The Genetic Association of VKORC1 (-1639G<A) Polymorphism with Warfarin Dose Requirements among Egyptian Patients with Venous Thromboembolism

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ABSTRACT

The aim of the study was *to estimate the frequency of VKORC1 (-1639G>A) genotypes and to figure out the effect of "vitamin K epoxide reductase complex subunit 1" (VKORC1) gene polymorphism (-1639G>A) on "warfarin dose requirement" in a sample of Egyptian patients with "venous thromboembolism".* ***Subjects & Methods:*** *A* ***case-control study*** *was carried out in "the Medical Research Institute hospital, Alexandria University, Egypt". Cases were patients with "venous thromboembolism"(VTE) admitted to the "Experimental and Clinical Surgery Department".* ***Controls were*** *healthy volunteers without history of VTE. Coming to the hospital as patients' relatives, within the same age range as cases. Both groups were included in the molecular study using PCR/RFLP technique for detection of (VKORC1) gene polymorphism (-1639G>A).* ***Results:*** *The study comprised 37 cases and 47 controls. Twelve patients (32.4%) had GG genotype, 19 patients (51.4%) had AG genotype and 6patients (16.2%) had AA genotype. There was a significant variation in "warfarin maintenance dose "in patients with different genotypes of (VKORC1) at position -1639 suggesting that,(VKORC1) polymorphism has an essential role on the "warfarin maintenance dose" requirement. In addition, a statistical significant difference between VKORC1 (-1639G>A) genotypes and daily "warfarin maintenance dose"was found among cases. No significant difference was detected in Genotype and allele frequencies of VKORC1 (-1639G>A) polymorphism between both cases and controls.* ***Conclusions:*** *In the Egyptian patients with VTE, both VKORC1-1639G and VKORC1-1639A alleles were found with allelic frequency (58 % and 42 %) respectively. There was a significant variation in" warfarin maintenance dose" among patients with different genotypes of (VKORC1) at position -1639, and that (VKORC1) polymorphism has an important influence on the" warfarin dose requirements".*

Key words: *"Venous Thromboembolism", "warfarin maintenance dose", VKORC1 (-1639G>A) genotypes, polymorphism.*

INTRODUCTION

"Venous Thromboembolism" (VTE) is the third most frequent cardiovascular disorder after stroke and myocardial infarction [(1)].Warfarin is the most widely used anticoagulants for prevention of "venous thromboembolism". Warfarin has a very narrow therapeutic index [(2)]. In addition, the dose required for anticoagulant effect of warfarin exhibits large inter-ethnic and inter-individual differences [(3)].

The dose variability of warfarin contributed to non genetic factors including:gender, age, weight, height, diet, smoking and medications, but genetic factors have been contributed as the largest to warfarin dose variability[(4)] .The most two genetic factors which are correlated with"warfarin dose requirements" are variations in the genes encoding the enzymes:cytochrome P450 2C9 (CYP2C9) and vitamin K epoxide reductase (VKOR) [(5)].

Wallin & Hutson[(6)] stated that (Warfarin produces its pharmacological effect by interfering with the synthesis of vitamin K dependent clotting factors via inhibition of "vitamin K epoxide reductase complexsubunit 1"(VKORC1). This

interferes with the post-translational gamma-carboxylation of glutamicacid residues on coagulation factors II, VII, IX, and X, plus the anticoagulant proteins C,S, and Z by gamma-glutamyl carboxylase (GGCX). Diminution of reduced vitamin K leads to manufacture of nonfunctional coagulation factors, causing anticoagulation).

"Vitamin K epoxide reductase complex subunit 1" gene (VKORC1) encoded VKORC1 protein. "Warfarin dose requirements" are influenced by genotype for several non-coding polymorphisms in this gene.The increased sensitivity to warfarin is associated with a common non-coding variant, -1639G>A.Thus, patients with the AA genotype require lower initial doses of Warfarin than those with the AG or GG genotype.The −1639G>A allele frequency varies among different races and explains the differences in standard dose requirements among Asians , blacks and whites[7].

