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Role of VKORC1 Gene Polymorphisms in Determining the Optimal Dose of Warfarin in a Group of Syrian Patients

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ABSTRACT:*The main goal of this study is to detect the frequency distribution ofsingle nucleotide polymorphisms (SNPs) of vitamin K epoxide reductase complex subunit 1(VKORC1) gene, and to determine its potential role in the control of warfarin dose inSyrian patients. The study included 125 patients with high risk of thrombosis of adults who visited the Heart Disease & Surgery Hospital (HDSH) and Aleppo University Hospital (AUH) and treated with warfarin as oral anticoagulant therapy, and the dose-corrected by the international normalized ratio (INR) at least three months ago. Genomic DNA was extracted from blood samples, and genotype analysis for VKORC1-1173C>T and VKORC1-1639G>Apolymorphismswas done by polymerase chain reaction-restriction fragment length polymorphism assay (PCR-RFLP). Data were analyzed using SPSS version 20. The results obtained in this study suggest that Genotype frequency distribution of VKORC1-1173C>T and VKORC1-1639G>A polymorphisms was found to be different from other populations and has significant effect on warfarin dose requirement(P<0.05). It is concluded that there is a need to include VKORC1polymorphisms detection tests in the warfarin dosing algorithm, as this has animportant role in reducing serious hemorrhagic or thrombotic complications, especially in patients with the VKORC1-1173C>T(TT) and VKORC1-1639G>A(AA)homozygous mutant genotypes.*

KEY WORDS: *VKORC1; Polymorphisms; Warfarin Dose; Syrian.*

1. Introduction

Treatment with warfarin remains a major challenge for the treating physician because it shows a wide variation in the therapeutic response and the adverse reactions in and between patients. A higher or lower dose than needed could lead to bleeding and thrombotic risk, respectively [1, 2].However, warfarin is the most widely used oral anticoagulant in the world and is prescribed to prevent the development and occurrence of thromboembolic events associated with certain pathologic conditions such as deep venous thrombosis (DVT), pulmonary embolism (PE), atrial fibrillation (AF), postoperative replacement of artificial heart valves and recurrent stroke [3].

Two-thirds of warfarin dose variation was due to environmental factors like age, body mass index, smoking status, gender, and diet, among others, while the remaining one-third is caused by genetic factors such as the CYP2C9 and VKORC1 genes [4-7]. Very recently, genetic variations within the gene encoding for a subunit of the vitamin K epoxide reductase complex, namely the VKORC1 gene, have been found to predict sensitivity to warfarin therapy. VKORC1 gene encodes the vitamin K epoxide reductase complex subunit 1 and it is a warfarin target. The vitamin K epoxide reductase complex subunit 1 normally catalyzes the carboxylation reaction of the vitamin K-dependent protein glutamic acid residues in order to activate it, the latter of which are responsible for catalyzing the clotting factor pathway [8].

The VKORC1 gene is located on chromosome 16p11.2 and it is locus spans about 5 Kbp, encompassing 3 exons and 2 introns, and encodes for a small protein of 163 residues [9]. The polymorphisms in the VKORC1 gene explain up to 25-50% of the variance in anticoagulant dose [10]. Out of these SNPs, 2 have shown significant effect on warfarin anticoagulant response. First one is a promoter polymorphism, VKORC1–1639G>A suggested to cause

changes in VKORC1 transcription-binding site leading to decreased VKORC1 mRNA expression in human liver cellsand decreased VKOR protein synthesis, and the other is VKORC1 1173C>T [11].

