# IMPACT OF CYP2C9 GENETIC POLYMORPHISM ON WARFARIN DOSE REQUIREMENTS IN PAKISTANI POPULATION

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**ABSTRACT**

Variations of cytochrome-P450 enzyme system (CYP2CP) are associated with impaired metabolism of warfarin. The objective of our study was to estimate the frequency of genetic and allelic variants of CYP2C9 in Punjabi population of Pakistan and their effects on warfarin dose requirement. One hundred and twenty unrelated Pakistani subjects belong to Punjab province, were randomly included from the registry of National Institute of Heart Disease Rawalpindi, Pakistan. The patients had stable international normalized ratio (INR) of 2 to 3 for last 3 months with warfarin therapy after heart valves replacement. The detection of CYP2C9 variant was done on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. Total 120 patients (73 males; 47 females) of mean age of 37 years participated in the study. Nine patients had mutant allele CYP2C9*3 (7.5%), one CYP2C9*2 (0.8%) and 110 patients exhibited wild type CYP2C9*1 (91.7%). The frequency of CYP2C9 genotype was *1/*1 (0.858) ; *1/*3 (0.117) ; 2/*20 (0.08 ) and *3/*3 (0.017) in our study population. A high dose of warfarin (42.2$\pm$9.56) mg/week is required for patients with *1/*1 genotype as compared to patients with *2/*2 (17.5$\pm$1.9) and *1/*3 (16.6$\pm$2.3) allele ($p<0.001$). Individuals with CYP2C9*3/3* need lowest (8.75±1.76 mg/week) daily warfarin dose. In conclusion, the genetic variations in the CYP2C9 occur in 14% of Punjabi ethnic group in Pakistan. Presence of CYP2C9*2 or *3 variants is an independent predictor of low warfarin dose requirement in our patients. CYP2C9 variants assay may be used in high risk groups for appropriate dose adjustment to avoid complications on long term basis.

**Keywords**: Gene frequency, CYP2C9, Pharmacogenetics, Polymerase chain reaction, Polymorphism, Warfarin, Pakistani population.

## INTRODUCTION

Cytochrome-P450 enzyme system has a key role in metabolizing variety of drugs including warfarin. Clinically warfarin is an oral anticoagulant drug, commonly prescribed in thromboembolic disorders (Sconce *et al*., 2005). Warfarin is metabolized primarily by the CYP2C9 hepatic microsomal enzyme system (Seng *et al*., 2003; Lee *et al*., 2002). Drug metabolizing enzyme activity is established genetically. Polymorphisms among CYP2C9 alleles are well known and markedly influence drug response and biotransformation of lipophilic compounds to polar metabolites (Zhu *et al*., 2007). The variable drug response due to CYP2C9 polymorphisms leads to change in efficacy and toxicity of drug among different individuals (Evans & Relling, 1999).

There are nine exons in CYP2C9 gene. It encodes a protein of 490 amino acids. Single nucleotide polymorphisms result in allelic variants that lead to exchange of amino acid. They differ only at few residues in coding area (Goldstein *et al*., 1994). The altered phenotype of allelic variants is due to mutations of CYP2C9 predominantly of CYP2C9* 2 and CYP2C9* 3. The enzymes encoded by CYP2C9 alleles reduce catalytic activity in comparison to wild type CYP2C9*1 (Higashi *et al*., 2002).

These diversities in allelic variants of CYP2C9 profoundly alter warfarin dose requirement (Cho *et al*., 2007). Patients with genetically variant alleles other than allele*1 need lower doses of warfarin to maintain INR within range. This is because of slower clearance of S-warfarin by the cytochrome enzyme system (Joffe *et al*., 2004). These polymorphisms (CYP2C9*2 and CYP2C9*3) are responsible for almost 50-60% alteration in warfarin dose (Wadelius *et al*., 2007). High degree of variability in drug response due to pharmacogenetic polymorphism and a narrow therapeutic index complicates warfarin therapy initiation and maintenance in the patients of cardiovascular diseases (Millican *et al*., 2007). There has been a serious risk of intracranial haemorrhage in different ethnic groups ranging between 2.04-4.06% of patients taking warfarin maintenance therapy (Shen *et al*., 2007). Warfarin requires careful clinical, laboratory and pharmacogenetic assessment to balance the risks of bleeding and clotting due to over-and under-anticoagulation therapy respectively.

