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V.Ya. Polyakov, Yu.A. Nikolaev, A.V. Gusev, E.V. Sevostyanova

FEATURES OF GENETIC MARKERS OF OXIDATIVE STRESS AND FUNCTIONAL LABORATORY PARAMETERS IN COMORBID PATIENTS WITH CAROTID ATHEROSCLEROSIS

The features of the dependence of the degree of atherosclerotic stenosis of the carotid arteries on the expression level of oxidative stress marker genes in patients with atherosclerosis and comorbid pathology were studied. The association of decreased expression of the genes encoding antioxidant defense factors *GSTP1, NRF2, HMOX1* with ultrasound signs with increasing severity of atherosclerotic stenosis of the carotid arteries; an inverse correlation between the expression of the *HMOX1* gene and the degree of stenosis of the right internal carotid artery, between the level of expression of the *GSTP1* gene and the degree of stenosis of the left internal carotid artery; a direct correlation between the diameter of the right common carotid artery and the level of total cholesterol and low-density lipoprotein cholesterol in the blood serum were revealed. The knowledge gained can be used to develop a new medical technology for predicting the progression of atherosclerosis.

Keywords. Carotid atherosclerosis, gene expression, antioxidant protection, dyslipidemia, ultrasound duplex scanning.

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Introduction. In modern clinics, the incidence of atherosclerosis (AS) occurring in comorbidity (CM) conditions is increasing [1, 3, 6]. Depending on the localization and severity of AS, certain clinical and morphological manifestations are formed [6, 7]. Starting with endothelial dysfunction followed by a specific cascade of intra- and intercellular reactions, AS can form one of the pathogenetic platforms of cardiovascular CM [9]. Risk factors for endothelial damage, early stages of AS include: hypercholesterolemia, hyperhomocysteinemia, elevated cytokine levels (interleukins-1 and -8, tumor necrosis factor alpha), and oxidative stress (OS) [6]. The study of genes – markers of oxidative stress (OS) and the antioxidant defense system, such as Nrf2, is actively developing. Nrf2 – (Nuclear factor etythroid 2-related factor 2) is a transcription factor with antioxidant effects associated with the pathogenetic mechanisms of some forms of cardiovascular pathology (CVP) [15]. Nrf2 regulates the biosynthesis, utilization and regeneration of glutathione, thioredoxin and NADPH, as well as the production of reactive oxygen species and NADPH oxidase to maintain cellular redox homeostasis [20]. Studies have shown the possibility of therapeutic effects in atherosclerosis by activating the NrF2-dependent anti-inflammatory effect [11] and the inhibitory effect of the Keap/Nrf2 system on macrophage ferroptosis [13]. Nrf2 can reduce the risk of chronic diseases associated with atherosclerosis due to the corrective effect on endothelial function [14]. In OS, destruction of cell membranes occurs due to lipid peroxidation processes, reduction of antioxidant activity, as well as phospholipase hydrolysis. When the endothelium is damaged, the production of active oxygen species is activated, which have a vasoconstrictor effect, providing an increased tone of smooth muscle cells. A vasomotor form of endothelial dysfunction (ED) occurs, which is important in the mechanisms of both a systemic increase in blood pressure and local angiospasm, which emphasizes its role in the pathogenesis of cardiovascular diseases; dyslipidemia is closely associated with the activation of lipid peroxidation mechanisms and radical formation. Hypercholesterolemia leads to a decrease in NO production and a decrease in its vasodilating effect. On the surface of LDL in the intima of blood vessels, the LOX-1 receptor is present, the elevated level of which in patients with hypercholesterolemia and arterial hypertension (AH) is combined with a decrease in NO-dependent vasodilation and, accordingly, the progression of vascular damage [9]. The most common clinical method for diagnosing atherosclerotic blood flow disorders in the main arteries is ultrasound duplex scanning of arteries [4]. However, this method is financially expensive and difficult to apply for developing approaches to predicting the progression of AS.

