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Original article

Association of Cytochrome P4502C9, Vitamin K Epoxide Reductase and Gamma-Glutamyl Carboxylase Gene Polymorphisms with Warfarin Dose Requirement Among Sudanese Patients

Rowida Babiker Eltayeb Ahmed [1], Salah Eldin Gumaa Elzaki [2], Amanda G. Elgoraish [3], Hussam Ali Osman [4],

[1]Faculty of Pharmacy, Ahfad University for Women, Omdurman, Sudan.
[2]Department of Epidemiology, Tropical Medicine Research Institute, National Centre for Research, Khartoum, Sudan.
[3]Department of Epidemiology, Tropical Medicine Research Institute, National Centre for Research, Khartoum, Sudan.
[4]Department of Biotechnology, Faculty of Pharmacy, Ahfad University for Women, Omdurman, Sudan.

*Corresponding authors: Rowida Babiker Eltayeb Ahmed; *rowida.babikir@yahoo.com*

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Abstract

Background: Warfarin is a common anticoagulant drug that has a narrow therapeutic index; a higher dose causes excessive bleeding and a lower dose leads to cerebrovascular clotting and stroke in patients. The management of warfarin therapy is challenging because there is variability in patient response due to many factors. Genetic factors that are most relevant, such as CYP2C9, and andVKOR, are the target site for warfarin. The study aimed to investigate the association between CYP2C9*2, VKORC1 (-1639 G>A), and GGCX T>G polymorphisms with warfarin daily dose in Sudanese patients on warfarin treatment referred to anti-coagulation clinics in Khartoum State, Sudan. ***Method:*** A cross-sectional descriptive study was conducted on randomly selected 107 patients on warfarin with different clinical indications. Their genotype was analyzed by Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) to determine the polymorphisms. ***Result:*** The study revealed that CYP2C9 genotype wild type was more frequent than heterozygote. For VKORC1 the frequency of the A allele was 61.8% and for the G allele was 38.2%, the GGCX genotype was observed only in wild-type and homozygous genotypes (89.7%& 10.3%, respectively). The t allele of GGCX was higher than the G alleles. ***Conclusion:*** The most common polymorphisms that revealed high significance on warfarin dose determination were VKORC1A/giving evidence to new guidelines dose requirements according to the patient genotype. These new dose requirement recommendations may lead to a significant improvement in the management of anticoagulant therapy in Sudan.

Keywords: ***Warfarin, VKORC1, GGCX, CYP2C9, Anticoagulation, INR***

Introduction

Warfarin is one of the most widely prescribed anticoagulants for the prevention of thromboembolic events associated with atrial fibrillation, and venous and arterial thrombosis, especially in patients with rheumatic heart disease with mechanic heart valve replacement. Most clinicians prescribe a 3-10 mg/day dose for the first 2–5 days, then followed by a maintenance dose based on frequent INR (International Normalized Ratio) monitoring [1]. The goal of warfarin therapy is an INR of 2 to 3 for most indications and an INR of 2.5 to 3.5 targeted for some mechanical valve replacement patients. Since warfarin has a narrow therapeutic index, it becomes imperative that the INR is maintained within the optimal range, and frequent monitoring is required [2]. The conventional pharmacological treatment, in some cases, leads to under or over-dosages of warfarin. Low-dose treatment may not reach effective anticoagulation and the high dose may increase the risk of bleeding in the initial therapeutic phase [3].

CytochromeP450 CYP2C9 is a liver enzyme required for warfarin metabolism. A series of genetic polymorphisms have been described within the cytochrome P450 CYP2C9, the gene variant (CYP2C9*2) rs 1799853 a substitution of a cysteine for arginine at position 144 within the exon 3 code for enzymes with approximately 12%of the enzymatic activity of the wild-type genotype CYP2C9*1. This variant allele has been associated with decreased warfarin dose requirements, more time to achieve stable dosing, a higher risk of bleeding during the initiation phase, and a significantly higher bleeding rate [4].

The VKORC1 gene which is the drug target encodes the vitamin K epoxide reductase enzyme and catalyzes the rate-limiting step in vitamin K recycling. The polymorphism occurs in the promoter region of VKORC1 -1639G>A (rs9923231) and alters a transcription factor binding site, leading to lower protein expression.

