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CLINICAL STUDY

Frequencies of polymorphisms in *CYP2C9* and *VKORC1* genes influencing warfarin metabolism in Slovak population: implication for clinical practice

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Abstract: *Objectives:* The study was aimed at establishing an effective molecular-genetic method for detecting polymorphisms in genes *CYP2C9* and *VKORC1*, which affect the pharmacogenetics of warfarin, and at determining their prevalence in Slovak population.
Background: Warfarin, derivative of coumarin, belongs to the most commonly prescribed oral anticoagulants with narrow therapeutic index. An insufficient dose of warfarin can result in failure to produce the antithrombotic effect, whereas an overdose increases the risk of bleeding. It was proven that genetic variability in two genes, *CYP2C9* a *VKORC1*, has a significant influence on the individual's response to the dosage of warfarin.
Methods: In a control group of 112 randomly selected individuals, we tested the frequency of selected single nucleotide polymorphisms including CYP2C9*2 (430C>T), CYP2C9*3 (1075A>C), VKORC1*2 (1173C>T) by allele-specific Real-Time PCR and VKORC1*2 (-1639G>A) by using PCR-RFLP.
Results: Due to the combination of frequent alleles CYP2C9*2, CYP2C9*3 and VKORC1*2 in Slovak population we determine that 25% of population need a standard 5-mg daily dose of warfarin, while 44%, 23%, and 8% need 4 mg, 3 mg and 2 mg of warfarin per day.
Conclusion: Slovak population is in Hardy-Weinberg equilibrium and frequencies of SNPs were in accordance with other published results in European populations *(Tab. 5. Fig. 3, Ref. 51)*. Text in PDF *www.elis.sk.*
Key words: warfarin, pharmacogenetics, CYP2C9, VKORC1, polymorphisms.

Warfarin as well as other 4-hydroxycoumarin derivates is used as an oral anticoagulant. It functions as an antagonist of vitamin K, which is a necessary factor of the blood clotting cascade (1). Nowadays it is one of the drugs most commonly used in prevention or therapy of some disorders. High risk of embolisms, heart attack, atrial fibrillation, some of inherited blood coagulation diseases, and conditions after surgery (e.g. heart valve) are treated with warfarin. Also, it is used as a prevention treatment in patients at high risk of stroke or in relapse of deep vein thrombosis (2). The essential anticoagulant effect of warfarin is induced by the inhibition of vitamin K epoxide reductase (VKOR), which leads to a decrease in vitamin K-dependent coagulation protein synthesis (3). The commercially used warfarin is a racemic mixture of S- and R-enantiomers. These isoforms have mutually different metabolic effects. S-warfarin is about five times more active than R-warfarin (4). After oral intake, warfarin is transported to the liver, where R enantiomer is metabolized by cytochromes (CYP1A1, CYP1A2 and CYP3A4) and more effective S enantiomer is metabolized by cytochrome P450 (CYP2C9) (5). Gamma-glutamyl carboxylase (GGCX) participates in the activation of the hypofunctional prothrombin clotting factors II, VII, IX, X to its active form (6). A reduced form of vitamin K influenced by VKOR takes part in the clotting process (Fig. 1). VKOR influences both warfarin enantiomers, which leads into a decrease in their efficiency (7).

The enzyme activity affecting the metabolism of warfarin can be expressed at different phenotypes levels, usually called as NM – normal metabolizers, IM – intermediate metabolizers, and PM – poor metabolizers. The time required to achieve stable concentration of warfarin in serum varies also in dependence on the genotype of each patients (8).

According to up-to-date knowledge, warfarin metabolism is mainly influenced by genetic factors such as polymorphisms affecting *CYP2C9* enzyme activity and pharmacokinetics (4) and pharmacodynamics of *VKORC1* (9).

