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ASSOCIATION OF THE GENETIC POLYMORPHISM E670G OF THE PCSK-9 AND THE SEVERITY OF THE CAROTID ATHEROSCLEROSIS IN PATIENTS WITH HETEROZYGOUS FAMILIAL HYPERCHOLESTEROLEMIA IN THE UZBEK POPULATION

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ABSTRACT

Objective: to study the severity of carotid artery atherosclerosis in patients with heterozygous familial hypercholesterolemia in the Uzbek population, depending on the level of PCSK-9 and the genetic polymorphism E670G of the PCSK-9 gene.

Material and methods. The study included 57 patients with chronic stable coronary artery disease (SCAD) and familial heterozygous hypercholesterolemia (HeFH, group I). The comparison group consisted of 144 patients with SCAD without HeFH divided into two subgroups: A – statin free before the research (n=63) and B (n=81) who took it as outpatients; control group consisted of 17 healthy people. The level of proprotein convertase subtilisin-kexin type 9 (PCSK-9) was measured with Human Proprotein Convertase 9/PCSK9 ELISA Kit (MULTI SCIENCE, China). The genetic typing of PCSK9 E670G (rs505151) polymorphism was performed by means of the PCR-RFLP method.

Results. A comparison of the results of duplex scanning of carotid arteries in patients with HeFH showed that the carotid intima-media thickness (CIMT) on the left (1.14±0.18 mm, P<0.01) and on the right (1.15±0.16 mm, P<0.01) was higher, than in the comparison group: 1.05±0.17 mm and 1.04±0.18 mm, respectively. The studies revealed a positive correlation between the incidence of Myocardial infarction (MI) in the history in patients with HeFH and the (r=0.38, P<0.05). The CIMT also correlated with an increase in the concentration of PCSK9 (r = 0.31, P <0.05) in the blood and the carriage of the G allele of polymorphism E670G (r = 0.39, P <0.05) of the PCSK9 gene.

Conclusion. In patients with heterozygous familial hypercholesterolemia in the Uzbek population a direct correlation was established between Myocardial infarction in the history, the carotid intima-media thickness, an increase in the concentration of PCSK-9 in the blood and the carriage of the G allele of E670G polymorphism of the PCSK9 gene, that allows them to be used as prognostic markers for the risk of development of cardiovascular complications.

Keywords: *heterozygous familial hypercholesterolemia, E670G polymorphism of PCSK-9 gene, carotid atherosclerosis.*

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AHeterozygous familial hypercholesterolemia (HeFH) is known to be one of the most common hereditary disorders in the world. 1 out of 500 people in the population suffer from it, while for the homozygous form the figure is 1 out of 1 million. In 90% of the cases, the disorder is associated with mutation in the LDL receptor-coding (LDL-R) gene, in 5–10% of the cases – in the apolipoprotein B gene, and 1-2% of the cases – in the proprotein convertase subtilisin-kexin Type 9 (PCSK9) gene (1). Increased blood PCSK9 level is common for all the HeFH genetic variations, and the use of monoclonal antibodies that inactivate PCSK9 is a successful treatment approach in hypercholesterolemia.

In 2003, Abifadel et al. discovered several variations of mutation in the PCSK9 gene, some of which were classified as «gain-of-function» (GOF), i.e., causing hypercholesterolemia, while the others were classified as «loss of function» (LOF), i.e., associated with reduction in cholesterol level and decrease in IHD risk [2,3,4,5].

PCSK9 plays an important part in blood cholesterol level regulation and thus affects atherosclerosis processes [6,7]. The loss of function (LOF) variations in PCSK9 genetic polymorphisms (Y142X and C679X) cause reduction in the PCSK9 synthesis or secretion in cell cultures, which is associated with a considerable decrease in plasma PCSK9 concentration [8]. While a number of studies have shown the association between the gain of function (GOF) of E670G genetic polymorphism of the PCSK-9 gene and the lipid profile and severity of coronary atherosclerosis [9,10], others failed to prove this [11,12].