Patients are -1639A carriers, and require lower initial and maintenance doses of the drug compared to −1639G carriers [5,6].

The GGCX enzyme catalyzes the biosynthesis of vitamin K-dependent clotting factors by carboxylation protein-bound glutamate residues [7]. GGCX oxidizing reduces vitamin K to vitamin K-2,3-epoxide while adding a carboxyl residue to the gamma carbon on selected glutamic acids to produce functional clotting factors II, VII, IX, and X and other vitamin K-dependent proteins [8].

Methods

A cross-sectional descriptive hospital-based study was carried out in the major cardiac hospitals in Khartoum State, Ahmed Gasim hospital, and Sudan Heart Institute, between 23rd October 2017 and 14th March 2018. Randomly 107 Sudanese patients on warfarin therapy, aged 17 ≥ years old with different cardiac indications, were selected. Clinical and personal data of selected participants were collected using a data sheet and from their medical files. Blood samples were taken from participants after given their written consent to participate in the study. One and a half milliliters of whole blood samples were collected from participants during routine blood collection and stored in containers at a temperature (of 3-4 °C). DNA was extracted and purified from the blood by QIAGEN (QIAamp DNA Blood Mini kit Cat. No.51104) according to manufacturing recommendation, DNA, then was quantified by Gene Quant (Amersham Biosciences, UK) and read at 260 nm and 280nm for protein the ratio of DNA to protein and the concentration of the DNA was taken as purity and quality. The ratio of 1.7-1.9 was taken as a good purity (DNA concentration ranged between 10-150 µg/ml). Nested PCR was run for the amplification of CYP2C9*2, VKORC1, and GGCX genes using specific primers sequences listed in Table (1) and the thermal cycler machine was programmed as shown in Table (2). Genotyping of CYP2C9*2, VKORC1 (-1639 G>A), and GGCX T>G were performed by PCR restriction fragment length polymorphism. The CYP2C9*2 allele was uncleaved by AvaII and remained at 454bp [**Figure1**]. The wild-type allele was cleaved into two fragments of 400 bp and 54 bp. For VKORC1, the amplified products (290 bp) were digested with MspI restriction enzyme which produced a 124-and a 166-bp fragment [**Figure 2**]. For GGCX, outer PCR products were 810 bp [**Figure 3A**], and nested PCR products were 129 bp [**Figure3b**] (The PCR product was digested with Alu I which cleave the normal allele and subjected to electrophoresis in 3% agarose.in 1x TBE buffer, incubated at 37 °C for 16 hours and Electrophoresis was done at 100 volts for 60 mins. Statistical analyses were conducted using SPSS (version 20; SPSS Inc., Chicago, IL) software. Descriptive analyses of percentages of categorical variables were reported using chi-square x2. Comparisons of continuous variables were made using Student's t-test for parametric data. The differences between the mean daily warfarin dose and the mean daily calculated dose were evaluated by paired samples t-tests. All single nucleotide polymorphisms were tested for deviations from the Hardy–Weinberg Equilibrium. A P-value<0.05 was considered statistically significant.

Table 1: Primers sequences and PCR fragments length used in this study

Polymorphism	Primer sequences (5'-'3)	Fragments length	References
CYP2C9*2	GTATTTTGGCCTGAAACCCATA GGCCTTGGTTTTTCTCAACTC	454bp	
VKORC1	GCCAGCAGGAGAGGGAAAT AGTTTGGACTACAGGTGCCT	290 bp	[9]
GGCX	E-IX-5 5'-CTG GTT TTG CAG CCC CTT CTT-3' E-X-3 5'- AAG CAA GGG CTG TTC ATC TTG G-3' IX-mut 5'-TAT AAC AAC TGG ACA AAT GAG C-3' 1370C 5'-CCC AGG GTT AAG GTA GCC-3'	810 bp 139 bp	[10]

