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Warfarin Pharmacogenetics : Polymorphisms of the *CYP2C9*, *CYP4F2* , and *VKORC1* loci in a genetically admixed Omani Population

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Abstract

This is the first study to evaluate the spectrum and prevalence of dose-predictive genetic polymorphisms of the *CYP2C9, CYP4F2* and *VKORC1* loci together, in a geographically-defined, ethnically admixed healthy adult Omani population sharing common life style/environmental factors. Since the present day Omani population is the result of an admixture of Caucasian, African and Asian ancestries, we compared the pharmacogenetic profile of these three loci in this population. Interestingly, the Omani pharmacogenetic profile, in terms of allele and genotype distribution, has values that are intermediate between Caucasians and African Americans, the African admixture further substantiated by the presence of *CYP2C9**8 allele. However, limitations and usefulness of such comparisons warrant caution, as the data from pharmacogenetic literature do not always represent bonafide population categories. Furthermore, definition of study population based on microgeographical scale would be more appropriate in pharmacogenetic research rather than the flawed racial, ethnic or social categorizations since pharmacogenetic variation is clinal, and genetic influences will be further altered by lifestyle and environmental factors.

Key Words: Pharmacogenetics, CYP2C9; CYP4F2; VKORC1; Warfarin; Omani

Introduction

In the current clinical practice, routine pharmacogenetic testing is performed only for a few drugs and an important candidate is warfarin, a commonly prescribed anticoagulant with narrow therapeutic index. Small warfarin dose variations result either in hemorrhagic or thrombotic complications[1]. Initially genetic polymorphisms in cytochrome P450 2C9 [*CYP2C9*] and vitamin K epoxide reductase complex 1 [*VKORC1*] have been shown to affect the warfarin dose and thereby influence the occurrence of complications[1-4]. Subsequently, a genetic variant of *CYP4F2* locus was also found to be associated with warfarin dose variability and time to reach therapeutic international normalized ratio (INR) through a linked polymorphism[5,6]. Since the risk of bleeding, due to over-anticoagulation, occurs mainly at the initiation of warfarin therapy[7], knowledge of a patient's genotype could be beneficial in planning an initiation protocol that is likely to avoid this complication. Accordingly, inclusion of *CYP2C9, CYP4F2* and *VKORC1* genotype data in the existing algorithms is expected to reduce the risk of bleeding during the warfarin induction phase.

Nevertheless, most pharmacogenetic models explain less than 50% of the variation in the warfarin dose amongst treated patients. This implies that other factors (genetic, concurrent disease, or environmental) participate in the intra and inter individual variations in warfarin dose requirement. However, three subsequent large genome-wide association studies failed to confirm the existence of additional genetic loci, other than *CYP2C9, CYP4F2* and *VKORC1*[8-10]. Therefore, the unexplained part of the variability is believed to be essentially due to life style and environmental factors (including dietary bioavailability of vitamin K).

At the population level, significant differences are reported in the performances of dosing algorithms between groups loosely designated as Caucasians and African Americans, with only marginal benefit for African Americans during warfarin initiation therapy[6,11]. Similarly, performance was quite variable even within the so called east Asian population groups[12]. Such differences are believed to be due mainly to significant differences in the prevalence of functional polymorphisms in the genes encoding the key enzymes involved in the pharmacokinetic(*CYP2C9* and *CYP4F2*) and pharmacodynamic (*VKORC1*) behavior of warfarin/ vitamin K although, as mentioned above, other non-genetic factors could still

contribute to some extent. Given the fact that the frequency distribution of polymorphisms in *CYP2C9* and *VKORC1* varies across populations, influence of these genes could affect different proportions of individuals in a given population. Thus a clear understanding of the spectrum and prevalence of functionally relevant mutant alleles of these loci in a given geographically-restricted population group could help in designing targeted screening strategies for markers of drug response. In fact it is striking to note that the present trend in labeling populations into ethnicity/race is not accurate and hence does not represent the bonafide genetic structure and drug response profiles of a given study population. Moreover, since human genetic variation is greatly influenced by geography it is more appropriate to study the genetic structuring of geographically–defined populations for evaluating the efficacy and safety of drugs with narrow therapeutic index such as warfarin rather than using the flawed concepts of (self identified) race/ethnicity. Accordingly, we thus undertook to analyze the frequency distribution of warfarin dose-predictive genetic polymorphisms in the *VKORC1*, *CYP2C9* and *CYP4F2* loci, for the first time, in a geographically defined, ethnically admixed Omani population.