In Syria, warfarin is empirically administered to a wide range of patients with repeated dose adjustment until the target INR is reached (2-3). A 5 mg/day warfarin dose is initially administered followed with a number of INR tests and dose adjustments. Attempts to reach a warfarin stable dose (WSD) are accompanied with a high risk of bleeding adverse effects. This is the first study in Syria to report the population-based frequencies of VKORC1-1173C>T and VKORC1-1639G>A polymorphisms and their impact on warfarin dose requirement in Syrian patients treated with warfarin.The main goal of this study is to detect the frequency distribution ofsingle nucleotide polymorphisms of VKORC1 gene and to determine its potential role in the control of warfarin dose in Syrian patients, and to compare the data obtained with existing published data for other populations in the Middle East and in many regions of the world.

2. Materials and Methods

2.1 Patients:

The study included 125 patients who were receiving warfarin therapy at Heart Disease & Surgery Hospital (HDSH) and Aleppo University Hospital (AUH). A complete clinical history was recorded for each patient in a research form. Informed consent of all patients to participate in the research was also documented in the research form. Therapeutic indications of treatment with warfarin and the patient's age, gender, length, weight, associated drugs and diseases,daily and total weekly dose (TWD) of warfarinwhich were measured after at least three months of treatment, and the therapeutic INR value were recorded.Patients with liver, renal or gastrointestinal diseases, smokers, alcoholics, taking vitamin K-containing drugs or drugs that interfere with warfarin treatment were excluded [12]. The other baseline characteristics of study population are given in Table 1.

Table 1. Baseline Characteristics of Study Population

Characteristics	Rang	Mean	Number (N)	Percentage %
Patients			125	
	Male		80	64%
	Female		45	36%
Age (years)	23-87	56		
	Valve replacement		54	43.2%
	Atrial fibrillation		29	23.2%
	Deep venous thrombosis		17	13.6%
	Mitral stenosis		10	8%
Therapeutic	Coronary artery bypass grafting		8	6.4%
Indication of	Myocardial infarction		2	1.6%
Warfarin	Pulmonary embolism		2	1.6%
	Aortic dissection		2	1.6%
	Left ventricular aneurysm		1	0.8%
TWD (mg)	7-87.5	40		
INR	1.8-3.5	2.65		

Some patients were monitored by prothrombin time (PT) for 2 weeks until they reached the INR therapeutic range of (2-3), and the dose of warfarin was stabilized and followed for at least three months to confirm the dose. The dose of warfarin in some patients has been fixed at values lower or greater than the INR therapeutic range because they were considered stable by their physicians at this value.

2.2 DNA extraction and genotyping:

Whole blood samples were collected in EDTA tubes. Genomic DNA was extracted usingGF-1 Blood DNA Extraction Kit (Vivantis) by columns method [13].The extracted DNA was genotyped for VKORC1-1173C>T and VKORC1-1639G>A polymorphisms using polymerase chain reaction-restriction fragment length polymorphism assay (PCR-RFLP).

The sequences of forward (5`-CTAAGATGAAAAGCAGGGCCTAC-3`) and reverse (5`-CTGCCCGAGAAAGGTGATTTCC-3`) primers used for VKORC1-1173C>T polymorphisms and forward (5`-GAGCCAGCAGGAGAGGGAAATAT-3`) and reverse (5`-GTTTGGACTACAGGTGCCTGCC-3`) primers for VKORC1-1639G>A polymorphisms were obtained from reference study [14].

The PCR was carried out for each sample in a final volume of 50 µL containing the concentrations and volumes of the reagents as given in Table 2. The thermal stages with their temperatures and number of cycles are given in Table 3. The amplified DNA fragment containing the VKORC1-1173C>T polymorphismswas digested with *Sty I* restriction enzyme (Thermo), whereas for VKORC1-1639G>A polymorphisms restriction enzyme *Msp I* was used (Thermo).