The prevalence of CYP2C9 mutant alleles varies in different human populations (Moridani *et al*., 2006). The percentage of CYP2C9 genetic variants is found to be

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different among Asian, Caucacian and African population. CY2C9*2 and CYP2C9*3 genetic variants are higher in Caucasian population as compared to African-American and Asian populations (Schwarz, 2003).

The CYP2C9 variant allele's frequency has not yet been explored in different ethnic population in Pakistan. The current study was aimed to identify common variants of CYP2C9 polymorphism in Punjabi population of Pakistan and to observe the impact of these genetic variants of CYP2C9 on warfarin dose requirement in these patients. We also compared allele frequency of our study population with Asian populations.

## MATERIALS AND METHODS

Pakistan is located in South Asia and has a population of 16 million. Punjab is one of the four provinces with major share in the population. We studied unrelated Punjabi subjects as first step to introduce pharmacogenomics in our clinical setup. This study project was accepted by the institution review committee of AM College (National University of Sciences and Technology), Rawalpindi, Pakistan.

One hundred and twenty Punjabi patients were randomly included after informed consent from the registry of National Institute of Heart Disease Rawalpindi, Pakistan. Sample size was calculated according to the average of most prevalent allele in various Asian populations in previous international studies by applying formula:-

$$N = \frac{Z^2 P (1-P)}{E^2} \quad \text{where } P = 0.915$$

The patients were on warfarin therapy either after heart valves replacement or venous thrombosis. They had stable INR in the therapeutic range from 2 to 3 for at least 3 month. The patient's age ranged from 12 to 65 years of either sex. The patients having any liver disease, coagulation disorders, taking any medication known to interfere with warfarin were excluded.

Clinical history and examination of each patient was carried out. Prothrombin time was measured by using manufacturer instruction on an automated CA 400 coagulometer (Germany) (Sirridge & Shannon, 1984). The INR of each patient was estimated as following INR= patient time/control time. The international sensitivity index (ISI) of this assay was 1.07. Patient's weekly warfarin dose was recorded.

***PCR amplification conditions for allele*2 and*3***

Five mL blood was taken in EDTA tubes and peripheral blood film leucocytes were used for DNA extraction. It was done by using in-house salting out method (Miller *et al*., 1988). Extracted DNA was kept at -40$^0$C till PCR analysis. PCR-RFLP assay was used for genotyping of CYP2C9*2 (Arg144Cys) and CYP2C9* 3 (Ile359Leu) (Moridani *et al*., 2006). Single chain PCR-RFLP technique was applied to identify *2 and *3 alleles by selecting forward as well as reverse primer (2F= 5'-GGA GGA TGG AAA ACA GAG ACT TA-3',2R= 5'-TGA GCT AAC AAC CAG GAC TCA T-3' and for allele *3 (3F=5'-GCT GTG GTG CAC GAC GTC CA GAGA TGC-3' ,3R=5'-ACA CAC ACT GCC AGA CAC TAG G-3'). One set of forward and reverse primers was used for allele *2 variant at exon number 3.The other set of primers was utilized to genotype allele *3 variant at exon 7.

PCR was done in 20 µl of reaction mixture for allele *2 and *3, containing 1 µl of genomic DNA (50µg), 0.4 µl deoxynucleoside triphosphates, 1 µl primers 2F and 2R each and, primers 3F and 3R each separately, 2 µl X PCR buffer with 3.2 µl of $MgCI_2$ and 0.2 µl of Tag DNA polymerase (Fermentas Life Sciences, North America) and 11.2 µl of deiodinized water. For activation of Tag polymerase, cycling conditions were set at 95$^0$C for 15 minutes, then at 94$^0$C/20 seconds, 60$^0$C/20 seconds and 72$^0$C/25 seconds. For each new cycle we added one second and reached final extension at 72$^0$c for 5 minutes. PCR was ended at 4$^0$C done on Gradient palm cycler (San Francisco, CA, 94107, USA). An amplicon of 396 bp was achieved for CYP2C9*2 and 297 bp for CYP2C9*3. We used AvaII for CYP2C9*2 and NsiI for CYP2C9*3 as restriction enzymes for DNA digestion. A total of 32 ul reaction mixture contained 10ul of PCR amplicon, 18 ul of nuclease free water, 2ul of 10 x reaction buffer, 2ul restriction enzyme AvaII (10U/µl) and NsiI (10U/µl) (Fermentas, USA). Digestion was done at 37$^0$C temperature overnight. The endproducts of digestion were analyzed by gel electrophoresis with 2% agarose. Ethidium bromide was used for staining.