In view of the above, **the Aim of the research was to** study the structural and functional changes in the carotid arteries in patients with heterozygous familial hypercholesterolemia, considering the level of PCSK-9 and of E670G genetic polymorphism of the PCSK-9 gene.

MATERIALS AND METHODS

57 patients with chronic stable coronary artery disease (SCAD) and heterozygous familial hypercholesterolemia (HeFH, group I) were included. The comparison group included 144 patients with IHD but without HeFH (group II); control group consisted of 17 healthy people.

To verify the diagnosis and assess the patients' clinical condition severity, the following risk factors were evaluated: increased blood pressure (BP), smoking, BMI, diabetes mellitus as well as structural and functional parameters: 12-lead ECG, echocardiography assessing the myocardium systolic and diastolic functions, ultrasound examination of brachiocephalic arteries, 24-hour ECG monitoring, stress test, and, where necessary, coronary angiography.

An ultrasound examination of the brachiocephalic arteries was conducted using SIEMENS Acuson X700 (Germany), by a linear sensor with a frequency of 7 MHz [20 m].

To assess the lipid profile and inflammation markers, the following parameters were studied: total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), triglycerides (TG), ultra-sensitive C-reactive protein, biochemical parameters (ALT, AST, CPK). A Daytona analyzer (Randox, Ireland) was used for this purpose. To determine the atherogenic index (AIX), the following equation was used: AIX = (TC – HDL-C)/HDL-C (ru). The level of proprotein convertase subtilisin-kexin type 9 (PCSK9) was determined by means of enzyme-linked immunoassay using the reagents Human Proprotein Convertase 9/PCSK9 ELISA Kit (MULTI SCIENCE, China).

The E670G (rs505151) polymorphism of PCSK9 was genotyped using the PCR-RFLP method. The genomic DNA was isolated from the peripheral blood using the DNA extraction kit DiatomTM DNA Prep 200: Isogene, Russia, in accordance with the manufacturer's protocol.

To identify the E670G polymorphism of the PCSK9 gene, forward 5`-CACGGTTGT GTCCCAAATGG-3` and reverse 5`-GAGAGGGACAAGTCGGAACC-3`primers were used (7). The PCR primers were synthesized by SibEnzyme Ltd (Novosibirsk, Russia).

Statistical analysis. The Statistica 6.0 advanced statistical analysis package was used for the statistical analysis of the data. The obtained data was presented as

mean and standard deviation (m±SD), where the statistical significance of the obtained measurements for compared mean values was determined by the Student's t-test (t) with calculated error probability (p) to check normality of the distribution. If the distribution of studied variables differed from the normal distribution, the non-parametric analysis tests, Wilcoxon signed-rank, to compare two related samples, matched samples, or repeated measurements, and Mann-Whitney T-test

for two samples was used. In order to find differences between qualitative statistical measures, the $\chi 2$ method was used together with the Fisher's exact test for small samples. The empirical genotype frequency distribution conformance to the theoretically expected Hardy-Weinberg equilibrium was checked by the $\chi 2$ test.

RESULTS

According to the comparison of the frequencies of E670G (rs505151) polymorphism genotypes and alleles in HeFH patients vs non-HeFH patients vs healthy persons (Table 1), the number of G alleles in Group I (13, 11.4%) was twice as high as in Group II (17, 6.0%) and threefold than in the healthy (1, 3.0%) group, but the differences were not significant.

