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Micro-RNA in Patients with Carotid Atherosclerosis

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Abstract—Atherosclerosis is a multifactorial disease and one of the leading causes of ischaemic stroke. In recent years, microRNA has become an important factor to consider in the pathogenesis of atherosclerosis. MicroRNAs are non-coding RNA sequences that are divided into proatherogenic and atheroprotective. This study evaluated the leukocyte expression of certain microRNAs (miR-126-(5p/3p), miR-29a-(5p/3p), miR-33a-5p and miR-21-(5p/3p)) in patients with carotid atherosclerosis. Statistically significant differences were found between patient groups in regard to the molecular levels, as well as correlations between several microRNAs. This indicates the potential use of microRNA in diagnosis and treatment, and confirms the role of microRNAs as important regulators of carotid atherosclerosis.

Keywords: microRNA, carotid atherosclerosis, cerebrovascular disease, atherogenic profile

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INTRODUCTION

Atherosclerosis is a multifactorial disease that can affect almost any arterial blood vessel in the body. Atherosclerosis in the carotid artery system (carotid atherosclerosis) is one of the leading causes of ischaemic stroke (37% of cases according to the literature [1]). The pathogenesis of atherosclerosis involves chronic inflammation, metabolic disturbances and lipid accumulation, oxidative stress, immune pathology, numerous non-hereditary risk factors, including environmental pollution, smoking, diet and lifestyle, as well as epigenetic regulation, in particular, the microRNA system [2].

MicroRNAs are small (about 22 nucleotides), single-strand non-coding RNA sequences thought to affect most biological processes [3]. They act as significant post-transcriptional modulators/regulators of gene expression. Typically, microRNAs suppress gene expression by interacting with the 3'-untranslated region of the target matrix RNA (mRNA), triggering its degradation or blocking gene product translation. One type of microRNA can modulate multiple mRNAs involved in various biological processes; at the same time, one type of mRNA can be regulated by several microRNAs [4]. MicroRNAs also play a regulatory role in adipose stem cell ontogenesis by supporting cell proliferation and differentiation. It is thought that some microRNAs directly control complex genetic networks involved in angiogenesis [5].

MicroRNAs are involved in almost all molecular steps of atherosclerosis pathogenesis (to a varying degree), including endothelial function, cholesterol metabolism disorders, monocyte activation and their invasion of the vascular wall, activation of platelets and endothelial smooth muscle cells, and plaque formation [6]. For example, miR-126-(5p/3p), miR-29a-(5p/3p), miR-33a-5p and miR-21-(5p/3p) molecules have been identified as microRNAs associated with atherosclerosis [7].

MiR-126 is one of the most widely expressed endothelial microRNAs, and has been shown to play a critical role in the regulation of inflammation and angiogenesis. Moreover, miR-126 is also involved in endothelial dysfunction through a reduction in VCAM-1 adhesion molecule expression, thereby reducing leukocyte adhesion to the arterial wall [8].

The level of miR-29a correlates with the intima-media complex thickness and is higher in patients with atherosclerosis [9]. One of the target genes for this microRNA are type I and III collagen genes, which, in turn, play an important role in atherosclerosis. It has previously been shown that using miR-29a antagonists (antagomirs) prevents atherosclerotic plaque remodelling [10].

The MiR-33a microRNA affects the activity of a range of target genes, including *ABCA1*, an important regulatory factor of cholesterol metabolism (specifically, ensuring its efflux from cells). MiR-33a and miR-33b are located on the intron part of genes encoding sterol regulatory element-binding proteins (SREBP-2 and SREBP-1), and regulate lipid homeostasis. MiR-33 suppresses the outflow of cellular cholesterol to apolipoprotein A1 and reduces circulating