CYP2C9 is located on chromosome 10 and consists of 9 exons and 8 introns. The *CYP2C9* gene is highly polymorphic. Two allelic variants, CYP2C9*2 (430C>T, rs1799853) and CYP2C9*3 (1075A>C, rs1057910) differing from the wild-type CYP2C9*1

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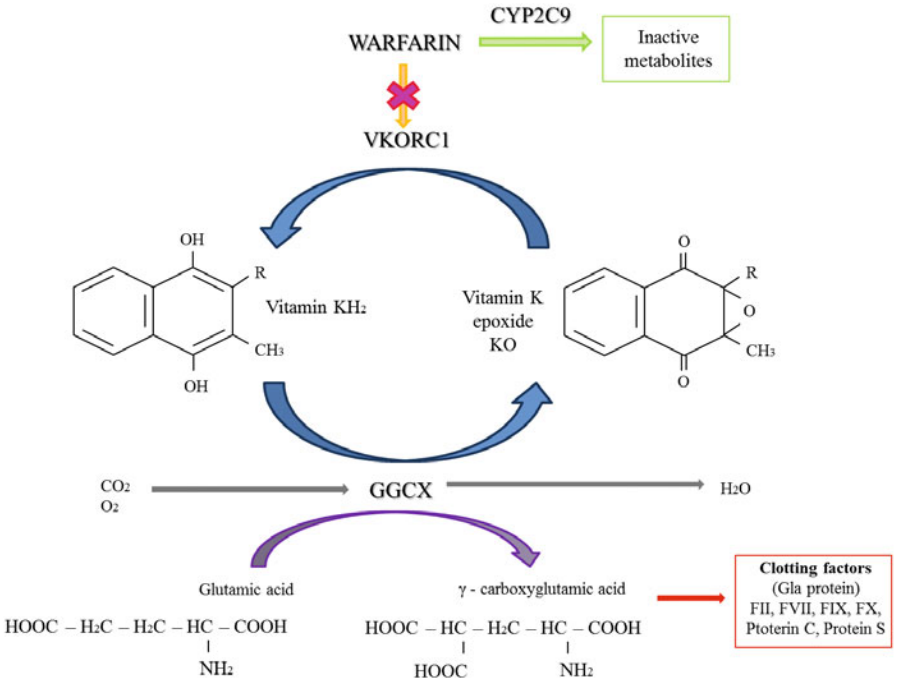


Fig. 1. Mechanism of action of warfarin and roles of CYP2C9 and VKORC1 in modulating anticoagulation. Warfarin exerts its pharmacological effect by inhibiting VKORC1. VKORC1 is the vitamin K cycle enzyme controlling regeneration of reduced vitamin K, an essential cofactor that drives formation of the clotting factors. CYP2C9 is the major P450 enzyme that metabolizes *S*-warfarin to inactive metabolites. CYP2C9 – Cytochrome P4502C9; VKORC1 – vitamin K epoxide reductase complex 1, GGCX – gammaglutamyl carboxylase.

by a single point mutation, have been associated with reduced metabolic clearance of CYP2C9 substrates (such as warfarin and phenytoin) as compared to the wild-type. Thus a standard dose may result in raised serum drug levels (10). CYP2C9*2 is located in exon 3 and the incidence of allele T in Caucasians is 10–20 %. CYP2C9*3 is located in exon 7 and the incidence of allele C in Caucasians is 8 % (11). Both of these allelic variants are less frequent in Asian and Afro-American people (12). Polymorphic forms of enzymes lead to a decrease in hydroxylation of S-warfarin *in vitro* and patients with this genotype belong to the PM phenotype. In the early stages of therapy, PM phenotypes are at risk of increased bleeding after warfarin treatment. These individuals require lower doses of warfarin to achieve the desired anticoagulant effect compared to patients with at least one CYP2C9*1 (WT) haplotype (13). The enzyme activity is reduced to 12 % (CYP2C9*2) and 5 % (CYP2C9*3), respectively to WT (14). Studies show that *CYP2C9* genotype is responsible for 12 % of interindividual variability in response to warfarin therapy (12).

