CYP4F2 1347C>T and GGCX 12970C>G Polymorphisms as Determinants of Stable Warfarin Dose in Sudanese Patients of Heart Valve Replacement

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Research Article

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Abstract

Purpose: This study intended to explore the contribution of *CYP4F2* 1347C>T and *GGCX* 12970C>G polymorphisms on warfarin dose requirements among Sudanese subjects.

Methods: A total of 136 Sudanese patients of heart valve replacement receiving stable warfarin dose were recruited for this study. Blood samples were collected; DNA was extracted using phenol chloroform method. Genotyping for *CYP4F2* 1347C>T and *GGCX* 12970C>G polymorphisms was performed using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method. The association of genotype with warfarin dose requirement was measured by Kruskal Wallis and Mann Whitney U tests. Genotype, age, gender, comorbidity and concurrent medication were tested as predictors of stable warfarin dose using univariate regression analysis. For all tests, p. values < 0.05 were considered statistically significant.

Results: Frequencies of *CYP4F2* 1347C>T genotypes were 22.8% CC, 61% CT and 16.2% TT. The frequencies of C, T alleles were 0.533, 0.466 respectively which are deviated from Hardy Weinberg equilibrium (*p*. value = 0.008). Regarding *GGCX* 12970C>G genotyping, 96.9% were CC and 3% were CG. The frequencies of C, G alleles were 0.984 and 0.015 respectively which are in accordance with Hardy Weinberg equilibrium (*p*. value = 0.859). Insignificant differences in the mean daily warfarin dose between different genotype groups were observed (all *p*. values > 0.05). None of the studied variables was significant predictor of stable warfarin dose in this study population (*p*. values > 0.05).

Conclusion: No significant contribution of *CYP4F2* 1347C>T and *GGCX* 12970C>G polymorphisms on warfarin dose requirements was observed in this study population.

Introduction

Warfarin remains the main stay oral anticoagulant worldwide even after the invention of direct thrombin inhibitors (DTIs). Its indicated for the prevention and treatment of pro thrombotic conditions including atrial fibrillation, prosthetic heart valves and venous thromboembolism [1]. Warfarin dosing is complicated by the variation in individuals' response to this therapy which is partially explained by clinical and genetic factors [2]. Among the warfarin pharmacogenetics, *CYP4F2* and *GGCX* polymorphisms have been described.

The *CYP4F2* gene locates in chromosome 19 p13.2, spanning 20.1 kilo bases, and possesses 20 exons; it is a member of the cytochrome P450 super family enzymes which catalyses many reactions including drug metabolism, synthesis of cholesterol and other lipids. *CYP4F2* variant (1347C > T, rs2108622) involves a V433M missense mutation with reduced enzyme activity and reduced Vitamin K metabolism. The V433M polymorphism in exon 11 was associated with higher warfarin dose requirements explaining 11% of dose variation in Asian population [3]. Variations in *CYP4F2* genes showed variable degrees of influence on warfarin dose requirements among different populations. Several studies had replicated this finding as illustrated by Liang *et al*. who compared individuals with the homozygous *CYP4F2* genotype

(CC) with carriers of the T variant allele either CT or TT genotypes which required 10% and 21% higher warfarin dose respectively [4]; However, there is no consensus on the impact of this SNP on warfarin dosage especially when combined to other variables such as age, race, or when compared to other polymorphisms [5]. In the same prospective and although it was significant, the effect of *CYP4F2* polymorphism was relatively small when compared to the effects of *CYP2C9* and *VKORC1* polymorphisms. This was demonstrated in a Chinese study which showed that; *CYP4F2* rs2108622 contributes on dose variation by only 3% compared to 15% contribution of *VKORC1* rs9923231 polymorphism [6]. Lower frequencies of C1347T variant allele was reported in Blacks compared with other ethnicities, this with lesser impact on warfarin dose requirements [7].

The *GGCX* gene locates in chromosome 2 p12, spanning 13.9 kilo bases and possesses 15 exons. It encodes for gamma glutamyl carboxylase, an integral membrane microsomal enzyme located in the rough endoplasmic reticulum. This enzyme catalyses the vitamin K dependant carboxylation of glutamate residues of clotting factors VII, IX, X and II in addition to proteins C and S. Reduced vitamin K is an essential cofactor for the activation of clotting factors by gamma-glutamyl carboxylase, which is encoded by GGCX. Common variants of *GGCX* gene have been noted to influence warfarin dose variability in different populations [8, 9]. Shikata *et al*. described a microsatellite marker in intron 6 of the *GGCX* gene that was associated with warfarin dose [10].

