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ORIGINAL ARTICLE

Frequency Distribution of *CYP2C9* and *VKORC1* Mutations among Bulgarian Patients and their Importance for Anticoagulant Therapy

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SUMMARY

Background: **Genetic polymorphisms of CYP2C9 and VKORC1 play a major role in pharmacokinetics and pharmacodynamics of coumarin anticoagulants. The purpose of our study was to assess the relative frequency of the above mutations in Bulgarian population in order to predict bleeding tendencies and precisely manage the anticoagulant therapy during the postoperative period after cardiac surgery with extracorporeal circulation.**
Methods: **Genomic DNA samples from 200 Bulgarian patients subjected to cardiac surgery with extracorporeal circulation were analyzed for VKORC1 1639G>A and CYP2C9*2&*3 polymorphisms by real-time polymerase chain reaction (PCR), then allele frequencies of various genotypes were calculated by Hardy-Weinberg Equilibrium.**
Results: **Median patients' age was 63.9 ± 10.8 years; 66.5% were male. Median BMI was 28.6 ± 5.4 kg/m^2. Genotype distribution for CYP2C9 was *1/*1 - 51%, *1/*2 - 21%, *1/*3 - 13.5%, *2/*3 - 4%, *3/*3 - 2%, and *2/*2 - 1.5%. The calculated frequency of CYP2C9*1 allele was 74.25%, CYP2C9*2 allele was 13%, and CYP2C9*3 allele was 12.75%, and all allelic frequencies were in Hardy-Weinberg equilibrium (p-value = 0.358). The major VKORC1 genotype was G/A - 47%, followed by G/G - 35.5% and A/A - 17.5%). Based on Hardy-Weinberg Equilibrium, there was no significant difference between observed and expected frequencies (X - -3.779), presumably as a result of the homogeneity in the population.**
Conclusions: **Analysis of the data obtained in the course of the study suggested that identification of homozygous carriers of VKORC1-1639 G>A (rs9923231) in Bulgarians may be useful in developing recommendations for personalized therapy. On the contrary, homozygous carriers of CYP2C9*2 or *3, included only 4.5% of the studied patients, thus indicating that this group would benefit less from dosing algorithms. Our results demonstrated good agreement with the results obtained in other studies conducted in the Caucasian population.**
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KEYWORDS

CYP2C9, oral anticoagulants, pharmacogenetics, VKORC1

INTRODUCTION

Currently, the adverse drug events in hospital settings demonstrate high frequency [1,2]. Medicines that have been particularly connected include antiplatelets, anticoagulants, cytotoxic drugs, immunosuppressants, diuret-

ics, antidiabetics, and antibiotics [3,4]. Fatal adverse drug reactions are often attributable to hemorrhage, the most common suspected cause on the background of antithrombotic and anticoagulant treatment [5-7], and incorrect dosage is the most common error [4,8]. The application of molecular techniques and data analyses using the personalized approach to treatment allows the preliminary selection of genetic polymorphisms that are associated with hypersensitivity to anticoagulant therapy [6,8,9].

Nowadays, the most promising tool of personalized medicine in regard to clinical practice is the pharmacogenetic testing and the application of pharmacogenetic-based dosing algorithms [10-14].

Some of the gene complexes determining drug metabolism are cytochrome P450 (CYP) genes [15-17]. Among the members of the *CYP2C* subfamily of cytochrome P450, CYP2C9 is the most abundant isoform in the liver, metabolizing a wide range of clinically relevant drugs. Functional polymorphisms in the *CYP2C9* gene, especially the *2 and *3 alleles result in decreased metabolism of *CYP2C9* substrates and are potential sources of alteration in initial anticoagulant treatment [18].

