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Vitamin K Epoxide Reductase Complex 1 (VKORC1) Gene Polymorphisms in Population of Eastern Croatia

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A B S T R A C T

Vitamin K epoxide reductase (VKOR) is a key enzyme in the γ-carboxylation of proteins associated with important bodily functions (coagulation, bone metabolism, etc.). This feature is particularly used in warfarin therapy, which blocks the VKOR enzyme and leads to production of dysfunctional coagulation proteins. Genetic factors, particularly vitamin K epoxide reductase complex 1 (VKORC1) gene, have greatest influence on warfarin therapy. The aim of this study was to determine the distribution of VKORC1 1173C>T and VKORC1 -1639G>A polymorphisms, which are most important for warfarin therapy. We investigated 420 unrelated healthy individuals, mostly blood donors, from region of the Eastern Croatia. Both investigated polymorphisms were in perfect linkage disequilibrium (LD) and showed identical results. 151 patients (36%) were homozygous for the wild-type (C/C and G/G), 196 (47%) were heterozygous (C/T and G/A) and 73 (17%) were homozygous for the variant allele (T/T and A/A). Number of normal alleles among individuals was 498 (59.3%), and number of variant alleles was 342 (40.7%). The data obtained are in good agreement with the results of studies in other European populations.

***Key words**: vitamin K, vitamin K epoxide reductase, gene frequency, linkage disequilibrium, SNP, genetic variation, pharmacogenetics, anticoagulants, warfarin, Croatia*

Introduction

Vitamin K is generic name for several related compounds with similar structure (phylloquinone [K_1], menaquinone [K_2] and menadione [K_3])[1,2]. Vitamin K acts as a cofactor to γ-Glutamyl carboxylase (GGCX), an enzyme that catalyzes posttranslational carboxylation of particular glutamic acid (Glu) to γ-carboxyglutamic acid (Gla) in number of body proteins, which is essential for their proper biological function. When reduced form of vitamin K (vitamin K hydroquinone – KH_2) participate in γ-carboxylation, it is simultaneously oxidized to vitamin K 2,3-epoxide (K>O). Due to the very limited amount of available vitamin K in the body, prompt reduction of K>O in vitamin K active form (KH_2) is required in order to maintain γ-carboxylation. This process, also known as the vitamin K cycle, is achieved with help of Vitamin K-epoxide reductase (VKOR), a key enzyme in recycling of available stocks of vitamin K[1–4]. It was observed that precisely this process of reduction of K>O is rate-limiting step in process of γ-carboxylation[5,6]. VKOR primarily catalyzes de-epoxidation of K>O to vitamin K, while, probably, some other enzymes catalyze reduction of vitamin K to KH_2 *in vivo*[5].

Availability of reduced vitamin K is essential for proper function of some very important body proteins (also known as vitamin K dependent proteins). Those proteins are: coagulation factor and proteins (FII, FVII, FIX, FX, Protein C, S and Z), bone metabolism proteins (matrix Gla proteins [MGP] and osteocalcin, which is main non-

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colagen protein of bone matrix and is associated with the mineralized matrix of bone)[1,7] and cell proliferation and apoptosis proteins (growth arrest specific gene 6 product [GAS6])[1,3–5,8,9]. Inadequate availability of vitamin K leads to insufficient γ-carboxylation of vitamin K dependent proteins, which results in formation of partially carboxylated or non-carboxylated proteins[10]. Biological activity of those proteins is usually significantly reduced.

Impact of reduced vitamin K availability on function of coagulation proteins is utilized in oral anticoagulation therapy. 4-hydroxycoumarin anticoagulants exert their anticoagulant action by blocking VKOR enzyme, which result in decreased amount of reduced vitamin K and, consequently, in production of hypofunctional or non-functional coagulation factors[1,4,10,11]. Warfarin is the most used 4-hydroxycoumarin anticoagulant, and most used oral anticoagulant worldwide[12]. It is commonly prescribed for prevention and treatment of thrombotic disorders[10,11,13–15]. Therapeutic effect of warfarin can be directly evaluated by monitoring of prothrombin time which is expressed as international normalized ratio (INR) units. Therapeutic range is usually kept between 2 and 3 INR, in most cases[10]. Although proven very effective, warfarin dosage is extremely challenging due to it's very narrow therapeutic index and wide interindividual variability[10,13,16]. Some of factors that influence warfarin response are: nutrition (vitamin K intake), some concomitant diseases, drug-drug interactions and, with greatest influence, genetic factors[10,13,17].