In this regard, the study of the characteristics of atherosclerotic changes and the state of the Nrf2-dependent antioxidant system in comorbid patients with AS is relevant.

The purpose of the study. To study the features of the dependence of the degree of atherosclerotic stenosis of the carotid arteries on the level of expression of genes - markers of oxidative stress in patients with atherosclerosis and comorbid pathology.

Material and methods of research. The study included 51 patients of the Clinic of the Federal Research Center for Fundamental and Translational Medicine (FRC FTM). The sample included: 29 patients with verified carotid atherosclerosis with stenosis of more than 30%, randomly selected according to the established criterion, 22 patients for comparative analysis, without carotid atherosclerosis or with minor atherosclerotic changes in the carotid arteries without stenosis. The age of the patients studied ranged from 30 to 80 years. The average age was 64.9 ± 7.2 years. 52% of patients included in the

Federal State Budgetary Budgetary Institution Federal Research Center for Fundamental and Translational Medicine: **POLYAKOV Vladimir Yakovlevich** – MD, visiting research fellow, vpolyakov15@mail.ru, ORCID: 0000-0002-9606-2331; **NIKOLAEV Yuri Alekseevich** – chief scientific researcher, head of the laboratory, uanikolaev@frcftm.ru, ORCID: 0000-0002-1690-6080; **GUSEV Artem Vladimirovich** – graduate student, fury76@mail.ru; **SEVOSTYANOVA Evgeniya Viktorovna** – senior researcher, luck.nsk@rambler.ru, ORCID: 0000-0003-1132-3801.

study were men, 48% were women. An assessment was made of the presence of diagnosed diseases reflected in the main and concomitant diagnoses in the medical records during hospitalization of patients, as well as a comparative analysis of the presence of pathologies in each of the two groups of patients. Verification of diagnoses in patients was carried out in accordance with current clinical guidelines. The patients underwent diagnostic duplex scanning of the neck vessels (Vivid E9, GE, USA) to determine the diameter of the common carotid arteries (CCA), internal carotid arteries (ICA), the thickness of the intima-media complex, peak systolic blood flow velocity and the percentage of stenosis of the CCA and ICA. The level of the lipid spectrum of the blood was determined in the serum taken in the morning on an empty stomach; the indicators of total cholesterol (TC), low-density lipoprotein cholesterol (LDL), alpha cholesterol and triglycerides (TG) were determined. The measurements were carried out on a biochemical analyzer "BECKMAN COULTER AU480"

The expression level of polymorphisms of the gene of the antioxidant defense system *NRF2, HMOX1, GSTP1, NQO1* was determined in a group of comorbid patients with atherosclerosis with varying degrees of verified stenosis of the carotid arteries, as well as in a comparison group by PCR (polymerase chain reaction), using a CFX96 amplifier (Bio-Rad Laboratories). Work on determining gene expression was carried out on the basis of the "laboratory of molecular mechanisms of free-radical processes" of the FRC FTM (head of the laboratory and chief researcher - MD Elena Bronislavovna Menshchikova). RNA was isolated using a special ready-made reagent kit ("Lira" from BioLabMix). The obtained RNA samples were subjected to the reverse transcription procedure to obtain cDNA using a special reagent kit "Reverta-L" ("Amplisens" by Helicon) according to the instructions. The comparative expression of mRNA of the genes *NRF2, HMOX1, GSTP1, NQO1* was studied using the real-time PCR method on a CFX96 amplifier (Bio-Rad Laboratories). The housekeeping gene *GAPDH1* was used as a reference. The amplification reaction was carried out as follows: a 20 μl PCR reaction mixture was prepared containing: 10 μl Biomaster HS-qPCR SYBR Blue (2x) reaction mixture (high-throughput recombinant HS-Taq DNA polymerase, deoxynucleoside triphosphate mixture, PCR buffer, MgCl, SYBR Green I, inert dye), forward and reverse primers, DNA template, sterile water. Amplification was performed according to the following program: 5 minutes at 95 °C for preliminary denaturation, then 40 cycles: 10 seconds at 95 °C for denaturation, 15 seconds at 60 °C for primer annealing, fluorescent signal collection, 20 seconds at 72 °C - elongation. The mRNA expression level of genes was calculated according to the 2-ΔΔCT method and normalized relative to the reference housekeeping gene Gapdh1. The 2-ΔΔCT method is widely used as a relative quantification strategy for quantitative analysis of real-time PCR data. The method used is a convenient way to calculate relative gene expression levels between different samples, allowing direct use of threshold cycles (Ct/Cq) obtained by the real-time PCR system on a thermocycler for calculation.