Vitamin K epoxide reductase catalyzes the formation of vitamin K hydroxyquinone, which is essential for the synthesis of coagulation factors II, VII, IX and XI, as well as proteins C and S [19]. The enzyme vitamin K epoxide reductase, which is the pharmacodynamic target of oral anticoagulants, is encoded by the *VKORC1* gene. Similar to *CYP2C*, the genetic variants of *VKORC1* have been noted to be predictive of patient's response in order to ensure the effective anticoagulation and to minimize the bleeding risk [20-22]. The polymorphisms of *VKORC1* and *CYP2C9* play an increasingly important role in the inter-individual variability in anticoagulant response during the treatment with oral anticoagulants (warfarin, acenocoumarol, etc.) [9,22-24].

The distribution of *CYP2C9* and *VKORC1* polymorphisms depend on interethnic variability and demographic factors [25,26]. That is why it is so important to evaluate the frequency of these allelic and genotype variability and to determine a scientific based pre-treatment approach. In the present study, we analyzed the frequency of these polymorphisms in a cohort of Bulgarians and compared it with similar data from other populations.

MATHERIALS AND METHODS

Design of the study

Peripheral blood samples from 200 unrelated pre-operative Bulgarian patients at University Hospital "St. Ekaterina" Sofia, who gave informed consent for the use of their DNA for research, were obtained in agreement with the Research Ethics Committee at Medical University-Sofia. Isolated DNA samples were tested anonymously.

Genotyping

For genotyping, DNA was isolated from EDTA anticoagulated peripheral blood samples using a silica membrane spin column based manual isolation procedure, using a starting volume of 200 µL, and an elution volume of 100 µL. The isolates were then analyzed by real-time polymerase chain reaction (PCR), in a reaction volume of 20 µL, using a commercially available diagnostic PCR kit for detection and discrimination of *CYP2C9*2* (rs1799853) allele, *CYP2C9 *3* (rs1057910) allele, and *VKORC1*-1639 G>A (rs9923231) allele (gb PHARM Warfarin, Generi Biotech, Czech Republic). The kit contains three distinct master mixes, each of which includes a primer-probe set for duplex detection of a specific mutation and its corresponding wild-type allele. Additionally, for each mutation site, the kit contains three positive controls (homozygous mutant = MUT, homozygous wild type = WT, heterozygous = HET) - a total of nine positive controls. The fact that for each patient sample, either the wild-type or the mutant allele was expected to produce a distinct signal, was used as a straight-forward and efficient inhibition and process control, and consequently no additional internal control assay was utilized. Contamination was controlled by following a unidirectional 3-zone workflow, and using an NTC to monitor for accidental contamination events in each PCR run. The experiments were performed on a 5-channel Rotor-Gene Q system (Qiagen, Germany). The results were analyzed for each of the three assays separately, using the allelic discrimination plugin on the instrument software. Within each duplex system, a signal in the FAM channel indicated a wild-type allele, and a signal in the HEX channel indicated a mutant allele. Presence of only one signal indicated a homozygous genotype, while presence of signals in both FAM and HEX was indicative of a heterozygote. The wild-type *CYP2C9* allele (*1) was assigned in case of absence of any detectable mutant alleles. After the analysis, allele frequencies were calculated.

Statistical analysis

Allele frequencies of various genotypes were calculated by Hardy-Weinberg Equilibrium (Genepop version 4.7.5) [27]. p-value of ≤ 0.05 was considered statistically significant.

RESULTS

Demographic characteristics

The demographic characteristics of the studied patient group are summarized in Table 1. There were a total of 133 (66.5%) men and 67 (33.5%) women. The median age was 63.9 ± 10.8 years.

Table 1. Demographic characteristics of the studied patient cohort.

Demographic characteristics (n = 200)		Mean (range or %)
Age (years)		63.9 ± 10.8
Gender	male	133 (66.5%)
	female	67 (33.5%)
Active smokers		18 (9%)
BMI (kg/m^2)		28.6 ± 5.4

Table 2. *CYP2C9* & *VKORC1* allele and genotype frequencies.

