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Genetic and nongenetic factors associated with warfarin dose requirements in Egyptian patients

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Abstract

Background and objective—Warfarin is a commonly used oral anticoagulant with a narrow therapeutic index and various genetic and clinical factors that influence interpatient variability in dose requirements. This study investigated the impact of genetic and nongenetic factors on warfarin dose requirements in Egyptians.

Methods—DNA was extracted from 207 patients taking warfarin for more than 2 months and genotyped for *VKORC1* (3673 G> A), *CYP2C9* *2*3*4*5*8, *CYP4F2* (V33M; rs2108622), *APOE* (rs429358, rs7412), and *CALU* (rs339097) gene polymorphisms. Linear regression modeling was conducted to identify the genetic and nongenetic factors that independently influence warfarin dose requirements.

Results—*VKORC1* 3673 AA or GA genotype ($P < 0.0001$), one or two variant alleles of *CYP2C9* gene ($P = 0.0004$), *APOE* ε2 haplotype ($P = 0.01$), and increasing age ($P < 0.0001$) were all associated with lower warfarin dose, whereas smoking ($P = 0.025$) and pulmonary embolism ($P = 0.0059$) showed association with higher warfarin doses. These factors explained 31% of the warfarin dose variability. This is the first independent confirmation of the association of the *CALU* rs339097 variant with higher warfarin dose requirement, although inclusion of this single nucleotide polymorphism in the multiple regression model failed to achieve significance ($P = 0.066$). *CYP4F2* (V33M) polymorphism was not significant ($P = 0.314$), despite its high frequency in the studied population (42%).

Conclusion—The study shows that *VKORC1*, *CYP2C9* polymorphisms, *APOE* ε2 variant, and several clinical/ demographic variables are important determinants of warfarin dose requirements in Egyptian patients. The percentage of variability explained by these factors is lower than in those of European ancestry, but similar to the variability explained in Asians and African ancestry.

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Keywords

APOE; *CALU*; *CYP2C9*; *CYP4F2*; Egyptian; polymorphism; warfarin; *VKORC1*

Introduction

Warfarin is ranked among the most prescribed drugs worldwide [1], but its use presents numerous challenges in clinical practice, as it is associated with considerable morbidity because of its narrow therapeutic range and large interpatient variability in dose requirements [2,3]. Numerous studies have described the genetic and nongenetic factors that influence warfarin dose requirements in those of European, Asian, and African ancestry [3,4]. Genetic variability in *VKORC1* and *CYP2C9* has been consistently replicated to associate warfarin dose. Several studies in those of European ancestry have also suggested a role for *CYP4F2*, a recent report suggests a role for *CALU* in those of African ancestry, and *APOE* has been variably associated [5]. However, there are limited data on associations in Middle Eastern or Mediterranean populations.

The primary objective of this study was to determine the genetic and nongenetic factors associated with warfarin dose requirements in Egyptians.

Methods

Study population

The study was approved by the Research Ethics Committee at the Faculty of Medicine, Ain Shams University, Cairo, Egypt. A total of 207 Egyptian patients signed the written informed consent and participated in this study. Eligible patients were enrolled at four sites in Cairo, one hundred patients were enrolled from an Egyptian Cardiology Clinic, sixty-three patients were enrolled from Nasser Institute Hospital, twenty-nine patients from the Cardiology Clinic at Al Azhar University, and fifteen patients from Demerdash Hospital.

Patients were considered eligible if they were taking stable weekly doses of warfarin (Marevan; GlaxoSmith Kline, Cairo, Egypt) for three consecutive clinic visits, occurring over a minimum time period of 2 months. A stable weekly maintenance dose of warfarin means a dose that did not vary by more than 10% between clinic visits. The international normalized ratio (INR) at each of the three visits had to be in the patient's specific goal INR range. As in earlier studies, one of the three INR readings was 0.2 units, which was above or below the goal INR range because of the inherent variability in the INR measurement [6]. Patients with liver cirrhosis, advanced malignancy, hospitalization within the earlier 4 weeks, and those who suffered from febrile/diarrheal illness within the past 2 weeks were excluded from the study.

