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CYP2C9 Gene Analysis of Some Iranian Hypersensitive Patients to Warfarin

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Abstract: For the first time in this study, the pharmacogenetic effects of *CYP2C9* polymorphism on warfarin sensitivity in some Iranian patients who are on warfarin treatment were shown. The study group consisted of clinically sensitive patients(21 patients) and the control group (37 adult patients). For detection of *CYP2C9*2* and *CYP2C9*3* variants, a protocol based on restriction fragment length polymorphism based polymerase chain reaction with *Eco47I* and *KpnI* was used. In clinically sensitive patients about 81% and in normal response patients about 24.3% carried variant genotypes.

Key words: *CYP2C9*, warfarin, hypersensitivity to warfarin

INTRODUCTION

Warfarin is the most commonly prescribed oral anticoagulant drug for the prophylaxis and treatment of venous and arterial thromboembolic disorders in many countries and is being prescribed to an increasing number of patients (Hirsh *et al*., 1998). The effectiveness and safety of warfarin therapy is critically dependent on maintaining the prothrombin time expressed as the International Normalized Ratio (INR), which is a ratio of the time required for the patient[7] s blood to coagulate relative to a standardized coagulation time, within the desired therapeutic range (Sconce *et al*., 2005).

The mechanism of warfarin action is inhibition of vitamin K1 2,3 epoxide reductase (VKOR). Warfarin therapy is problematic due to a narrow therapeutic index and frequent bleeding complications. Certainly, the estimation of the optimal dose of warfarin in this narrow interval is difficult because of significant interpatients' variability in warfarin sensitivity (Brukner, 2009) The interindividual variability in anticoagulant response is multifactorial. Patient and environmental factors including genetics, body size, dietary vitamin K status, concurrent diseases and medications have been shown that affect on anticoagulant response to warfarin (Kamali, 2006).

Warfarin has two enantiomeric forms, R- warfarin and S-warfarin, with different pharmacodynamic and pharmacokinetic properties (Stubbins *et al*., 1996). *CYP2C9* is the principal enzyme that catalyzes the conversion of S-warfarin to inactive 6-hydroxy and 7-hydroxy metabolites whereas the oxidative metabolism of R-warfarin is mainly catalyzed by CYP1A2 and CYP3A4. Since the potency of (S)-warfarin is much higher than (R)-warfarin, about three to five fold, any change in the activity of *CYP2C9* gene is likely to have a significant influence on the anticoagulant response. The existence of genetic polymorphism in *CYP2C9* that causes functionally significant effects on enzyme activity is now well established. Based on several *in vitro* and *in vivo* studies, the residual activity of *CYP2C9*2* and *CYP2C9*3* enzymes are estimated to be 12 and 5%, respectively, of the wild type enzyme (Bhasker *et al*., 1997).

The most common allele is designated as *CYP2C9*1* and is considered the wild-type allele. A cytosine to thymine (C→T) exchange at nucleotide 430 produces Arg^{144} Cys variant allele (*CYP2C9*2*) in exon three. An adenine to cytosine (A→C) exchange at nucleotide 1075 produces the Ile^{359}Leu variant allele (*CYP2C9*3*) in exon seven (Lee *et al*., 2002).

Significant differences in *CYP2C9*2* and *CYP2C9*3* allelic frequencies among individuals of distinct ethnicity have been described (Xie *et al*., 2002).

The aim of this study was to determine the pharmacogenetic effects of *CYP2C9* polymorphism on warfarin sensitivity in some Iranian patients who were on warfarin treatment.

MATERIALS AND METHODS

This project has been conducted at Alzahra University since 2005.

