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CONTENTS

ORIGINAL ARTICLE

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ORIGINAL ARTICLE

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ANALYSIS OF THE BLOOD HYPERCOAGULATION RISK IN PATIENTS WITH ISCHEMIC ATHEROTHROMBOTIC STROKE DEPENDING OF THE VDR GENE POLYMORPHISMS

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ABSTRACT

Aim: of our study was the analysis of the blood hypercoagulation risk in patients with ischemic atherotrombotic stroke depending of the VDR gene polymorphisms.

Materials and Methods: Blood of 170 patients with ischemic atherothrombotic stroke (IATS) and 124 healthy individuals (control group) was used for genotyping. Four polymorphisms (FokI, BsmI, ApaI, TaqI) of gene VDR were examined with PCR-RFLP methodology. Statistical analysis was performed by using SPSS-17.0 program.

Results: Among patients with IATS who are carriers of the f/f genotype, FokI polymorphism of VDR gene by high thrombin time and a decrease in the rate of spontaneous fibrinolysis was registered. In individuals with the B/B genotype homozygous for the polymorphic variant, BsmI had significantly lower mean values of prothrombin and thrombin time and increased the rate of spontaneous fibrinolysis. The homozygotes for the A-allele ApaI polymorphism have 2.7 times higher risk of developing blood hypercoagulation than homozygotes for the a-allele was found.

Conclusions: Biochemical signs of hypercoagulation syndrome among patients with IATS who are carriers of the f/f genotype of the FokI polymorphic variant and among B/B homozygotes of the BsmI polymorphic variant and homozygotes for the A-allele of the ApaI polymorphism of the VDR gene were registered.

KEY WORDS: blood coagulation, ischemic atherothrombotic stroke, gene polymorphism, VDR

INTRODUCTION

The ischemic atherothrombotic stroke (IATS) accounts for 50% of cases and is the cause of disability in the middle-aged working population. Vascular disorders can be associated with changes in the structure of many genes. Given the presence of a hereditary component in the etiology of this disease, global scientific research is focused on identifying candidate genes, the products of which can lead to dysfunction of the vascular endothelium and disruption of the hemostasis system [1, 2, 3].

Among the main risk factors for complications of the atherosclerotic process, the factors that determine the hypercoagulable activity of the blood occupy an important place. Thus, an increase in the level of plasma fibrinogen is considered an independent factor that increases the risk of exacerbation of coronary heart disease by 3-4 times [4, 5, 6]. The vitamin D receptor (VDR) gene serves as a good candidate gene for susceptibility to essential hypertension. The gene regulates the renin angiotensin system by influencing blood pressure regulation. Around 3% of the human genome is regulated by the vitamin D endocrine system. Several studies are known to have reported mixed results regarding the relationship between the VDR gene and hypertension [7].

Calcifications of cerebral arteries are an unfavorable prognostic factor for the occurrence of fatal events stroke complications. One of the central links in the protection of blood vessels from ectopic calcification is the matrix protein Gla-protein (MGP), the presence of which in tissues prevents both the initiation of pathological calcification and its spreading Today, the factors involved in the regulation of MGP gene expression and possible mechanisms through which the anticalcinogenic properties of the protein are realized. This gives reason to talk about the MGP functional system, to which, in addition to the protein itself, may include such factors as the vitamin D receptor (VDR), enzymes that take participation in biochemical transformations of MGP, – vitamin K-oxidoreductase (VKOR) and γ-glutamylcarboxylase (GGCX), – and also possible targets for MGP, particularly bone morphogenetic protein 2 (BMP-2). The effective operation of these systems depends on many factors, one of which is the polymorphism of the genes encoding the structure of the corresponding proteins. A number of generally accepted indicators, including prothrombin time and prothrombin index, thrombin time, fibrinogen content in blood plasma, and the intensity of spontaneous fibrinolysis, assessed the state of blood coagulation processes in patients with IATS [8, 9].

AIM

The aim of our study was the analysis of the blood hypercoagulation risk in patients with ischemic atherothrombotic stroke depending of the VDR gene polymorphisms.