Researchers performing Genome-Wide Association Studies (GWAS) determined that single nucleotide polymorphisms (SNPs) of two genes have greatest influence on warfarin dose[18,19]. First gene is CYP2C9 (OMIM 601130), which encodes hepatic microsomal enzyme cytochrome P450 2C9, and whose activity represents the major route of metabolism of warfarin[20–22]. Most important CYP2C9 alleles for warfarin therapy are CYP2C9*1 (wild-type allele, most common in the population), CYP2C9*2, (430C >T) and CYP2C9*3 (1075A>C)[9,23–25]. Individuals with CYP2C9*2 and CYP2C9*3 alleles have lower warfarin dose requirements and are more susceptible to over-anticoagulation and bleeding complications[23,25,26]. Second gene, named vitamin K epoxide reductase complex subunit 1 gene (VKORC1 – OMIM 608547), was discovered recently (in 2004) by two independent teams[27,28]. VKORC1 gene is located on short arm of chromosome 16, its spans 5139 base pairs and encodes a 163 amino acid integral membrane protein – VKOR enzyme responsible for regeneration of vitamin K[29]. Association of VKORC1 SNPs with the therapeutic dose of warfarin was observed soon after the discovery of the gene[30–32]. Although multitudes of VKORC1 SNPs were tested for association with warfarin therapy, only few of them showed positive correlation with warfarin dose and also significant minor allele frequency (MAF) in population. Several authors attempted to explain VKORC1 polymorphisms influence on warfarin dose by combining them into haplotypes[31,33]. Later researches showed that all VKORC1 haplotypes could be replaced with only two SNPs which explain almost all of dose variability[31,34–36]. Those SNPs are VKORC1 1173C>T (rs9934438) and VKORC1 -1639G>A (rs9923231)[18,34,35]. VKORC1 1173C>T represents nucleotide substitution in first intron of VKORC1 and it was first SNP associated with lower doses of warfarin[30,33]. VKORC1 -1639G>A is a SNP taking place in promoter region of VKORC1 gene at the second nucleotide in E-box. This polymorphism impacts transcription factor binding site which affects expression of gene and carriers of variant alleles had lower values of mRNA and presumably fewer functional copies of VKORC protein[31,37,38]. It was observed that carriers of VKORC1 1173C>T and VKORC1 -1639G>A variant alleles require significantly lower doses of warfarin, compared with normal, wild-type homozygote. Also, some authors observed an association of VKORC1 polymorphisms (-1639G>A and 1173C>T) with an increased risk of adverse effects in therapy (INR>4, bleeding etc.)[39–43], time needed to reach the target INR, time spent above the therapeutic INR[44,45] or below therapeutic INR[46]. One of most important findings was that VKORC1 SNPs frequencies in population vary significantly depending on race and ethnicity[36,47].

The aim of this study was to determine variant allele frequency distribution of two VKORC1 polymorphisms: VKORC1 1173C>T (rs9934438) and VKORC1 -1639G >A (rs9923231) in Croatian population and to find out if they are in linkage disequilibrium (LD) like in other populations[32,36,37,41,48,49].

Subjects and Methods

This study included 420 unrelated healthy individuals: 269 male and 151 female, aged 21–79 (median age 41), predominantly blood donors, from region of Eastern Croatia. Study was performed according to Declaration of Helsinki. During the recruiting process, all subjects were informed about the purpose of this study and they all signed informed consent. Ethical Committee of the Institute of Public Health for Osijek-Baranja County and Ethical Committee of the University Hospital Centre Osijek approved this study.

A 5 ml peripheral blood sample was collected from each participant into Vacutainer EDTA containing tubes (Becton Dickinson, Erembodegem, Belgium). Genomic DNA was isolated from peripheral leukocytes from anticoagulated whole blood using High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany), according to manufacturer instructions.

Real-time PCR instrument Light Cycler 1.5 (Roche, Mannheim, Germany) was used for detection of polymorphisms. Genotyping for VKORC1 1173C>T and VKORC1 -1639G>A was achieved using LightMix for the detection of human VKORC1 C1173T and VKORC1 G-1639A detection kits (TIB Molbiol, Berlin, Germany). Melting curve analysis was performed to distinguish between polymorphisms. Positive and negative controls were included in each run and random 10% of samples were re-analyzed with 100% concordance with previous analyses.

Allele frequencies were estimated by gene counting. Hardy-Weinberg equilibrium testing, χ^2 and Fisher's exact tests were performed using R software version 2.15.3 (http://www.r-project.org/). Significance level of 0.05 was used for all tests.

