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ORIGINAL ARTICLE

The effect of GGCX and CYP4F2 gene polymorphisms in genotype-guided dosing of warfarin in patients with a history of cardiac surgery

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Abstract Cytochrome P450 4F2 (CYP4F2) and γ-glutamyl carboxylase (GGCX) have small but significant roles in the maintenance dose of warfarin, an oral anticoagulant. *CYP4F2* 1347 C > T and *GGCX* 12970 C > G polymorphisms have been used in the pharmacogenetic dosing algorithms of warfarin for Caucasians, Chinese, Turkish and Indian populations. There are no reports about the genotype frequencies of these polymorphisms in Iranian population. In the present study, we aimed to find out the genotype frequencies of *CYP4F2* 1347 C > T and *GGCX* 12970 C > G polymorphisms and the impression on warfarin dosage in an Iranian-Azari population. *CYP4F2* 1347 C > T and *GGCX* 12970 C > G polymorphisms were genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) in 100 cases receiving warfarin after cardiac surgery. No significant differences were found between prescribed and protocol doses of warfarin based on variants of *CYP4F2* 1347 C > T and *GGCX* 12970 C > G polymorphisms. Patients with combination genotypes 1347CC/12970CG and 1347TT/12970CG demonstrated significantly different prescribed and protocol doses of warfarin. Age of patients was negatively correlated with prescribed dose of warfarin. Variations of *CYP4F2* 1347 C > T and *GGCX* 12970 C > G polymorphisms play a role in determining the required dose of warfarin in patients with cardiac surgery of Iranian-Azari population.

Keywords CYP4F2 · Cardiac surgery · GGCX · Polymorphism · Warfarin

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Introduction

Gene variations in the response of patients to medications have been noticed and documented since 1950s (Carson et al. 1956). This observation has also been partially explained by factors such as age, body size, race, concurrent diseases, and other medications (Wadelius and Pirmohamed 2007). Almost 50% of cases of adverse drug reactions or lack of therapy efficacy is caused by the genetic makeup of a patient (Pirmohamed and Park 2001). Polymorphisms in genes coding for Drug Metabolizing Enzymes (DMEs) have also been shown to significantly influence the quality of patient's response towards drugs (Wadelius and Pirmohamed 2007). Direct (phenotypic) determination of the concentration change rate of a drug or its metabolites in the blood is a time-consuming and complicated procedure, in addition to requiring the patient to take the relevant medication. Determining allelic variants (genotype) of biotransformation genes, however, allows for the identification of patients who are more likely to develop adverse drug reactions (Iskakova et al. 2014).

Warfarin is an oral anticoagulant that is widely prescribed for the prevention of various thromboembolic

events (Büller et al. 2004; Geerts et al. 2004; Harrington et al. 2004; Salem et al. 2004; Singer et al. 2004). The key enzymes involved in the pharmacodynamics and pharmacokinetics of warfarin include cytochrome P450 2C9 (CYP2C9), vitamin K epoxide reductase complex1 (VKORC1), CYP4F2 and GGCX (Schalekamp et al. 2006; Sconce et al. 2005; Wen et al. 2008; Wu et al. 2008). Warfarin exerts its anticoagulant effect by inhibiting VKORC1 enzyme. The vitamin K cycle is critical to anticoagulation because it controls c-carboxylation of glutamic acid residues on vitamin K-dependent proteins (clotting FII, FVII, FIX, FX, protein C, S, and Z) (Hirsh et al. 2001). This process is carried out by GGCX (Presnell and Stafford 2002), which acts in concert with VKORC1. GGCX consumes vitamin K hydroquinone (K1H2) as a cofactor in carrying out the carboxylation reaction, producing vitamin K1 2,3 epoxide (KO) in the process, which then must be recycled back to K1H2 by VKORC1. Rare non-synonymous mutations in *GGCX* gene lead to congenital deficiency of the vitamin K-dependent clotting factors (VKDCF type 1), consistent with an important role of GGCX in this pathway (Rost et al. 2004, 2006). A *GGCX* variant, namely rs11676382 SNP, has been shown to have a small but significant effect on warfarin maintenance dose, accounting for 2% of variations in determining warfarin dose in Caucasian population (Rieder et al. 2007).