Table 2. Polymerase chain reaction reagents and its concentrations and volumes used in present study

Serial NO.	Reagents with Their Concentrations	Quantity Used in µL for VKORC1- 1173 C> T	Quantity Used in µL for VKORC1- 1639 G> A
1	dH2O	37.7	28
2	10 X PCR Buffer	5	5
3	50 mmol/L MgCl2	2.5	12
4	10 mmol/L dNTPs	1	1
5	20 Pmol/L forward primer	0.75	0.75

Table 3. Thermal cycles protocol of polymerase chain reaction used in present study

Thermal Stages		**Temperature**	**Time**	**Number of Cycles**
Stage 1				
Initial denaturation	94°C		10 minutes	1
Stage 2 Denaturation	94°C			
Annealing	58°C	for VKORC1- 1173 C>T	1 minutes	35
	68°C	for VKORC1- 1639 G>A	1 minutes	
Extension	72°C			
			1 minutes	
Stage 3				
Final extension	72°C		10 minutes	1
Storage	4°C			

The digestion was carried out by adding 1 µL of respective enzyme (10 U/µL) and 2µL 10X digestion buffer to 10 µL of the PCR product and adjusting the final volume to 30 µL with dH_2O. The mixture was incubated at 37C for 16 hours. The restriction enzyme digested products were separated on 3% agarose gel and visualized with ethidium bromide under UV illumination. The size of the RFLP bands was depicted with 50 bp DNA ladder (Thermo). A representative gel picture for VKORC1 genotypes are given in Fig. 1.

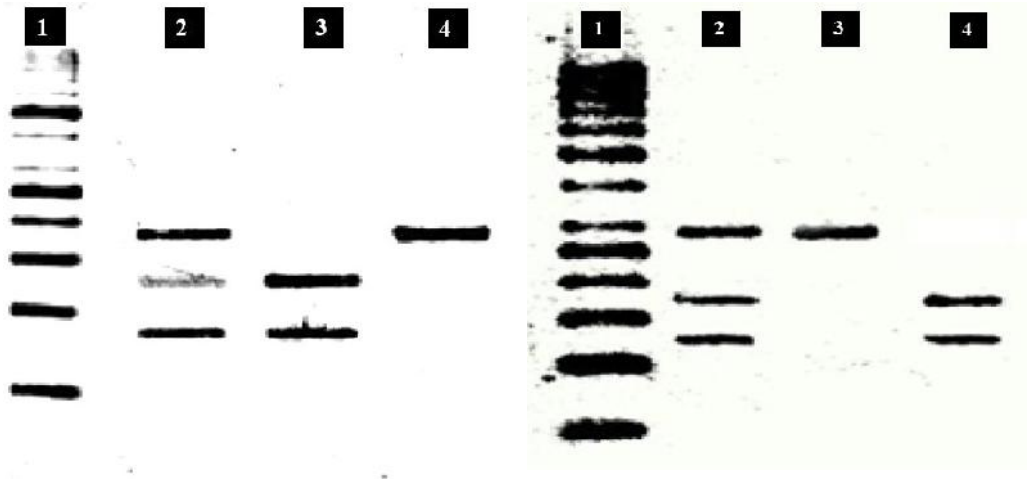

Fig. 1: Representative Agarose gel (3%) picture showing VKORC1 genotypes.On the left: Lane 1, 50 base pair DNA ladder; Lane 2, Heterozygous for VKORC1-1173C>T (CT); Lane 3, Wildtype genotype for VKORC1-1173C>T (CC); Lane 4, Homozygous mutant for VKORC1-1173C>T (TT).**On the right**: Lane 1, 50 base pair DNA ladder; Lane 2, Heterozygous for VKORC1-1639G>A (GA); Lane 3, Homozygous mutant for VKORC1-1639G>A (AA); Lane 4, Wildtype genotype for VKORC1-1639G>A (GG). The amplified PCR product was resolved at 201 base pair (bp) in case of VKORC1-1173C>T polymorphism. The PCR fragment containing C allele was digested into 2 fragments of 127 bp and 74 bp, whereas T allele was not digested and resolved at 201 bp. The amplified PCR product was resolved at 291 bp for VKORC1-1639G>A. The PCR fragment containing G allele was digested into 2 fragments of 167 bp and 124 bp, whereas A allele was not digested and resolved at 291 bp.