Table 2: PCR temperature profile for CYP2C9*2, VKORC1, and GGCX genes

Gene / Condition	CYP2C9*2	VKORC1	GGCX	
			Outer	Nest
Initial Denaturnation	94 °C /9 min	94 °C/3 mins	94 °C for 3 min	94 °C for 3 min
Denaturation	94 °C /30 sec	94 °C/1 mins	94 °C for 45 sec	94 °C for 60 sec
Annealing	58 °C /30 sec	59 °C /1 min	57 °C for 30 sec	55 °C for 45 sec
Extension	72 °C /30 sec	72 °C/1 min	72 °C for 60 sec	72 °C for 5 sec
Final extension	72 °C / 5 mins	72 °C/7 mins	72 °C for 3 min	72 °C for 3min
Holding Temperature	4 °C ∞	4 °C ∞	4 °C ∞	4 °C ∞
No of cycles	35	35	30	30

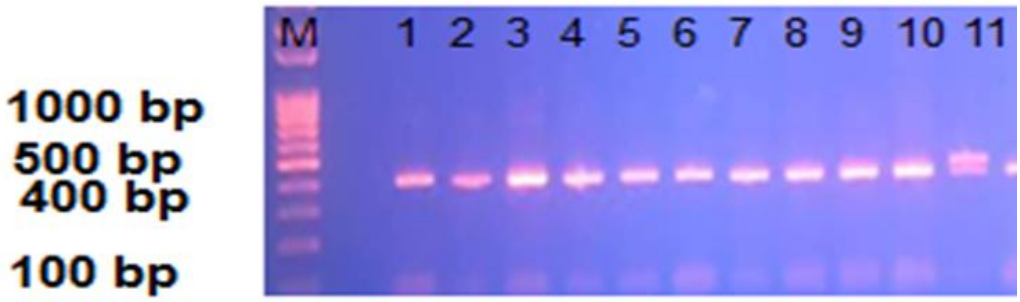

Figure 1: The figure shows the analysis of CYP2C9*2 polymorphism by electrophoresis of the digested PCR using AvaIIenzyme. Lane M contains DNA ladder 100 bp while lanes 1-10 contain the wild type of CYP2C9 (1*/1*), lane 11contain the heterozygous sample 1*/2(fragments of 454 and 400 bp), and the smaller 54 fragments were not detected in the gel)

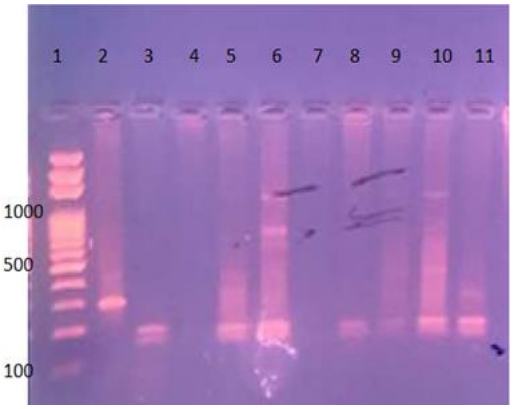

Figure 2: This figure shows the digestion of vitamin K in the promoter region by the Msp1 restriction enzyme. The first lane from the left contains a 100 bp ladder. Lane two contains the PCR product (undigested 290). The samples from lanes 3-11 contain the product that was digested by the enzyme numbers 4 and 7 (no PCR product)

