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The Impact of CYP2C9 and VKORC1 Polymorphism In Patient's Response to Warfarin and Acenocoumarol

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ABSTRACT

Warfarin is commonly prescribed as oral anticoagulant medication for Libyan patients, the wide inter-individual variation between the patients in their response to oral anticoagulants is attributed to genetics factors, mainly polymorphisms in CYP2C9 and VKORC1. This study was aimed to assess the impact of genetic (CYP2C9*2, *3 and VKORC1- 1639G>A/ and 1173 C>T polymorphism), and non- genetic factors: age, and body mass index (BMI) in the response of Libyan patients using oral anticoagulants.A total of 100 patients with stable maintenance dose of warfarin or acenocoumarol were recruited during their routine follow up in anticoagulant clinic at Tripoli Medical Centre. CYP2C9 and VKORC1 variant alleles were screened by (HRM) real-time PCR, followed by DNA sequencing.The variant allele frequencies of CYP2C9*2, CYP2C9*3, and VKORC1 -1639G>A/1173C>T were 9.5%, 4.0%, 4.5%, and 37.0%, respectively.Carriers of VKORC1 (-1639 G>A, 1173 C>T) variant alleles required a significantly lower doses of oral anticoagulants compared with carriers of wild type, P value =0.04, and 0.019, respectively. No significant difference in dose requirement was found between carriers of wild type, and CYP2C9*2 and *3 variant alleles, P value =0.11 and 0.98, respectively.The multivariate regression model including age, BMI, VKORC1, and CYP2C9 genotype produced weak model for estimating the drug dosage (R^2= 8.6%); and neither genetic nor non-genetic factors could be used as a predictor for estimation of oral anticoagulant dosage.Our data showed that VKORC1 variant alleles but not CYP2C9*2, *3 variant alleles significantly contributed to oral anticoagulant dose variability.

Key words: Libya–CYP2C9–VKORC1–Warfarin.

1 INTRODUCTION

Warfarin is a commonly prescribed oral anticoagulant medication for the treatment of many thromboembolic events. Its narrow therapeutic index remains the main challenge for physicians to prescribe the right dose with minimum side effects. The wide inter-individual variation among pa-tients is attributed to genetic and non-genetic factors, where the most common studied single nucleotide polymorphisms (SNPs) that cause warfarin sensitivity are CYP2C9*2 and 3, and VKORC1-1639G>A/ and 1173 C>T [1, 2].

Coumarins are drugs that act by inhibition of vitamin K epoxide reductase enzyme (VKOR) with resultant blocking of biologically active clotting factors [3].

These drugs are administered as racemic mixtures consisting of 50% of each enantiomer [4]. Although the mecha-nism of action of these drugs are similar, there are some differences in pharmacokinetics between warfarin and acenocoumarol [3].

S-warfarin (the most active form) is mainly metabolized by CYP2C9 into inactive metabolite 6, 7-hydroxy warfarin. R-warfarin is metabolized by several other CYP iso-forms like CYP1A2, CYP3A4, and CYP2C19 into inactive metabolite 6, 8, and 10-hydroxy warfarin [5]. The CYP2C9 is the principal metabolizing enzyme of both acenocoumarol enantiomers [6, 7].

For many reasons, warfarin is considered an ideal drug for studying the pharmacogenetics; due to its narrow therapeutic index, adverse drug reaction, wide inter-individual variation in dose requirement, and the influence of many of genetic variants on its effect or metabolism [8, 9].

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Several studies have been conducted on the role of CYP2C9 and VKORC1 in warfarin pharmacology. Flock-hartet al. [10] showed that there are at least 35 alleles of CYP2C9 documented. However, the effects of most of these alleles on warfarin dosage are yet to be charac-terized [11]. Functional significance of the allelic variants CYP2C9*2 (R144C; rs1799853) in exon 3, and CYP2C9*3 (I359L; rs1057910) in exon 7 was demonstrated in expres-sion assays, providing evidence that both reduced-function variants resulted in impaired metabolism of S-warfarin, and decreased its clearance [12, 13]. Other researchers have re-ported similar findings [14–20].

It is worth mentioning here that CYP2C9*2, *3 have similarly been associated with reduced S-acenocoumarol clearance, lower steady state acenocoumarol dose require-ments [21, 22].

The VKORC1 (VKOR complex, subunit 1) geneencoding the VKOR catalytic subunit (VKORC1) was identified in parallel by two independent groups [23, 24].

