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Abstract

ABSENCE OF ASSOCIATION BETWEEN G3730A POLYMORPHISM OF VKORC1 GENE AND ISCHEMIC ATHEROTHROMBOTIC STROKE IN THE NORTH-EASTERN REGION OF UKRAINE

Introduction. Vitamin K epoxide reductase complex, subunit 1 (VKORC1), which encodes the catalytic subunit of the vitamin K epoxide reductase complex (VKOR), is necessary for activation of vitamin K-dependent coagulation factors (II, VII, IX, X), anticoagulation factors (Protein C, S, Z) and protein with anti-calcification properties (Matrix Gla-protein) in the vitamin K cycle. Disruption of these proteins can lead to circulatory disorders, thrombosis and identify themselves by the calcification of the middle layer of the vessel wall (Mönckeberg's arteriosclerosis) and (or) by the deposition of calcium in atheromatous plaques. Considering that VKOR probably brings effects on artery calcification development and on clot formation, which are the main causes of acute ischemia development, the aim of present work was to perform a case-control study on representatives of the north-eastern region of Ukraine in order to assess the possible association of G3730A VKORC1 gene polymorphism with ischemic atherothrombotic stroke (IAS).

Materials and methods. The study group included 170 unrelated Ukrainian patients with a mean age of 64.8 ± 9.5 years who had IAS. The control group consisted of 124 clinically healthy individuals with the absence of cardio-vascular pathologies. Allelic polymorphism of 3'UTR region G3730A (rs7294) of the VKORC1 gene was determined by amplification and subsequent restriction fragment. The $\chi 2$-test was used to assess the deviations from the Hardy–Weinberg equilibrium for genotype frequencies, and it was also used for comparison of the allele and genotype frequencies between different studied subgroups. The differences were considered statistically significant with a P-value <0.05. All statistical analyses were performed using the Statistical Package for Social Science program (SPSS for Windows, version 17.0, SPSS Inc., Chicago, IL).

Results. The distribution of homozygous carriers of major allelic variant (G/G), heterozygous (G/A) and homozygous minor allele (A/A) variants in IAS patients was 31,8 %, 50,0 % and 18,2 %, respectively. The corresponding distribution of variants in the control group were 36,3 %, 50,8 %, 12,9 % (P > 0.05 by $\chi 2$-test). In analyzing the genotype frequencies for G3730A polymorphism of the VKORC1 gene in the two sexes is not found significant differences in their correlation (P > 0.05 by $\chi 2$-test).

Conclusion. There is no association of G3730A single nucleotide polymorphism of the VKORC1 gene with ischemic atherothrombotic stroke in representatives of the Ukrainian population.

Keywords: ischemic atherothrombotic stroke, vitamin K-epoxide reductase, single nucleotide polymorphism.

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Резюме

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G3730A ПОЛІМОРФІЗМ ГЕНА VKORC1, НЕ ПОВ'ЯЗАНИЙ З ІШЕМІЧНИМ АТЕРОТРОМБОТИЧНИМ ІНСУЛЬТОМ У ПІВНІЧНО-СХІДНОМУ РЕГІОНІ УКРАЇНИ

Метою дослідження було дослідження наявності можливого зв'язку G3730A-поліморфізму гена VKORC1 із розвитком ішемічного атеротромботичного інсульту (ІАІ) серед населення північно-східного регіону України.

Матеріали і методи дослідження. Об'єктом дослідження були 170 пацієнтів з ІАІ і 124 практично здорових особи. Для визначення поліморфізму гена VKORC1 використовували метод PCR–RFLP. Статистичний аналіз реалізовували із використанням пакета програм SPSS 17.0.

Результати дослідження. Розподіл гомозигот за основним алелем (G/G), гетерозигот (G/A) та гомозигот за мінорним алелем (A/A) у пацієнтів і ІАІ становив 31,8, 50,0 та 18,2 %. Відповідний розподіл у групі контролю був таким: 36,3, 50,8 та 12,9 % ($P > 0,05$ за χ^2–критерієм). Зроблено висновок, що в українській популяції поліморфізм 3'UTR ділянки гена VKORC1 не асоційований з розвитком ІАІ ні у жінок, ні у чоловіків.