Statistical analysis of the data was performed using the statistical software package "STATISTICA 10.0" (StatSoft Incorporated, USA). Normal distribution of indicators was estimated using the Kolmogorov-Smirnov criterion. Parametric and nonparametric statistics methods were used. Quantitative parameters were described by calculating arithmetic means and standard error of the mean (M±SE). Qualitative parameters were described by calculating the frequency of occurrence of a feature (n (%)). The t-test for comparison of independent variables was used to analyze the statistical significance of differences in quantitative indicators. Spearman correlation analysis was performed. Differences were considered statistically significant at a significance level of $p<0.05$.

Results and discussion. In the main group of patients, the average values of expression of the antioxidant defense genes *Nrf2* (0.67±0.21) and *HMOX1* (0.75±0.21) were statistically significantly lower than in the comparison group (Table 1).

In the main group of examined patients, a more frequent occurrence of expression of the antioxidant defense genes *NRF2, GSTP1, HMOX1* below the reference values than within or above the reference values was revealed: *NRF2* - in 57% (Fig. 1, a), *GSTP1* - in 86% (Fig. 1, b), *HMOX1* - in 77% (Fig. 1, c). The level of expression of the *NQO1* gene in the main group was higher than the reference values in 60% of those examined.

A comparative analysis of the incidence of comorbid pathology in the examined patients was performed: hy-

Table 1

Differences in gene expression in the main and comparison groups

Groups	*NRF2**	*HMOX1**
Main group	0.67±0.21	0.75±0.21
Comparison group	1.20±0.38	2.05±0.21

Note. * - statistical difference between the parameters of the main and comparison groups, $p<0.05$.

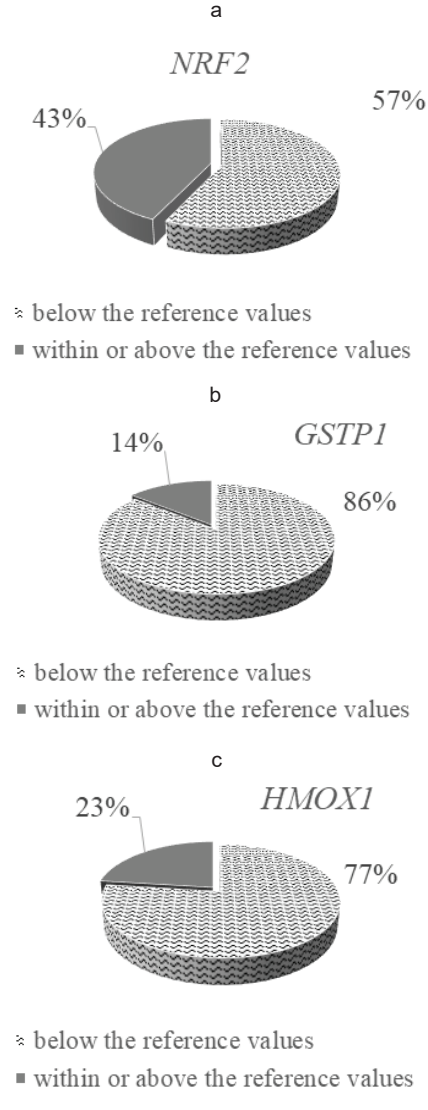

Fig. 1. Distribution of the expression level of antioxidant defense genes in relation to reference values in the main group of patients: a - *NRF2*, b - *GSTP1*, c - *HMOX1*