Two common polymorphisms were found to be associated with warfarin sensitivity in VKORC1 gene are-1639G>A in promoter, and 1173C>T intron1.In a meta-analysis, the difference in warfarin dose in relation to genotype for the−1639 polymorphism was compared for a Caucasian and an Asian population [25]. In this study, it was found that Cau-casian patients with one −1639A allele required a 25% lower dose and patients with two −1639A alleles a 50% lower dose than patients without this variant allele. This effect was also present in Asian patients, although to a lesser extent (14 and 38% lower doses, respectively) [25].

To the best of knowledge, evaluation of response to anti-coagulant therapy based on genotyping has not been previ-ously studied in Libyan patients, and so this study was con-ducted to evaluate the impact o of CYP2C9 and VKORC1 polymorphism on patient response to warfarin and acenocoumarol.

2 MATERIALS AND METHODS

2.1 Subjects

The study was approved by the cardiology board at Tripoli Medical Centre. A total of 119 Libya patients signed the written informed consent and participated , attending anticoagulant clinic at Tripoli Medical Centre (TMC) taking either warfarin (marevan®) or acenocoumarol (sinthrom®), 100 patients were on treatment for at least more than two months, and on stable maintenance dose one month based on INR results, the study participants were recruited in the period from November 2015 to September 2016. 19 cases were excluded from this study; because they did not fit our criteria, a questionnaire was used, and retrospective clinic reviews were performed. Patients were excluded from the study under the following conditions: renal failure (defined as abnormal serum creatinine or urea level), liver dysfunction (defined a s presence of chronic hepatic disease or biochemical evidence of significant hepatic impairment), cancer, and pregnant women.

Data were collected regarding age, body weight, height, lifestyle habits such as smoking and vitamin K intake, oral anticoagulant daily dose, indication for use and date of ini-tiation, past medical history, concomitant medications in-take, and history of bleeding events while on oral anticoagulant.

2.2 DNA extraction and genotyping

Two ml of venous blood was collected in EDTA vials. Ge-nomic DNA was extracted using a QIAamp® DNA blood mini kit (Qiagen, Valencia, CA) according to the manufac-turer's instructions. The genetic analysis was carried out in the laboratory of the National Center for Disease Control (NCDC) in Tripoli.

HRM analysis was used to determine the genotypes for CYP2C9 (*2 and *3) and VKORC1 (G>A and C>T) variants.

2.2.1 PCR-HRM

HRM analysis was performed on a Rotor-Gene Q real-time rotary analyzer (QIAGEN GmbH). The primers used for CYP2C9 C430T polymorphism detection were C430TF 5'CCTCATGACGCTGCGGAA-3' as a for-ward and C430TR 5'-GCCTTGTGGAGGAGTTGAG -3' as a reverse primers, while those used for CYP2C9 A1075C HRM CYP92F 5'-CCACATGCCCTACACAGATG-3' as a forward, and CYP92R 5'-TGCCCCATGCAGTGACCTG-3' as a reverse primers, for VKORC1 (-1639G>A, 1173C>T) the forward primers were: CTCAAGTGATCCACCCACCT-3', 5'-5'-

GGGTGGAACCAGGTTAGGAC-3' and the reverse primers were: 5'-ACAGACGCCAGAGGAAGAGA-3' and 5'-AGGGGAGGATAGGGTCAGTG-3' respectively.

Reaction mixes of 25 µl contained 12.5 µl 2x mi-real-time EvaGreen® Master (Taq Polymerase: 0.05 u/µl, dNTPs (dATP, dCTP, dGTP, dUTP) (200 µM), reaction buffer with KCl and MgCl2 (3 mM), EvaGreen®, stabilizers) from (Metabion, Martinsried- Germany), 0.75 µl of 10 pmol/µl of each primer, and 10-60 ng genomic DNA. The thermal profile for the first gene: CYP2C9C430T polymorphism contained one cycle of Taq polymerase activation at 95^{O}C for 2 min followed by 40 cycles of 95^{O}C for 5 s, 55^{O}C for 10 s, and 72^{O}C for 20 s. After amplification, HRM analysis data were collected from 70^{O}C to 90^{O}C, with each step raised by 0.05^{O}C, followed by a waiting time of 1 s.

The thermal profile for A1075C polymorphism contained one cycle of Taq polymerase activation at 95^{o}C for 2 min followed by 40 cycles of 95^{o}C for 5 s, 54^{o}C for 10 s, and 72^{o}C for 20 s. After amplification, HRM analysis data were collected over a temperature range of 84^{o}C and 90^{o}C in 0.05^{o}C increments, followed by a waiting time of 1 s.