Materials & methods

Study population

The study was initiated after obtaining formal approval from the institutional ethics committee. Study subjects were unrelated healthy adult blood donors attending the blood bank at the Sultan Qaboos University Hospital, Muscat, Oman. A written informed consent was obtained from each participant as per our study protocol. A total of 240 subjects (180 males and 60 females) agreed to participate. Their mean age was 28.6($\pm$7.2 SD) years.

A 5ml blood sample was collected in tubes containing EDTA. Genomic DNA was isolated using the semi-automated ABI PRISM™ 6100 Nucleic Acid Prep Station, [Applied Biosystems, Foster city, CA, USA] and samples were stored at -20^{0}C pending analysis. All the DNA polymorphisms were studied by direct sequencing of the relevant PCR- amplified genome segment on ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems, Foster city, CA, USA) using home-designed primers.[available on request]

Genotyping

CYP2C9 & CYP4F2*:*

DNA segments encompassing exons 3 and 7 of *CYP2C9* and exon 11 of *CYP4F2* genes were amplified by PCR and the PCR products were submitted for DNA sequencing to explore the following single nucleotide polymorphisms(SNPs); *CYP2C9* *2 (430C>T), *3 (1075A>C), *4 (1076T>C), *5 (1080C>G), *8(449G>A),*11(1003C>T) and CYP4F2*3(1297G>A) respectively using a sequencing protocol provided by the manufacturer of the commercial kit. (Applied Biosystems, Foster city, CA, USA)

VKORC1:

The DNA segment containing the g.-1639G>A polymorphism (rs 9923231) and 6484 C>T or g.1173 C>T (rs 9934438) of the *VKORC1* locus (GeneBank accession number AY587020) were PCR-amplified and analyzed by DNA sequencing of the PCR product as above.

Statistical analysis:

Frequency distribution of the *CYPC2C9*, *CYP4F2* and *VKORC1* polymorphisms were compared by chi square and Fishers exact test. A p value of < 0.05 was considered as statistically significant. The observed allele frequencies were used in Hardy-Weinberg's equation for analyzing the degree of deviation, if any, between the observed and expected genotype frequencies by using weighted least square estimates of allele frequencies and chi-square goodness-of-fit tests, using SPSS software (ver.15) for all statistical analysis.

Results

***CYP2C9* allele & genotype frequencies**

Amongst a total of 240 subjects enrolled, genetic polymorphisms of *CYP2C9*, *CYP4F2*, and *VKORC1* loci were analyzed in 220,(Table 1) 192(Table 2) and 157(Table 3) samples respectively and the combined frequencies for all the three loci was available in 157 subjects(Table 4). The results obtained in this study were compiled and compared with the data reported for the Caucasian, African American and Asian populations[6]. The combined *CYP2C9* mutant allele (*CYP2C9* *2,*3 and *8) frequency for Omanis was 0.16, a value intermediate between that of Caucasians and African Americans. Similarly, the frequency of

warfarin-sensitive homozygotes or compound heterozygotes for the *CYP2C9* mutant allele was also of intermediate value between Caucasians and African Americans/Asians. The *CYP2C9* *4, *5, and *11 alleles were not found in the studied Omani subjects [direct sequencing of exons 3 and 7].

CYP4F2 allele & genotype frequencies

The variant allele and genotype frequencies of the *CYP4F2* locus are compiled with data for Caucasian, African American and Asian populations[6]. The observed *CYP4F2**3 allele and genotype frequency in Omanis was not different from that observed in Caucasians/Asians, but was quite significantly higher ($p<0.05$) than that reported for African Americans (3 and 10 fold respectively).

VKORC1 allele and genotype frequencies

The frequency of the minor allele *g.-1639A* in Omanis showed intermediate values between African Americans and Caucasians, but nevertheless threefold higher than African Americans and around three fourths of the frequency reported in Caucasians[6]. Nevertheless, Asians had the highest frequency of *VKORC1* variant allele (1.5 to 2 fold of Caucasians and Omanis respectively). A similar trend was noted for the homozygosity for this warfarin sensitive *VKORC1* allele.