CYP4F2 is a primary liver vitamin K1 oxidase that catalyzes the metabolism of vitamin K1 to hydroxyl vitamin K1 and removes vitamin K from the vitamin K cycle. It acts as an important counterpart to VKORC1 in limiting excessive accumulation of vitamin K (Whirl-Carrillo et al. 2012). Caldwell et al. reported that an exonic polymorphism in *CYP4F2* gene was responsible for approximately 2% of variations in maintaining the required dose of Warfarin through a genome wide association study (GWAS) carried out on Caucasian population (Whirl-Carrillo et al. 2012).

In Iranian population, not much has been reported about the frequency distribution of polymorphisms in *GGCX* and *CYP4F2* genes and their associations with required doses of oral administration of warfarin. On the other side, HapMap data show wide ethnicity specific differences in the allelic distribution of these two polymorphisms (Barrett et al. 2005). Therefore, an attempt was made in the present study to evaluate the allele and genotype frequencies of *CYP4F2* 1347 C > T and *GGCX* 12970 C > G in an Iranian-Azari population. Moreover, the effect of genotypes on dose of warfarin was evaluated in subjects under cardiac surgery.

Patients and methods

Study subjects

In this study, a total of 100 patients including 45 males and 55 females, were requited from Imam Reza Hospital, Tabriz, Iran during 2009–2014. Subjects were under warfarin therapy due to cardiac surgery. Demographic data of cases were collected through medical history records. The local Ethical Committee of Tabriz University of Medical Sciences approved the study protocol and written informed consent was endorsed through all subjects.

Sampling and genotyping

About 5 ml of peripheral blood was collected from each patient in ethylenediaminetetraacetic acid (EDTA)-anticoagulated venoject tubes. Afterwards, genomic DNA was extracted from peripheral blood using phenol–chloroform method. The extracted DNA samples were stored at −20 °C. The allele and genotype frequencies of *CYP4F2* 1347 C > T and *GGCX* 12970 C > G genes were determined by Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method. The primers used in the PCR assay were based on the sequence of the human gene and designated by Oligo7 software. Sequence of the primers and restriction enzymes are shown in Table 1. PCR-RFLP on *CYP4F2* 1347 C > T SNP was performed with 100 ng of the genomic DNA in 25 ml reaction volume containing 0.5 ml of 10 pmol/ml forward primer, 0.5 ml of 10 pmol/ml reverse primer, 0.2 ml of 10 mmol/l dNTPs, 1 U of Taq DNA polymerase and 2.5 ml of 10× PCR buffer. PCR was performed under the conditions as follows: initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 60 s. A final extension at 72 °C for 7 min was carried out. The 439-pb PCR product was digested with 10 U of *Pvu*II (Fermentas

Table 1 SNP characteristics, enzymes, break points and primers

SNP	Reference SNP	Position	Restriction enzyme	PCR primer	Break point
CYP4F2 1347 C > T	rs2108622	19p13 Exon 2	*Pvu*II	F: 5′-CGGAACTTGGACCATCTACA-3′ R: 5′-CCTACTCTCCCACAGGCATTA-3′	5′…CAG*CTG… 3′
GGCX 12970 C > G	rs11676382	2p11.2 Intron	*Hin*dIII	F: 5′-CCATTTCTTCTCCCTTGTCAGT-3′ R: 5′-CAAGTGACCCTCCATCTCCC-3′	5′…AAG*CTT… 3′

Inc., Burlington, ON, Canada) at 37 °C overnight and was resolved on a 2.5% agarose gel using Gel Green DNA Staining (Table 1; Fig. 1a). The PCR-RFLP on *GGCX* 12970 C > G was done with 100 ng of the genomic DNA in a 25 ml reaction volume containing 0.6 ml of 10 pmol/ml forward primer, 0.6 ml of 10 pmol/ml reverse primer, 0.2 ml of 10 mmol/l dNTPs, 1 U of Taq DNA polymerase and 2 ml of 10× PCR buffer. Thermal cycling conditions were 94 °C for 4 min followed by 30 cycles of 94 °C for 40 s, 60 °C for 40 s and 72 °C for 25 s. A final extension at 72 °C for 5 min was carried out. The 840-pb PCR product was digested with 10 U of *Hind*III (Fermentas Inc.) at 37 °C overnight and was resolved on a 2.5% agarose gel with Gel Green DNA Staining (Table 1; Fig. 1b). The sizes of specifically digested fragments were determined as follows: in the case of *CYP4F2* 1347 C > T: 379 bp for C allele and 439 bp for T allele; *GGCX* 12970 C > G: 840 bp product digested to 580 and 260 bp. If the product was digested, the allele was identified as C; if not, it was identified as G (Table 2; Fig. 1).