The thermal profile for the second gene: VKORC1 G3673A polymorphism contained 1 cycle of Taq polymerase activation at 95^{O}C for 2 min followed by 40 cycles of 95^{O}C for 5 s, 58^{O}C for 10 s, and 72^{O}C for 20 sec. After amplifica-tion, HRM analysis data were collected over a temperature range of 70^{O}C to 90^{O}C, with 0.05^{O}C increments, followed by a waiting time of 1 s, and the thermal profile for C6484T

polymorphism contained 1 cycle of Taq polymerase activation at 95°C for 2 min followed by 40 cycles of 95°C for 5 s, 60°C for 10 s, and 72°C for 20 s. After amplification, HRM analysis data were collected from 84°C to 90°C in 0.05°C increments, followed by a waiting time of 1 s.

2.3 DNA sequencing

PCR products of samples that showed variation using HRM analysis were further subjected to automated sequencing of the region of interest. Exons 3 and 7 of CYP2C9 gene were amplified by conventional PCR using specially designed primers C430T_F 5'-CCTCATGACGCTGCGGAA-3', C430T_R 5'-GCCTTGTGGAGGAGTTGAG-3' for the amplification of exon 3 producing an amplicon of 194bp, and A1075C_A 5'-CCACATGCCCTACACAGATG-3', and A1075C_B 5'-TGCCCCATGCAGTGACCTG-3' for the amplification of exon 7 producing an amplicon of 208bp. While the mutations of the VKORC1 were amplified by conventional PCR using specially designed primers G3673A A-F5'-CTCAAGTGATCCACCCACCT- 3', G3673A- R5'-ACAGACGCCAGAGGAAGAGA- 3', producing an amplicon of 166bp, and C6484T F5'-GGATAGGGTCAGTGACATGGAAT-3', C6484T R5'-GCCCGAGAAAGGTGATTT-3', producing an amplicon of 138 bp. The last step in sequencing were resultant pellet was resuspended in 20 µl of Hi-Di formamide, loaded in 96-well plate, and analyzed with an ABI Prism 3130 genetic analyzer. The resultant sequences were edited, and the database analysis were carried out using BLAST (http ://www.ncbi.nlm.nih.gov/BLAST).

2.4 Statistical analysis

Allelic frequencies were calculated by a gene-counting method. One-way ANOVA was carried out using Microsoft Excels software 2013. One-way analysis of variance was used to assess for the effect of CYP2C9 and VKORC1 genetic polymorphisms on the mean weekly dose of the oral anticoagulant.

One -way ANOVA was also used to assess the relation-ship between the non-genetic factors (age, and BMI), and mean weekly dose of the oral anticoagulant. Results were shown as mean ± SEM. A p value of <0.05 was considered statistically significant.

Multiple linear regression analysis was calculated by SPSS version 21, to predict the effect of genetic factors (VKORC1, and CYP2C9 polymorphisms), and nongenetic factors such as age, and body mass index, on the final oral anticoagulant weekly maintenance dose.

3 RESULTS

3.1 Demographic data and genotyping

In this study a total of 119 patients were enrolled. 19 patients were excluded because they did meet the inclusion criteria. 100 patients were included in the study; 90 patients were on warfarin and 10 on acenocoumarol. 80 pa-tients (80%) were women and 20 (20%) were men. For the 100 patients recruited in our study, the primary reason for anticoagulation therapy was heart valve replacement their INR ranges from 2-4 depends on the patient's status. Table 1 summarizes the clinical characteristics of the study population. The genetic characteristics of the study popu-lation are summarized in Table 2.

3.2 Effect of VKORC1 and CYP2C9 genetic polymorphism on the mean weekly dose of oral anticoagulant

3.2.1 VKORC1

VKORC1 -1639G>A variant allele had a significant effect on the mean weekly dose requirements of oral anticoagulant. The dose of the anticoagulant for the GG carriers was 4.30± 3.3 mg /week, whereas the comparable dose in the AA (2 cases) carriers was 2.00 ± 0.0 mg/week, surprisingly we found that in heterozygote GA carriers (5 cases), the mean weekly dose was 5.94 ± 9.5 mg/week, which was higher than GG carrier, and significantly higher than AA carriers (P = 0.04). Moreover, VKORC1 1173 C>T variant allele showed a significant effect on mean dose/week, with P value = 0.019, the average dose of CC carrier was 4.78± 4.6 mg\week which was higher than 4.35 ± 2.8 mg\week in CT carrier and 3.08 ± 2.1 mg\week in TT carrier.