Results

Total of 420 individuals were analyzed to determine polymorphisms distribution of VKORC1 1173C>T and VKORC1 -1639G>A single nucleotide polymorphisms (SNPs) in population of Eastern Croatia. Both polymorphisms were in perfect LD, and showed identical results in our study. 151 individuals (36%) were homozygous for wild type (C/C and G/G), 196 (47%) were heterozygous (C/T and G/A) and 73 (17%) were homozygous for mutation (T/T and A/A). Allele distribution was as follows: 498 alleles (59.3%) were normal alleles (C and G) and 342 alleles (40.7%) were mutated alleles (T and A). All genotypes were in Hardy-Weinberg equilibrium (p=0.495).

We have also analyzed distribution of VKORC1 1173C >T and VKORC1 -1639G>A according to gender. Among male individuals 103 of them (38%) were homozygous for wild type (C/C and G/G), 124 (46%) were heterozygous (C/T and G/A) and 42 (18%) were homozygous for mutation (T/T and A/A). Among female individuals 48 of them (32%) were homozygous for wild type (C/C and G/G), 72 (48%) were heterozygous (C/T and G/A) and 31 (21%) were homozygous for mutation (T/T and A/A). There were no statistically significant differences in polymorphisms distribution in relation to gender (χ^2=2.534; p= 0.282). Obtained results are in concordance with published results for European Caucasian population. Worldwide VKORC1 1173C>T and VKORC1 -1639G>A polymorphism distribution is presented in Table 1.

Discussion

Large proportion of interindividual and interethnical variability in warfarin response can be attributed to genetic factors which regulate pharmacokinetics and pharmacodynamics of drug. VKORC1 SNPs can contribute up to 37% of complete variability[50]. It is known that warfarin dose requirements vary across ethnic groups worldwide.

Clinical observations shows that individuals of Asian descent require lower doses, while individuals of African descent require higher doses of warfarin, compared to population of European descent[51,52]. This can be explained with distribution of VKORC1 1173C>T and VKORC1 -1639G>A variant alleles between different ethnic groups. As it can be seen from Table 1, frequency of variant allele (T and A), which is marker of low warfarin dose phenotype, in Asian populations is predominant. Among Chinese, Japanese and Korean it accounts for over 90% of all alleles[53–55]. This very high proportion of low dose phenotype allele could explain lower dose requirements among Asians. Only exception in Asia is Indian population, which displays completely different fre-

TABLE 1
VKORC1 1173C>T AND VKORC1 -1639G>A VARIANT ALLELE FREQUENCY DISTRIBUTION IN CROATIAN POPULATION AND DIFFERENT POPULATIONS WORLDWIDE

Population	Sample size	Variant allele (T and A) frequency (%)	p value*	Reference
Europe				
Croatian	420	40.7	–	Present study
Austrians	206	43.0	0.605	[64]
Danes	244	41.8	0.806	[65]
French	115	41.3	0.915	[67]
Germans	200	41.5	0.862	[33]
Greek	98	48.5	0.175	[68]
Hungarians	510	39.0	0.638	[69]
Italian	147	39.8	0.922	[30]
Dutch	1525	39.3	0.329	[66]
Norwegians	212	36.6	0.344	[48]
Romanians	332	42.2	0.710	[70]
Slovenians	165	43.3	0.577	[71]
Spaniards	175	41.4	0.927	[72]
Turks	292	40.4	0.938	[73]
UK (English)	297	47.3	0.080	[49]
South America				
Argentines	101	50.5	0.092	[62]
Brazilians	196	37.8	0.536	[63]
North America				
USA (African)	300	10.8	<0.001	[58]
USA (Asian)	102	66.7	<0.001	
USA (Caucasian)	106	40.6	1.000	
USA (Hispanic)	101	43.6	0.653	
Africa & Middle East				
Egyptians	200	72.3	<0.001	[59]
Iranians	126	55.6	<0.001	[60]
Izrael (Arab)	102	60.3	<0.001	[61]
Izrael (Druze)	180	53.3	<0.001	
Izrael (Jew)	162	57.4	<0.001	
South Africans (Black)	993	3.5	<0.001	[57]
Asia				
Chinese	178	91.6	< 0.001	[53]
Indian	102	14.2	< 0.001	[56]
Japanese	341	91.8	< 0.001	[54]
Korean	265	94.0	< 0.001	[55]

* Fisher's exact test – frequency of variant allele (T and A) of Croatian *vs.* other populations

quency distribution: only around 14% are carriers of variant allele[56].