Since warfarin treatment may associate with haemostatic complications related to improper dosing, therefore identification of dose predictors may help avoiding such events. Moreover; as the influence of pharmacogenetics on warfarin dosing in Sudanese subjects is poorly investigated, the aim of this study was to explore the contribution of *CYP4F2* 1347C > T and *GGCX* 12970C > G polymorphisms on Sudanese subjects' response to warfarin therapy.

Material And Methods

This study was conducted in Sudan heart institute in Khartoum – Sudan. It involved 136 patients of heart valve replacement receiving stable warfarin dose for at least 3 months' prior the time of this study, and their previous INRs were within the therapeutic range. The study was approved by the research committee of Alzaiem Al-Azhari University and informed consent was taken from each subject before enrolment in the study. Data on patients' demographics, warfarin dose, INR values, thrombotic and or bleeding events, other diseases and medications were collected by questionnaire. Venous blood samples (1.8 ml) were collected in tri-sodium citrate tubes; buffy coat was collected after centrifugation and stored at -20°C for subsequent DNA extraction. DNA extraction was performed on Buffy coat samples using Phenol chloroform method as described by Sambrook *et al*. [11] DNA samples were then kept at -20° C until usage.

Genotyping:

Genotyping of all samples for *CYP4F2* 1347 C>T was performed by PCR-RFLP as described by Natarjan *et al*. [12] with some modifications. Briefly; PCR was performed in premix tubes (iNtRON Biotechnology, Korea) in a 25µl volume containing 3µl genomic DNA, 15 picomol of each primer (F:5'-GTCTCCTGGGTAGGAAGAG-3' and R: 5'-GTTGTGTGTGTCTTTGAG-3'; Macrogen, Seoul, Korea). Thermocycling profile consisted of the first step held at 94°C for 5 minutes followed by 40 cycles at 94°C for 30 seconds, at 61°C for 30 seconds, at 72°C for one minute and a 10 minutes' final extension at 72°C. The resulted 243 bp was digested for 2 hours with 20 units of *PvuII* enzyme in 1X *NEBuffer3.1* (NEW ENGLAND BioLabs Inc, UK) at 37°C. Digestion product was then analysed by electrophoresis on 2.5% agarose (iNtRON Biotechnology, Korea) stained with Ethidium bromide.

For *GGCX* 12970C>G polymorphism, genotyping was performed by PCR-RFLP method as described by Rathore *et al*. [13] Briefly; PCR was performed in premix tubes (iNtRON Biotechnology, Korea) in a 25µl volume containing 3µl genomic DNA, 15 picomol of each primer (F:5'-GCTTCTTGTTGCGAAAGCTCTAT-3' and R: 5'-CAAACACTTGGGAACAGTTAGCT-3'; Macrogen, Seoul, Korea). Thermocycling profile consisted of the first step held 94°C for 5 minutes followed by 30 cycles at 94°C for 30 seconds, at 57°C for 30 seconds, at 72°C for one minute and a 10 minutes' final extension at 72°C. The resulted 1288 bp was digested for 4 hours with 10 units of *HindIII* enzyme in 1X *NEBuffer2.1* (NEW ENGLAND BioLabs Inc, UK) at 37°C. Digestion product was then analysed by electrophoresis on 2% agarose (iNtRON Biotechnology, Korea) stained with Ethidium bromide.

Statistical analysis:

The distribution of alleles for their accordance with Hardy Weinberg equilibrium was calculated by Chi square test using one degree of freedom (df), *p*. values of < 0.05 were considered deviated from the equation. Comparison of mean daily warfarin dose between different genotype groups was calculated by Mann-Whitney U or Kruskal-Wallis tests. The effect of patients' variables on warfarin dose was measured. These variables include age, gender, co-morbid disease, concurrent medications and genotypes of *CYP4F2* 1347C>T and *GGCX 12970C>G*. Univariate regression analysis was applied using each one of these factors as independent variable to predict the mean daily warfarin dose as a dependent variable.