Aim of the Study

Was to estimate the frequency of VKORC1 (-1639G>A) genotypes and to figure out the effect of "vitamin K epoxide reductase complex subunit 1" (VKORC1) gene polymorphism (-1639G>A) on "warfarin dose requirement" in a sample of Egyptian patients with" venous thromboembolism".

SUBJECTS AND METHODS

Study Design

A **case -control** study.

Setting

"Medical Research Institute hospital, Alexandria University, Egypt".

Subjects

1. Cases:

This group included patients with" venous thromboembolism" (VTE) comprising deep vein thrombosis (DVT) and pulmonary embolism (PE). Patients were recruited from the "Experimental and Clinical Surgery Department ", within the period from November 2014 to July 2016.

Inclusion criteria:

Age range from 15 to 70 years old and a stable "warfarin dose requirement" for three consecutive times with dose titration to an international normalized ratio (INR) target range of 2–3.

Exclusion criteria:

All patients with hepatic dysfunction, cancer, advanced heart failure, renal disease, hypothyroidism, and diseases with bleeding tendency.

2. Control:

Controls were healthy volunteers without history of VTE. Coming to the hospital as patients' relatives within the same age range as cases.

Methods:

This study protocol was approved by the "Ethical Committee of the Medical Research Institute, Alexandria University, Egypt". All patients and controls provided signed informed consent prior to their enrollment to the study.

Patients and controls were subjected to the following:

I- History taking and clinical examination:

Information about name, gender, age, weight, smoking status, and co-medication was recorded.

INR measurement and daily warfarin dose of every patient were also recorded.

II- Molecular genetics study for detection of (VKORC1) Gene Polymorphism (1639G>A): (PCR/RFLP) according to the methodology of Aomori et al 2009 [8].

Analysis of the results:

Table 1: PCR/RFLP for detection of VKORC1 (-1639G>A) Gene Polymorphism using NciI restriction enzyme[8]**:**

Gene	*Polymorphic allele*	*PCR product (bp)*	*VKORC1-1639 G/G digest (bp)*	*VKORC1-1639A/A digest (bp)*	*VKORC1-1639 A/G digest (bp)*
VKORC1	-1639G>A	636	472, 50 and 114	522 and 114	522, 472, 114 and 50

III. Statistical analysis: [9, 10]

Statistical analysis was performed using SPSS version 20.0 and Med Calc. v 12.4.0. Qualitative variables (gender, smoking and gene polymorphism) were described by their frequency and percent. Quantitative variables (age, warfarin dose) were described by the arithmetic mean and standard deviation after a normality test which revealed normality of their distributions. (Shapiro- Wilk test $p > 0.05$).

Comparison of warfarin dose among different gene polymorphisms was performed through one way ANOVA and Pairwise comparisons were followed using Gabriel test due to the equality in groups' variances (Levene's test $p > 0.05$) and difference in the groups' sample sizes.

Comparison of warfarin dose among; males and females, smokers and non-smokers was done using the independent t test. The association between the age and warfarin dose was tested through the Pearson correlation coefficient (r).

The simple linear regression model was used to quantify the percent variation in the warfarin dose (as dependent variable) which is accounted for by the variation in the VKORC1 genotype (as independent variable) and was presented as R^2.

Testing differences in gene polymorphism distributions between cases and controls was done using the Pearson's chi-squared test. The population of the studied sample was explored to find its equilibrium with Hardy-Weinberg equation. Differences between observed and expected frequencies were tested through the Pearson's chi-squared test.

RESULTS

The study included 37 patients with "venous thromboembolism" (VTE) and 47 healthy volunteers without history of VTE as a control group.