Table 1. MicroRNA expression in the study and the control group

MicroRNA	Group with CA ($\times 10^6$ copies)	Group without CA ($\times 10^6$ copies)	*P*
miR-126-5p	1.31 [1.23; 1.35]	2.24 [2.16; 2.43]	**0.000000003**
miR-126-3p	1.35 [1.28; 1.37]	2.26 [2.19; 2.44]	**0.000000003**
miR-29-5p	2.55 [2.41; 2.82]	2.64 [2.50; 3.28]	0.09
miR-29-3p	2.63 [2.39; 2.88]	2.67 [2.50; 3.26]	0.12
miR-33a-5p	4.44 [4.19; 4.88]	3.67 [3.16; 4.10]	**0.000008**
miR-21-5p	25.64 [22.26; 28.56]	31.13 [29.48; 31.99]	**0.00009**
miR-21-3p	26.15 [23.56; 29.60]	31.46 [29.64; 31.85]	**0.00009**

high-density lipoprotein (HDL) cholesterol levels in mice and primates. A number of in vivo studies have shown that miR-33 antagonism or genetic deficiency leads to increased HDL levels, which potentially slows down atherosclerosis progression [11].

MiR-21 is considered by some researchers to belong to mechanosensitive microRNAs, that is, microRNAs that are predominantly expressed in the arterial wall and activated under shear stress associated with turbulent blood flow, which is an important component of atherosclerotic plaque formation [12].

The **aim** of this pilot study was to evaluate the leukocyte expression of the above listed microRNAs in a cohort of patients with haemodynamically significant carotid atherosclerosis.

PATIENTS AND METHODS

The study group included patients ($n = 25$, average age: 67 years, 60% men) with atherosclerotic stenosis of the internal carotid artery (>50% of the vessel lumen), without evidence of previous cerebrovascular disease (the carotid atherosclerosis or CA group). The comparison group consisted of 11 patients comparable in gender and age, with no evidence of carotid artery disease. Carotid atherosclerosis was verified using ultrasound duplex scanning and the ECST method was used to assess the degree of stenosis. The exclusion criteria were decompensated chronic disease, tumour and paraneoplastic processes, and blood disorders.

Blood samples were obtained via cubital venipuncture in the morning, on an empty stomach. To isolate microRNA from patient blood plasma samples, the MagMAX™ mirVana™ total RNA isolation kit (Thermo Fisher Scientific, Finland) was used, designed to isolate total RNA, including microRNA, from different sample types. The kit uses MagMAX magnetic bead technology to ensure reproducible extraction of high-quality RNA, which we used to detect TaRMan™ miRNA. Further, a reverse transcription polymerase chain reaction (40 amplification cycles) was performed using the standard PCR technique, with the appropriate set of primers (Thermo Fisher Scientific, Finland).

Statistical processing was performed using the Statistica software (version 12.0); nonparametric methods were used, including the Mann-Whitney U test to compare intergroup differences, as well as descriptive statistics (median, interquartile interval), and the Spearman correlation coefficient.

RESULTS AND DISCUSSION

Statistically significant differences between patient groups were found for most of the studied microRNAs (Table 1). The most significant differences between the patient groups were obtained for miR-126, miR-33a and miR-21. The only microRNA that was significantly higher in the study group was miR-33a-5p (4.44 vs. 3.67). The atheroprotective role of miR-126-5p, miR-126-3p, miR21-5p and miR-21-3p was indicated by their higher levels in the group without carotid atherosclerosis. Neutral results were obtained for miR-29-5p and miR-29-3p. Correlation analysis revealed certain associations between several microRNAs (Figs. 1–3), but only in the group with carotid atherosclerosis. An increase in miR-126-5p expression levels was associated with a decrease in miR-33a-5p levels, while miR-126-5p and miR-21-5p expression levels correlated directly with each other.

It was found that miR-33a-5p and miR-21-5p expression levels show an inverse correlation in patients with atherosclerosis, but this was not observed in the comparison group.