The frequency of the genotypes studied corresponded to the Hard-Weinberg equilibrium distribution (P>0.05).*- Non-significant differences available

Table 1. E670G (rs505151) Polymorphism Genotype and Alleles of PCSK9 Gene in HeFH Patients vs Non-HeFH Patients vs Healthy Persons (n, %)*

Genotypes and alleles	I, HeFH (n=57)	II, non-HeFH (n=144)	All patients (n=201)	III, healthy (n=17)
AA	46 (80.7%)	128 (89.0%)	174 (86.6%)	16 (94.1%)
AG	9 (15.8%)	15 (10.3%)	24 (11.9%)	1 (5.9%)
GG	2 (3.5%)	1 (0.7%)	3 (1.5%)	0
A	101 (88.6%)	271 (94.0%)	372 (92.5%)	33 (97.0%)
G	13 (11.4%)	17 (6.0%)	30 (7.5%)	1 (3.0%)

**- Non-significant differences available*

According to the results of carotid artery ultrasound examination in HeFH patients (Table 2), the carotid intima-media thickness (CIMT) on the left (1.14±0.18, P<0.01) and on the right (1.15±0.16, P<0.01) exceeded the same values of the comparison group: 1.05±0.17 and 1.04±0.18, respectively. Moreover, the HeFH patients had more atherosclerotic plaques in the left (66.7% vs 50.0%, P<0.05) and right (68.4% vs 43.0%, P<0.01) common (CCA) and internal (ICA) carotid arteries. Stenosis % on the left (43.4%) and on the right (42.3%) was also higher than in the comparison group (28.3% and 27.2%, respectively, P<0.05).

The study of structural changes in the common and internal carotid arteries considering the specifics of distribution of PCSK-9 gene E670G polymorphic variants revealed that besides the higher frequency of cardiovascular complications (History of myocardial infarction, stroke, coronary artery bypass grafting) the HeFH patients – G allele carriers had significantly higher incidence of atherosclerotic plaques in the carotid arteries: 11 (100%, P<0.05) vs 28 (61%), which also indicates higher severity of multifocal atherosclerosis (Table 3).

Table 2. Comparative Analysis of the Structural and Functional Parameters of Brachiocephalic Artery Changes in HeFH and non-HeFH patients with IHD (M±SD, n (%)

Parameters	I, HeFH (n=57)	II, non-HeFH (n=144)
CIMT on the left, mm	1.14±0.18**	1.05±0.17
CIMT on the right, mm	1.15±0,16**	1.04±0.18
Atherosclerotic plaque on the left (CCA, ICA)	38 (66.7%)*	72 (50.0%)
Atherosclerotic plaque on the right (CCA, ICA)	39 (68.4%)**	62 (43.0%)
stenosis % on the left (CCA, ICA)	43.4%*	28.3%
stenosis % on the left (CCA, ICA)	42.3%*	27.2%

*Note: *,**,*** - P<0.05, P<0.01, P<0.001 significance of differences between Groups I and II*

The importance of assessing the structural and functional condition of carotid arteries in HeFH patients is confirmed by correlation analysis. As is seen from Table 4, there was a positive correlation (r=0.38, P<0.05) between CIMT and MI history in HeFH patients, which makes it possible to use the CIMT value as an important predictive marker for atherosclerosis progression. Increased glucose level and blood PCSK-9 concentration and the PCSK-9 gene E670G polymorphism G-allele carriage also represent important predictive markers for carotid artery lesions and thus, for the risk of cardiovascular complications (Table 4).

DISCUSSION

The polymorphisms of LDL-receptor-, PCSK9- and ApoB-regulation genes are known to be the most frequent genetic causes of HeFH development. According to published data, in most cases, loss-of-function (LOF) genetic polymorphisms associated with a PCSK9 level decrease, particularly, R46L (rs11591147), are considered the leading regulators of its action [13,14].