Clinical data were collected and double-checked from different sources to get the most precise data for each case. A brief interview with each patient was conducted in which they were asked to document the following information: age, body weight, height, sex, smoking status, alcohol history, indication for warfarin use, weekly prescribed warfarin dose, earlier history of hemorrhage, past medical history, and concomitant medications taken during the past three clinic visits. Finally, a blood sample for DNA isolation and genotyping was obtained from each patient.

Genotyping procedure

Blood samples (5 ml) were taken from each patient for INR measurement and DNA extraction. Genomic DNA was extracted from samples of peripheral blood leukocytes using

either a QIAamp DNA Blood Mini Kit (183 samples; QIAGEN, Cairo, Egypt) or automated QIAcube device (24 samples; QIAGEN, Cairo, Egypt) according to the manufacturer's guidelines. *CYP2C9* polymorphisms; *CYP2C9**2 (Arg144Cys), *CYP2C9**3 (Ile359Leu), *CYP2C9**4 (Ile359Thr), *CYP2C9**5 (Asp360Glu), *VKORC1* 3673G>A, and *APOE* (rs429358 and rs7412) genes were genotyped in by PCR followed by pyrosequencing based on a published assay from our laboratory [6–8]. *CYP4F2* (Val433Met) was genotyped by TaqMan assay [9–11]. The genotyping of *CYP2C9**8 (Arg150His) was performed by PCR-restriction fragment length polymorphism method, using *AciI* restriction endonuclease enzyme and with assay validation confirmed by direct sequencing and known *CYP2C9**8 carrier samples used as positive controls [12–13]. The *CALU* (rs339097) single nucleotide polymorphism (SNP) was genotyped by PCR followed by pyrosequencing, in which the following PCR and sequencing primers were used: PCR forward: 5'-GGATTCTGAATCTGGCCAATAC-3'; reverse: 5'-biotin GGATTCTG AACAGGGACTCAGTTT-3'; and forward sequencing primer: 5'-TGAATCTGGCCAATACT-3'. The standard PCR reaction mixture used for the amplification of the target sequence consisted of 12.5 µl including 6.5 µl of ABI PCR master mix with Taq DNA polymerase, 1 µl dimethylsulfoxide (Sigma-Aldrich, St Louis, Missouri, USA), 1 µl of each primer (Invitrogen, Carlsbad, California), 1.25 µl of water for DNA, and 2 µl of genomic DNA.

Statistical analysis

Baseline demographics were analyzed using descriptive statistics. Numerical variables were represented as mean ± standard deviation or median and interquartile range as appropriate. Categorical variables were presented as percentages. The mean weekly warfarin dose was calculated by taking the average of the warfarin dose of the patient, which was documented at each of the three consecutive clinic visits. Hardy–Weinberg equilibrium was tested by allele counting and χ^2 analyses with one degree of freedom. Analysis of variance was used to compare the average warfarin dose between the genotype groups. Haplotype of *APOE* SNPs rs429358 and rs7412 was constructed using PHASE v2.1 software [14].

Mean weekly warfarin dose differences by genotype or haplotypes were evaluated by nonparametric methods. A stepwise linear regression model selection was used to assess the mean weekly warfarin dose in relation to genetic factors (*VKORC1*, *CYP2C9*, *CYP4F2*, *APOE*, and *CALU* genotypes), and nongenetic factors (sex, age, body surface area, use of aspirin, indication for warfarin, concomitant diseases, and smoking status). The genetic factors were coded as, (i) *VKORC1* 3673: AA genotype as 2, G genotype as 1, GG genotype (wild type) as 0, (ii) *CYP2C9*: *1/*1 genotype (wild type allele) as 0; heterozygous variant genotype (*1/*2, *1/*3, *1/*4, *1/*5, *1/*8) as 1, and homozygous variant genotype (*2/*2, *2/*3, *3/*3, *5/*5) as 2, (iii) *CYP4F2* (Val433Met): CT and TT genotypes as 1, CC genotype (wild type) as 0, (iv) *APOE* (rs429358 and rs7412): haplotype TT was coded as ϵ 2, TC as ϵ 3, and CC as ϵ 4 alleles, respectively; and (v) *CALU* (rs339097): CT and CC genotype as 1, TT genotype as 0. Nongenetic factors that may contribute to warfarin dose requirements were also included in the model. These variables included age (decades), sex, indication for warfarin therapy, smoking status, and concurrent interacting medications. Square root transformation of the dose was applied to improve model fit and limit heteroscedasticity. *P* value of less than 0.05 was considered significant. All statistical analyses were carried out with SAS (version 9.2; SAS Institute) and SPSS software (version 17.0 for windows; SPSS Inc., Chicago, Illinois, USA).