Combined *CYP2C9*, *CYP4F2* and *VKORC1* frequencies

The combined frequency data for the *CYP2C9, VKORC*1 and *CYP4F*2 loci which allow the prediction of probable outcome if exposed to warfarin[6]. In these 157 Omani subjects, for whom we had the complete data for all the analyzed loci, individual warfarin sensitive genotype frequency of *CYP2C9* as well as *VKORC1* were seen in 3.7% and 14% respectively. However, none of these subjects were homozygous for the *CYP4F*2*3 allele (Table 4), which confers relative warfarin resistance[5,6]. Furthermore, amongst these 157 subjects, only 2 cases (1.2%) were bearing warfarin-sensitive genotype for both *CYP2C9* and *VKORC1* loci together.

Discussion

This is the first study documenting the frequencies of *CYP2C9, CYP4F2,* and *VKORC1* in healthy adult Omani subjects. Oman, is located in the southeastern part of the Arabian Peninsula with a native population of around 2 million. The Omani population consists of an admixture of Caucasian, African and Asian ancestries. Each of these populations, constituting the present day Omanis, had previously been shown to require a distinct average warfarin dose corroborating with the relative prevalence of warfarin sensitive alleles.

CYP2C9

CYP2C9 is the principal drug metabolizing enzyme that catalyzes the hydroxylation of warfarin[13,14]. The *CYP2C9* enzyme accounts for 80%–85% of the metabolism of the pharmacologically potent S-warfarin enantiomer. Seven principal alleles of *CYP2C9*, including *CYP2C9**2 (430C>T), *3 (1075A>C), *5 (1080C>G), *6 (818delA) *8(449G>A) , (1003C>T)*11 *and* (269T>C)*13 (http://www.cypalleles.ki.se)[15], are associated with a decreased metabolic efficiency of the *CYP2C9* enzyme[15-17]. Although more than 34 different *CYP2C9* variants have been described, only two common variants, *CYP2C9*2* and *CYP2C9*3* have consistently been shown to be polymorphic in various populations and have been demonstrated to show significant clinical variability in warfarin dose requirements with propensity for bleeding complications[1-3].

Direct sequencing of the two exons of *CYP2C9* was our option (instead of RFLP-based assessment of the two SNP's [i.e. *CYP2C9**2 and *3] as, by sequencing the exons 3 and 7 of the *CYP2C9* gene, we were able to study the status of six of the seven principal alleles responsible for significant reduction in the enzyme activity namely *CYP2C9**2,*3,*5, *6,*8 and *11 alleles[15-18] with the last four alleles occurring almost exclusively in Africans . This is relevant, as the present Omani population is known to have African admixture as Zanzibar was under Omani rulers. In our study, we observed that homozygotes and compound hetrozygotes for the mutant *CYP2C9**2,*3 and *8 alleles were present in about 3.7% of the Omani subjects with a heterozygote rate of 24.2% (Table 1) comparable with a previous report on Omani patients on warfarin therapy[19]. However, that study did not explore the

polymorphic state of the *VKORC1* and *CYP4F2 loci*, which are also associated with warfarin dose variability.

CYP4F2

The *CYP4F*2 locus has been shown to code the primary human liver microsomal vitamin K_1 oxidase and the *CYP4F*2*3 allele is associated with decreased steady-state hepatic concentrations of the enzyme with consequent reduction in the metabolism of vitamin K_1 and a higher warfarin requirement for therapeutic anticoagulant effect. Indeed subjects homozygous for *CYP4F2**3 allele require warfarin of about 1.2mg more than the wild type genotype[5]. Since *CYP4F*2*3 allele frequency in African American subjects is distinctly low they are believed to benefit less from the dosing algorithms that include this variant[6,20]. Furtheremore, since Omani subjects have a comparable frequency of *CYP4F2**3 allele with that of Caucasians, its effect in the algorithm will be similar, but quite marginal as we failed to find homozygous CYP4F2*3 allele in any of the subjects bearing the warfarin sensitive genotype for *CYP2C9* and *VKORC1*(Table 4). On the contrary, we speculate that *CYP4F2* typing will be particularly beneficial for east Asian population given the high frequency of both the *VKORC1* warfarin sensitive genotype and the relative warfarin resistant *CYP4F2* *3/*3 genotype.