Ключові слова: ішемічний атеротромботичний інсульт, вітамін К-епоксидредуктаза, поліморфізм поодиноких нуклеотидів.

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G3730A ПОЛИМОРФИЗМ ГЕНА VKORC1, НЕ СВЯЗАННЫЙ С ИШЕМИЧЕСКИМ АТЕРОТРОМБОТИЧЕСКИМ ИНСУЛЬТОМ В СЕВЕРО-ВОСТОЧНОМ РЕГИОНЕ УКРАИНЫ

Целью исследования было изучение наличия возможной связи G3730A-полиморфизма гена VKORC1 с развитием ишемического атеротромботического инсульта (ИАИ) среди населения северо-восточного региона Украины.

Материалы и методы исследования. Объектом исследования были 170 пациентов с ИАИ и 124 практически здоровых человека. Для определения полиморфизма гена VKORC1 использовали метод PCR-RFLP. Статистический анализ реализовывали с использованием пакета программ SPSS 17.0.

Результаты исследования. Распределение гомозигот по основному аллелю (G/G), гетерозигот (G/A) и гомозигот по минорному аллелю (A/A) у пациентов и ИАИ составило 31,8, 50,0 и 18,2 %. Соответствующее распределение в группе контроля было таким: 36,3, 50,8 и 12,9 % ($P > 0,05$ по χ2-критерию). Сделан вывод, что в украинской популяции полиморфизм 3'UTR участка гена VKORC1 не ассоциирован с развитием ИАИ ни у женщин, ни у мужчин.

Ключевые слова: ишемический атеротромботический инсульт, витамин К-эпоксидредуктаза, полиморфизм единичных нуклеотидов.

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Вступ

Atherosclerosis and its complications are the most frequent cause of death not only of senile people, but also of working age people [1].One of the most serious complications of atherosclerosis is a brain stroke. WHO research shows that stroke

mortality completes 12–15 % of total mortality [2] Acute circulatory disorders, caused by cerebrovascular atherosclerosis is dominated among brain strokes [3]. According to different authors, the share of atherothrombotic stroke accounts from 60 % to 75 % [3, 4, 5].

Like most other diseases caused by atherosclerosis stroke belongs to a group of multifactorial diseases. One of the main etiological factors of its pathogenesis is genetic predisposition [5]. Today, a significant amount of accumulated data on the participation of various polymorphic genes in the formation of multifactorial pathology [6, 7].One of them is gene of vitamin K epoxide reductase complex, subunit 1 (VKORC1), which encodes the catalytic subunit of the vitamin K epoxide reductase complex (VKOR). This enzyme is necessary for activation of vitamin K-dependent coagulation factors (II, VII, IX, X), anticoagulation factors (Protein C, S, Z) and protein with anti-calcification properties (Matrix Gla-protein) in the vitamin K cycle [8]. Disruption of these proteins can lead to circulatory disorders, thrombosis and identify themselves by the calcification of the middle layer of the vessel wall (Mönckeberg's arteriosclerosis) and (or) by the deposition of calcium in atheromatous plaques [9].

Considering that VKOR probably brings effects on artery calcification development and on clot formation, which are the main causes of acute ischemia development, the aim of present work was to perform a case-control study on representatives of the north-eastern region of Ukraine in order to assess the possible association of the VKORC1 gene polymorphism with ischemic atherothrombotic stroke (IAS). Single nucleotide polymorphism G3730A of the 3'UTR region was chosen for the study.