Results

A total of 207 Egyptian patients participated in this study; they are considered urbanites living in Cairo or other surrounding cities. Patients' demographics, indication for warfarin,

goal INR range, most common concomitant disease, and most common concurrent interacting medications taken are shown in Table 1.

The study included 93 men and 114 women, with a mean age of 47.4 ± 14.7 years. Indication for warfarin therapy varied, with mitral valve replacement as the most common indication for warfarin therapy ($n=109$, 52.7%). The mean warfarin dose was 36.8 ± 17.9 mg/week, median was 35, and an interquartile range of 24.5–42 mg per week. The median of the mean INR for the whole study population was 2.25, with interquartile range of 2.1–2.4.

Complete genotyping data were available for 195 patients. All genotypes were in Hardy–Weinberg equilibrium (Table 2). DNA concentrations from the remaining 12 patients were too low to be genotyped. The minor allele frequencies (MAFs) of the SNPs study are contrasted between the Egyptian population studied here and population or published values for four other populations (Turkish, Asian, African–American, and Caucasian) (Table 2). These data suggest that MAFs among Egyptians cannot be assumed to be close to a specific continental population. For example, we observed variant alleles at *CYP2C9* *4, *5, and *8, along with *CALU* rs339097, all of which are generally considered nonvariant in Caucasians, and observed primarily in those of African ancestry. In contrast, the MAF for *CYP2C9* *2 and *3 was similar to Caucasians; *CYP4F2* V433M was substantially higher than any other population reported to date, whereas *APOE* rs429358 was lower than typically observed in African–Americans or Caucasians. Thus, the MAFs for these various SNPs exhibit no consistent pattern in comparison with specific continental populations, even within a gene.

Dose requirements for each genotype group are shown in Table 3. For statistical comparison of warfarin dose by genotype, *CYP2C9* polymorphisms, *CYP2C9* genotypes were separated into three groups: wild type genotype (*1/*1), heterozygous variant genotype (*1/*2, *1/*3, *1/*4, *1/*5, *1/*8), and homozygous variant genotype (*2/*2, *2/*3, *3/*3, *5/*5). Among 122 wild-type homozygotes, 60 variant heterozygotes, and 13 variant homozygotes, the mean warfarin doses were 40.3 ± 19.5 , 31.7 ± 14.8 , and 32.0 ± 14.1 mg per week, respectively ($P=0.0028$). Warfarin doses were also significantly different by *VKORC1* genotype ($P<0.0001$), *CALU* ($P=0.04$), and marginally significant for *APOE* $\epsilon 2$ ($P=0.08$), but not by *CYP4F2* genotype ($P=0.314$; Table 3; Fig. 1).

Results of the regression analysis are presented in Table 4. The R^2 of the final regression model was 31.02%. The model shows that genetic and nongenetic factors were both predictors of warfarin dose requirement. Lower warfarin dose was associated with variants of *VKORC1* 3673, *CYP2C9*, and *APOE* $\epsilon 2$ and age, whereas smoking and pulmonary embolism, as indicated, were associated with higher warfarin dose. *CALU* (rs339097) was marginally significant, but did not meet the criteria to stay in the final model ($P=0.066$).