I. Patient's characteristics and VKORC1 genotyping:

The age of the studied patients ranged from 15 to 70 years old (mean 46.6±14.19 years). Seventeen (45.9%) were males and twenty (54.1%) were females.Twenty eight (75.7%) were non-smokers and nine (24.3%) were smokers .The mean "warfarin maintenance dose" was 5.4 ± 1.47 mg/day, and ranged from 2.5 to 9.5 mg/day .Twelve patients (32.4%) had GG genotype, 19 patients (51.4%) had AG genotype and 6 patients (16.2%) had AA genotype , figures(1,2).

PCR-RFLP Gel Electrophoreses Results:

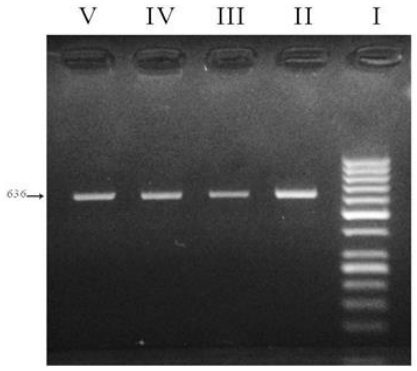

Fig. (1): PCR amplification of VKORC1 gene (-1639G>A polymorphism), resolved on 2% agarose gel.
Lane I: Molecular weight marker (50 bp DNA ladder).
Lane II-V: 636 bp PCR products.

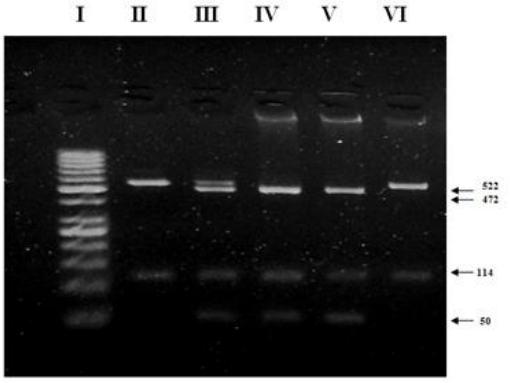

Fig. (2): Restriction digestion of PCR products of VKORC1 gene (-1639G>A polymorphism) using NciI enzyme resolved on 3% agarose gel
Lane I: Molecular weight marker (50 bp DNA ladder).
Lane IV& V: show bands at 472, 114 and 50 bp representing GG genotype
Lane III: show bands at 522, 472, 114 and 50 bp representing AG genotype
Lane II &VI: show bands at 522, and 114 bp representing AA genotype

II. Association of genetic and non-genetic factors with warfarin dose requirements:

There was no linear relation between age and daily "warfarin maintenance dose"(r= 0.03, p=0.884). The mean warfarin dose in male patients was 5.3 ±1.61 mg/day, while in female patients it was 5.5 ± 1.36 mg/day. This difference was not statistically significant (t= 0.53, p= 0.598). The mean warfarin dose in smokers was 5.6 ± 0.99 mg/day, while it was 5.3 ± 1.59 mg/day in non-smokers.This difference also was not statistically significant (t=0.48, p= 0.635) table 2.

Table 2: Gender and smoking status versus Mean daily "warfarin maintenance dose"

Factors	*Warfarin dose (mg/day) Mean ± SD*	*p value*
Gender		0.598
Male	5.3 ±1.61	
Female	5.5± 1.36	
Smoking status		0.635
Smokers	5.6 ± 0.99	
Non-smokers	5.3 ± 1.59	

III. Effect of VKORC1(-1639G>A) genotype on "warfarin dose requirements":

The mean warfarin dose among GG patients was 6.2±1.59 mg/day, in AG patients it was 5.3±1.19 mg/day and it was 4.3±1.33 mg/day among AA patients, figure 3. Warfarin doses were significantly different per VKORC1 genotype (F= 4.16, p= 0.024). (Table 3).

Multiple comparisons revealed significant difference in "warfarin dose requirements" between GG and AA patients (Mean difference = 1.9 mg, p= 0.02).