VKORC1 is located on chromosome 16 and consists of 3 exons and 2 introns (1). Common polymorphisms in the regulatory region of *VKORC1* gene correlate strongly with warfarin response across the normal dosing range (15). Polymorphisms in *VKORC1* gene significantly change the pharmacodynamics and dosage adequacy of warfarin (16). The most frequent and most deeply studied polymorphisms are VKORC1 -1639G>A and 1173C>T. These SNPs, in the promotor and first intron of *VKORC1* gene influence the enzymatic activity of VKOR (17) which is an enzyme in vitamin K cycles and the pharmacological target of coumarins (18). VKORC1 1173C>T, rs9934438 is in almost complete linkage disequilibrium with the polymorphism of VKORC1 -1639G>A, rs9923231 where both associate with increased sensitivity to warfarin (16). These SNPs are responsible for 30 % of interindividual variability in warfarin treatment (7). Haplotype VKORC1*2 is present in 42 % of Europeans (19) and is relatively rare in Asian population (20).

The management of warfarin therapy is challenging because on the one hand there is a large variability in dose requirements (0.5–60 mg per day) necessary for effective therapy and on the other hand there is the risk of life-threatening bleeding (21). The range of warfarin doses for individual genotypes is based on numerous conducted studies. To determine the warfarin dose, we have to take into account age, race, weight, height, sex, other medications, as well as polymorphisms in genes *CYP2C9* and *VKORC1* (22). The prediction of dosing based on genotypes is presented in Table 1 (23).

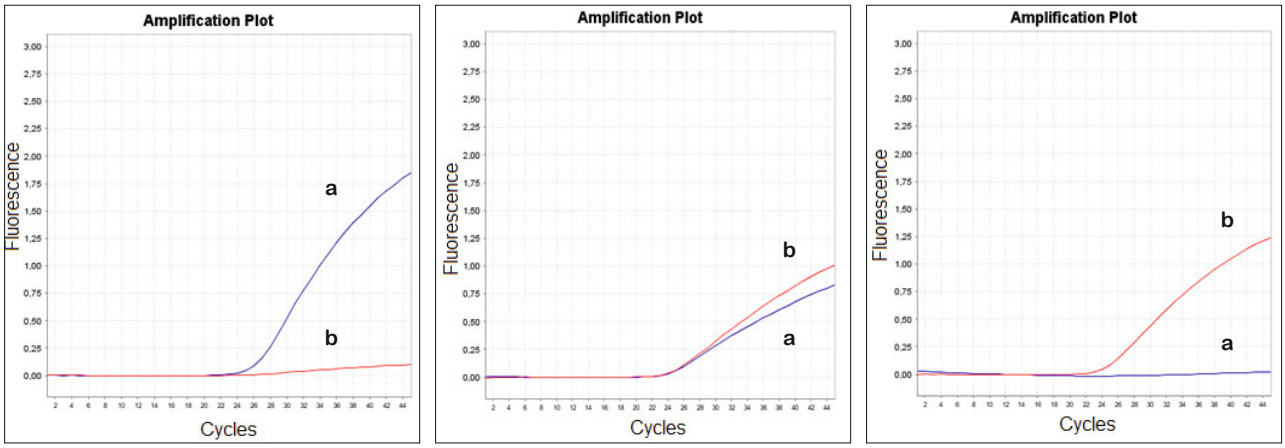
Material and methods

The control group consists of 112 individuals, all healthy subjects of Caucasian origin. Participants signed an informed consent before the study. They were randomly selected and never treated with warfarin. Blood samples for DNA extraction were collected in 3-mL tubes containing potassium EDTA. DNA was extracted from 200 µl whole blood by using DNA NucleoSpin Blood Kit (Macherey-Nagel). The frequencies of selected single

Tab. 1. Influence of genotype effect on warfarin dose per day.