Finally, we compared the prescribed dose (a dose of warfarin that is prescribed according to the patients INR and patient's response to drug without knowing the genotype of individuals) and protocol dose (a dose of warfarin that is obtained from the pharmacogenetic algorithm available on http://www.warfarindosing.org and it is based on genotypes of individuals) in subjects, after genotyping.

DNA sequencing

The products of PCR amplification of *CYP4F2* and *GGCX* genes were subjected to DNA sequencing. A total of 6 DNA samples from each locus were used for DNA sequencing in order to ascertain whether the PCR amplification products and digestion results were the authentic target fragments. It was observed that sequencing results were in accordance with the standard DNA sequences of target DNA, provided by NCBI gene tool, and digestion results.

Statistical analysis

Experimental data were expressed as mean ± standard deviation (SD) and analyzed by SPSS v.21 and PLINK softwares. Hardy–Weinberg law of genetic equilibrium was employed to detect the goodness of fit-test of genetic balance. The genotypes and allele frequencies were calculated using χ^2-test. A level of $P \leq 0.05$ was considered as statistically significance.

Table 2 Length of restriction fragments of CYP4F2 and GGCX gene products

Restriction enzyme	Genotype	Length of restriction fragments (bp)
*Pvu*II for CYP4F2	C/C	379 and 60
	C/T	439–379 and 60
	T/T	439
*Hind*III for GGCX	C/C	580 and 260
	C/G	840–580 and 60

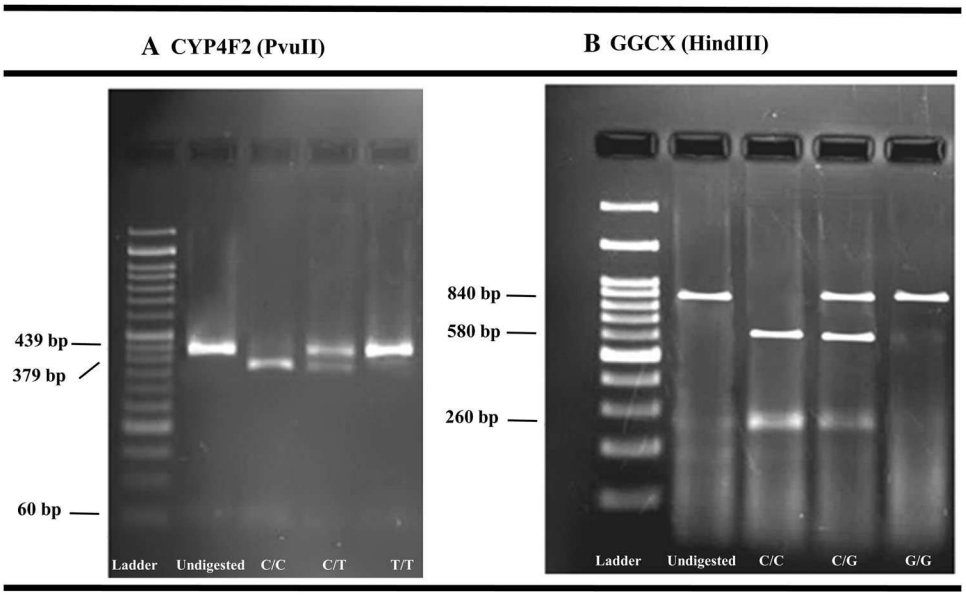

Fig. 1 Gel electrophoresis patterns of CYP4F2 1347 C > T and GGCX 12970 C > G SNPs. **a** The amplified fragments of CYP4F2 1347 C > T were digested with *Pvu*II, the PCR product was 439 bp. If the product was digested, creating two fragments of 379 and 60 bp, the allele was identified as *C*; if there was no digestion, it would be identified as *T*. **b** The amplified fragments of GGCX 12970 C > G were digested with *Hind*III, the PCR product size was 840 bp. If the product was digested, creating two fragments of 580 and 260 bp, the allele was identified as *C*; if there was no digestion, it would be identified as *G*