For all tests, *p.* values < 0.05 were considered statistically significant. All analysis was carried out using statistical package for social sciences (SPSS) version 21 (IBM Japan Ltd., Tokyo, Japan).

Results

A total of 136 Sudanese patients of heart valve replacement were included in this study. They were 59% females and 41% males with a mean age of 42 years old. All of them receive warfarin for life with a mean dose of 5.4±2.2 mg/day, while the mean INR was 3.4±1.9. Comorbidity was reported in 11.5% of participants and this includes hypertension, Diabetes mellitus, Hyperthyroidism and hyperlipidemia. About 20.6% of total patients receive medication alongside with warfarin.

Genotyping results:

Among the 136 patients; 22.8% were homozygous for the wild type allele (CC) of *CYP4F2* gene, 61% were heterozygous (CT), while 16.2% were homozygous for the mutant allele (TT). The frequency of C allele is 0.533, while the T allele frequency is 0.466. These alleles frequencies are deviated from Hardy Weinberg equilibrium (*p*. value = 0.008).

Regarding *GGCX* genotyping; results were available for 132 sample, the four samples were excluded from further analysis of this polymorphism. Out of 132 samples, 96.9% were homozygous for the wild type allele (CC), while 3% were heterozygous (CG). The frequency of C allele is 0.984, while the G allele frequency is 0.015. These frequencies are in accordance with Hardy Weinberg equilibrium (*p*. value = 0.859).

The effect of genotype on warfarin dose requirements:

Mean daily warfarin doses were compared between *CYP4F2* CC, CT and TT genotype groups and the effect of additive and dominant models on warfarin dose was studied. Insignificant differences in the mean daily warfarin dose between different *CYP4F2* genotype groups was observed among the studied population (*p*. value > 0.05) (Table 1). When TT genotype group was compared with CC, CT genotype groups after exclusion of patients with *VKORC1*-1639 AA genotype, the TT genotype group associated with higher warfarin dose (7.0±1.3mg) than CC, CT genotype groups (5.3±1.9mg) and the difference was statistically significant (*p*. value = 0.002) (Table 1).

Regarding *GGCX* genotypes, comparison of the mean daily warfarin dose between the two genotype groups using Mann-Whitney U test revealed an insignificant difference between CC and CG groups in warfarin dose requirements (*p*. value = 0.811) (Table 1).

Univariate analysis revealed that, neither *CYP4F2* 1347C>T nor *GGCX 12970C>G* genotype were significant predictors of stable warfarin dose in this study population (*p*. values > 0.05) (Table 2).

Table 1

The effect of *CYP4F2* additive and dominant models and *GGCX* genotypes on the mean daily warfarin dose:

Gene	Reference allele frequency*	Minor allele frequency *	Data		
			AA**	AB**	BB**
CYP4F2	C(51)	T(49)	18(20) [5.7±2.0]	55(62) [5.2±1.8]	16(18) [5.7±2.4]
GGCX	C(98.8)	G(1.2)	84(98) [5.3±1.9]	2(2)[5.5]	
P. values		**AA vs AB vs BB**	0.316***	0.811****	
		AA vs AB + BB	0.498***	NC	
		AA + AB vs BB	0.246***	NC	

*Values given in the parenthesis are percentages

**Values for AA, AB and BB are given in sequential order as: number of patients, followed by percentages in parenthesis, followed in squared brackets by the mean daily warfarin dose

***Values are calculated by Kruskal Wallis or Mann-Whitny U tests for *CYP4F2* genotypes

****Values are calculated by Kruskal Wallis or Mann-Whitny U tests for *GGCX* genotypes

Table 2

Results of univariate regression analysis:

Predictor	R^2	Significance*
Age	0.026	0.101
Gender	0.000	0.926
Co morbidity	0.021	0.144
Concurrent drug	0.003	0.575
CYP4F2 genotype	0.000	0.991
GGCX genotype	0.000	0.885

Discussion

The aim of this study was to investigate the contribution of genetic polymorphisms on the variability of Warfarin dose requirements in Sudanese patients. Firstly, regarding Warfarin doses and INR values, Participants in this study showed a wide range of Warfarin dose requirements (1.0—13.0 mg) and INR values (1.0—13.6). These values give further evidence that Warfarin is a drug with wide inter-subject

variability in dose requirements and anti-coagulation effect respectively, which is in accordance with previous reports on different ethnic groups.