Patients: This is a case-control study. The study group clinically sensitive patients (21 patients) were selected and referred by participating centers according to the following criteria:

- Patients who need less than 10.5 mg $week^{-1}$ of warfarin to keep the INR in therapeutic range (INR = 2-3)

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- Patients had been used usual dosage of warfarin (5-15 mg day^{-1}) but they had INR>4 even in seven days after cessation of warfarin or three days after receiving 3 mg day^{-1} vitamin K1

The first criterion was chosen from previous reports (Linder, 2001) and the second one arbitrary chosen by our group while encountered such patients in clinic.

The control group (37 adult patients) was selected randomly from patients taking warfarin for various cases and seemingly showed normal response to warfarin.

Methods: For each patient a form was provided and collected clinical data including weight, height, dose of warfarin, concomitant medications, indication for anticoagulant, length of therapy, complications during therapy and INR results. At the first visit, a non-fasting blood sample was obtained on the day of the index visit approximately 12 h after the last dose of warfarin and was aliquoted for PT, INR and blood clotting factors (FII, FV, FVII, FVIII and FIX) measurements and *CYP2C9* genotyping.

Genomic DNA was isolated from whole blood using DNG™ Plus solution. For detection of *CYP2C9*2* and *CYP2C9*3* variants, a protocol based on PCR technique and endonuclease digestion with *Eco47I* and *KpnI* was respectively used (fermentas) (GenBank accession numbers: L16877 to L16883). Polymerase Chain Reaction (PCR) was performed with primers, 5′ tacaaatacaatgaaaatatcatg 3′(forward) and 5' ctaacaaccagactcataatg 3′ (reverse) for exon three and for *CYP2C9*2* variants and 5' tgcacgaggtccagaggtac 3'(forward) and 5′acaaacttaccttgggaatgaga 3′(reverse) for exon seven and for *CYP2C9*3* variants. A forced mismatch was included in forward primer create a restriction site for *KpnI* digestion for *CYP2C9*3* variant and exon seven. The annealing temperature was 58°C and PCR reactions were run for 30 cycles. The PCR product from this amplification is 691-bp fragments for exon three and 105-bp fragments for exon seven. The PCR products were digested with *Eco47I* for detection of the Arg144Cys polymorphism and *CYP2C9*2* variants in exon three and with *KpnI* for detection of the Ile359Leu polymorphism and *CYP2C9*3* variants in exon seven. Digestion occurred in a total volume of 30 μL containing 15 μL of PCR product, 5 units restriction enzyme and 3 μL 10X buffer(supplied with the enzyme) at 37°C for a minimum of 1 h. Digestion products were separated on 2% agarose gels (Xie *et al*., 2002).

After digestion with *Eco47I* there were 527 and 164-bp fragments for *CYP2C9*1*1, 691-bp fragments for *CYP2C9*2*2 and 691, 527, 164-bp fragments for *CYP2C9*1*2 genotypes.

RESULTS

Among 21 patients who were clinically assigned to be sensitive according to the study criteria (mentioned above), 17 patients (81%) finally proved to carry some variant alleles (*1*2 or *2*2) and four patients (19%) had normal allele (wild type *1*1). The PCR products for exon three and exon seven have been shown in Fig. 1 and 2, respectively.

The PCR products from exon three after digestion with *Eco47I* have been shown in Fig. 3.

Among 37 patients with normal response to warfarin, nine patients (24.3%) had variant alleles (*1*2 or *2*2) and 28 patients (75.7%) had wild type allele (*1*1) (Table 1).

None of our study group or control group patients showed allele *3 variant. About 66.7 and 14.3% of study group and 21.6 and 2.7% of control group carried *CYP2C9*1*2* and *CYP2C9*2*2* genotypes, respectively.