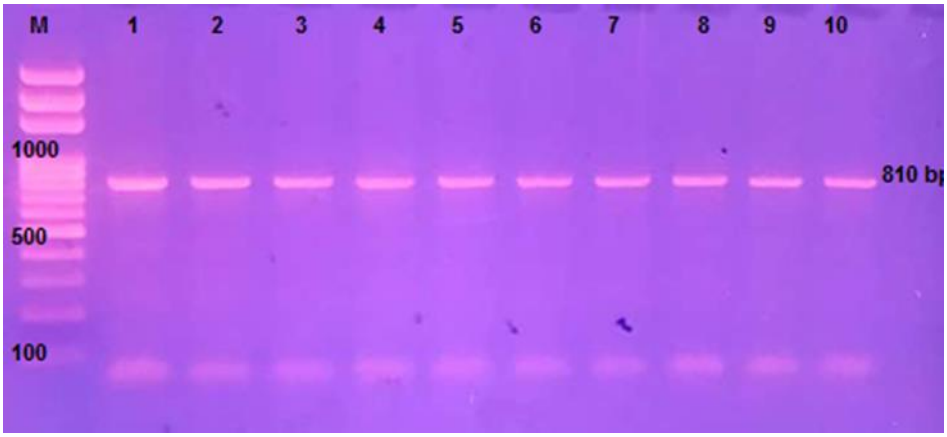

Figure 3A: The outer PCR products GGCX gene

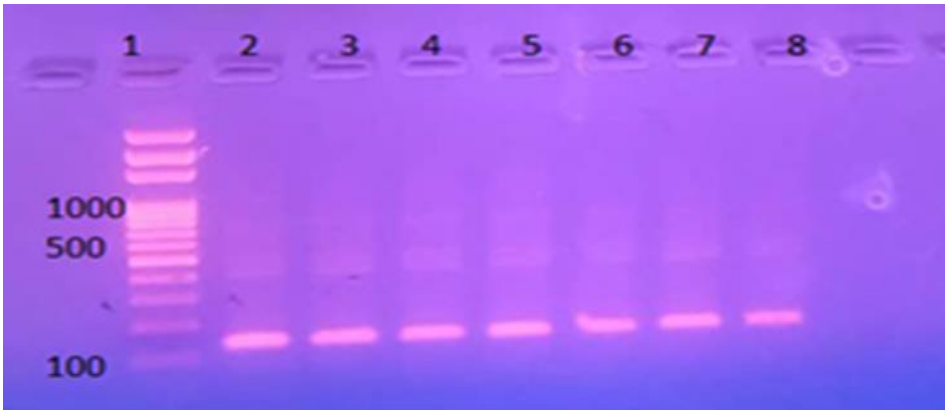

Figure 3B: The nested PCR products of the GGCX gene

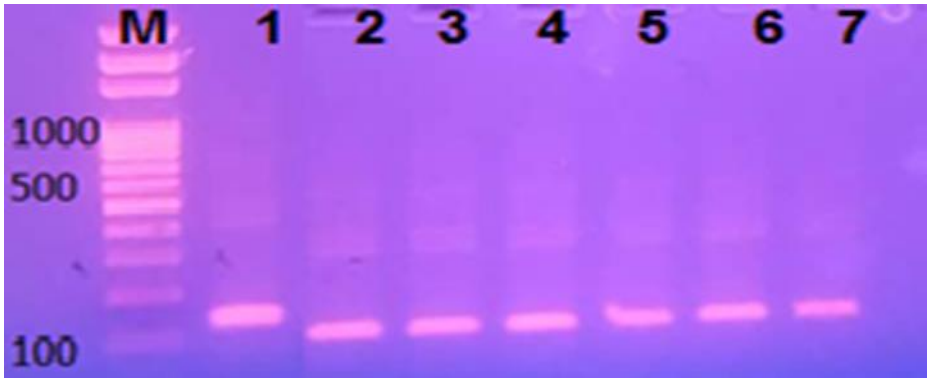

Figure 3C: Digested PCR products of nested GGCX with Alu1 enzyme

Digested products of GGCX with Alu1 enzyme, Lane M (molecular weight marker), lane 1 undigested PCR products of Nested PCR of GGCX gene, lane 2-7 showed fragments of 90 bp and smallest products of 39 was not seen in the gel.

Results

Clinical characteristics of the study population

The 107 study participants had an average age of 49.96+18.14 ranging between 17 to 86 years old. The gender distribution of the patients was (42.1%) males and (57.9%) females. The mean of the INR for the study subject was 2.55±0.98, ranging between 1.30 to 5.0. 34% of those who were of normal body weight. Indications for warfarin therapy varied, with deep vein thrombosis as the most common indication (47.7%). The clinical characteristics of the patients were listed in Table (3).

Genetic characteristics of anticoagulation of patients

The observed CYP2C9 genotype showed wild type 1*/1* (80.7%) more frequently than heterozygous. The frequency of the CYP2C9*2 allele was lowest (9.7%) than that of CYP2C9*1 (90.3%). For VKORC1, (48.2%) of subjects were with homozygous AA genotype and the frequency of allele A was 61.8% and for allele, G was 38.2%. GGCX genotype observed only wild type and homozygous mutant genotypes (89.7%& 10.3%, respectively). T allele (89.7%) was higher than the G allele in Table (4).