VKORC1 genotype	CYP2C9 genotype					
	*1/*1	*1/*2	*1/*3	*2/*2	*2/*3	*3/*3
*1/*1	5 – 7 mg	5 – 7 mg	3 – 4 mg	3 – 4 mg	3 – 4 mg	0.5 – 2 mg
*1/*2	5 – 7 mg	3 – 4 mg	3 – 4 mg	3 – 4 mg	0.5 – 2 mg	0.5 – 2 mg
*2/*2	3 – 4 mg	3 – 4 mg	0.5 – 2 mg	0.5 – 2 mg	0.5 – 2 mg	0.5 – 2 mg

The range of expected warfarin dosage based only on polymorphisms in genes *CYP2C9* (CYP2C9*2, 430C>T, rs1799853 and CYP2C9*3, 1075A>C, rs1057910) and *VKORC1* (VKORC1*2, -1639G>A, rs9923231) and their combination (23). To determine the warfarin dose, we have to take into account age, race, weight, height, sex and other medications.



A – Wild type **B** – Heterozygous **C** – Mutant

Fig. 2. Schematic pictures of Allele-specific Real-Time PCR of polymorphisms in genes *CYP2C9* (430C>T, rs1799853 and 1075A>C, rs1057910) and *VKORC1* (1173C>T, rs9934438) (a – Wild-type allele, b – mutant allele).

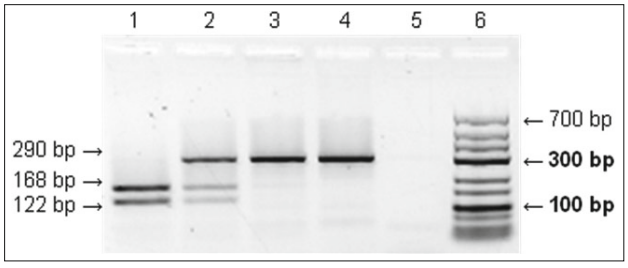


Fig. 3. PCR-RFLP of *VKORC1* -1639G>A, rs9923231 by *HpaII*. 1 – Wild-Type with GG genotyp (168 + 122 bp), 2 – Heterozygot with GA genotyp (290 + 168 + 122 bp), 3 – Mutant with AA genotyp (290 bp), 4 – Undigested control (290 bp), 5 – Negative control, 6 – Molecule marker - O´GeneRuler Low Range DNA Ladder (700, 500, 400, 300, 200, 150, 100, 75, 50 and 25 bp).

nucleotide polymorphisms CYP2C9*2 (430C>T), CYP2C9*3 (1075A>C), VKORC1*2 (1173C>T) were tested in a control group (112 randomly selected individuals) by allele-specific Real-Time PCR. The reaction was performed in 10-µl volumes containing approximately 100 ng of DNA. The assay for identification of CYP2C9 and VKORC1 alleles were obtained from Applied Biosystems: C__25625805_10 for CYP2C9*2 (430C>T) rs1799853, C__27104892_10 for CYP2C9*3 (1075A>C) rs1057910 and C__30204875_10 for VKORC1*2 (1173C>T) rs9934438. The amplification of PCR consisted of an initial denaturation step at 95 °C for 10 min followed by 45 amplification cycles (95 °C for 15 sec and 60 °C for 60 sec). The frequencies of SNP VKORC1*2 (-1639G>A) rs9923231was tested in a control group (112 randomly selected individuals) by using PCR-RFLP. The PCR reaction was performed in 20 µl volumes containing approximately 100 ng of DNA, 2xPCR Master Mix (Fermantas), 0.5 µM of each primer (Forward 5´ GCCAGCAGGAGAGGGAAATA 3´ and Rewerse 5´AGTTTGGACTACAGGTGCCT 3´). The amplification of PCR consisted of an initial denaturation step at 95 °C for 2 min followed by 35 amplification cycles (95 °C for 15 sec, 59 °C for 30 sec, 72 °C for 60 sec) and final polymerization step at 72 °C for 7 min. After PCR reaction, all PCR products were digested by restriction enzyme *HpaII* (Fermentas) which cuts when -1639G>A polymorphisms is not present, and visualized by agarose gel electrophoresis.