Discussion

Populations in the Mediterranean represent some of the first populations to migrate out of Africa, which make them particularly interesting for genomics research [15]. Historical records show the Egyptian population is one that has undergone genetic admixture and racial mixing, which created a heavily mixed population of modern Egyptians including several ethnic groups such as Bedouins, Peasants, Nubians, Berbers, and urbanites [16]. The former ethnic groups each make up a relatively small percentage of the population and live along the coast, or in rural areas. Approximately 98% of the population lives within the Nile Valley and delta in towns or cities, the largest of which is Cairo. The population of the Nile valley and the delta forms a fairly homogeneous group whose dominant physical characteristics are the result of the admixture of the indigenous African population with those of Arab ancestry. Within urban areas (the northern delta towns especially), foreign

invaders and immigrants, Persians, Romans, Greeks, Crusaders, Turks, and Circassians, long ago left behind a more heterogeneous mixture of physical types. Blond and red hair, blue eyes, and lighter complexions are more common there. Thus, this is an admixed population that has not been extensively investigated in genetic studies.

The aim of this study was to investigate the contribution of genetic and nongenetic factors on the variability of warfarin dose requirements in the admixed Egyptian population. The comparison of the MAFs of the SNPs studied showed no consistent patterns, with MAFs of SNPs ranging from being similar to Caucasians, to being similar to Africans, to being different from all earlier studied populations. These data highlight that polymorphism frequencies cannot be presumed to mirror those of European Caucasians or any other continental population. This is perhaps not surprising, given the presumed historical ancestral admixture of Egyptians. We are not aware of any population genetics studies that clearly define the ancestral makeup of Egyptians, and so the findings in this study, as it relates to MAFs, further supports recent recommendations of the importance of genomics research in the Mediterranean region [15].

Not surprisingly, we observed the highly replicated effects of *VKORC1* and *CYP2C9* on warfarin dose requirements. The contribution of *CYP2C9* to variability in Egyptians was similar to that observed in Caucasians, while the contribution of *VKORC1* to variable dose was much lower than in Caucasians, and more similar to what has been observed in African-Americans [8].

Studies of the *CYP4F2* (V33M; rs2108622) polymorphism in whites, blacks, and Brazilians have shown an association with the warfarin dose requirement [9,17,18]. Despite the high frequency of *CYP4F2* polymorphism in the Egyptian population (42%), we observed no significant association between the *CYP4F2* (V33M; rs2108622) polymorphism and warfarin dose requirement ($P=0.314$). However, a trend for dose differences by genotype was observed, with the direction being consistent with the literature.

We also studied the *CALU* SNP that was recently defined to lead to an approximately 11% higher warfarin dose for each variant allele in a primarily African-American population [5]. We are the first to confirm the association of the rs339097 variant with higher warfarin dose requirement, notably in a non-African-American cohort. We observed that variant allele carriers required 14.1 mg/ week more warfarin than TT homozygotes. Although inclusion of this SNP in our multiple regression model failed to achieve significance ($P=0.066$) this is likely an issue of power, as we only had nine variant carriers in our cohort. These data suggest, however, that *CALU* is likely to be an important contributor to high dose, and may be particularly relevant in non-Caucasian populations.

The literature on *APOE* is inconsistent. Some studies have shown the *APOE* $\epsilon 4$ allele to be associated with higher therapeutic warfarin dose [19,20]. In contrast, other studies have shown no association with warfarin dose and *APOE* genotype [8,19,21,22]. We observed that the *APOE* $\epsilon 2$ variants were significantly associated with lower warfarin dose requirements in the linear regression model ($P=0.01$), although of borderline significance when considered alone.

This study also assessed the contribution of genetic and nongenetic factors to the interindividual variability of warfarin dose requirements. In the Egyptian population, 31% of variability in warfarin dose was explained by a combination of genetic and nongenetic factors. This is much lower than observed in most studies of Caucasians, although similar to the amount of variability explained by such factors in African-Americans and Asians [6,23,24]. For example, in the 5700 patient cohort that comprised the International Warfarin Pharmacogenetics Consortium, the model developed by the International Warfarin