3.2.2 CYP2C9

Unlike VKORC1 genotypes, the CYP2C9*2 CT carriers re-quired a lower mean weekly dose of oral anticoagulant (3.70± 3.2 mg) than CC carriers (4.50 ± 3.7 mg) with P value (0.11), which was statistically insignificant. In addition, the CYP2C9 *3 AC variant alleles had no significant effect on mean weekly dose of the drug, with no significant difference in dose between the two groups; 4.36 ± 3.7 mg for wild type and 4.35 ± 3.8 mg for heterozygote, with P value (0.98).

3.3 Relation between age and mean dose per week of oral anticoagulant

Among study population in older patients (those over 70 years), the mean weekly dose of oral anticoagulant signif-icantly decreased in comparison to other age groups. The mean weekly dose to achieve stable anticoagulation in the five age groups was: 5.58 ± 6.86; 4.40 ± 3.4; 4.14 ± 3, 4.61± 4.2; 2.98 ± 1, respectively by analysis of variance (P= 0.14). No significant difference in the mean weekly dose of oral anticoagulant was found between all age groups.

Table 1. Clinical characteristic of study population

		All (n= 100) (%)	Warfarin (n=90) (%)	Aceno-coumarol (n=10) (%)
Weekly dose, (mg)	Mean ± SD	4.35 ± 1.92	4.33 ± 1.91	4.45 ± 1.96
Age, y	Mean ± SD	54.51 ± 9.6	54.4 ± 9.65	54.8 ± 9.54
BMI, kg/m2	Mean ± SD	29.62 ± 6.1	29.6 ± 6.1	29.8 ± 6.08
Sex	Male	20 (20)	17 (85)	3 (15)
	Female	80 (80)	73 (91)	7 (8.7)
Family history	Yes	25 (25)	22 (88)	3 (12)
	No	73 (73)	67 (91.7)	6 (8)
Smoking	Yes	0	0	0
	No (current)	100 (100)	90 (90)	10 (10)
Level of physical activity	Physically active	20 (20)	18 (90)	2 (10)
	Not physically active	79 (79)	71 (89.8)	8 (10)
Indica-tion	Atrial Fibrillation + others	14 (14)	13 (92.8)	1 (7)
	Mitral Valve Replacement + others	53 (53)	48 (90.5)	5 (9)
	Aortic Valve Replacement + others	25 (25)	21 (84)	4 (16)
	Double valve replacement	18 (18)	16 (88.9)	2 (11)
	Tricuspid valve replacement and regurgitation	2 (2)	2 (100)	
Co-medications	NSAIDS	1 (1)	1 (100)	0
	Thyroid Hormones	7 (7)	7 (100)	0
	statins	10 (10)	8 (80)	2 (20)
Con-comitant diseases	Nothing	55 (55)	48 (87)	7 (12.7)
	Diabetes mellitus	18 (18)	18 (100)	0
	Hypertension	23 (23)	21 (91)	2 (8.7)
	Hypothyroidism	6 (6)	6 (100)	0
	Rheumatism	3 (3)	2 (66.7)	1 (33)
	Anaemia	1 (1)	1 (100)	0
	Bronchial Asthma	1 (1)	1 (100)	0

Table 2. Genetic characteristics of our (anticoagulated) patients

Polymorphism	Allele	N (%)	Genotype	N (%)
VKORC1				
3673G>A	G	187 (95.4)	GG	91 (91)
	A	9 (4.5)	GA	5 (5)
			AA	2 (2)
1173C>T	C	126 (63)	CC	39 (39)
	T	74 (37)	CT	48 (48)
			TT	13 (13)
CYP2C9				
430C>T	C	181 (90.5)	CC	81 (81)
	T	19 (9.5)	CT	19 (19)
			TT	0%
1075A>C	A	190 (95)	AA	91 (91)
	C	8 (4)	AC	8 (8)
			CC	0%

Table 3. Multiple linear regression analyses for variables responsible for stable oral anticoagulant dose (mean dose/week).

predictors	Unstandardized Coefficients			Standardized Coefficients	P value
	B	Std. Error	R Square	Beta	
(Constant)	7.085	1.639			0
BMI	-.042-	0.033	1.92%	-.134-	0.2
Age	-.015-	0.021	1.68%	-.076-	0.464
VKORC1(G>A)	-.171-	0.486	0.40%	-.036-	0.726
VKORC1 (C>T)	0.703	0.395	3.30%	0.179	0.078
CYP2C9*2	-.735-	0.503	2.60%	-.148-	0.147
CYP2C9*3	0.106	0.724	0%	0.015	0.884