The long history of studying atherosclerosis is full of various facts concerning its origin, progression and development of corresponding clinical signs. Significant immunological activity, lipid accumulation, smooth muscle cell proliferation, cell apoptosis, necrosis and fibrosis are an incomplete list of what accompanies atherosclerosis. Atherosclerosis is caused by a combination of multiple factors, including genomics and epigenetic modifications, as well as

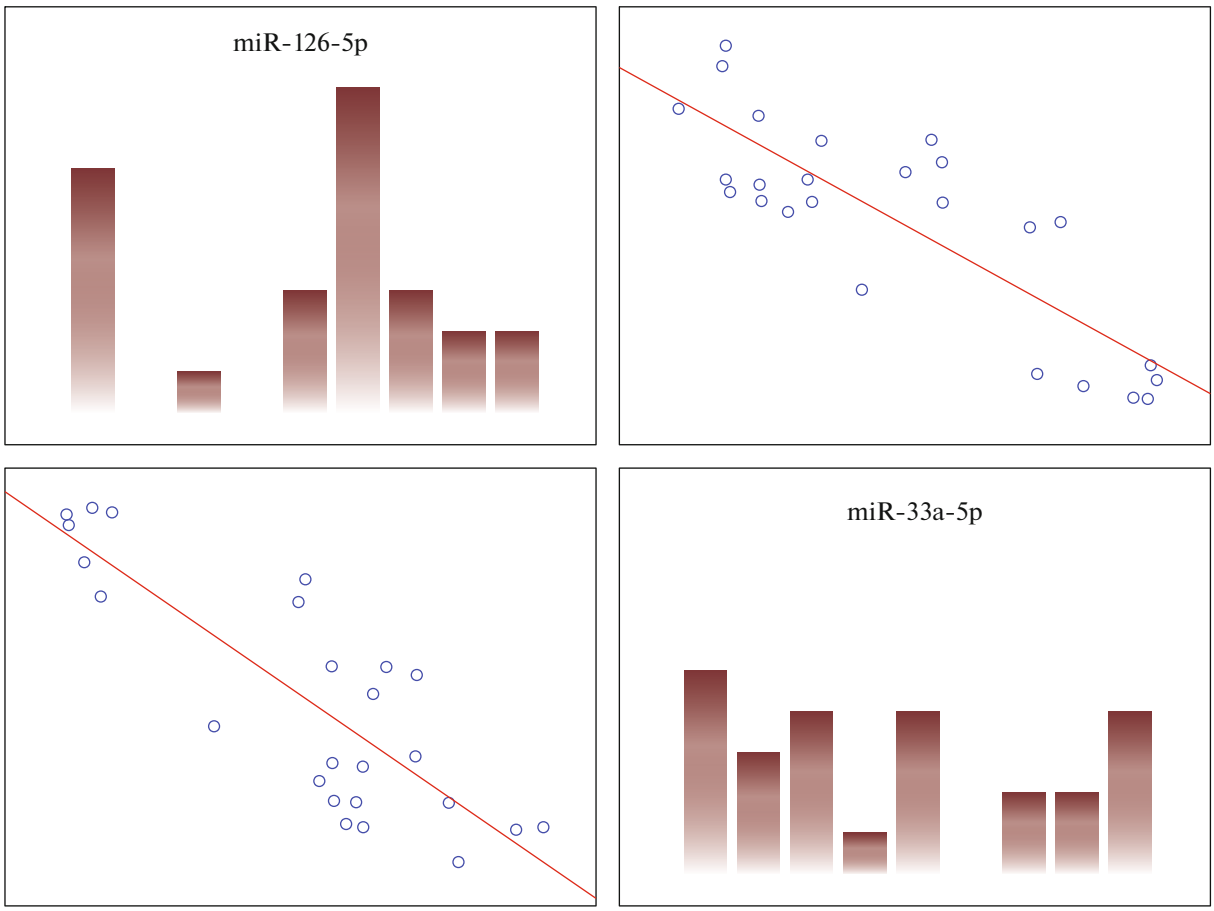

Fig. 1. Relationship between miR-126-5p and miR-33a-5p expression in patients with carotid atherosclerosis (Spearman coefficient −0.79, $p < 0.05$).

non-hereditary risk factors (environmental pollution, smoking, diet and lifestyle).