Distribution of patients with increased INR after the current dose

Out of these 107 cases, there were only 3 cases with INR of 3.83 during the current dose of 7mg. All these comparisons showed no significant statistical difference between warfarin doses (P-value >0.05) Table (5).

Warfarin dose according to Genotypes

The comparison for all patients of the mean daily warfarin doses with those of the estimated ideal doses was shown in Table (6), and these differences were not statistically significant (P-value>0.05). The mean daily warfarin dose with the wild type CYP2C9*1 and CYP2C9*2 heterozygous were not significantly different from those with daily mean calculated dose (P-value >0.05). The mean daily warfarin dose in patients with wild-type VKORC1 (AA genotype) and VKORC1 mutant GG genotype was highly significantly different from those of the mean daily calculated dose (P-value<0.001). In contrast, there was no statistically significant difference between the doses in patients with the VKORC1 heterozygous alleles. Patients with homozygous mutants for GGCX should increase their daily warfarin dose compared to the current daily dose, but this was not statistically significantly different (P-value>0.05). The mean daily warfarin dose with the wild-type GGCX was also not statistically significantly different (P-value>0.05).

Daily warfarin dose with bleeding events

According to bleeding events Table (7), the mean warfarin dose requirement was 5.0 mg and this was statistically significantly different (P-value<0.05).

Table 3: Characteristics of studied patients.

Characteristics		Patients
Age in years means		49.96±18.14
Gender N (%)	Male	45(42.1)
	Female	62(57.9)
Height cm mean±SD		165.02±9.60
Weight k/g mean±SD		68.38±19.66
INR mean±SD		2.55±0.98
Body Mass Index (kg/m^2) N (%)	Underweight	12(11.2)
	Normal	46(34.0)
	Overweight	29(27.1)
	Obese	20(18.7)
Bleeding N (%)	Yes	5(4.7)
	No	102(95.3)
Indication for anticoagulation N (%)	AF	10(9.3)
	AV	4(3.7)
	DVT	51(47.7)
	MVR	5(4.7)
	PE	10(9.3)
	RHD	17(15.9)
	Others*	10(9.3)

*SD= Standard deviation; N= number of study subjects; *others (DC, IHD, MI)*

Table 4: Distribution of CYP2C9, VKORC1 and GGCX genotypes in patients receiving warfarin

Genotype		N (%)
CYP2C9	1*/1*	71(80.7)
	1*/2*	17(19.3)
	Alleles 1*	159(90.3)
	Alleles 2*	17(9.7)
VKORC1	AA	41(48.2)
	GA	23(27.1)
	GG	21(24.7)
	Alleles A	105(61.8)
	Alleles G	65(38.2)
GGCX	TT	87(89.7)
	GG	10(10.3)
	Alleles T	174(89.7)
	Alleles G	20(10.3)

Table 5: Distribution of patients with increased INR after the current dose

Number of patients	Daily warfarin dose (mg)	INR mean±SD
17	2	2.89±1.05
16	3	2.43±0.879
5	4	2.80±1.26
54	5	2.48±0.903
9	6	2.16±0.833
3	7	3.83±2.02

P-value=0.095

Table 6: Association of genetic polymorphism with warfarin dose requirements in Sudanese

Genotype		Daily warfarin dose (mg) mean	Calculate dose(mg) mean±SD	P-value
CYP2C9	1*/1*	4.29±1.39	4.30±1.31	0.976
	1*/2*	4.06±1.48	3.84±1.51	0.808
VKORC1	AA	4.23±1.39	3.12±0.76	0.000*
	GA	4.65±1.15	4.54±0.98	0.728
	GG	4.05±1.47	5.73±1.22	0.000*
GGCX	TT	4.35±1.38	4.15±1.34	0.329
	GG	3.4±1.43	4.65±1.58	0.116
All patients		**4.29±1.36**	**4.23±1.32**	**0.742**