Table 3 Genotype and allele frequencies of CYP4F2 1347 C > T and GGCX 12970 C > G SNPs in patients

SNP	Allele/genotype				
GGCX 12970 C > G	C	G	CC	CG	GG
Frequency	151 (75.5%)	49 (24.5%)	52 (52%)	47 (47%)	1 (1%)
CYP4F2 1347 C > T	C	T	CC	CT	TT
Frequency	146 (73%)	54 (27%)	52 (52%)	42 (42%)	6 (6%)

Table 4 Comparison of mean prescribed dosages of warfarin and protocol dosages warfarin according to genotypes of the CYP4F2 and GGCX gene polymorphisms

Gene	Genotype (%)	Mean of prescribed dosage (mg/day)	Mean of protocol dosage (mg/day)	*P* values
CYP4F2	CC (52)	3.48 ± 1.54	4.17 ± 0.60	0.082
	CT (42)	4.05 ± 1.74	4.03 ± 0.48	0.541
	TT (6)	3.75 ± 1.25	4.23 ± 0.63	0.610
GGCX	CC (52)	4.03 ± 1.81	3.99 ± 0.55	0.741
	CG (47)	3.41 ± 1.33	4.27 ± 0.53	0.071
	GG (1)	N.A*	3.6**	–

*The GG genotype had missing data for prescribed dosage

**The GG genotype had only one data for protocol dosage

Results

Allele and genotype frequency

The frequencies of *GGCX* (CC, GG and CG), *CYP4F2* (CC, TT and CT) genotypes in the study population are presented in Table 3. Regarding the *GGCX* gene, the C and G alleles were found in 75.5 and 24.5% of patients, respectively. The CC genotype was the most common variation with 52% frequency. Moreover, CG and GG genotypes were observed in 47 and 1% of patients, respectively. On the other side, C and T alleles of *CYP4F2* gene were represented in 73 and 27% of cases, respectively. For this position, CC genotype was more prevalent (52%) among subjects and CT and TT genotypes were found in 42 and 6% of patients, respectively.

Genotype distribution according to warfarin dosage

As shown in Table 4, comparison of mean of protocol and prescribed warfarin dosages according to various genotypes of *CYP4F2* and *GGCX* gene polymorphisms resulted that there was no statistically significant difference between two dosages. The comparison of warfarin protocol and prescribed dosages based on different combined genotypes for CYP4F2 1347 C > T and GGCX 12970 C > G polymorphisms demonstrated that 25 patients with 1347CC/12970CG combined genotype had significant difference in warfarin prescribed and protocol dosages (3.1 ± 1.3 vs. 4.36 ± 0.54; $P \leq 0.033$). Alternately, significant difference in prescribed and protocol warfarin dosages (3.7 ± 1.25 vs. 4.23 ± 0.63; $P \leq 0.044$) was observed in subjects (six cases) with 1347TT/12970CG combined genotype. Frequencies of the combined genotypes and the values of both prescribed and protocol warfarin dosage are illustrated in Table 5.

Correlation between warfarin dosage and clinical manifestations

Correlation between age, weight and height of patients with protocol and prescribed dosages of warfarin demonstrated that age of patient was negatively correlated with protocol dosage ($r = -0.87$; $P = 0.0374$). However, age of the studied cases had no correlation with the prescribed dosage of warfarin ($r = -0.131$; $P = 0.465$). Moreover, no significant correlation was observed between weight of the patients and both protocol and prescribed dosages of warfarin ($r = 0.214$; $P = 0.314$ and $r = 0.245$; $P = 0.215$,

Table 5 Comparison of mean of prescribed and protocol warfarin dosages according to the combination genotypes of CYP4F2 1347 C > T and GGCX 12970 C > G polymorphisms