To find out if the Slovak population is in to the Hardy-Weinberg equilibrium the results were evaluated by chi-square test. Allele's frequencies of different populations were determined and compared using Z-test.

Results

We determine the genotypes of CYP2C9*2 (430C>T) rs1799853, CYP2C9*3 (1075A>C) rs1057910 and VKORC1*2 (1173C>T) rs9934438 by allele-specific Real-Time PCR (Fig 2) and VKORC1*2 (-1639G>A) rs9923231 by PCR-RFLP (Fig. 3). The frequency rate of selected polymorphisms in genes *CYP2C9* and *VKORC1* influencing the pharmacogenetics of warfarin was tested in the control group of 112 randomly selected individuals.

Tab. 2. Haplotype frequency of *CYP2C9* a *VKORC1* in the control group of 112 randomly selected subjects.

	*1/*1	*1/*2	*2/*2	*1/*3	*2/*3	*3/*3
CYP2C9	77	16	2	14	3	0
VKORC1	41	58	13	–	–	–

Tab. 3. Genotype frequency of *CYP2C9* a *VKORC1* in the control group of 112 randomly selected subjects.

Gene and substitution	*CYP2C9* 430C>T	*CYP2C9* 1075A>C	*VKORC1* -1639G>A	*VKORC1* 1173C>T
Wild-Type	CC - 91 (81.25%)	AA - 95 (84.82%)	GG - 41 (36.61%)	CC - 41 (36.61%)
Heterozygous	CT - 19 (16.96%)	AC - 17 (15.18%)	GA - 58 (51.79%)	CT - 58 (51.79%)
Mutant	TT - 2 (1.79%)	CC - 0 (0.00%)	AA - 13 (11.61%)	TT - 13 (11.61%)

We defined the haplotypes (Tab. 2) and genotypes (Tab. 3) frequencies. To detect significant differences in the frequency of alleles between Slovak and other populations (Tabs 4 and 5) we used a statistical test method Z.

Discussion

The implementation of molecular-genetic diagnostic methods in pharmacology has brought forward the fact that the drugs with a narrow therapeutic index are now safer for setting up a proper dose in view of individual genome variations and individualized therapy. Warfarin belongs to this group of drugs, and their effect is influenced by polymorphisms in genes *CYP2C9* and *VKORC1*. Detection of genotype before initiating the therapy could help avoid complications right at the beginning but also during long-term treatment.

Tab. 5. Percentage frequencies of polymorphisms VKORC1*2 in different population.

Population	n	%	p	References
		VKORC1*2	VKORC1*2	
Slovak	112	38		
British	297	47	0.10	(22)
Americans	1119	37	0.84	(43)
Afro-Americans	378	10	**0.00**	(43)
Italian	147	40	0.74	(7)
Canadian	126	39	0.87	(44)
Japanese	828	91	**0.00**	(32)
French	563	42	0.43	(45)
Swedish	181	39	0.86	(38)
Chinese	273	92	**0.00**	(17)
Turkish	205	50	**0.04**	(41)
Holland	1756	39	0.83	(42)
European-Americans	216	50	0.44	(46)
Ethiopian	154	20	**0.00**	(47)
Lithuanian	67	35	**0.04**	(48)
Iranian	126	55	0.43	(49)
Caucasians	92	49	0.14	(17)
Greek	98	48	0.77	(50)
Romanian	332	42	0.13	(51)

Frequencies of polymorphisms in gene *VKORC1* (VKORC1*2) and comparison of our results with frequencies in other populations. Statistic evaluation of results at significance level of $p<0.05$. Significant differences between Slovak and other populations are underlined.