On the other hand, in black Africans population and populations of African origin (e.g. African-American), almost over 90% of individuals are carriers of normal allele and only up to 10% of variant allele[57,58], which could explain higher warfarin dose requirements in black populations compared to Asians and Caucasians. Regarding other African nations that have been investigated, Egyptians are similar to Asian model (over 70% are carriers of variant allele)[59]. In region of Middle East, VKORC1 1173C>T and VKORC1 -1639G>A variant allele frequencies from different populations are also available[60,61].

In South America, Argentinian and Brazilian population variant allele frequencies are similar to European population distribution[62,63]. In North America, among African-American population variant allele frequency is 10.8%, while among Asian-American population variant allele frequency is 66.7%. Caucasian population and Hispanics exhibit same variant allele distribution as Europeans (variant allele frequency around 41%)[58].

Among European populations tested for VKORC1 1173C>T and VKORC1 -1639G>A polymorphisms, almost all showed similar frequency of variant allele (36.6 – 48.5%)[30,33,48,49,64–73].

In our study, we have found that frequency of variant allele in population of Eastern Croatia is 40.7%. When compared to other European populations, there is no statistically significant difference (Fisher's exact test, $p > 0.05$) between VKORC1 1173C>T and VKORC1 -1639G>A variant allele distribution of Croatian population and those of other European populations (see Table 1). There were also no significant differences compared to South American and North American Caucasian and Hispanics populations. Statistically significant difference (Fisher's exact test, $p < 0.05$) was observed when Croatian population was compared to Asian (and Asian-American), African (and African-American) and Middle East populations.

Conclusion

To the best of our knowledge, this report is first to address frequency distribution of VKORC1 1173C>T and VKORC1 -1639G>A polymorphisms in Croatian population. Distribution found in our study is in good agreement with those found in other European populations. Our results confirms that VKORC1 1173C>T and VKORC1 -1639G>A are in perfect LD in Croatian population, similar to results of other studies conducted on Caucasian and Asian populations[32,36,37,41,48,49]. Considering those results, future studies of above mentioned polymorphisms in Croatian population should be limited to genotyping only one of them.

According to our data, 47% of Croatian population are carriers of one variant VKORC1 1173C>T and VKORC1 -1639G>A allele, which means that those individuals will have up to 36% lower warfarin dose requirements[38,49,74]. Another 17% are homozygous for variant allele, which means that those individuals will have up to 60% lower dose requirements[38,49,74]. This could imply that those individuals will probably be more prone to overanticoagulation and adverse events. With knowledge of VKORC1 genotype data before commencing of therapy, and with combining them with CYP2C9 genotype data and other clinical and environmental factors, warfarin dose could be adjusted which could prevent occurrence of adverse events.

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POLIMORFIZMI VITAMIN K EPOKSID REDUKTAZA KOMPLEKS 1 (VKORC1) GENA U POPULACIJI ISTOČNE HRVATSKE

S A Ž E T A K

Vitamin K epoksid reduktaza (VKOR) je ključan enzim u γ-karboksilaciji proteina povezanih s važnim tjelesnim funkcijama (koagulacija, metabolizam kostiju i dr.). Ta značajka je posebno iskorištena u terapiji varfarinom koja blokira VKOR enzim i uzrokuje stvaranje nefunkcionalnih koagulacijskih proteina. Na terapiju varfarinom najveći utjecaj imaju genetski čimbenici, posebno vitamin K epoksid reduktaza kompleks 1 (VKORC1) gen. Cilj ovog istraživanja bio je utvrditi raspodjelu dvaju polimorfizama najznačajnijih za terapiju varfarinom: VKORC1 1173C>T i VKORC1 -1639G>A. Ispitano je 420 zdravih osoba, pretežno dobrovoljnih davatelja krvi, iz područja istočne Hrvatske. Rezultati su pokazali da su oba ispitivana polimorfizma u savršenoj neravnoteži povezanosti (eng. linkage disequilibrium – LD) i pokazali su identične rezultate. Za 151 ispitanika (36%) potvrđeno je da su homozigoti za divlji tip (C/C i G/G), 196 ispitanika (47%) su heterozigoti (C/T i G/A) a 73 (17%) su homozigoti za varijantni alel (T/T i A/A). Broj normalnih alela među ispitanicima bio je 498 (59,3%), dok je varijantnih alela pronađeno 342 (40,7%). Dobiveni podaci se dobro slažu s rezultatima studija u drugim Europskim populacijama.