Genotype	Percent/frequencies, n = 200	Gender		Allelic frequencies
		m/f	Significance	
***CYP2C9* allele & genotype**				
*1/*1	57.0 (57.3) &	70/44	NS	Allele (*1) = 74.25% Allele (*2) = 13% Allele (*3) = 12.75%
*1/*2	21.0 (20.3) &	30/12	NS	
*1/*3	13.5 (14.1) &	20/7	NS	
*2/*2	1.5 (1.8) &	2/1	NS	
*2/*3	4.0 (5.0) &	7/1	NS	
*3/*3	2.0 (1.4) &	4/2	NS	
***VKORC1*-1639 G>A genotype**				
G/G	35.5 (35.4) &	42/29	NS	Allele (G) = 59% Allele (A) = 41%
G/A	47.0 (48.2) &	66/28	NS	
A/A	17.5 (16.4) &	25/10	NS	

& - Values, obtained by Hardy-Weinberg Equilibrium, m - male, f - female.

Table 3: Combined *CYP2C9* & *VKORC1* frequencies.

Genotype	Number (%), n = 200	Gender	
		m/f	Significance
G/G *1/*1	48/200 (24%)	21/17	NS
G/G *1/*2	13/200 (6.5%)	10/3	p = 0.004
G/G *1/* 3	7/200 (3.5%)	3/4	NS
G/G *2/*2	0/200 (0%)	0/0	NS
G/G *2/*3	6/200 (3%)	5/1	NS
G/G *3/*3	4/200 (2%)	4/0	NS
G/A *1/*1	56/200 (28%)	40/16	NS
G/A *1/*2	20/200 (10%)	12/8	NS
G/A *1/*3	12/200 (6%)	11/1	NS
G/A *2/*2	2/200 (1%)	2/0	NS
G/A *2/*3	2/200 (1%)	2/0	NS
G/A *3/*3	2/200 (1%)	0/2	p = 0.048
A/A *1/*1	19/200 (9.5%)	12/7	NS
A/A *1/*2	10/200 (5%)	8/2	NS
A/A *1/*3	6/200 (3%)	5/1	NS
A/A *2/*2	0/200 (0%)	0/0	NS
A/A *2/*3	0/200 (0%)	0/0	NS
A/A *3/*3	0/200 (0%)	0/0	NS

Table 4: Genotype frequencies of *CYP2C9* and *VKORC1* gene polymorphisms in different ethnic groups.

Genotype	Frequency (%)					
	Present study	African-American	Asian	Caucasian	Significance (p-value)	References
***CYP2C9* allele & genotype frequencies**						
*1/*1	57.0	75.7	86.3	67.0	NS Present study/Asian p = 0.014	[30-34,36]
*1/*2	21.0	4.3	3.9	17.1	p < 0.001	
*1/*3	13.5	3.3	6.9	13.0	p = 0.046	
*2/*2	1.5	0.3	1.0	3.0	NS	
*2/*3	4.0	0.3	0.0	0.0	NS	
*3/*3	2.0	0.0	0.0	0.0	NS	
***VKORC1*-1639 G>A genotype frequencies**						
G/G	35.5	80.3	22.5	39.0	p < 0.001	[21,29,30,32-36]
G/A	47.0	17.7	21.6	47.0	p < 0.001	
A/A	17.5	2.0	55.9	14.0	p < 0.001	

CYP2C9 & VKORC1 allele and genotype frequencies

The *CYP2C9*2* (rs1799853) allele, *CYP2C9*3* (rs1057910) allele, and *VKORC1*-1639 G>A (rs9923231) gene frequencies are summarized in Table 2.

Approximately 57% (114/200) of the studied population had the wild type of *CYP2C9* genotype (*1/*1). The remaining patients had mutant variants with *1/*2 being the most prevalent genotype variant (42/200, 21%) followed by *1/*3 (27/200, 13.5%), *2/*3 (8/200, 4%), *3/*3 (6/200, 2%) and *2/*2 (3/200, 1.5%). Based on Hardy-Weinberg Equilibrium, there was no significant difference between observed and expected *1/*1, *1/*2 and *2/*2 allele frequencies (X - -3.693), but the observed allele *3/*3 frequencies were higher than expected (X - -1.468). The calculated frequency of the wild-type *CYP2C9*1* allele was 74.25%, *CYP2C9*2* allele was 13%, and *CYP2C9*3* allele was 12.75%, and all alleles were in Hardy-Weinberg equilibrium (p-value = 0.358).