## STATISTICAL ANALYSIS

Statistical analysis was done with SPSS (version 16.0, Chicago IL). Mean, SD and range were calculated for age, INR and weekly warfarin dose. For CYP2C9 alleles *1, *2 and *3 variants frequency and percentage was calculated. Fisher test were applied for comparison of allele frequency. Patients with and without CYP2C9 polymorphisms were compared for warfarin dose requirement by using one way ANOVA. The p -value less than 0.05 were taken as significant.

## RESULTS

A total of 120 unrelated Punjabi patients comprising 73 males, 47 females with mean age of 37.8 ± 13.0 years participated in the study. The percentage of CYP2C9 allelic variants in Punjabi inhabitants in Pakistan are

given in table 1. The percentage did not deviate significantly from H-Weinberg equation. Most frequently identified percentage of CYP2C9*3 was 0.075 (95% CI; 0.042-0.108). The CYP2C9*2 allelic variant was 0.008 (95% CI; 0.003-0.019).

**Table 1**: Alleles and genotype frequencies of CYP2C9 among Pakistani (Punjabi) population (n=120).

| **Alleles** | **n** | **Frequency** | **95% CI** |
|---|---|---|---|
| CYP2C9*1 | 110 | 0.916 | 0.858-0.954 |
| CYP2C9*2 | 1 | 0.008 | 0.003-0.019 |
| CYP2C9*3 | 9 | 0.075 | 0.042-0.108 |
| **Genotype** | | | |
| CYP2C9*1/*1 | 103 | 0.858 | 0.814-0.902 |
| CYP2C9*1/*3 | 14 | 0.117 | 0.075-0.156 |
| CYP2C9*2/*2 | 1 | 0.008 | -0.003-0.019 |
| CYP2C9*3/*3 | 2 | 0.017 | 0.0008-0.032 |

DNA restriction-digested fragments of normal, variant allele *2 (a; 396bp; lane 6) and variant allele *3 (b; 274bp; lane 1&2) are shown in Representative gel (fig. 1). The CYP2C9*1/*3 genotype was present in high percentage amongst all the other variants (table 1). Genetic variations in the CYP2C9 *1/*3,*2/*2 and *3/*3 occur in 14% of Punjabi patients in Pakistan.

We compared the allelic frequencies of Punjabi population with other Asian populations. CYP2C9* 3 genetic variant in Punjabi living in Pakistan is close to Indian Kerala province and Tamilians but differ significantly from Iranians, Chinese and Korean (P<0.05).

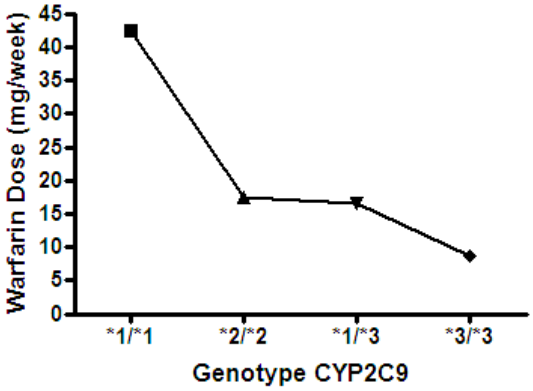


**Fig. 2**: The comparison of mean weekly warfarin dose and genotypes of CYP2C9 revealed variant of *1*3, *2*2 and *3/3 require lower dose than wild type.

The dose of warfarin needed to get a therapeutic range was remarkably different. CYP2C9 genotype relative to average weekly warfarin dose requirement is shown in fig. 2. CYP2C9*1/*1 genotype required a higher dose of warfarin (42.2±9.56) mg/week as compared to patient