** P-value<0.001 highly significantly different*

Table 7: Daily warfarin dose to bleeding events

Bleeding	Daily warfarin dose	P-value
Yes	5.80±1.09	0.011
No	4.22±1.34	

Discussion

The study findings of the genotypes CYP2C9*2 and CYP2C9*1/*1 which represented 19.3% and 80.3% of participants were in agreement with the previous study [11]. The role of genotype CYP2C9*2 as a noticeable effect or of warfarin dose variations compared to genotype CYP2C9*1/*1 leads to recommendations of lower doses. The CYP2C9 polymorphisms, also, demonstrated variations according to different ethnic groups. This study showed evidence comparable to most recent studies of variations in frequencies of different alleles of Caucasians, Asians, and Africans, by 10-14%, 1-2%, and 0.5-1% respectively. A Romanian study, showed that the CYP2C9*2 allele was present in 11.3% of participants [12].

Moreover, the genotype VKORC1 AA and GA were present in participants in 48.2% and 27.1% respectively, heterozygous while 24.7% had a normal allele. Various studies demonstrated the significant role of VKORC1 -1639G/A polymorphism in warfarin dose requirements. Some other studies suggested that SNPs in VKORC1 were more likely to contribute to dose variations than those SNPs in CYP2C9 [13]. A Chinese study investigating the relationship between the VKORC1 haplotype and warfarin dose requirements concluded that low doses were more prevalent in Chinese than in Caucasians and the African population [14]. GGCX gene polymorphism, homozygous mutant, was shown to be present in, only, 10.3% of participants while the rest had normal alleles. These genes' characteristic effects on dose requirements variation led to the significant conclusion that only 4.7% of participants developed bleeding events with the mean dose recommended.

Conclusion

The significance of the study results is that special warfarin dose requirements for genotypes VKORC1 A/A and G/G should be over-emphasized to avoid unnecessary bleeding. This result would open the door for more patient-personalized dose requirements as part of the appreciation of gene expression in pharmacological treatment. Gene differences and mutations in different ethnic groups have significant implications on warfarin dose requirements. Understanding Pharmacogenetics involved in the anti-coagulation drug variability guides clinicians to prescribe patient-personalized doses to prevent anticipated bleeding cases. As the results of this study, we can conclude that it is useful to investigate the VKORC1 genotype presence in patients as part of the diagnostic effort to avoid unfavorable responses (elevated INR during initial dose) to come up with safe standard care, which is not known before this study. Future studies, need to investigate in a more detailed manner the effect of different gene mutations on present 'fit for all' pharmacological treatment. Future studies could include clinical and epidemiologic studies designed to investigate whether CYP2C9 and VKORC1 polymorphisms and other genotypes are more important genetic determinants of warfarin dosage among patients with bleeding disorders, such as hemophilia, compared with those without bleeding disorders

Abbreviations

CYP2C9: Cytochrome P450 2C9
VKOR1: Vitamin K epoxide reductase complex subunit1
GGCX Gamma-glutamyl Carboxylase
PCR: Polymerase Chain Reaction
RFLP: Restriction Fragment Length Polymorphism
BP: Base Par
SNP: Single nucleotide polymorphism
INR: international normalization Ratio
TBE: Tris-borate EDTA
AF: Artial fibrillation
AV: Artioventricular
DVT: Deep vein thrombosis
MVR: mitral valve replacement
RHD: Rheumatic heart disease; PE: Pulmonary Embolism

Declarations

Availability of data and materials

All data generated or produced from this study are included in this manuscript. Raw data from the study will be available upon request after publication

Competing interests

None declared

Funding statement

None

Authors' contributions

RBE and SGE conceived and carried out the practical part of the study, writing the manuscript draft AGE participated in the study design, statistical analysis, and interpretation. HAO revived the draft of the manuscript. All the authors coordinated and participated in the design of the study and the drafting of the manuscript and approved it.

Ethical approval and consent to participate in the Study

The study received ethical clearance from the ethical research committee at the Federal Ministry of health; Sudan at its meeting on 19th October 2017. All participants provided signed informed consent before enrolment.

Consent for publication

Not applicable

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