Variables are defined as follow: VKORC1 (G>A) genotype: input 1 for GG, 0 for GA, AA; VKORC1(C>T) genotype input: 1 for CC, 0 for CT, TT; CYP2C9*2 genotype: input 1 for CC, 0 for CT; CYP2C9*3 genotype: input 1 for AA, 0 for AC; Age: input age in years; body mass index: input BMI in kg/m2:

3.4 Relationship between BMI and mean dose per week of oral anticoagulant

To determine the relation between stable dose and BMI, we used one-way ANOVA, our results indicated that there was no statistical difference among the different groups (P =0.66) i.e BMI has no effect on mean weakly dose requirements of the anticoagulant.

3.5 Multiple linear regression analyses for variables responsible for stable oral anticoagulant doses (mean dose/week)

The variability explained by the model of the multiple linear regression test was 8.6% using the genetic and non-genetic variables: VKORC1 (-1639G>A), VKORC1 (1173C>T), CYP2C9*2, CYP2C9*3, age, and body mass index (BMI) This means that all these predicted variables account for only 8.6% in dose variation, the F value was 0.20 which means that results were statistically insignificant.

4 DISCUSSION

The oral anticoagulant warfarin is more commonly prescribed to Libyan patients than acenocumarol for the treatment of many thromboembolic events like atrial fibrillation, valve heart diseases, and others.

The wide inter-individual variation in dose requirements of this medication was the main reason for us to investigate the contributing factors for such variation i.e. genetic factors including selected SNPs of CYP2C9 and VKORC1 polymorphism, and non-genetic factors such as age, and BMI.

The impact of CYP2C9*2, CYP2C9*3 and VKORC1 (-1639G>A) genetic polymorphism on oral anticoagulant dosage, has been previously studied [26–30].

The effect of VKORC1 1173C>T genotype had a more significant effect on oral anticoagulant dosage than VKORC1 (-1639 G>A); because patients carrying one or two variant alleles needed lower doses compared to wild type. These results are consistent with D'Andreaet al., [31], Moon et al., [32] , and Limdi et al. [33]. As for VKORC1 G>A genotypes, our results were consistent with those de-scribed by other researchers [26–29, 34–38].

In our study we found that GA carriers needed higher doses of oral anticoagulant than GG and AA carriers. This could be attributed to the small number of cases included in our study who carry GA, or AA variant alleles (5, and 2 cases respectively).

Although there are very few studies are done in African population, the majority of them are done in West African.

In addition, our results demonstrated no significant effect of CYP2C9 *2 and *3 variants bearers on oral anticoagulant dosage. These findings are discordant with those reported by others [26, 28, 32, 33, 37–40].

Several studies have reported that age plays an important role in dose requirement of oral anticoagulants. However, our results demonstrated no effect of age on drug dosage, this was in agreement with Wattanachaiet al. [27].

Height, BMI and weight have all been reported to be predictive factors for warfarin dosage , Alrashidet al. [38], Namaziet al. [41], Miao et al. [42], and Loebsteinet al. [43]).

Wattanachaiet al. [27] reported that the maintenance dose of warfarin was significantly and positively correlated with body weight, and BMI but height had no significant correlations with stable dose. Our results showed no effect of BMI on drug dose variability.

Multiple linear regression analyses of our results showed no significance effect of the combined genetic (VKORC1 and CYP2C9 polymorphisms), and non-genetic factors (age, BMI) on predicting the dose of oral anticoagulant. However, for the non-genetic factors our findings were in agreement with those of Wattanachaiet al. [27] and, Esmerianet al. [40] but were inconsistent with Alrashidet al. [38] Considering the genetic factors as predictors for drug dosage, our results are inconsistent with many studies including those of Ghozlanet al., and Esmerianet al. [29, 40].

In our patients, oral anticoagulant dosing was related to genetic variations, in particular VKORC1 1173C>T more than other factors (age and BMI).

In conclusion, this is the first time that the association between oral anticoagulant use and VKORC1, CYP2C9 genes polymorphism has been investigated in Libyan patients. Our study indicated that unlike VKORC1 variant alleles, the effect of CYP2C9*2 and *3 variant alleles on drug dosage was insignificant. The effect of VKORC1 1173CT was more prominent than VKORC1 -1639GA. Furthermore, neither the genetic nor the non-genetic factors could explain the inter-individual variability to the oral anticoagulants dose; and so, cannot be used as predictors to prescribe the right dose for Libyan patients.

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6 CONFLICT OF INTEREST

No conflict of interest.

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