Pharmacogenetics Consortium explained 26% of the variability in African-Americans/blacks, and 33% of the variability in Asians [23]. In this study, *VKORC1* 3673G>A polymorphisms explained 10% in the warfarin dose variability, also much less than observed in most studies of Caucasians, but similar to the African-Americans [23]. *CYP2C9* polymorphism explained 5% of the dose variability, which is at the lower end of the range of variability explained in many studies in Caucasians [6,24]. This shows that the inter-individual variability in warfarin dose requirements in the Egyptian population is due, at least partially, to additional genetic and nongenetic factors that need further investigation. This study population was generally younger, and had more patients with aortic valve replacement than those in other warfarin pharmacogenetic studies. This may have influenced the amount of variability in warfarin dose we could explain. In addition, the average dose in the five patients with the pulmonary embolism diagnosis was 70mg per week, although their INRs were not unusually high, averaging approximately 2.5. It is unclear why they would have required higher doses than the rest of the population and so significance of pulmonary embolism in the model may represent a chance finding. Finally, it was a surprise that a measure of body size (BSA in this case) was not a significant predictor of dose. We also modeled height and weight, with and without smoking, but no measures of body size were significant. It is possible that this is because of the minimal variation in height, which varied in the population by less than 4%.

In conclusion, this is the first report detecting the genetic associations of *VKORC1*, *CYP2C9*, *CYP4F2*, *APOE*, and *CALU* polymorphisms with warfarin dose requirements in Egyptian population. This study shows that genetic polymorphisms in *VKORC1*, *CYP2C9*, *APOE*, along with nongenetic factors are determinants of warfarin dose requirements. These factors explain less variability than has been shown in Caucasians, suggesting other important determinants of response variability remain to be determined.

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Fig. 1. Effect of *VKORC1*, *CYP2C9*, *CYP4F2*, *APOE* ε2, and *CALU* genetic polymorphisms on warfarin dose requirements in Egyptians. Bars represent mean warfarin dose required. Error bars represent standard deviation. ($P<0.05$ is considered significant, test done by analysis of variance). *APOE* ε2 was coded as 0, 1, and 2 copies.

Table 1

Baseline demographics of warfarin-treated patients

Demographic characteristics	All patients (<i>n</i> = 207)
Age (years)	47.4 ± 14.7
Sex	
Male	93 (44.9%)
Female	114 (55.1%)
Race	
Egyptian	207 (100%)
Height (cm)	166.7 ± 8
Weight (kg)	77.9 ± 15.7
Indication for warfarin ^a	
Atrial fibrillation	2 (0.96%)
Mitral valve replacement	107 (51.7%)
Deep vein thrombosis	34 (16.4%)
Pulmonary embolism	5 (2.4%)
Cerebrovascular accident	22 (10.6%)
Aortic valve replacement	26 (12.6%)
Cardiomyopathy	5 (2.4%)
Others	7 (3.36%)
Most common concomitant disease ^b	
Hypertension	41 (19.8%)
Hyperlipidemia	39 (18.8%)
Diabetes	24 (11.6%)
Most concurrent interacting medications ^c	
Aspirin	34 (16.4%)
Glimiperide	9 (4.3%)
Goal INR range	
1.5–2.0	9 (4.33%)
2.0–3.0	197 (95.19%)
2.5–3.5	1 (0.48%)

INR, international normalized ratio.

Data are expressed as mean ± standard deviation or *n* (%).

^aPatients might have more than one indication for warfarin therapy.

^bPatients might have more than one concomitant disease.

^cPatients might have taken more than one concomitant interacting medication.

Table 2

Comparison of CYP2C9 (*2, *3, *4, *5, *8), VKORC1 (3673G > A), APOE and CALU CYP4F2 minor allele frequencies in Egyptian, Turkish, Asian, African-American, and Caucasian populations