The major *VKORC1* haplotype was G/A (94/200, 47%), followed by G/G (71/200, 35.5%) and A/A (35/200, 17.5%). Based on Hardy-Weinberg Equilibrium, there was no significant difference between observed and expected frequencies (X - -3.779), presumably as a result of the population's homogeneity. No significant differences between male and female individuals were observed. The calculated allelic (A) frequency was 59% and that of the wild-type allele (G) was 41%, and both alleles were in Hardy-Weinberg equilibrium (p-value = 0.751).

Based on *CYP2C9* and *VKORC1* genotype analyses, no significant differences between male and female individuals were observed.

Combined CYP2C9 & VKORC1 frequencies

Table 3 summarizes the combined CYP2C9 and VKORC1 genotype frequencies for all studied individuals. Different allelic combinations of both genes were obtained. Fifty-six (56/200, 28%) individuals had both CYP2C9 polymorphisms and AG/AA haplotype of VKORC1 gene.

DISCUSSION

In the present study, we evaluated the frequency of polymorphisms of *VKORC1* and *CYP2C9* genes in Bulgarian population. According to the published data, the patients who carry the A allele at position -1,639 *VKORC1* and the variants *CYP2C9 *2* and **3* require a significantly lower mean daily dose of oral vitamin K antagonists [28].

VKORC1-1639 G>A (rs9923231), an allele critical for pharmacogenetic-based dosing, was found to be quite prevalent in Bulgarian population, occurring in about 64.5% of the subjects, comprising 35 homozygous and 80 heterozygous carriers out of 200 studied individuals. In contrast to African - Americans and Asians [30-32], the *VKORC1*-1639 G>A genotype frequency in Bulgarian individuals or in individuals of European descent, was more consistent ($p < 0.001$ for all *VKORC1*-1639 G>A genotypes) [29,36]. In the context of the Bulgarian population, heterozygous *VKORC1*-1639 G>A would appear to be the most critical genetic factor for consideration in dosing of vitamin K inhibitors, owing to its prevalence. Based on these frequencies, approximately four out of 20 Bulgarian individuals are *VKORC1*-1639 G>A (rs9923231) homozygotes who may benefit from the assessment of this allele using pharmacogenetic-

based dosing algorithms. Consequently, upward adjustment of maintenance doses to achieve an optimal clinical response would seem logical when such responses are not observed.

Similar to the Caucasian population [36] (Table 4), the presence of *CYP2C9*2* and **3* allelic variants in Bulgarian population predicts a *CYP2C9* intermediate enzymatic activity. In contrast with *VKORC1*-1639 G>A (rs9923231) allelic frequency, only 4.5% (approximately one in 20) individuals were *CYP2C9*2* or **3* homozygotes, indicating that this population would benefit less from dosing algorithms that include this pharmacogenetic variant. Genotype analyses showed that the homozygous *CYP2C9*2/*2* and *CYP2C9*3/*3* and compound *CYP2C9*2/*3* genotypes have a low frequency in both the Bulgarian population and the indicated ethnic groups [30-34,36].

CONCLUSION

In conclusion, our findings suggest that heterozygous *VKORC1*-1639 G>A and *CYP2C9*1/*2* are prevalent genotypes, which may influence the pharmacokinetics of anticoagulant therapy with vitamin K inhibitors in Bulgarian individuals. Additionally, the presence of combined genotypes clearly makes a strong case for genetic screening prior to anticoagulant treatment, and drug response monitoring afterwards until stability in patients is achieved. In that manner, genotype-guided therapy would be more effective to ensure a sufficient anticoagulant effect in a specific patient cohort and to minimize bleeding incidences.

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Declaration of Interest:
VN is an employee and shareholder in SGP Bio Dynamics Ltd., which has provided the instrument and reagents used for conducting the experiment.

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