Table 3: Association of genetic polymorphism with" warfarin dose requirements"

Gene	*Genotype (n)*	*Warfarin dose (mg/day)Mean ± SD*
VKORC1 (-1639G>A)	GG (12)	6.2±1.59
	AG (19)	5.3±1.19
	AA (6)	4.3±1.33

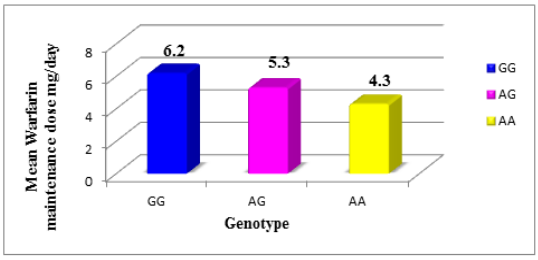

Fig. 3: Mean daily "Warfarin maintenance dose" of the studied patients according to genotype

The simple linear regression R^2 demonstrated that19.6% of change in warfarin daily dose was attributed to the variation in VKORC1 (1639G>A) genotypes (R^2=19.6%, p value=0.006), table 4.

Table 4: Regression analysis between mean daily warfarin dose as a dependent variable and VKORC1 (1639G>A) genotype as an independent variable

Factor	*Coefficient*	*P value*
constant	7.1397	0.000
VKORC1 (-1639G>A)genotype	-0.9437	0.006

R^2=19.6%, R^2 (adj) = 17.3%

IV. Genotype and allele frequencies of VKORC1 (-1639G>A) among patients and controls:

As shown in table 5and figure 4, GG genotype was found in 32.4 % of patients, while in 21.3% of controls. AG genotype was found in 51.4 % of patients and in 51.1%of controls. AA genotype was found in 16.2 % of patients, while in 27.7 %of controls.

No significant difference was detected in the gene distribution between patients and controls (χ2= 2.18, p = 0.336).

Though the frequency of G allele was higher among cases than controls (58 % of cases compared to 47 % of controls) and the frequency of A allele was higher among controls than cases (53% of controls compared to 42 % of cases) but these differences were not statistically significant (χ2= 1.69, p= 0.194), table 5and figure 5.

Table 5: Genotype and allele frequencies of VKORC1 (-1639G>A) among patients and controls

VKORC1 (-1639G>A) gene	*Patients*		*Controls*		*χ2*	*p value*
	Number (n=37)	**Percentage**	**Number (n=47)**	**Percentage**		
Genotype						
GG	12	32.4 %	10	21.3 %		
AG	19	51.4 %	24	51.1 %	2.18	0.336
AA	6	16.2 %	13	27.7 %		
Allele frequencies						
G	43	58 %	44	47 %	1.69	0.194
A	31	42 %	50	53 %		

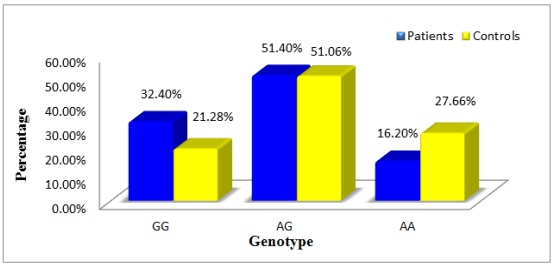

Fig. 4: Genotype frequency of VKORC1 (-1639G>A) polymorphism among patients and controls

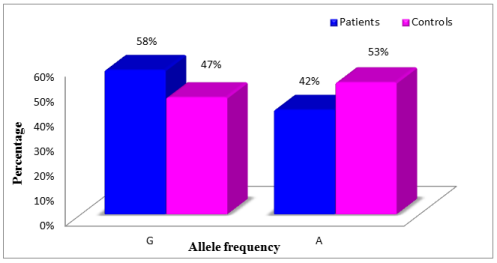

Fig. 5: Allele frequency of VKORC1 (-1639G>A) polymorphism among patients and controls

As no significant differences were detected between observed and expected frequencies in both cases (X^2 = 0.11, p = 0.946) and controls (X^2 = 0.03, p = 0.984). Thus, the observed genotype frequencies were in Hardy-Weinberg equilibrium. table 6.