VKORC1

Some common SNPs of *VKORC1* locus defining the *VKORC*1 haplotypes have been associated with clinically significant differences in warfarin maintenance dose[3]. Indeed a single SNP, either g.-1639 G>A in the *VKORC1* promoter or g.1173 C>T in intron 1 behaved as a tag SNP in distinguishing the warfarin sensitive *VKORC*1*2 haplotype from the other haplotypes[3,4,20]. Our study revealed that this warfarin sensitive tag-SNP g.-1639A or g.1173T was found in approximately 44% of the Omani population, with 14% in homozygous state.

Population Comparison of *CYP2C9*, *VKORC1* and *CYP4F2*polymorphism

Genetic polymorphisms in the *CYP2C9, VKORC1* and/or *CYP4F2* loci are thus far the only important genetic determinants for individual warfarin dose variations[8,9]. As the frequency distribution of these polymorphisms in the three genes varies across populations

(https://www.pharmgkb.org/), the relative contribution of the *CYP2C9, CYP4F2* and *VKORC1* to the warfarin dose will obviously be different among population groups[21]. Given the extensive admixture of the present-day Omani population with gene flow from (east) Africa, Europe and Asia, it is tempting to compare the prevalence data of Omanis with the above mentioned population groups (Tables 1-3). Indeed the allele and genotype distribution has values intermediate between Caucasians and African Americans apparently complying with the documented Caucasian and African admixture of the Omani population[22], and noticeably by the presence of *CYP2C9**8 allele in Omanis, a prevalent allele in the African population. However, limitations and usefulness of these comparisons and interpretations warrant caution as the terms Caucasians, African Americans and Asians used in pharmacogenetic literature do not represent population genetic categories, but rather a flawed concepts of racial, ethnic and social categories quite often self declared. This is an important consideration because human genetic variation is clinal and greatly influenced by geography[23]. Our study concerns a fully characterized distinct geographically-defined Omani population group, with unbiased racial, ethnic or social considerations. Indeed, regardless of admixture, individuals from this population share common lifestyle / environmental factors, which are also important determinants of warfarin dose response besides genetic factors.

GENETIC INTERACTIONS

It is interesting to note that although genetic polymorphisms in *CYP2C9*, *VKORC1* and/or *CYP4F2* are important, homozygosity or compound hetrozygosity for the major mutant alleles in *CYP2C9 [*2 and *3]* and *VKORC1* [g.-1639AA] contribute to warfarin sensitivity whereas, homozygosity for the mutant allele of *CYP4F2* [*3] confers relative warfarin resistance[3-5,10,14]. Thus, from a population perspective, the expected contribution of the *CYP2C9* and *VKORC*1 on the one hand and *CYP4F2* on the other will be opposite in determining the warfarin requirement. Indeed, homozygosity for the *CYP4F*2*3 has previously been associated with a relatively higher warfarin dose requirement in the Caucasian subjects[5]. Nevertheless, the population prevalence of homozygosity for the mutant allele *CYP4F2*3* in all studied Caucasian populations as well as the Omani population being similar, influence of this locus across such populations will be similar, leaving the *CYP2C9* and *VKORC1* loci as the main warfarin dose predictive markers in the Omani population.

This complies with the previous population studies wherein only *CYP2C9* and *VKORC1* were found to be the two major loci influencing the warfarin dose requirement[8]. Recently a paper by Limdi NA et al[24] concluded that warfarin-dosing algorithm incorporating *VKORC1* g.-1639G>A improved dose prediction across 3 racial groups namely Asian, Black and white subjects recruited across several countries in the cadre of IWPC. It was then concluded that although at the population level, the contribution of *VKORC1* towards warfarin dose requirements is higher in whites than nonwhites, genotype predicts similar dose requirements across racial groups.[24]

In our population, only 1.2% of Omanis showed warfarin sensitive *CYP2C9* and *VKORC1* genotypes together and hence were at the highest risk of bleeding complications if exposed to warfarin. None of these subjects however possessed the warfarin relative resistance *CYP4F2* genotype (*3/*3). Thus, altogether around one fourth of the Omani subjects are susceptible to warfarin treatment- related bleeding complications as compared to one fifth of the Caucasians based on the independent homozygosity for *CYP2C9* and *VKORC1* mutant genotypes (Tables 1 & 3).