In this study, the impact of clinical factors on Warfarin dose requirements was evaluated. Among the tested variables, only indication of Warfarin therapy had a significant impact on Warfarin dose (*p*. value = 0.028), while the other factors including patient's age, gender, co-morbid diseases and concurrent medications had minor effect on Warfarin dose requirements. This finding is in accordance with the previous findings of Sherif NE *et al* in Sudanese patients [14] and Shahin *et al* in Egyptian patients [15]. They reported minor effect of the studied patients' variables on Warfarin dose, suggesting that further clinical factors may have an influence on Warfarin dose. At the other hand, our finding disagrees with previous observations of associations between old age and female gender with lower Warfarin dose requirements [16, 17]. In the current study age factor explained only 2.6% of dose variation while gender factor had no impact on Warfarin dose, so this discrepancy could be explained by the relatively younger ages of participants in this study (Mean 42.2 years), while the effect of gender might be diluted by the effects of other clinical and genetic factors.

The *CYP4F2* 1347 G > A minor allele frequency (MAF) in our population is high (0.466), this is close to the MAF reported in Egyptian (0.42) [15] and Indians (0.43) [13], while it is substantially higher than the frequencies reported in American Indians, Mozambicans and Brazilians (4.4, 8.7 and 24 per cent respectively) [18]. *CYP4F2* 1347 genotypes frequencies reported in this study are deviated from Hardy Weinberg equilibrium (Chi square *p*. value = 0.008), and as there were no previous reports of MAF on this SNP in Sudanese, these findings raise the possibility of evolutionary impact on the frequency of this SNP, including the effects of genetic mutations, recombination, gene drifts and natural selections. So collectively; alleles' frequencies observed in this study showed some sort of genetic similarity between Sudanese and Egyptian, African and some Asian populations, while at the other hand, a genetic discrepancy exists between Sudanese and Caucasians, and some Asian populations.

In the current study population, despite the high frequency of *CYP4F2 1347C > T* polymorphism, no significant impact of this polymorphism on Warfarin dose was observed (*p*. value = 0.316), this is in accordance with Shahin *et al*. [15] findings in Egyptians, who reported a high frequency of *CYP4F2 1347C > T* polymorphism with an insignificant impact on Warfarin dose (*p*. value = 0.314). Alternatively; in other studies, a minor but still significant effect of this SNP was observed in Asians and Caucasians [13,19—22]. Among these studies, the percentage effect of this SNP on dose variation ranged between 1.5% and 11%. This discrepancy may be attributed to variations in genetic makeup between different ethnic groups, but generally in all population groups; the impact of this SNP on Warfarin dose variation, if any, is very small and this could be related to the fact that CYP4F2 enzyme is not directly involved in the vitamin K cycle.

The MAF of GGCX 12970 polymorphism observed in this study is 0.015 which is comparable to the global MAF reported by NCBI, and those reported in Indian population (0.014)[13] but it is quite lower than what had been observed in African American (0.025) [23]. This SNP showed no significant impact on

Warfarin maintenance dose among this study population. This finding is in accordance with the findings of Schelleman *et al*. [25] in African Americans and Boxia *et al*. [24] in European patients, both of them reported no significant effect of *GGCX 12970 C > G* polymorphism on Warfarin dose. While other reports demonstrated an association of this SNP with Warfarin sensitivity in Indians and Caucasians with the percentage effect on the variation in Warfarin dose explained by this SNP ranging between 0.3 up to 2% [25, 26]. The small contribution of this SNP on Warfarin dosing, could be related to the low frequency of the minor allele in all populations.

Conclusion

No significant contribution of *CYP4F2* 1347C > T and *GGCX* 12970C > G polymorphisms on warfarin dose requirements was observed in this study population.

Declarations

Ethical approval:

Approval was granted by the research committee of Alzaiem Al-Azhari University.

Consent to participate:

Informed consent was obtained from all individual participants included in the study.

Human and animal ethics:

This study was performed in line with the principles of the Declaration of Helsinki.

Consent for publication:

Not applicable.

Availability of supporting data:

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests:

The authors have no relevant financial or non-financial interests to disclose.

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Authors contribution:

Ahmed, Elwaleed and Abdelrahim designed the study. Ahmed performed the research, NassrEldin analysed the data, Ahmed and Mai wrote the paper. All authors read and approved the final version of the paper.

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