Materials and methods

Study subjects

Our study group included 170 unrelated Ukrainian patients with a mean age of 64.8 ± 9.5 years who had IAS and had been under medical surveillance and outpatient treatment at the 5th Sumy City Clinical Hospital. A final diagnosis of IAS was established on the basis of clinical, computed tomography and magnetic resonance imaging investigations. Each case of IAS was assessed according to the TOAST criteria [10]. The patients with ischemic stroke of cardioembolic origin and undetermined etiology were excluded from the studied group. The control group consisted of 124 clinically healthy individuals with the absence of cardiovascular pathologies, as confirmed by anamnesis, ECG examination, and measurement of arterial pressure and biochemical data. The control group and a group of patients did not differ by age and the ratio of persons of both sexes ($P > 0,05$ for the χ^2-test).

The study had been previously approved by the Ethic Committee on Medical Research of the Medical Institute of Sumy State University. An appropriate informed consent was obtained from all patients. Blood sampling for genotyping was performed under sterile conditions into 2.7 ml S-Monovette («Sarstedt», Germany) containing EDTA potassium salt as an anticoagulant, the samples were frozen and stored at –20 °C.

Amplification and genotyping

DNA for genotyping was extracted from venous blood using commercially available kits («Isogene Lab Ltd», Russian Federation) according to the manufacturer's protocol. Allelic polymorphism of 3'UTR region G3730A (rs7294) of the *VKORC1* gene was determined by amplification and subsequent restriction fragment. The sequence of nucleotides in specific primers were as follows: upstream (sence) – 5`-TTTAGAGACCCTTCCCAGCA-3`, downstream (antisense) – 5`-AGCTCCAGAGAAGGCAACAC-3`. For amplification were 50–100 ng added to the DNA mixture which containing 5 µl 5 PCRbuffer, 1.5 mM magnesium sulfate, 200 µM of each dNTP, 20 pM of each primer and 0.5 U of Taq DNA polymerase («Fermentas», Lithuania). Amplification fragment was consisted of 33 cycles: denaturation – 94 °C (50 sec), hybridization of primers – 64.5 °C (45 sec) and elongation – 72 °C (1 min). For restriction analysis 6 µl amplification product was incubated at 37 °C for 18 hours with 2 U *SsiII* in the Tango buffer. If the 3730th position *VKORC1* gene contained guanine, amplificate which consisted of 674 base pairs digested with *SsiII* with three fragments of 117, 216 and 341 base pairs. In case of replacement guanine to adenine restriction site for *SsiII* was lost and visualized two fragments of 117 and 216 base pairs. The restriction fragments were separated by electrophoresis (0,1 A; 140 V), performed for 40 min and analyzed on the ethidium bromide-stained 2.5 % agarose gel using ultraviolet transillumination.

Statistical analysis

The normal distribution and homogeneity of variances were tested before further statistical analyses. The χ^2-test was used to assess the deviations from the Hardy–Weinberg equilibrium for genotype

frequencies, and it was also used for comparison of the allele and genotype frequencies between different studied subgroups. The differences were considered statistically significant with a P-value < 0.05. All statistical analyses were performed using the Statistical Package for Social Science program (SPSS for Windows, version 17.0, SPSS Inc., Chicago, IL).

Results

The frequency of the three possible genotypes for the studied *VKORC1*gene polymorphism, and verification of compliance the distribution of major and minor alleles of the Hardy–Weinberg equilibrium are presented in Table 1. Verification of distribution genotypes for the G3730A polymorphism for compliance Hardy–Weinberg law is revealed that in control group, and in the main group deviation from the established equilibrium is not statistically significant. It was found that the ratio of alleles in both groups were not significantly different from the expected (P > 0.05).