It is estimated that seven million people are treated with warfarin worldwide. In Europe, it is approximately two million patients. However, more than 20 % of them are hospitalized in the first six months because of bleeding complications caused by anticoagulant treatment (9). The pharmacogenetics of warfarin is significantly influenced by genetic factors such as the prevalence of polymorphisms in genes *CYP2C9* and *VKORC1*.

Tab. 4. Percentage frequencies of polymorphisms CYP2C9*2 and CYP2C9*3 in different population.

Population	n	%		p		References
		CYP2C9*2	CYP2C9*3	CYP2C9*2	CYP2C9*3	
Slovakia	112	10	8			
British	948	13	7	0.32	0.71	(22)
American	935	11	6	0.74	0.46	(27)
Italian	365	13	11	0.37	0.32	(7)
Taiwanese	37	0	3	0.00	0.19	(28)
Malaysian	27	0	11	0.00	0.65	(29)
Israel	384	13	8	0.37	1.00	(30)
Canadian	189	11	8	0.78	1.00	(31)
Japanese	828	0	2	0.00	0.02	(32)
Russian	62	9	5	0.83	0.43	(33)
Uruguayan	53	13	11	0.58	0.55	(34)
French	126	12	9	0.62	0.78	(35)
Croatian	181	17	7	0.08	0.75	(36)
Singapore	59	0	3	0.00	0.14	(37)
Swedish	1487	11	7	0.73	0.71	(38)
Chinese	178	0	4	0.00	0.18	(39)
Brazilian	103	1	1	0.00	0.01	(40)
Turkish	205	13	10	0.42	0.55	(41)
Holland	1525	13	6	0.31	0.45	(42)

Frequencies of polymorphisms in gene *CYP2C9* (CYP2C9*2 and CYP2C9*3) and comparison of our results with frequencies in other populations. Statistic evaluation of results at significance level of $p<0.05$. Significant differences between Slovak and other populations are underlined.

We determined the SNPs genotype *CYP2C9*2, CYP2C9*3* and *VKORC1*2* of 112 individuals by using allele-specific Real-Time PCR and PCR-RFLP. Frequencies of alleles with polymorphisms found in the current study are 10 % (CYP2C9*2), 8 % (CYP2C9*3) and 38 % (VKORC1*2). Similar results were described by Takahashi et al 2006 (24), who determined the frequency of the same alleles was 14 %, 11 % and 42 % in 115 subjects of Caucasian population. Polymorphisms in genes *CYP2C9* and *VKORC1* play an important role in the dosage of warfarin. Based on the results mentioned above, 25 % of Slovak population was found to need a standard dose of warfarin (5 mg per day). Taking into account a combination of the most severe polymorphisms CYP2C9*2, CYP2C9*3 and VKORC1*2, 44 %, 23 % and 8 % of individuals need 4 mg, 3 mg and 2 mg of warfarin per day, respectively based on the general therapeutic scheme implemented by Kitzmiller et al 2011 (23). Despite large advances in the prediction of stable warfarin dose, approximately 50 % of the dose variability remains unexplained. Inter- and intraindividual variability is a big challenge in warfarin treatment. The anticoagulant effects of warfarin are modified also by diet and foods containing vitamin K, although the dietary influences tend to be of more short-term character (25).

VKORC1*3 haplotype (3730G>A, rs7294) was also defined and is present in 38 % of Europeans (13). It causes an increase in the dosage of warfarin (AA) up to 30 % compared with WT (7). The frequency of this allele in the Slovak population is 41 % (26).

Slovak population is in Hardy-Weinberg equilibrium. The frequencies of selected SNPs correlate with other European populations (7, 22, 33, 35, 36, 38, 42) and thus with Caucasians (17). This type of study was done for the first time in Slovakia.

Knowledge of the genotype of individuals allows physicians to adequately set up the warfarin dosage and avoid undesirable complications and life-threatening conditions, especially at the beginning of the warfarin therapy. The pharmacogenetic test is useful also for patients who are treated with warfarin in long term and suffer from serious side effects. FDA (Food and Drug Association) recommends genotype testing before the start of therapy.

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