Combined genotype	No. (% of patients)	Prescribed dosage (mg/day)	Protocol dosage (mg/day)	*P* value
1347CC/12970CC	27 (27%)	3.86 ± 1.69	4.02 ± 0.63	0.091
1347CC/12970CG	25 (25%)	3.1 ± 1.3	4.36 ± 0.54	0.033
1347CT/12970CC	25 (25%)	4.21 ± 1.95	3.96 ± 0.48	0.101
1347CT/12970CG	16 (16%)	3.8 ± 1.35	4.17 ± 0.48	0.057
1347CT/12970GG	1 (1%)	N.A*	3.60**	–
1347TT/12970CG	6 (6%)	3.7 ± 1.25	4.23 ± 0.63	0.044

*The CTGG haplotype had missing data for prescribed dosage

**The CTGG haplotype had only one data for protocol dosage

Table 6 Correlation between clinical manifestations and warfarin protocol and prescribed dosages

Parameter	Protocol dosage		Prescribed dosage	
	Pearson correlation coefficient (r)	P value	Pearson correlation coefficient (r)	P value
Age	−0.87	0.0374	−0.131	0.465
weight	0.214	0.314	0.245	0.215
Height	0.145	0.741	0.105	0.835

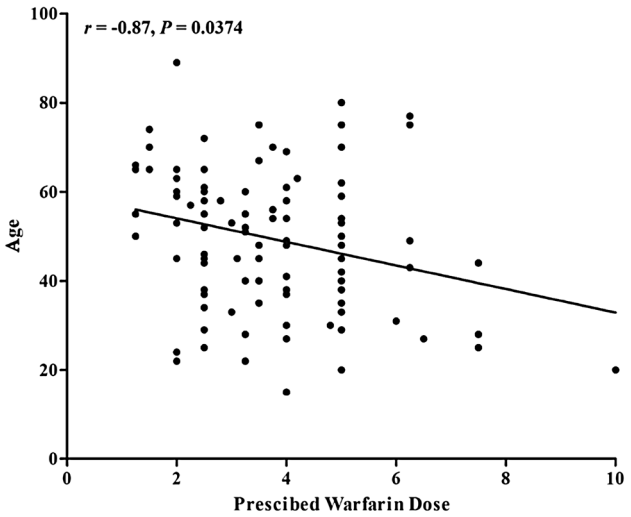

Fig. 2 Relationship between prescribed warfarin dosage and age

respectively). In addition, height of the cases had no correlation with both protocol and prescribed dosages of warfarin ($r=0.145$; $P=0.741$ and $r=0.105$; $P=0.835$, respectively; Table 6; Fig. 2).