pertension, coronary heart disease; previous acute myocardial infarction, acute cerebrovascular accident, type 2 diabetes mellitus (Fig. 2). The average comorbidity index for the above-mentioned diseases (1 point for each nosological form) was calculated for each patient and the average value in the groups was calculated. In the main group, the comorbidity index was statistically significantly ($p<0.05$) higher (3.24±0.27) than in the comparison group (1.06±0.25) (Fig. 2). The incidence of nosological forms in the groups was as follows: in the main group, hypertension was present in all subjects, in the comparison group - in 62.5% of patients, which is 37.5% more than in the main group. Ischemic heart disease was 69.5% more common in the main group than in the comparison group: 88.2% and 18.7%, respectively. Acute myocardial infarction in the anamnesis was more common in the main group than in the comparison group (12.5%) (70.5%). Acute cerebrovascular accident in the anamnesis was 16.8% of cases in the main group, which is 10.55% more common than in the comparison group (6.25%). Type 2 diabetes mellitus was 41.2% in the main group; there were no patients with type 2 diabetes in the comparison group (Fig. 2).

Maximum degree of stenosis of the carotid arteries in the main group of examined patients was up to 81%, the average degree of stenosis of the common and internal carotid arteries was from 31% to 45%.

In the main group, the average lipid spectrum values did not exceed the reference values: TC 3.65 mmol/l [3.27; 4.74], LDL 2.4 mmol/l [1.94; 2.91], TG 1.41 mmol/l [0.87; 2.67], at the same time, significant direct correlations were revealed between the level of TC, LDL in the blood serum and the diameter of the common carotid artery, the level of TG and the diameter of the internal carotid artery (Table 2), which is explained by an increase in the rigidity of the vascular wall with a shift in lipid values towards the proatherogenic side, even within the reference values. In the main group, an inverse correlation was found between the expression of the *GSTP1* gene and stenosis of the left internal carotid artery ($r = -0.59$; $p <0.05$) (Table 2), an inverse correlation was found between the expression of the *HMOX1* gene and the severity of stenosis of the right internal carotid artery ($r = -0.45$; $p <0.05$).

The study showed the relationship between the degree of atherosclerotic stenosis of the carotid arteries, the level of expression of antioxidant defense genes

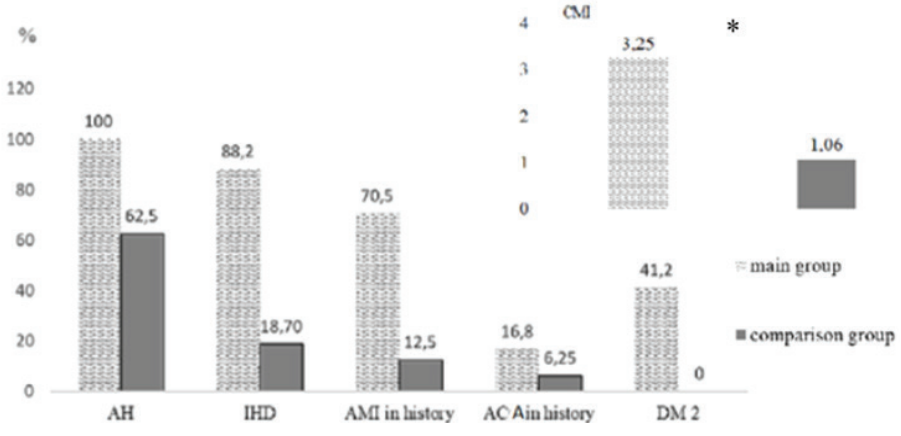

Fig. 2. Incidence of comorbid pathology in the main group and the comparison group: ICI – comorbidity index (in points), AMI – acute myocardial infarction; CHD – ischemic heart disease: ACC – acute cerebrovascular accident: DM 2 – diabetes mellitus type 2, * - $p < 0.05$