MATERIALS AND METHODS

SUBJECTS

The studied group included 170 patients with IATS (42.4% women and 57.6% men) aged 40 to 85 years (mean age 64.7 ± 0.73 years). The ischemic nature of the stroke according to the anamnesis and clinical picture of the disease, the results of an MRI study of the brain was established. The pathogenic variant of stroke was determined according to TOAST criteria [10], based on the anamnesis and features of the clinical course of the disease, ultrasound Doppler ultrasound, ECG. The control group consisted of 124 patients in whom the absence of cardiovascular pathology was confirmed by anamnestic data collection, electrocardiogram withdrawal and blood pressure measurements. The control group and the group of patients with IATS did not differ in the ratio of persons of different sex (P = 0.294 on the $\chi 2$ criterion), but the mean age of the first (76.7 ± 0.93 years) was significantly higher than the second was (P <0.001) [11].

AMPLIFICATION AND GENOTYPING

As described in our earlier research DNA for genotyping from the venous blood using commercially available kits (Isogene Lab Ltd) according to the manufacturer's protocol was extracted. Determination of polymorphisms *Fok*I- (rs 2228570), *Bsm*I- (rs1544410), *Apa*I- (rs7975232), and *Taq*I- (rs731236) of the *VDR* gene using the polymerase chain reaction method followed by restriction fragment length analysis upon detection by agarose gel electrophoresis was performed. Primers synthesized by Metabion (Germany) and enzymes (Taq polymerase and restrictase) by Thermo Scientific (USA) were used. PCR in a GeneAmp PCR thermocycler System 2700 ("Applied Biosystems", USA) was performed [12].

Detection of the restriction products by horizontal electrophoresis in 2.5% agarose gel (Sigma-Aldrich, USA) was performed containing ethidium bromide (Sigma-Aldrich, USA). The results in ultraviolet rays using an automatic video reading system "Vi-Tran" in a transilluminator ("Biocom") were visualized [11, 12].

STATISTICAL ANALYSIS

We performed statistical analysis using the SPSS-17 program. Before testing the statistical hypotheses, an analysis of the normality of the distribution of values in the samples was carried out, by determining the asymmetry and excess coefficients using the Willkie-Khan-Shapiro and Lilliefors criteria using the algorithms implemented in SPSS-17. The significance of the differences between the two samples was determined using Student's t test (t). Based on the magnitude of t and the number of degrees of freedom (l = n1 + n2-2), the difference between the two samples (P) was found on the Student's distribution table. The difference was considered significant if the probability of a random difference did not exceed 0.05 ($p < 0.05$). Non-parametric criteria to estimate differences in mean trends and independent samples were used, namely the Fisher exact method for a four-field table (TMF). The use of nonparametric criteria made it possible to find out significant differences in cases where the criterion t did not reveal them [12].

RESULTS

A number of commonly accepted indicators, including prothrombin time and prothrombin index, thrombin time, fibrinogen content in blood plasma, and the intensity of spontaneous fibrinolysis, assessed the state of blood coagulation processes in patients with IATS. In *Fok*I polymorphism homozygotes for the f-allele (f/f) the average value of thrombin time was slightly higher (P=0.047), and spontaneous fibrinolysis was lower (P=0.046) than individuals with genotypes (*F/F* and *F/f*). Other indicators did not depend on the genetic factor that was the subject of our analysis and were not statistically significant (P>0.05) (Table 1).

When the *Bsm*I polymorphism is studied, that homozygotes for the *B*-allele are characterized by lower average values of prothrombin (P=0,045) and thrombin time (P=0,048) and greater spontaneous fibrinolysis in the blood plasma (P=0.036), that is, they have biochemical signs of hypercoagulation syndrome (Table 1).

Data on blood clotting indicators in patients with IATS depending on their genotype according to the *Apa*I polymorphism of the *VDR* gene are also given (Table 1). It can be seen that homozygotes for the *A*-allele are characterized by lower average values of prothrombin time (P=0.020) and prothrombin index in blood plasma (P=0.011), that is, they have biochemical signs of hypercoagulation syndrome. And a data on blood coagulation parameters in patients with IATS depending on their genotype according to the *Taq*I polymorphism were also studied. It can be seen in Table 1 that the *Taq*I genotype does not affect the studied indicators.