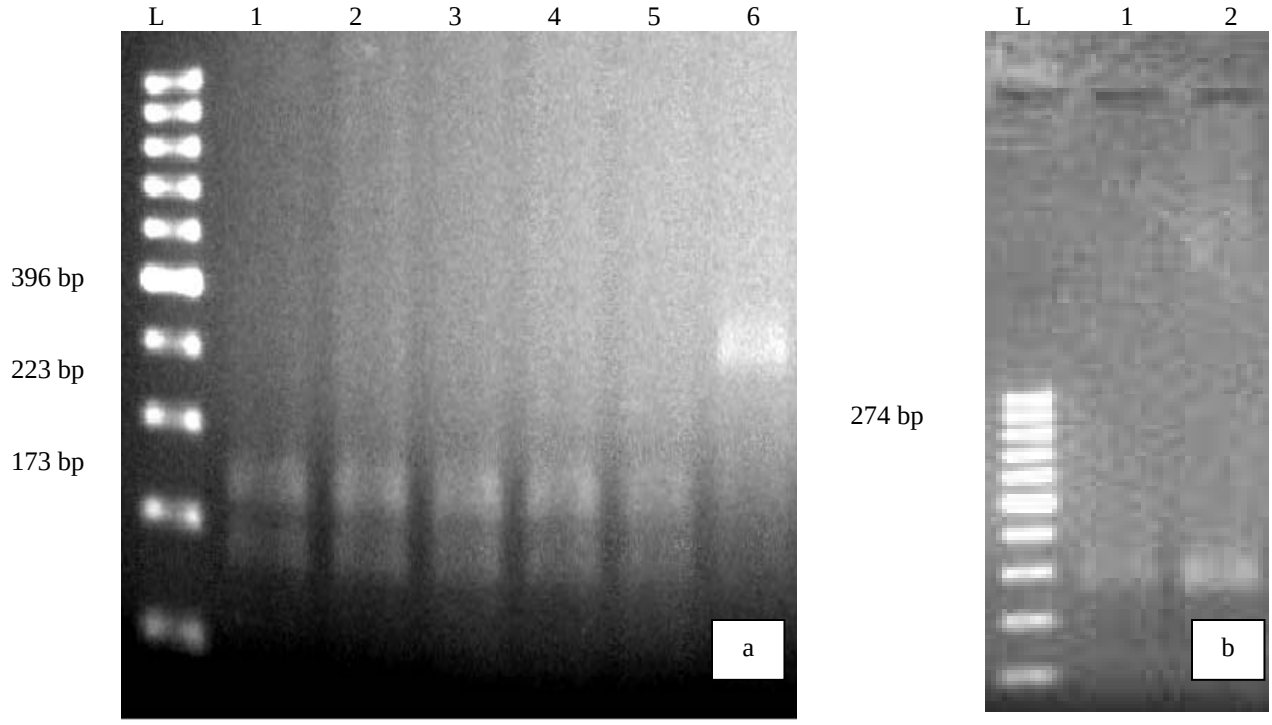


**Fig. 1**: Representative gels showing separation of DNA restriction-digested fragments allowing identification of (a) normal 173 bp, 223 bp (lane 1-5) and variant allele *2 (396 bp; lane 6), (b) allele *3 variant (274 bp; lane 2).

**Table 2**: The Alleles and genotype frequencies of CYP2C9 in the Pakistani population (Punjabi) as compared to other Asian population (%)

| Ethnicity | n | Alleles | | | Genotype | | | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *1 | *2 | *3 | *1/*1 | *1/*2 | *1/*3 | *2/*2 | *2/*3 | *3/*3 | |
| Pakistan | 120 | 91.6 | 0.8 | 7.5 | 85.8 | 0 | 11.7 | 0.8 | 0 | 1.7 | Present study |
| Iranian | 200 | 87.2 | 12.7 | 0 | 87.2 | 10.5 | 0 | 7.5 | 0 | 0 | Zand *et al* 2007 |
| Tamilians | 135 | 90.7 | 2.6 | 6.7 | 82.3 | 4.4 | 12.7 | 0 | 0.7 | 0 | Adithan *et al* 2003 |
| Kerala | 120 | 90.0 | 2.0 | 8.0 | 81.0 | 4.0 | 14.0 | 0 | 0 | 1.0 | Jose *et al* 2005 |
| Chinese | 115 | 98.3 | 0 | 1.7 | 97.0 | 0 | 3.0 | 0 | 0 | 0 | Wang *et al* 1995 |
| Korean | 574 | 98.9 | 0 | 1.1 | 97.7 | 0 | 2.3 | 0 | 0 | 0 | Yoon *et al* 2001 |

with genotype CYP2C9*2/*2 (17.5±1.9), CYP2C9*1/*3 (16.6±2.1mg/week) and genotype CYP2C9*3/3* (8.75±1.76) ($p<0.001$). Individuals with *3*3 needed lowest daily warfarin dose.