2.3 Statistical analysis:

Statistical analysis of data was performed using SPSS version 20. The observed allele frequency was recorded, and the confidence interval for each allele was calculated at 95% confidence level.The expected frequency of alleles and genotypes for their accordance with Hardy Weinberg equilibrium was calculated by χ^2 test. ANOVA analysis was performed in order to assess the differences in the mean total weekly dose of warfarin among groups of patients with different genotypes of VKORC1 gene, and *P* value less than 0.05 was considered statistically significant. ANOVAanalysis was followed by post hoc Tukey test for pairwise comparison between VKORC1 different genotypes if ANOVA gave a *P* valueless than 0.05.

3. Results

A total of 125 patients (80 males and 45 females) who were on warfarin therapy were included into the study. The other baseline demographics are given in Table 1.The genotypic frequencies for the two VKORC1 polymorphismsare given inTable 4. Both SNPs werefound in accordance with Hardy Weinberg equilibrium (*P* value > 0.05). The effect of VKORC1-1173C>T and VKORC1-1639G>Apolymorphisms on warfarin dose was determined, and the comparison has been summarized in Table 5. There was statistically significant effect of different VKORC1-1173C>T and VKORC1-1639G>A genotypes on warfarin dose requirement (*P* value <0.05).

Table 4. The genotypic frequencies of VKORC1 gene polymorphisms.

Genotype	Number of participants (%)	Observed frequency %	95 % Confidence interval	Expected H-W frequency, %	*P* Value
VKORC1-1173C>T	125 (100)	-	-	-	
CC	14 (11.2)	11.2	5.67-16.72	15.4	**P*=0.080
CT	70 (56)	56	47.30-64.7	47.7	
TT	41 (32.8)	32.8	24.57-41.03	37	
VKORC1-1639G>A	125 (100)	-	-	-	
GG	10 (8)	8	3.23-12.75	5	**P*=0.086

GA	36 (28.8)	28.8	20.87-36.73	34.8
AA	79 (63.2)	63.2	54.75-71.65	60.2

Abbreviation: H-W, Hardy-Weinberg. *Consistent with HWE.

Table 5. Mean weekly dose of warfarin and its relationship with VKORC1 genotypes

Genotype	Number of participants (%)	Warfarin Dose (mg/w), Mean ±SD	*P* Value
VKORC1-1173C>T	125 (100)	-	
CC	14 (11.2)	57.16±24.91	*P=0.003
CT	70 (56)	42.78±17.01	
TT	41 (32.8)	31.35±15.93	
VKORC1-1639G>A	125 (100)	-	
GG	10 (8)	50.25±22.28	*P=0.03
GA	36 (28.8)	49.00±19.17	
AA	79 (63.2)	36.97±16.67	

Abbreviation: SD, standard deviation. *Significant.

On the basis of pairwise comparison of different VKORC1-1173C>T and VKORC1-1639 G>A genotypes, The results of the present study showed that patients who have homozygous mutantVKORC1-1173C>T (TT) and VKORC1-1639G>A (AA) genotypes required lesser warfarin dose when compared to homozygous wildtypeVKORC1-1173C>T (CC) and VKORC1-1639G>A (GG) genotypes, as the difference in dose requirement was statistically significant (*P* value <0.05), Fig. 2.