Gene	Variant	Genotype	Frequency [n (%)]	Minor allele frequency			Racial groups			
				Minor allele	Egyptian (study population)	Turkish ^a	Asian ^a	African-American ^a	Caucasian ^a	
CYP2C9	*2	CC	151 (77.43%)	T	0.117	0.106	0	0.02	0.143	
		CT	42 (21.54%)							
		TT	2 (1.03%)							
	*3	AA	161 (82.56%)	C	0.092	0.100	0.036	0.02	0.109	
		AC	32 (16.41%)							
CYP2C9	*4	CC	2 (1.03%)							
		TT	194 (99.49%)	C	0.003	0.01	0	0	0	
		TC	1 (0.51%)							
	*5	CC	192 (98.46%)	G	0.01	0	0	0.03	0	
		CG	2 (1.03%)							
VKORC1	*8	GG	1 (0.51%)							
		GG	192 (98.46%)	A	0.008			0.06	0	
		GA	3 (1.54%)							
	3673	GG	50 (25.51%)	A	0.462	0.50	0.87	0.12	0.43	
		GA	111 (56.63%)							
CYP4F2	V433M	AA	35 (17.86%)							
		CC	68 (35.42%)	T	0.42		0.26	0.07	0.27	
		CT	87 (45.31%)							
	rs429358	TT	37 (19.27%)							
		TT	168 (86.67%)	C	0.067			0.100	0.149	
APOE	rs7412	CT	26 (13.40%)							
		CC	169 (86.67%)	T	0.074			0.040	0.087	
		CT	23 (11.79%)							
	rs339097	TT	3 (1.54%)							
		TT	186 (95.38%)	C	0.023			0.195	0	
CALU		CT	9 (4.62%)							

Table 3
Association of genetic polymorphism with warfarin dose requirements in Egyptians

Gene	Variant	Genotype	Frequency (n = 207)	Mean $\pm$ SD warfarin dose (mg/week)	P value ^a
<i>CYP2C9</i>		*1/*1	122 (62.56%)	40.3 $\pm$ 19.5	0.0028
		*1/*2	34 (17.44%)	35.0 $\pm$ 16.9	
		*1/*3	23 (11.79%)	26.6 $\pm$ 10.4	
		*1/*4	1 (0.51%)	35	
		*1/*5	2 (1.03%)	28.0 $\pm$ 10	
		*1/*8	3 (1.45%)	39.7 $\pm$ 10.7	
		*2/*2	2 (1.03%)	29.8 $\pm$ 2.5	
		*2/*3	8 (4.10%)	32.4 $\pm$ 17.2	
		*3/*3	2 (1.03%)	33.3 $\pm$ 17.3	
		*5/*5	1 (0.51%)	31.5	
<i>VKORC1</i>	3673G > A	GG	50 (25.51%)	43.4 $\pm$ 21.6	< 0.0001
		GA	111 (56.63%)	38.1 $\pm$ 16.4	
		AA	35 (17.86%)	25.6 $\pm$ 12.3	
<i>CYP4F2</i>	V433M	CC	68 (35.42%)	35.2 $\pm$ 16.1	0.31
		CT	87 (45.31%)	37.3 $\pm$ 18.7	
		TT	37 (19.27%)	41.1 $\pm$ 20.9	
<i>APOE</i>		ϵ 2/ ϵ 2	3 (1.54%)	21.0 $\pm$ 3.5	ϵ 2 haplotype 0, 1, 2; P = 0.08
		ϵ 2/ ϵ 3	20 (10.26%)	31.0 $\pm$ 15.1	
		ϵ 2/ ϵ 4	3 (1.54%)	49.0 $\pm$ 18.2	
		ϵ 3/ ϵ 3	146 (74.87%)	38.2 $\pm$ 17.9	
		ϵ 3/ ϵ 4	23 (11.79%)	35.6 $\pm$ 22.0	
<i>CALU</i>	rs339097	TT	186 (95.38%)	36.5 $\pm$ 17.9	0.04
		CT	9 (4.62%)	50.6 $\pm$ 21.3	

SD, standard deviation.

^aThese P values were generated using nonparametric methods (Kruskal–Wallis or Wilcoxon Rank-sum test as appropriate).

Table 4

Linear regression stepwise modeling association between mean weekly warfarin dose (square root transformed) as a dependent variable and genetic and nongenetic factors as independent variables

Predictor	Coefficient	Standard error	Partial R^2 (%)	P value
Intercept	8.06	0.32		
VKORC1 3673 genotype	−0.63	0.13	10.33	< 0.0001
Age (decades)	−0.30	0.06	8.11	< 0.0001
CYP2C9	−0.41	0.14	5.17	0.0004
Pulmonary embolism	1.44	0.56	3.0	0.0059
APOE ε2	−0.60	0.22	2.53	0.01
Smoking status	0.58	0.26	1.88	0.025
Model R^2			31.02	< 0.0001