## DISCUSSION

CYP2C9 genotype encodes the main enzyme responsible for the metabolism of Warfarin (Hermans & Thijssen, 1993; Herman *et al*., 2007). CYP2C9 belongs to the CYP2C subfamily, and it constitutes 20% of the hepatic cytochrome P450 enzyme expressed in humans (Aynacioglu *et al*., 1999). Various genetic variants of CYP2C9 are identified and the most common allelic variants identified are CYP2C9*2 and CYP2C9*3 (Seng *et al*., 2003; Adithan *et al*., 2003). In CYP2C9 polymorphism the alteration in the structure of enzyme leads to its reduced activity up to 95%, and this result in decreased metabolism of S-warfarin (Zhu *et al*., 2007; Gaedigk *et al*., 2001). This is the first study on CYP2C9 alleles and its genotype in Pakistani Punjabi population. Genetic CYP2C9*3 variant is the most abundantly detected allele in the study population. CYP2C9*1/*3 genotype was detected in high percentage amongst all the other variants. Our special consideration was on CYP2C9 allele *3 due to its reduced activity in clearing the warfarin from the circulation thus making its action more potent and prolonged, whereas wild type genotype has a more rapid action on warfarin clearance acquiring the dose adjustment of warfarin (Bae *et al*., 2005). It has also been shown that there is an ethnic variation in prevalence of different variant alleles of CYP2C9 (Seng *et al*., 2003).

Our results were different when compared with Asian population, the most frequent variant allele in Pakistani Punjabi population is *3 where as in Caucasians it was allele*2 (Gaedigk *et al*., 2001; Aithal *et al*., 1999). When compared with Chinese the occurrence of *2 and *3 variants were more frequent in Pakistani population (Wang *et al*., 1995). Iranian population showed allele *2 more frequently than our population while allele *3 was more in our population showing marked difference in results (Zand *et al*., 2007). Occurrence of variant allele *2 and *3 in Tamilians population has been almost closer to our results (Adithan *et al*., 2003).

As far as the genotype results were concerned, frequency of *3/*3 was 1.7% which was only seen previously in Indian Kerala population by Jose *et al* (2005). While *1/*3 was similar to Tamilians and all populations of Southern India (Adithan *et al*., 2003; Jose *et al*., 2005), but it was markedly different from Iranians, Chinese and Koreans (Wang *et al*., 1995; Zand *et al*., 2007; Yoon *et al*., 2001). CYP2C9* 3 allelic variant frequency in Punjabi is same as of Indian but differ significantly from Iranian, Chinese and Korean.

In our study, subjects with *3/*3 genotype required significantly lower warfarin dose when compared with *1/*1 wild type genotype. Therefore, caution must be used when attempting to extrapolate these data to other ethnic groups in Pakistan. Warfarin dose is significantly related to genotype (Aithal *et al*., 1999). Very recently, individuals requiring low dose of warfarin were shown to carry more abundantly the CYP2C9*2 and/or the CYP2C9*3 allele than the wild type allele (CYP2C9*1) while comparing the clinical controls (Margaglione *et al*., 2000). The significance of CYP2C9*3 is much more than that of CYP2C9*2 because it shows the major decline in the activity of enzyme. On the other hand CYP2C9*2 allele causes medium fall in activity as compared to CYP2C9*1 (Lee *et al*., 2002). Because warfarin acts by inhibiting VKOR, a genetic variation in this enzyme increases a patient's sensitivity to warfarin and decreases the patient's dose requirement. In addition to these there are several other variants which contribute to impaired drug metabolism and its toxicity (Scott *et al*., 2008). These individuals carrying mutant alleles are at risk and this partially explains the widespread Interindividual variability in response to warfarin dose (Sconce *et al*., 2005).

Pre-treatment detection of CYP2C9 genetic variant will help the clinicians in optimizing warfarin dose. This study contributed in finding out the variability in warfarin-dose requirements, with the view to introduce a novel individualized dosing regimen in our clinical practice. The warfarin therapy management based on pharma-cogenetics is life saving and cost effective in the patients on long term anticoagulant therapy.

The acceptance of pharmacogenetics-oriented warfarin management by clinicians depends on clinical evidence. The present study is an example of decision analysis to undertake large-scale studies by simulating the magnitude of the clinical effectiveness and cost of an intensified anticoagulation services required for a CYP2C9 genotype-guided treatment scheme to translate into a cost-effective clinical strategy for all ethnic groups in the country. A limitation of our study is that most patients were Punjabi and number of subjects are relatively small. Therefore, caution must be used when attempting to extrapolate these data to other ethnic groups in Pakistan.

## CONCLUSION

Genetic variations in the CYP2C9 *1/*3,*2/*2 and *3/*3 occur in 14% of Punjabi patients in Pakistan with predominant mutant allele 3. Presence of at least one CYP2C9*2 or *3 polymorphism is an independent predictor of low warfarin dose requirements. CYP2C9 variants assay may be used in high risk groups for prediction of appropriate dose in order to avoid adverse effects especially at initiation of therapy aiming for rational and individualized pharmacotherapy.

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