The division of patients with IATS into two subgroups according to the presence and absence of functional and biochemical signs of blood hypercoagulation (patients with a prothrombin time < 9 seconds and a prothrombin index < 80% were considered prone to accelerated blood clotting) did not reveal any influence of the studied genetic a marker for the risk of developing hypercoagulable syndrome. Patients with a normal level of coagulation according to the *F/F* genotype were 23.1%, *F/f* genotype – 60.4% and *f/f* genotype – 16.5%. Among patients with IATS who have hypercoagulation syndrome, carriers of such genotypes were 24.1%, 45.6%, and 30.4%, respectively. Therefore, the distribution of allelic variants of the *VDR* gene according to the *Fok*I polymorphism is not significantly different in patients with IATI with a normal level of coagulation and hypercoagulation syndrome ($\chi 2=5.323$; P=0.007).

*Bsm*I polymorphism in patients with IATS was not associated with this syndrome. Thus, among patients with a normal level of coagulation, there were 49.4% with the

Table 1. Blood coagulation indicators in patients with IATS depending on the variants of the genotype according VDR gene polymorphisms (M±m)

	Prothrombin time, sec	Prothrombin index, %	Thrombin time, sec	Fibrinogen, g/L	Spontaneous fibrinolysis, min
			***Fok*I polymorphism**		
F/F (n=40)	9.26±0.27	82.6±2.07	16.4±0.59	3.92±0.20	484.6±6.5
F/f (n=91)	9.32±0.22	83.0±1.64	16.1±0.33	4.04±0.13	482.4±3.6
f/f (n =39)	10.05±0.34	88.3±2.17	17.7±0.65	3.70±0.18	466.8±5.5
F	2.088	2.108	3.107	1.056	3.135
P	0.127	0.125	0.047	0.350	0.046
			***Bsm*I polymorphism**		
b/b (n=71)	9.84±0.25	86.47±1.88	17.1±0.45	3.78±0.15	471.1±4.0
b/B (n=74)	9.39±0.23	83.51±1.63	16.6±0.41	3.91±0.13	483.9±4.2
B/B (n=25)	8.69±0.35	79.32±2.45	15.0±0.48	4.41±0.25	489.2±8.3
F	3.157	2.332	3.103	2.529	3.393
P	0.045	0.100	0.048	0.083	0.036
			***Apa*I polymorphism**		
a/a (n=45)	9.92±0.33	86.6±2.53	16.8±0.50	3.82±0.17	474.8±4.68
a/A (n=85)	9.58±0.22	85.6±1.57	16.9±0.42	3.82±0.14	476.9±3.92
A/A (n=40)	8.74±0.27	78.1±1.68	15.4±0.44	4.29±0.18	489.5±6.4
F	3.997	4.608	2.583	2.366	2.129
P	0.020	0.011	0.079	0.097	0.122
			***Taq*I polymorphism**		
T/T (n=68)	9.67±0.26	85.5±1.99	16.8±0.45	3.82±0.15	473.8±4.18
T/t (n=82)	9.49±0.22	84.5±1.51	16.6±0.38	3.90±0.13	480.2±3.98
t/t (n=20)	8.77±0.40	78.1±2.57	15.4±0.76	4.46±0.28	494.5±9.12
F	1.521	2.061	1.220	2.230	2.591
P	0.222	0.131	0.298	0.111	0.078

Note: n – the number of patients

b/b genotype, 40.5% with the *b/B* genotype, and 10.1% with the *B/B* genotype. Among patients with IATS who have hypercoagulation syndrome, carriers of such genotypes were 35.2%, 46.1%, and 18.7%, respectively. Thus, the distribution of allelic variants of the *VDR* gene according to the *Bsm*I polymorphism is not significantly different in patients with IATS with a normal level of coagulation and hypercoagulation syndrome ($\chi 2=4.457$; P=0.108).

It should be noted that the differences in the relationship between polymorphic variants of the VDR gene and the development of blood hypercoagulation are very close to the level of statistical significance. Thus, the ratio of genotypes a/a, a/A and A/A in patients without signs of blood hypercoagulation was 30.4%, 54.4% and 15.2%, while in patients with reduced indicators of prothrombin time and prothrombin index – 32.1%, 46.2% and 30.8% ($\chi 2 =5.794$, P1=0.055).