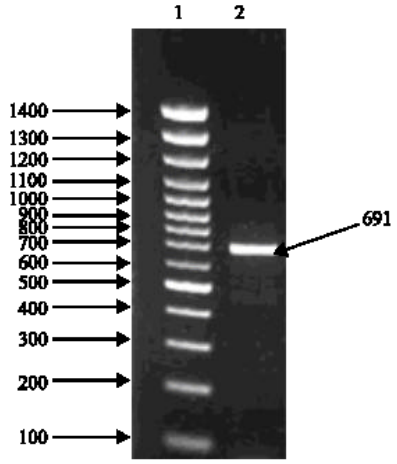

Fig. 1: PCR product, Lane 1: DNA ladder, lane 2: The PCR fragment from exon three

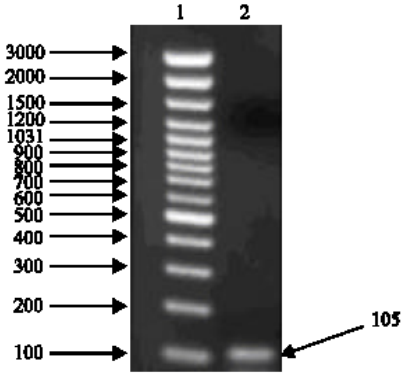

Fig. 2: PCR products, lane 1: DNA ladder, lane 2: The PCR fragment from exon seven

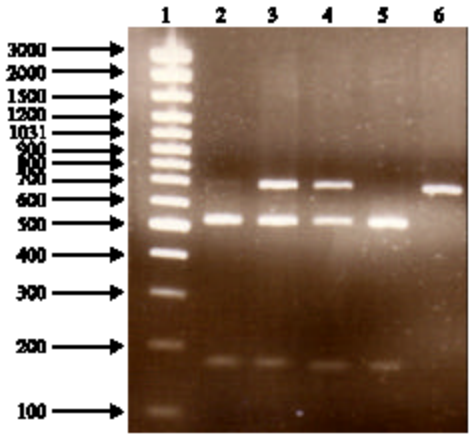

Fig. 3: RFLP based PCR products, lane1: DNA ladder, lane 2, 5: *CYP2C9*1*1, lane 3,4: *CYP2C9*1*2, lane 6: *CYP2C9* *2*2

Table 1: Cross tabulation genotypes

	Genotypes			
Group	*1*1	*1*2	*2*2	Total
Sensitive response				
count	4	14	3	21
%	19.0	66.7	14.3	100.0
Normal response				
count	28	8	1	37
%	75.7	21.6	2.7	100.0
Total				
count	32	22	4	58
%	55.2	37.9	6.9	100.0

DISCUSSION

The use of the same fixed dose of warfarin for all patients is not correct, because the responsiveness of different patients to warfarin is highly variable. The therapeutic effect for warfarin can vary up to 120 -fold between individuals (Takeuchi *et al*., 2009). For patients who are warfarin-sensitive, there is higher risk of life-threatening bleeding during the initiation phase of treatment. Traditionally, body weight and interactions with concomitantly taken drugs are the expected modifiers of warfarin dose requirements (Absher *et al*., 2002). It is now also well known that genetic polymorphism in warfarin metabolizing enzyme *CYP2C9* (Higashi *et al*., 2002) and in the warfarin sensitive enzyme *VKORC1* (D'Andrea *et al*., 2005) explain increased sensitivity to warfarin, but the amount of this relationship is race dependent (Brukner, 2009).

For the first time this study has found a significant and meaningful association between *CYP2C9* genotype and sensitivity to warfarin, high INR and bleeding complications in some Iranian sensitive patients to warfarin. Because most of our sensitive patients (81%) had an association between *CYP2C9* variant genotypes and warfarin sensitivity (*1*2 = 66.7 and *2*2=14.3), high INRs and bleeding complications, even though this correlation is not complete absolute or exact and linear. Approximately, 24% of normal responding patients had variant alleles (*1*2= 21.6% and *2*2=2.7%). Why this recent group have not shown execs or exaggerated response to warfarin could not be explained, but it could be due to wide range of warfarin therapeutic doses, coinheritance of prethrombotic genes, concurrent disease or medications that modifies warfarin response.

So, in future, more studies about the other effective factors that are important for warfarin sensitivity (mentioned above) are necessary.

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