The multistaged paradigm stresses that atherosclerosis is affected by the interaction between blood cells and endothelial cells, the lipid profile, oxidative stress, and disorders of carbohydrate metabolism and haemostasis. Studies of the genetic basis of atherosclerosis have revealed gene-related risk factors for atherosclerosis, such as single-nucleotide polymorphisms, mutations in atherosclerosis candidate genes, changes in DNA methylation, changes in gene expression, as well as the influence of non-coding RNA.

The results of this study are in line with current research regarding the difficulties of studying microRNA in cerebrovascular pathology. The examined microRNAs can be divided into proatherogenic (miR-33a) and atheroprotective (miR-126-5p, miR-126-3p, miR-21-3p, miR-21-5p).

MiR-33 are important regulators of lipid metabolism (particularly HDL) through the modulation of the *SREBP-1* and *SREBP-2* genes. For example, miR-33 alters the expression of *ABCA1* and *ABCG1* genes, preventing cholesterol release from cells [13]. One study [14] demonstrated increased HDL levels and decreased atherosclerotic damage 4 weeks after the administration of anti-miR-33 oligonucleotides. In addition, the use of miR-33 antagomirs in mice with hypercholesterolemia has been shown to slow down atherosclerotic progression by increasing the number of anti-inflammatory M2 macrophages around the atherosclerotic plaque [15]. Previously, however, no significant increase in miR-33 expression levels was observed in patients with carotid atherosclerosis. This observation confirms the pathogenetic (and potentially, the diagnostic and therapeutic) role of this miRNA in the given pathology.

A decrease in miR-126 expression was found in several studies of patients with atherosclerosis in various locations [16, 17]. MiR-126 is an endothelial-specific microRNA, associated with vascular wall integrity [18]. The inverse correlation between miR-126-5p and miR-33a-5p expression levels was significant only in patients with atherosclerosis. This may indicate an etiopathogenetic correlation and the inclusion of multidirectional regulatory mechanisms in the development and progression of atherosclerotic plaques.