In conclusion, this study for the first time establishes the allele and genotype frequencies of the three main genetic loci that are associated with the pharmacokinetic (*CYP2C9* & *CYP4F2*) and pharmacodynamic (*VKORC1*) variables for warfarin dose variations in a geographically explicit manner for the Omani population.

This study represents a resource for warfarin pharmacogenetic variations in a geographically-defined Arabian Gulf population, namely the Omanis. Since within this Gulf region, the history of population migration and the local effective population size differ, results obtained herein cannot be directly extrapolated to neighboring populations and requires perspective studies with structured sampling at a microgeographic scale[25]. At the global level, since many regions are still underrepresented in pharmacogenetic research, especially those like Africa and Asia where high disease burden and diversity prevails, cost-effective and safe pharmacologic interventions are needed.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Table 1: ALLELE AND GENOTYPE FREQUENCIES FOR *CYPC2C9* IN OMANI SUBJECTS AND THEIR COMPARISON WITH OTHER POPULATION GROUPS.

(a) Allele frequencies				
Allèle	**Caucasian[6][n=106]**	**Omani [n=220]**	**Afr. American[6][n=300]**	**Asian[6][n=102]**
*1	0.79	0.84	0.87	0.92
*2	0.15	0.08	0.03	0.03
*3	0.06	0.07	0.02	0.04
*8	0.00	0.01	0.05	0.01
*others	0.00	0.00	0.03	0.00
Mu	**0.21**	**0.16**	**0.13**	**0.08**
(b)Genotype frequencies (+expected %)				
Genotype				
Extensive metaboliser				
*1/*1	66.0(62.1)	72.1(71.1)	75.7(75.1)	86.3(84.9)
Intermediate metaboliser				
*1/*2, *1/*3, *1/*8 *others	25.5(33.4)	24.2(26.47)	22.7(23.1)	11.8#(14.5)
Poor metaboliser[Warfarin sensitive]				
*2/*2, *2/*3, *3/*3 *others	8.5#(4.4)	3.7(2.5)	1.7#(1.0)	2.0#(0.6)

Key: Mu – Mutant alleles (*CYP2C92,*3, *8 &*others together), * others – (*4,*5,*6,*11&*13); n=Number of subjects. Data from Ref. no. 6 is used for comparison**

 # p<0.05 when compared with Omani subjects. + Predicted Hardy-Weinberg frequencies.

Table 2: ALLELE AND GENOTYPE FREQUENCIES FOR *CYP4F2* IN NORMAL OMANI CONTROLS AND COMPARISON WITH OTHER POPULATION GROUPS.

(a) Allele frequencies

Allele	Caucasian[6]	Omani	Afr. American[6]	Asian[6]
*1	0.66	0.65	0.88	0.70
*3	0.34	0.35	0.12[#]	0.30

(b) Observed Genotype frequencies ([+]expected %)

Genotype	Caucasian	Omani (n=192)	Afr. American	Asian
CYP4F2 *1/*1	42.7 (43.3)	41.0(41.6)	78.0(78)[#]	48.0(48.3)
CYP4F2 *1/*3	46.3(45.0)	47.9(45.6)	20.7(20.6)[#]	42.0(42.4)
CYP4F2 *3/*3	11.0(11.7)	11.0(12.5)	1.3(1.4)[#]	9.0(9.3)

Data Compiled from Ref. No. 6 is used for comparison

 # p<0.05 when compared with Omani subjects.
[+] Predicted Hardy-Weinberg frequencies, n=Number of subjects.

Table 3: ALLELE AND GENOTYPE FREQUENCIES FOR *VKORC1* IN NORMAL OMANI CONTROLS AND COMPARISON WITH OTHER POPULATION GROUPS.