Table 1 – The frequency of allelic variants and alleles for the G3730A polymorphism of the VKORC1 gene in the control group and patients with IAS

	Control group,n (%)	*IAS group,n (%)*
Homozygotes G/G	45 (36.3)	54 (31.8)
Heterozygotes G/A	63 (50.8)	85 (50.0)
Homozygotes A/A	16 (12.9)	31 (18.2)
A-allele	0.62	0.57
G-allele	0.38	0.43
χ^2	0.7	0.06
P	> 0.05	> 0.05

Note: n – number of subjects; χ^2 i P – reflecting deviations in each group from Hardy–Weinberg equilibrium

Comparison of the frequency of allelic variants of the *VKORC1* gene for G3730A polymorphism in patients with IAS and the control group suggests no difference in different types of genotype distribution between of patients with atherothrombotic ischemic stroke and healthy patients (P > 0.05). Analyzing the genotype frequencies for G3730A polymorphism of the *VKORC1* gene in the two sexes did not reveal any significant differences in their correlation (Table 2).

Table 2 – **The frequency of allelic variants and alleles for the G3730A polymorphism of the VKORC1 gene in the control group and patients with IAS depending on gender**

Genotype	Females		Males	
	Control group	IAS group	Control group	IAS group
G/G	15 (33.3 %)	25 (34.7 %)	30 (38.0 %)	29 (29.6 %)
G/A	23 (51.1 %)	33 (45.8 %)	40 (50.6 %)	52 (53.1 %)
A/A	7 (15.6 %)	14 (19.4 %)	9 (11.4 %)	17 (17.3 %)
	P =0.815; χ^2 = 0.410		P =0.363; χ^2 = 2.029	

Note: P – the significance of differences in the distribution of genotypes between control group and IAS group

Similar studies in this direction are not numerous and contradictory. Porojan et al. investigated the association of allelic polymorphisms of the *VKORC1* genes and KLOTHO with atherosclerosis and calcification. The authors discovered that the C1173T polymorphism of the first intron *VKORC1* gene is associated with calcification of blood vessels and is an important genetic factor for atherosclerosis [11].

Wang et al. by studying distribution of genotypes for polymorphisms T2255C of the *VKORC1*gene have discovered that the presence of C allele increases the risk of coronary heart disease and hemorrhagic stroke by more than two times and

the risk of aortic dissection – by more than three times [12]. However Hindorff et al., which explored the association of polymorphisms of the gene VKORC1, among which T2255C is, with the development of myocardial infarction and other cardiovascular diseases have shown that none of the investigated SNP was associated with the development of heart disease and blood vessels studied [13].

In 2010, Shyu et al. investigated the relationship of genetic polymorphism of genes *GGCX* (Gln325Arg), *VKORC1* (G3730A) and *NQO1* (Pro187Ser) with a risk of atherothrombotic ischemic stroke. Researchers found a statistically significant protective effect of these polymorphisms concerning the risk of ischemic stroke. Synergism of the investigated loci was more expressed in patients who did not drink alcohol and were not smokers [14].

It is noteworthy that our research is the first one dedicated to the study of association of the G3730A VKORC1 gene polymorphism with the development of atherothrombotic ischemic stroke in Ukrainian population.

Implementation mechanisms of action of genetic factor that we studied may be associated not only with the influence on the process of calcification of the coronary arteries and brain, but on the process of blood coagulation [15]. It is known that vitamin K epoxid reductase carries out post translational modification of the vitamin K-dependent procoagulating proteins thereby affecting the process of blood clotting. The latter fact is of great importance in the pathogenesis of coronary and cerebral thrombosis. Investigations of some researchers suggest antagonistic nature of the interaction between coagulation and calcification of vascular wall [16], and VKOR can be considered as a link in these processes. Today there is a perception that an active, well-regulated nature of the process calcification [17] can indicate its adaptive significance in the pathogenesis of atherosclerosis. Is not excluded that calcification is an optimal variant of ending pathological process in the vascular wall and the factors delaying the laying of calcium should be regarded as risk-factors of destabilization of atherosclerotic plaques. Of course, the assumption requires both experimental and clinical evidence, and therefore makes it necessary to continue research in this direction. At this stage it is important to conclude that the *VKORC1* gene polymorphism can be considered one of the genetic factors of cardiovascular disease.

Висновки

There is no association of G3730A single nucleotide polymorphism of the VKORC1 gene with ischemic atherothrombotic stroke in representatives of Ukrainian population.

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