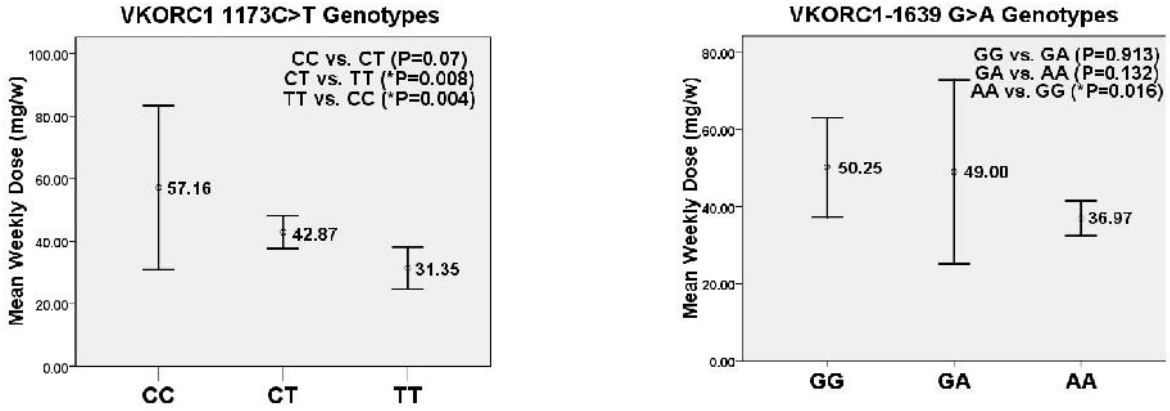

Fig.2.Pairwise Comparison of Warfarin Dose among VKORC1-1173C>T and VKORC1-1639G>A Genotypes. *Significant.

Discussion

Since VKORC1 breakthrough in 2004, several single-nucleotide polymorphisms (SNPs) have been identified in VKORC1 but very few of them have been found to be affecting the warfarin dose requirement [10]. This is the first study in Syria to report the population-based frequencies of VKORC1-1173C>T and VKORC1 1639G>Apolymorphisms and their impact on warfarin dose requirement in Syrian patients treated with warfarin. Several studieshave found an association between the presence of VKORC1 gene polymorphisms and a reduced dose of warfarin. Most of these studies demonstrated that VKORC1 polymorphisms accounted for most of the variation in warfarin dose requirements compared to its CYP2C9 counterpart [15-17].

The results of the current study showed that VKORC1-1639G>A (AA)homozygous mutant genotype was the dominant genotype in the Syrian population with 63.2%, followed by VKORC1-1173 C>T (CT) heterozygous genotype with 56%, while was the wildtype genotype of VKORC1-1639G>A (GG) and VKORC1-1639C>T (CC) the least common in the Syrian population with8% and 11.2% respectively as shown in Table 5.This indicates that most patients in Syrian population have a high risk of serious adverse reactions related to warfarin hypersensitivity. Therefore, these patients need lower doses of the drug or otherwise, they are vulnerable to serious life-threatening hemorrhages.

The allele's frequency distribution in Syrian population was different from other reported studies carried out in different populations as shown in Table 6. Compared with Arab countries, the allele frequency of VKORC1-1639A allele in our study was the higher with 77.6%. However, these results are consistent with the results obtained in the Egyptian study conducted by Ekladious and his colleagues [18]. The results of the current study showed that the allele frequency of theVKORC1-1639T allele was also high in the Syrian population with 60.8%. However, there was a higher frequency of this allele in an Egyptian study carried out by El Dinand his colleagues [19]. Apart from this study, published data on the frequency and distribution of VKORC1-1173T allele in Arab countries were very few as shown in Table 6.

Table 6. Minor allele frequencies (MAF) of VKORC1 gene in different ethnic groups

Populations	**Minor Allele Frequencies of VKORC1 (%)**		References
	-1173T	**-1639A**	
Syrian	60.8	77.6	Present Study
Lebanese	NA	52	[20]
Palestinian	NA	46.5	[21]
Egyptian	77	30-72.05	[18,19,22-24]
Sudanese	NA	37	[25]
Saudi	NA	42.7	[26]
Omani	NA	35	[27]
Kuwaiti	NA	40	[28]
Turkish	NA	40-51	[29-32]
Iranian	41.35	56	[33,34]
Pakistani	50.1	48.2	[35]
Indian	10-17	9.3	[36,37]
Malaysian	82.15	85.2	[38]
Chinese	81.5	89	[39,40]
Japanese	91.9	90	[41,42]
Italian	34.85	49.8	[43]
American	35.1	41	[44,45]