(a) Allele frequencies for *VKORC1 (g.-1639 G>A)*

	Observed frequency			
Allele	**Caucasian[6]**	**Omani**	**Afr. American[6]**	**Asian[6]**
-1639 G	**0. 594**	**0. 683**	**0.892**	**0.333**
-1639 A	**0. 406**	**0.317**	**0.108**	**0.667**

(b)Genotype frequencies for *VKORC1 (g.-1639 G>A)*

	Observed frequency ([+]expected %)			
Genotype	**Caucasian**	**Omani (n=157)**	**Afr. American**	**Asian**
G/G	**36.8 (35.3)**	**40.1 (46.6)**	**80.3 (79.5)**	**22.5 (11.1)**
G/A	**45.3 (48.2)**	**45.9 (43.3)**	**17.7 (19.3)**	**21.6 (44.4)**
A/A*[Warfarin sensitive]*	**17.9 (16.5)**	**14.0 (10.1)**	**2.0 (1.2)**	**55.9 (44.4)**

Data Compiled from Ref. No. 6 is used for comparison

[+] **Predicted Hardy-Weinberg frequencies, n=Number of subjects.**

Table 4: COMPARATIVE POPULATION FREQUENCIES FOR *CYP2C9*, *VKORC1* g.-1639G>A AND *CYP4F23 GENOTYPE**

CYP2C9	*VKORC1* g.-1639G>A	*CYP4F2**3 c.1297G>A	[#]Caucasian Freq. (%)	Omani [n=157] Freq. (%)	[#]Amer. African Freq. (%)	[#]Asian Freq. (%)
Extensive Metabolizer	**G/G**	**G/G**	**12.3**	**13.4**	**47.5**	**4.9**
		G/A	**9.4**	**12.1**	**11.2**	**3.9**
		A/A	**0.0**	**0.0**	**0.7**	**3.9**
	G/A	**G/G**	**11.3**	**11.5**	**10.9**	**10.8**
		G/A	**16.0**	**19.1**	**4.0**	**6.9**
		A/A	**3.8**	**1.9**	**0.0**	**1.0**
	A/A	**G/G**	**5.7**	**4.5**	**0.7**	**26.5**
		G/A	**5.7**	**4.5**	**0.7**	**21.6**
		A/A	**1.9**	**1.9**	**0.0**	**6.9**
Total			**66.1**	**72.1**	**75.7**	**86.4**
Intermediate Metabolizer	**G/G**	**G/G**	**4.7**	**7.0**	**15.9**	**3.9**
		G/A	**4.7**	**3.2**	**4.0**	**2.0**
		A/A	**0.9**	**0.6**	**0.4**	**2.0**
	G/A	**G/G**	**3.8**	**4.5**	**2.5**	**2.0**
		G/A	**5.7**	**5.7**	**0.4**	**0.0**
		A/A	**0.9**	**1.3**	**0.0**	**1.0**
	A/A	**G/G**	**1.9**	**0.0**	**0.7**	**0.0**
		G/A	**2.8**	**1.9**	**0.0**	**1.0**
		A/A	**0.0**	**0.0**	**0.0**	**0.0**
Total			**25.4**	**24.2**	**23.9**	**11.9**
Poor Metabolizer	**G/G**	**G/G**	**0.9**	**0.0**	**0.0**	**1.0**
		G/A	**3.8**	**0.6**	**0.0**	**1.0**
		A/A	**0.0**	**0.0**	**0.0**	**0.0**
	G/A	**G/G**	**2.8**	**0.6**	**0.4**	**0.0**
		G/A	**0.9**	**1.3**	**0.0**	**0.0**
		A/A	**0.0**	**0.0**	**0.0**	**0.0**
	A/A	**G/G**	**0.0**	**0.6**	**0.0**	**0.0**
		G/A	**0.0**	**0.6**	**0.0**	**0.0**
		A/A	**0.0**	**0.0**	**0.0**	**0.0**
Total			**8.4**	**3.7**	**0.4**	**2.0**

Extensive metabolizer: *CYP2C9**1/*1;
Intermediate metabolizer: *CYP2C9**1/variant;
Poor metabolizer: *CYP2C9* variant/variant

[#]Data combined from ref No 6 is used for comparison; n = number of subjects.