and some indicators of lipid metabolism in patients with comorbid pathology. In modern clinical practice, the incidence of cardiovascular pathology increases in conditions of comorbidity, which is most important in patients with cardiovascular pathology [8]. Combined pathology is associated not only with an increase in the number of patients with several diseases, but also the difficulties of organizing diagnosis and treatment, with the severity of the condition of these patients, "masking" different nosologies with similar syndromes, predicting the course of diseases and the difficulties of preventing complications, therefore accurate diagnosis is important using new modern methods [2]. Hypertension, the most common CM in the examined patients, is a multifactorial disease [5]. The pathogenesis of hypertension is closely related to atherosclerosis and lipid metabolism disorders [17]. Oxidation of low-density lipoproteins to oxidized LDL (oxLDL) under conditions of oxidative stress is one of the stages of the pathogenetic cascade of atherosclerosis [10], which is a manifestation of the pathogenetic relationship between OS and dyslipidemia. Increased secretion of chemokines by endothelial cells and increased expression of adhesion proteins on their surface allow them to recruit monocytes into the intima of the arteries, and monocytes differentiate into macrophages, which subsequently phagocytize lipids and form foam cells that undergo necrosis and apoptosis, forming the lipid necrotic nucleus of atherosclerotic plaque. Damaged endothelial cells during OS secrete growth factors to activate smooth muscle cells (SMC), which migrate through fenestration in the inner elastic membrane and phagocytize lipids mediated by surface lipoprotein lipase receptors, forming foam cells derived from SMC. In the late stages of AS, SMCs secrete extracellular matrix (collagen and elastin), forming fibrous capsules that increase the instability of atherosclerotic plaques [18]. An important role in regulating the cell response to OS is played by the Nrf2, which is a member of the Cap'collar (CNC) family of transcription factors – Bzyb (basic leucine lightning proteins) with 7 functional domains, participating in the regulation

Table 2

Correlations of ultrasound, laboratory parameters and the level of gene expression in the main group of patients

Indicators	expression of *GSTP1*	Diameter of the right CCA	Diameter of the left ICA
Stenosis of the left internal carotid artery	-0.59*	-0.51	0.37
Total serum cholesterol	-0.35	0.55*	0.26
Low-density lipoproteins	-0.13	0.53*	0.45
Triglycerides	0.02	0.32	0.58*

Note. CCA – common carotid artery; ICA – internal carotid artery; * - statistical significance of the indicator $p <0.05$

of their stability or transcriptional activity. [19]. Nrf2 is associated with the ubiquitin ligase complex Keap1/Cu13 (Kelch-like epichlorohydrin-associated protein 1/ Cullin 3, which inhibits its activity. Under oxidative stress or similar stimuli, cysteine residues are modified in Keap1 and protein binding with the Nrf2 is stopped. After exiting the complex, Nrf2 enters the nucleus and forms a heterodimer with the small protein Maf (Nrf2-Maf) [16]. The formed heterodimers combine with the elements of the antioxidant response (ARE) in the initiation domain in a sequence-specific manner [19], promoting the transcription of antioxidant enzymes.

Thus, the fact that a decrease in the expression of antioxidant protection genes contributes to the development and progression of atherosclerosis in CVP has been confirmed in studies, however, there is little clinical evidence on the involvement of the Keap1/Nrf2/ ARE regulatory system in atherogenesis in comorbid patients with atherosclerosis. The results obtained in this study of a decrease in the expression of the Nrf2 transcription factor gene, and the genes of antioxidant protection proteins as arterial stenosis increases clinically confirm the hypothesis of their involvement in the pathogenesis of atherosclerosis.

Conclusion. Low expression rates of genes *Nrf2, GSTP1, HMOX1* are associated with a greater severity of atherosclerotic stenosis of the carotid arteries in patients with comorbid pathology. In a group of patients with carotid atherosclerosis and severe or moderate stenosis of the carotid arteries, an inverse correlation was found between the expression of the *GSTP1* and *HMOX1* genes and the degree of stenosis. The new fundamental data obtained can be used to develop a new medical technology for predicting the progression of atherosclerosis.

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