NA: Not assayed

The average worldwide frequency of the VKORC1-1639A variant allele is ranging from (30-77.6%) in Middle East Arab populations, (40-51%) in Turkish population and (49.8%) in Italian population, (56-90%) in Asian populations and (41%) in American population. While the average worldwide frequency of the VKORC1-1173T variant allele is ranging from (10-91.9%) in Asian populations and (34.85%) in Italian populations, and (35.1%) in American population, and this also reflects an obvious difference in the allele frequency of VKORC1-1639A and VKORC1-1173T variant alleles in different ethnic and geographic groups. Thus, the term "Arab population" does not reflect a homogenous genetically population, so each region should be treated as an individual entity.

The results of the current study showed that, the mean weekly dose of warfarin in patients with homozygous mutant genotype VKORC1-1173C>T (TT)was statistically significant less if compared with the mean weekly dose needed by patients with homozygous wild type genotype VKORC1-1173C>T (CC), (31.35 mg, 57.16 mg respectively, P=0.004), and heterozygous genotype VKORC1-1173C>T (CT), (31.35 mg, 42.87 mg respectively, P=0.008). But the weekly dose requirement of heterozygous genotype VKORC1-1173C>T (CT) was not significantly different from homozygous wildtype genotypeVKORC1-1173C>T (CC) weekly warfarin dose (42.87 mg, 57.16 mg respectively, P=0.07).

In this study, same significant effect on warfarin dose requirement was seen in patients with homozygous mutant genotype VKORC1-1639G>A (AA). The mean weekly dose of warfarin in patients with homozygous mutant genotype VKORC1-1639G>A (AA) was statistically significant less if compared with the mean weekly dose needed by patients with homozygous wildtype genotype VKORC1-1639G>A (GG), (36.97 mg, 50.25 mg respectively, P=0.016), but the weekly dose requirement of heterozygous genotype VKORC1-1639G>A (GA) was not significantly different from either homozygous wildtype genotype VKORC1-1639G>A (GG) (49 mg Vs. 50.25, P=0.913),or homozygous mutant genotype VKORC1-1639G>A (AA) (49 mg Vs. 36.97 mg, P=0.132).

Several studies have shown such statistically significant relationship between VKORC1-1173T and VKORC1-1639A variant alleles and warfarin dose requirement [15-17, 23, 43]. However, few other studies have not recorded such relationship [11, 33, 46, 47]. This difference in results can be explained by presence of other genetic and non-genetic factors that play a greater role than these variants alleles in determining the optimal dose of warfarin in some populations. These factors are still under investigation in many studies [36, 48-50]. According to our results, VKORC1-1173T and VKORC1-1639A variant alleles play an important role in determining the optimal dose of warfarin in Syrian patients, Therefore, Patients with these variant alleles should be given a lower dose of warfarin. Further studies to verify this relationship, and to determine the role played by other genetic and non-genetic factors in determining the warfarin dose requirements are required.

Conclusion

The frequency distribution of VKORC1-1173T and VKORC1-1639A variant alleles in Syrian population was significantly different from that recorded in theother Arab countries and in theother populations around the world, indicating that, each population group should conduct an Independent study to determine the optimal dose of warfarin that corresponds to the genetic variations of the VKORC1 gene in this population group. The determination of the optimal dose of warfarin in our study was associated with the presence of VKORC1-1173T and VKORC1-1639A variant alleles, and this correlation was statistically significant (P<0.05). This suggests that the detection of these variants alleles should be included in the context of the treatment plan for the newly-developed patients on oral warfarin anticoagulant therapy, which minimizes hemorrhagic complications of treatment.

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