Table 6: The observed and expected values of the genotype frequencies among patient and control groups

Genotype	*Observed frequency*	*Expected frequency*	c^2	*p value*
Patient's group (n=37)				
GG	12	12.44	0.11	0.946
AG	19	18.02		
AA	6	6.52		
Control group (n=47)				
GG	10	10.38	0.03	0.984
AG	24	23.42		
AA	13	13.2		

DISCUSSION

"venous thromboembolism" is a disorder that can occur in all races and ethnicities, all age groups, and both genders [2,11]. Large inter-individual variabilityand narrow therapeutic range are challengingfor warfarin dose accuracy during the initiation phase .[3]. The treatment efficacy and safe use of warfarin is measured byprothrombintime as the international normalized ratio (INR)[3].Low dose will lead to thrombosis, while high dose increases bleeding [9].

The present study was designed to estimate the frequency of VKORC1 (-1639G>A) genotypes in an Egyptian sample and to figure out the effect of "vitamin K epoxide reductase complex subunit 1" (VKORC1) gene

polymorphism (-1639G>A) on "warfarin dose requirement"in patients with "venous thromboembolism". The effects of patient specific factors such as age, gender, and smoking on daily warfarin dosage were also assessed. Our decision to genotype for VKORC1–1639G>A polymorphism was based on data published by Yuan et al. [(12)] which showed that, this promoter polymorphism is believed to be the functional SNP in (VKORC1). Also, Rieder et al. [(13)] showed that, haplotypes of (VKORC1) were not more informative than any of the five segregating (VKORC1) SNPs (381, -1639, 6484, 6853, and 7566).

In the present study,the daily"warfarin maintenance dose" varied among patients with doses ranging from 2.5 mg to 9.5 mg / day and the mean" warfarin maintenance dose" was 5.4 ± 1.47 mg/day. Genotypes for (VKORC1) gene polymorphism (-1639G>A) correlate significantly with daily warfarin doses (p=0.006). The percentage of variability in" warfarin maintenance dose" accounted for by VKORC1 was 19.6 %.Consistent with previously published data [(14,15)], this study shows that, VKORC1-1639G>A polymorphism plays an important role in inter-individual warfarin dose variability.

Also, the carriers of the homozygous wild-type (GG) genotype for VKORC1, were given higher daily warfarin dosages (6.2 ± 1.59 mg/day), than both carriers of the heterozygous (AG) genotype (5.3 ± 1.19 mg/day) and carriers of the homozygous mutant (AA) genotype (4.3 ± 1.33 mg/day). There was a statistically significant difference in daily "warfarin maintenance dose" between GG and AA patients (Mean difference = 1.9 mg/day, F= 4.16, p= 0.024).

Several studies have reported similar findings. In Oner Ozgon et al. [(14)]study which was conducted on Turkish patients, it was found that patients with the AA genotype, on average, required 25.83 mg/week, which was 17.35 mg less warfarin per week than patients with the GG genotype (43.18 mg/week) (p < 0.0001). Another Study on Turkish patients Ozer et al. [(15)] also found that the mean daily dose in patients with VKORC1 -1639 GG genotype (5.95 ± 2.12 mg) was significantly higher than that of patients with VKORC1 −1639 AA genotype (2.49 ± 1.11 mg) (p < 0.01).

In Miao et al. [(16)] study on Chinese patients, it was found that patients with the GG and AG genotype received significantly higher doses of warfarin (3.32±1.02 mg/day; p<0.001) than those with the AA genotype (1.76±0.57 mg/day).

