencing. The sequencing results were read and the SNP genotypes were validated. DNA sequencing electropherogram of CYP2C9*2 and CYP2C9*3 polymorphism have been shown in fig. 1 and 2 respectively.

Analytical procedure for S- and R-warfarin enantiomers

S- and R-warfarin enantiomers extraction from plasma was accomplished by a validated HPLC method described by Qayyum *et al* (2015). A brief account of method is given here. One milliliter of plasma sample was acidified by addition of 700µL of 1N sulphuric acid. After mixing, 3mL of diethyl ether was added to extract S- and R-warfarin. The organic layer was separated and evaporated to dryness under a stream of nitrogen. The residual sample was reconstituted in 300µL of acetonitrile and 40µL was injected onto the HPLC system. The High Performance Liquid Chromatography (HPLC) system by Agilent 1100 Series with autosampler and fluorescence detector was used along with LiChroCART® 250-4 ChiraDex® (250x4mm, 5µm particle size) column and LiChroCART® 4-4 ChiraDex® (4x4mm, 5µm particle size) guard column. The fluorescence detector was set at an excitation wavelength of 300nm and an emission wavelength of 390nm. The mobile phase consisted of acetonitrile: glacial acetic acid: triethylamine (1000: 3: 2.5, v/v/v). The mobile phase was pumped at a flow rate of 1ml/min. All analyses were done at room temperature. The method was validated according to ICH Guidelines on validation of analytical procedures (ICH, 2005). All the parameters were found to be within acceptable range.

STATICAL ANALYSIS

Data was analyzed using SPSS version 20.0 (IBM Corporation, USA). Descriptive statistics was used to describe the data. Mean and standard deviation (SD) were calculated for quantitative variable like S/R ratio. Frequency and percentages were calculated for qualitative variable like genotypes. Analysis of variance (ANOVA) was applied to compare S/R ratio among different CYP2C9 genotypes. A *p*-value of less than 0.05 was taken as statistically significant. ANOVA was followed by Post-hoc Tukey's test for pair-wise comparison if ANOVA gave *p*-value of less than 0.05.

RESULTS

The concentration of S-warfarin was significantly different among different CYP2C9 genotypes as shown by *p*-value of less than 0.05 (p=0.018) whereas there was no effect of CYP2C9 genotypes on plasma concentration of R-warfarin (p=0.134). The effect of different CYP2C9*2 and CYP2C9*3 genotypes on S/R warfarin ratio was determined. The results are summarized in table 1. There was statistically significant effect of different genotypes on S/R warfarin ratio (p=0.000). Because of high standard deviation (SD) in S/R warfarin ratio data, normality of data was checked by One-sample Kolmogorov Smirnov test. A *p*-value of more than 0.05 for S/R warfarin ratio among different genotypes confirmed the normality of data. For pair-wise comparison with Post Hoc Tukey's test, CYP2C9*2/*2 was not included as there was only one subject in this group which cannot undergo statistical comparison. On the basis of pair-wise comparison of different CYP2C9 genotypes, it has been inferred that subjects possessing homozygous polymorphic CYP2C9*3/*3 genotype possessed significantly higher S/R ratio as compared to rest of the CYP2C9 genotypes (p=0.00). Heterozygous polymorphic genotypes including CYP2C9*1/*3, *2/*3, *3/*3 were having significant higher S/R warfarin ratio as compared to homozygous wild-type CYP2C9*1/*1 genotype. The results are summarized in table 2.

DISCUSSION

The effect produced by CYP2C9 genotypes on warfarin dose is because of their effect on S-warfarin metabolism through their action on CYP2C9 enzymatic activity (Kamali *et al*., 2004; Gong *et al*., 2011; Mahajan *et al*., 2013; Hernandeza *et al.,* 2015; Huang *et al.,* 2017). In order to assess this effect we analyzed warfarin

enantiomers plasma levels. In present study, the S-warfarin plasma levels were significantly affected by different CYP2C9 genotypes whereas there was no significant effect on R-warfarin levels. This supports the existing view that S-warfarin is metabolized by CYP2C9 enzyme whereas R-warfarin is metabolized by other enzymes (Moyer *et al*., 2009; Mahajan *et al*., 2013). Warfarin S/R enantiomeric ratio was found to be affected by CYP2C9 genotypes. Our data displays large variability in S/R ratio among individuals due to possession of different CYP2C9 genotypes by the patients. Some studies also have demonstrated such variations but they did not carry out CYP2C9 genotyping (Chan *et al*., 1994; Locatelli *et al*., 2005; Shaul *et al.,* 2017). In our study the S/R ratio was significantly affected by CYP2C9*3 variant allele increasing this ratio to 2.75:1 for homozygous variant genotypes as compared to 0.46:1 seen in subjects possessing wild-type genotype. CYP2C9*2 variant allele did not significantly affected the S/R ratio, although it was raised to 1:1 for homozygous variant genotypes as compared to 0.46:1 seen in subjects possessing wild-type genotype. This is in accordance with reported data of effect of CYP2C9 genotypes on warfarin dose (Kamali *et al*., 2004; Herman *et al*., 2005; Obayashi *et al*., 2006; Xue *et al.,* 2016). As already reported, CYP2C9*3 variant allele has demonstrated significant effect on warfarin dose whereas CYP2C9*2 did not (Qayyum *et al*., 2016). This observation in present study supports the fact that CYP2C9 gene influences CYP2C9 enzymatic activity in turn affecting S-warfarin levels and warfarin dose requirement. The number of studies conducted to see the impact of CYP2C9 genotypes on warfarin enantiomers concentration are much less than those studying their effect on warfarin dose (Chang *et al.,* 2016; Kubo *et al.,* 2016; Shalia *et al.,* 2016). Two USA-based studies conducted in Caucasians reported S/R ratio increased to 4: 1 for CYP2C9*3 variant allele as compared to 0.5: 1 for wild-type genotype thus significantly affecting warfarin enantiomeric levels (Steward *et al*., 1997; Henne *et al*., 1998). Another study conducted in Caucasians in Slovenia observed S/R ratio increased to 1.43:1 with variant genotype as compared to 0.45:1 in wild-type (Herman *et al*., 2005). Some studies were carried out in Asian regions as well. A study conducted in Japanese showed warfarin S/R ratio to be significantly different between CYP2C9*1/*3 and *1/*1 genotypes (0.52:1 vs 1.25:1) (Obayashi *et al*., 2006). An Indonesian study has also reported warfarin S/R ratio to be significantly different between CYP2C9*1/*3 and *1/*1 genotypes (Rusdiana *et al*., 2013). A study conducted in Israeli population have also established significant difference in warfarin S/R enantiomers ratio among different CYP2C9 genotypes with ratios of 0.35:1, 0.54:1 and 1.02:1 for wild-type, heterozygous and homozygous variants respectively (Caraco *et al*., 2008). To our best of knowledge, no such study till to-date is available in the neighboring countries like Iran and India. This is the first study carried out in Pakistan demonstrating the effect of CYP2C9 genotypes on warfarin enantiomers concentration.

CONCLUSION

It is concluded that polymorphisms in CYP2C9 gene influences CYP2C9 enzymatic activity in turn affecting S-warfarin levels but not R-warfarin levels. This in turn leads to different S/R warfarin enantiomers ratio among different CYP2C9 genotypes. So ultimately warfarin dose adjustments have to be made in patients possessing polymorphic alleles.

ACKNOWLEDGEMENT

The authors thank National University of Sciences and Technology, Islamabad for providing opportunity to carry out this study.

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