Table 4. Assessment of the Correlation between Initial Clinical Parameters and Intima-Media Complex Thickness in HeFH Patients

Item	Parameters	Spearman r	P
1	Age	-0,09	НД
2	Male sex	0,28	НД
3	Diabetes mellitus	0,25	НД
4	Myocardial infarction history	0,38	<0,05
5	Total cholesterol	0,07	НД
6	LDL-C	0,15	НД
7	TG	0,02	НД
8	HDL-C	-0,07	НД
9	AI	0,21	НД
10	Blood glucose	0,29	<0,05
11	Hs-CRP	0,05	НД
12	PCSK-9	0,31	<0,05
13	G-carriage	0,39	<0,05

Meanwhile, the single-nucleotide E670G (rs505151) polymorphism of the PCSK9 gene has been recognized in many studies as the leading «gain-of-function» (GOF) mutation in the rise in blood LDL-C concentration, especially in males [9,10]. For example, Chen et al. [9] studied genetic polymorphisms enhancing the PCSK9 function (GOF) in patients who participated in a study of Fluvastatin-LCAS efficiency. According to their data, the 23968A>G (E670G) genotype G-allele carriage is associated with increased LDL-C level and coronary atherosclerosis. They confirmed their observation in the TexGen study of a population with normal LDL level (average LDL-C level of 108 mg/dL), in which the G allele frequency was lower than in the LCAS study (0.043 vs 0.074; χ^2=5.5; P=0.019), and GG-genotype was absent in the sample. In another study, Evans and Beil [10] studied the frequency of PCSK9 gene 23968A>G (E670G) polymorphism in patients of the outpatient department of University Medical Center Hamburg-

Table 3. Comparison of Initial Clinical Hemodynamic Parameters in HeFH and non-HeFH Patients as a Function PCSK9 Gene E670G (rs505151) Polymorphism Allele G Carriage (M ± SD, n (%)

Parameters	I, HeFH (n=57)		II, non-HeFH (n=144)	
	AA (n=46)	AG + GG (n=11)	AA (n=128)	AG + GG
Mean age, years	48.7±10,1	51.8±9,0	61.6±9.8	59.4±9.0
Gender (male/female)	17/29 (37%/63%)	7/4 (64%/36%)	59/69 (46%/54%)	11/5 (69%/31%)
AH, n (%)	41 (89%)	11 (100%)	111 (87%)	15 (94%)
History of myocardial infarction, n (%)	21 (45.6%)	9 (82.0%)*	37 (29.0%)	5 (31.0%)
History of stroke, n (%)	3 (6.5%)	3 (27.3%)*	3 (2.3%)	1 (6.2%)
Coronary artery bypass grafting	1 (2.2%)	4 (36.4%)***	8 (6.25%)	2 (12.5%)
Percutaneous coronary interventions	13 (28.0%)	5 (45.5%)	21 (16.4%)	3 (19.0%)
CIMT on the left, mm	1.11±0.15	1.17±0.21	1.04±0.14	1.06±0.20
CIMT on the right, mm	1.13±0.14	1.17±0.18	1.03±0.17	1.05±0.19
Atherosclerotic plaque on the left (CCA, ICA)	28 (61.0%)	10 (91.0%)	65 (50.8%)	7 (43.8%)
Atherosclerotic plaque on the right (CCA, ICA)	28 (61.0%)	11 (100%)*	55 (43.0%)	7 (43.8%)
stenosis % on the left (CCA, ICA)	42.2%	45.7%	25.2%	31.4%
stenosis % on the left (CCA, ICA)	39.8%	46.9%	24.9%	29.5%

*Note: *,**,*** – P<0.05, P<0.01, P<0.001 significance of differences between AA- and G-carriers*

Eppendorf. The researchers found the association between G-allele carriage and increased LDL-C level for the European population (males only). A number of studies also revealed the association between 23968A>G (E670G) genotype G-allele carriage with increased CIMT [15] and coronary atherosclerosis severity [16]. However, this has not been confirmed in some of the studies [17]. There are also reports on the association between PCSK9 gene E670G polymorphism and increased risk of stroke caused by large-artery atherosclerosis [18].

CONCLUSION

The patients of the Uzbek population with heterozygous familial hypercholesterolemia showed a direct correlation between the history of myocardial infarction, carotid intima media complex thickness, increased blood PCSK-9 concentration and the carriage of the PCSK-9 gene E670G polymorphism G-allele, which makes it possible to use these as prognostic markers for the risk of cardiovascular complications.

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