Moreover, an Iranian study conducted by Namazi et al [(17)] , found that the mean weekly" warfarin dose requirement "was (39.2 ± 16.2 mg) in patients with the VKORC1 (–1639) GG genotype. This was significantly higher than the weekly dose among those patients with the AA genotype (26.5 ± 13.6 mg; p = 0.007).

Al-Jaibeji[(18)] study on Emirati patients, reported that for homozygous AA genotype patients a smaller dose was required (2.6 mg/day, SD 1.022) in comparison to the heterozygous AG genotype (4.75 mg/day, SD1.7). A higher dose was needed for the homozygous GG genotype (7 mg/day, SD 2.7) with a p-value < 0.0001. In Zahran[(19)] study on Jordanian patients, among the 147 patients warfarin doses differed significantly by VKORC1 - 1639 genotype (p <0.0001). Patients with the AA genotype, on average, required 26.9 mg/week, which was 23.1 mg less warfarin dose per week than patients with the GG genotype. (50 mg/week).

The present study also assessed the influence of patient age, gender, and smoking on daily" warfarin maintenance dose". Patient age was not statistically significant with daily"warfarin maintenance dose" (r =-0.93, p = 0.4). Seventeen patients were males (45.9 %) while twenty (54.1 %) were females. Also, patient gender was not statistically significant with daily" warfarin maintenance dose" (p= 0.62). Twenty-eight (75.7 %) patients were non-smokers while nine (24.3 %) were smokers. Patient smoking status was not statistically significant with daily "warfarin maintenance dose" (p= 0.62).

In agreement with our study, Ahorhorlu[(20)]found that warfarin dose was negatively correlated with patient age and statistically not significant (r = -0.024, p = 0.09), also women were found to be taking a higher mean daily warfarin dose of 5.75 mg (95% CI (5.174-6.326) compared to men who were administered 5.460 mg (95% CI (4.907-6.022), p = 0.479), although this was not statistically significant.

Similar findings have been published by Whitley et al.[(21)] who found that a very weak correlation existed between gender and "warfarin dose requirement", although not statistically significant. Also, he did not find a significant

relationship between smoking status and warfarin dose.

On the contrary, Sconce et al[3]showed that median daily "warfarin dose requirements "varied significantly with sex (2.9 mg females; 3.7 mg, males; P =.009). While warfarin doses were negatively correlated with age (r=0.40; P =0.001).Similarly, Aquilante et al [22] and Oner Ozgon et al [14] stated that the variables associated with lower" warfarin dose requirements" included increasing age (p< 0.0001).

In the present study, GG genotype was found in 32.4 % of patients, while in 21.28 %of controls. AG genotype was found in 51.4 % of patients and in51.06 % in controls. AA genotype was found in 16.2 % of patients, while in 27.66 % in controls. There were no statistical significant differences between the patients and control groups in the distribution of VKORC1 -1639G>A genotype as determined by a chi-square test (p-value = 0.336). This indicates that these genotypes are not associated with the susceptibility to "venous thromboembolism".

Though the frequency of G allele was higher among cases than controls (58 % of cases compared to 47 % of controls) and the frequency of A allele was higher among controls than cases (53% of controls compared to 42 % of cases) but these differences were not statistically significant ($\chi 2$= 1.69, p= 0.194).

This is in accordance with Yoshizawa et al [23] who reported that, the allele frequencies of VKORC1 –1639 among patient group and control subjects were not significantly different (p=0.3977).Allele frequencies of VKORC1 –1639 in patients (n=259) were 0.100 for G allele and 0.900 for A allele, and those for control subjects (n=341) were 0.082 and 0.918, respectively. Thus, VKORC1 –1639 genotype was not associated with altered risk of thromboembolic disease.

Similarly, Al-Jaibeji[18] found that there were no significance differences in the prevalence of GG,AG and AA genotypes for rs9923231 variants between the patients and control groups as determined by a chi-square test (p-value = 0.9296).