According to the data of the conducted regression analysis on the dependence between the *Apa*I polymorphism and blood hypercoagulation in patients with IATS, it was found that homozygotes for the *A*-allele have 2.7 times higher risk of developing blood hypercoagulation than homozygotes for the a-allele (Table 2).

The *Taq*I polymorphism in patients with IATS was not associated with this syndrome. So, among patients with a normal level of coagulation, there were with the *T/T* genotype 43.0%, *T/t* genotype 49.4%, and *t/t* genotype 7.6%. Among patients with IATS who have hypercoagulation syndrome,

Table 2. Analysis of the blood hypercoagulation risk in patients with IATS depending on the genotype by ApaI polymorphism of the VDR gene (logistic regression method)

	Genotype	CR	SE	WS	P	OR	95% CI for OR lower	95% CI for OR uppper
Blood hypercoagulation	a/A	0,110	0,369	0,089	0,766	1,116	0,541	2,302
	A/A	0,981	0,456	4,618	0,032	2,667	1,090	6,524

Note: A-allele homozygotes (A/A) are compared with a-allele carriers (a/A+a/a). CR – regression coefficient, SE – standard error, WS – Wald statistic, P – statistical significance, OR – risk ratio, CI – confidence interval.

carriers of such genotypes were 37.4%, 47.3%, and 15.4%, respectively. Thus, the distribution of allelic variants of the *VDR* gene according to the *Taq*I polymorphism is not significantly different in patients with IATS with a normal level of coagulation and hypercoagulation syndrome ($\chi 2 = 2.561$; $P = 0.278$) (Table 1).

DISCUSSIONS

During the study of blood coagulation indicators in patients with cerebrovascular pathology, biochemical signs of hypercoagulable syndrome characterized by a higher content of fibrinogen and lower average values of prothrombin time and prothrombin index were found in homozygotes for the B-allele of the *Bsm*I polymorphism and homozygotes for the A-allele of the *Apa*I polymorphism of the VDR gene.

The research of other scientists on this issue is ambiguous. Thus, Watzka et al. found no association between polymorphic variants of the *VKORC1* and *GGCX* genes and the level of vitamin K-dependent coagulation factors in the German population [13]. Marieke et al., examining patients with deep vein thrombosis, did not find an association between haplotypes *VKORC1*, *NQO1* and *GGCX* (polymorphisms rs6738645, rs699664, rs10179904, rs11676382, rs17026447, rs2028898) and the risk of venous thrombosis. Only for the H1 haplotype of the *GGCX* gene, according to the specified polymorphisms, a connection with a decrease in the activity of the blood coagulation factor II was found [14]. Vanakker et al. showed that the genetic polymorphism of the 8th exon Arg325Gln of the GGCX gene reduces carboxylase activity and induces deficiency of vitamin K-dependent blood coagulation factors. Scientists concluded that genetic variability of GGCX is a risk factor for severe neonatal bleeding [15]. Kimura et al. studied the effect of polymorphisms of GGCX, VKORC1 and CALU genes on the activity of C- and S-proteins among Japanese people. Women who were homozygotes for the major allele (Arg325Gln polymorphism of the GGCX gene) had significantly higher levels of protein C activity than heterozygotes and homozygotes for the minor allele [16]. When dividing patients into groups based on the presence and absence of blood hypercoagulation syndrome, we did not establish any relationship between the studied polymorphisms of the MGP system genes and the development of blood hypercoagulation syndrome in patients with ACS. Only in some groups of patients such an association was still found: in homozygotes for the minor allele (A/A) for the *G-7A* polymorphism of *MGP* gene with BMI≥25 kg/m^2, without obesity and with diabetes, and in f/f homozygotes for the *Fok*I polymorphism of the *VDR* gene with atherogenic dyslipoproteinemia and without diabetes, the hypercoagulation syndrome occurs more often.

CONCLUSIONS

Biochemical signs of hypercoagulation syndrome among patients with IATS who are carriers of the f/f genotype of the *Fok*I polymorphic variant and among B/B homozygotes of the *Bsm*I polymorphic variant of the *VDR* gene were registered. In A/A homozygotes of the *Apa*I polymorphic variant who have a stroke, the risk of hypercoagulable blood changes is 2.7 times higher than in patients with the a/a genotype.

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CONFLICT OF INTEREST
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