Discussion

The clinical use of pharmacogenomic/pharmacogenetic findings prior to warfarin administration can help us to predict a better starting dose for individual patients and potentially shorten the period required to achieve a stable warfarin dose. In the recent years, various studies have been carried out to identify genetic factors that may be associated with therapeutic dose of warfarin. It has previously been suggested that *GGCX* 12970 C > G and *CYP4F2* 1347 C > T gene polymorphisms are associated with interindividual differences on the anticoagulant response to warfarin. Therefore, this study was performed to determine the frequencies of *GGCX* 12970 C > G and *CYP4F2* 1347 C > T gene polymorphisms in Iranian-Azari patients and to evaluate the effects of gene variations on required dosage of warfarin therapy in order to ascertain the genetic basis of dose variation. We found that the frequency of CT and TT genotypes for CYP4F2 1347 C > T polymorphism in patients were 42 and 6%, respectively (Table 3). In Turkish population, the frequencies of (CC, CT and TT) genotypes for CYP4F2 1347 C > T polymorphism in patients were reported 37, 46 and 17%, respectively. These frequencies show an almost similar pattern with Middle East population that the frequencies of (CC, CT and TT) genotypes for CYP4F2 1347 C > T polymorphism were reported 36, 48 and 16%, respectively by Ross et al. (Ross et al. 2010). Our findings revealed that subjects carrying homozygous wild-type (CC) genotype for CYP4F2 1347 C > T polymorphism required a mean dose of warfarin (4.17 ± 0.60 mg/day), while patients with TT genotype require higher doses (4.23 ± 0.63 mg/day). However, this difference is not significant. Our results are in accordance with the findings of several previous studies that have been published in this field. It has previously been reported that patients carrying heterozygote and mutant genotypes of CYP4F2 1347 C > T polymorphism require a higher daily warfarin dose in comparison to individuals carrying wild-type (Özer et al. 2013). They revealed that patients with the wild-type genotype for CYP4F2 1347 C > T polymorphism required a mean dose of (4.53 ± 1.73 mg/day), while individuals with heterozygote and mutant genotypes were found to require higher doses (5.58 ± 2.24 and 5.42 ± 1.10 mg/day) respectively (Özer et al. 2013). In the Ghanaian population, the carriers of the homozygote genotype (TT) for CYP4F2 1347 C > T polymorphism were treated with higher daily warfarin dosages (6.88 ± 0.41 mg/day) than carriers of the heterozygous genotype (CT) (6.13 ± 0.22 mg/day). Patients with the homozygote wild-type genotype (CC) were given intermediate daily warfarin dosages of 6.16 ± 0.55 mg/day (Ahorhorlu 2014). These findings was slightly different from a study by Singh et al. who reported that carriers of CT and TT genotypes of CYP4F2 1347 C > T polymorphism required a 25% higher warfarin dosage than carriers of the wild-type genotype (CC) (Singh et al. 2011). Although, no compelling reasons were given for these inconsistencies but, these contradictory findings may be partly explained by racial differences of populations, differences in number of studied subjects and multigenic nature of warfarin maintenance dosing. However, further studies may be required to disclose other possible genes involving in warfarin dosing.

Regarding the GGCX 12970 C > G polymorphism, we found that the frequencies of CC, CG and GG genotypes in studied subjects were 52, 47 and 1%, respectively (Table 3). Similarly, the genotype frequencies of CC, CG and GG genotypes for GGCX 12970 C > G polymorphism have found to be 84.3, 14.8 and 0.9%, respectively in Slavic patients (Wypasek et al. 2014). A

mutant variation of the GGCX 12970 C > G polymorphism occurs at a frequency of 10% in Caucasian populations and nearly never occurs in the Asian and African-American populations (King et al. 2010). In Russian population, the genotype frequencies of CC, CG and GG genotypes for GGCX 12970 C > G polymorphism were found to be 84, 16, and 0%, respectively (Iskakova et al. 2014). Krishna Kumar et al. observed that frequencies of GGCX wild-, hetero-and mutant-type patients were 97.9, 2.1, and 0%, respectively in the Indian population (Kumar et al. 2014). In addition, our results showed that patients with wild- and hetero-type genotypes for GGCX 12970 C > G polymorphism required 3.99 ± 0.55 and 4.27 ± 0.53 mg/day warfarin, respectively (Table 3). Also, patients with 1347CC/12970CG and 1347TT/12970CG combined genotypes required significantly high doses of warfarin (Table 5). Similar data was obtained from a study by Kumar et al. in Indian population. They have revealed that among the genetic determinants of warfarin dose, GGCX genetic variants accounted for 6% of variability. The GGCX genetic variants were found to be rare in Indian population, however its effect on dose variation was significant (Kumar et al. 2014). In another study, GGCX 12970 C > G polymorphism was found to have lesser contribution (~1.7%) in north India according to specific dosing algorithm (Rathore et al. 2012).

We also observed that warfarin protocol dose was negatively correlated with age of patients. In an African–American population, it has been reported that total weekly required warfarin dosage was 2.4 mg less for each additional decade of patient's age (Whitley et al. 2007). Furthermore, none of the other indexes, namely weight and height, were correlated with protocol or prescribed dosage of warfarin.

Taken together, comparison of prescribed and protocol dosages of warfarin in variants of *CYP4F2* 1347 C > T and *GGCX* 12970 C > G polymorphisms showed significantly association with the warfarin dosage in patients with cardiac surgery in Iranian-Azari population. However, multi-ethnic nature of Iranian population suggests for more such studies in different parts of the country to confirm our results. Collectively, this will help scientists and clinicians to develop better personal treatment protocols for each patient based on their genetic makeup.

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