In the present study, in the patients group it was found that, the frequency of the GG genotype of the VKORC1 –1639 G>A variant is 32.4 %, the AG genotype is 51.4 %, and the AA genotype is 16.2 %. While the frequency of A allele in the VKORC1–1639G>A was 0.42 and the G allele frequency was 0.58.

The value of the minor allele frequency in the present study is in accordance with other published values in different populations. For Lebanese population [24] the A allele frequency was 0.528, the frequency of the GG genotype was 26.1 %, the AG genotype was 39.7 %, and the AA genotype was 34.2 %. For Turkish population [14] the A allele frequency was 0.5, the frequency of the GG genotype was 28.8 %, the AG genotype was 42.4 %, and the AA genotype was 28.8 %. For Argentine population [25] the A allele frequency was 0.5.The frequency of 20% was found for AA homozygotes, 60% for AG heterozygotes and 19% for GG homozygotes. For British population [3] the A allele frequency was 0.43, the frequency of the GG genotype was 25 %, the AG genotype was 56 %, and the AA genotype was 19 %.

However, allelic frequencies of -1639A in our study was higher compared to African-Americans [26] (0.12), while it was lower compared to Chinese [12] (0.89), Japanese[23] (0.9) and Malays[27] (0.74).

In the study conducted by Wu et al. [26] , the A allele frequency was lowest in the African-Americans (0.12), intermediate in the Caucasians and Hispanic-Americans (0.41 and 0.48) respectively, and highest among Asians (0.88).

Ethnicity appears to be an important factor in the determination of adequate "warfarin maintenance dose"[28]. "Warfarin dose requirements "vary significantly by race, with higher mean maintenance doses in African-Americans and lower mean doses in Asians compared with doses in Caucasians. This ethnic variability may be attributed to genetic differences [29].

In Limdi et al.[30] study among whites, Asians and blacks, VKORC1 –1639G>A polymorphism has shown a significant influence on warfarin response, accounting for 22.5 %, 18.4% of the variability in dose in whites and Asians respectively. While among North American blacks, VKORC1 –1639G>A polymorphism accounted for 4.2 % of the variability in dose. Although possession of the VKORC1 −1639A allele is associated with a similar decrease in the individual "warfarin dose requirement "irrespective of race, the variability in dose explained by (VKORC1) at a population level

varies by race. The -1639G>A allele frequency is the major allele (91%) in Asians. It is also common in whites (37%) and less common in North American blacks (10%). American blacks required the highest mean dose of warfarin (40 mg/week), while Asians required the lowest mean dose of warfarin (21 mg/week). Intermediate warfarin dose (31.5 mg/week) was required by whites.

Overall, the frequency of A allele in the VKORC1–1639G>A in Egyptian population seems to be intermediate between the relatively high frequency reported from Asian countries and the relatively low frequencies in African Americans populations and likethe frequency in Caucasian populations.

CONCLUSIONS

1. In the Egyptian VTE patients, both VKORC1-1639G and VKORC1-1639A alleles were found with allelic frequency (58 % and 42 %) respectively.
2. There was a significant variation in daily" warfarin maintenance dose "among patients with different genotypes of (VKORC1) at position -1639, and that VKORC1 polymorphism has an important influence on the "warfarin dose requirements'.

RECOMMENDATIONS

1. Further molecular studies using a larger sample size are recommended to make more reproducible results about VKORC1–1639G>A polymorphism and its influence on daily "warfarin maintenance dose "in the Egyptian population.
2. More researches are required to unravel other genes which may have an effect on "warfarin dose requirements" in the Egyptian population.
3. Further studies are desired to assess other important factors that improve dosing algorithm for personalizing warfarin dose during both the initiation and maintenance therapy to determine the cost-effectiveness of genotype-guided warfarin dosing in Egyptian patients.
4. This study could be adapted as a model for the application of genetic testing in clinical practice for other drugs and genes.

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