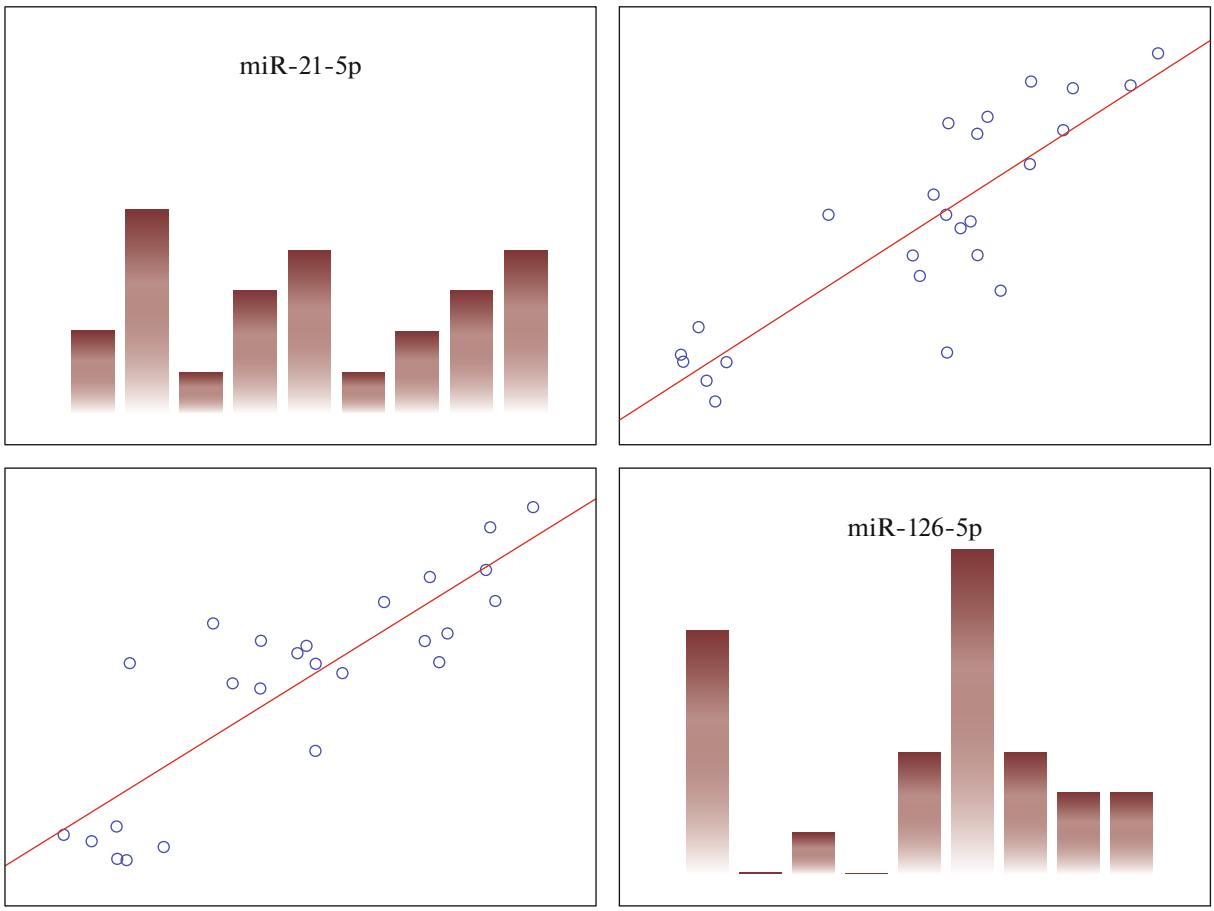

Fig. 2. Relationship between miR-126-5p and miR-21-5p expression in patients with carotid atherosclerosis (Spearman coefficient 0.82, $p < 0.05$).

It is worth noting the miR-21-5p and miR-21-3p expression levels, since they were significantly lower in the atherosclerosis group in our study, leading us to conclude that they play an atheroprotective role. Despite this, other studies we analysed demonstrate that this microRNA plays a dual role, including as a proatherogenic molecule. For example, miR-21 is known to inhibit translation of the Pdcd4 protein both in endothelial smooth muscle cells (suppressing apoptosis and stimulating proliferation) and in macrophages (suppressing inflammatory mechanisms and, consequently, atherogenesis). In addition, analysis of miR-21 expression in vascular endothelial cells showed that it is elevated in all conditions involving the endothelium (atherosclerosis, arteriosclerosis and aortic aneurysm) [19].

CONCLUSIONS

The expression of several microRNAs, associated with various steps in atherogenesis, was analysed in this pilot study of a cohort of patients with haemodynamically significant carotid atherosclerosis. Significant differences were found between the groups, allowing us to divide microRNAs into potentially proatherogenic (miR-33a) and atheroprotective (miR-126-5p, miR-126-3p, miR-21-3p and miR-21-5p). This observation was confirmed by the identified correlations between microRNA expression levels.

MicroRNAs are one of the important regulators of carotid atherosclerosis, which suggests their potential use in diagnosis and treatment (targeting antagomirs to proatherogenic molecules and/or administering atheroprotective microRNAs). These differences require further confirmation in larger population studies.

FINANCING

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COMPLIANCE WITH ETHICAL STANDARDS

The study was conducted in accordance with the ethical standards set out in the Declaration of Helsinki 1964 and its subsequent revisions, and approved by the Local Ethics

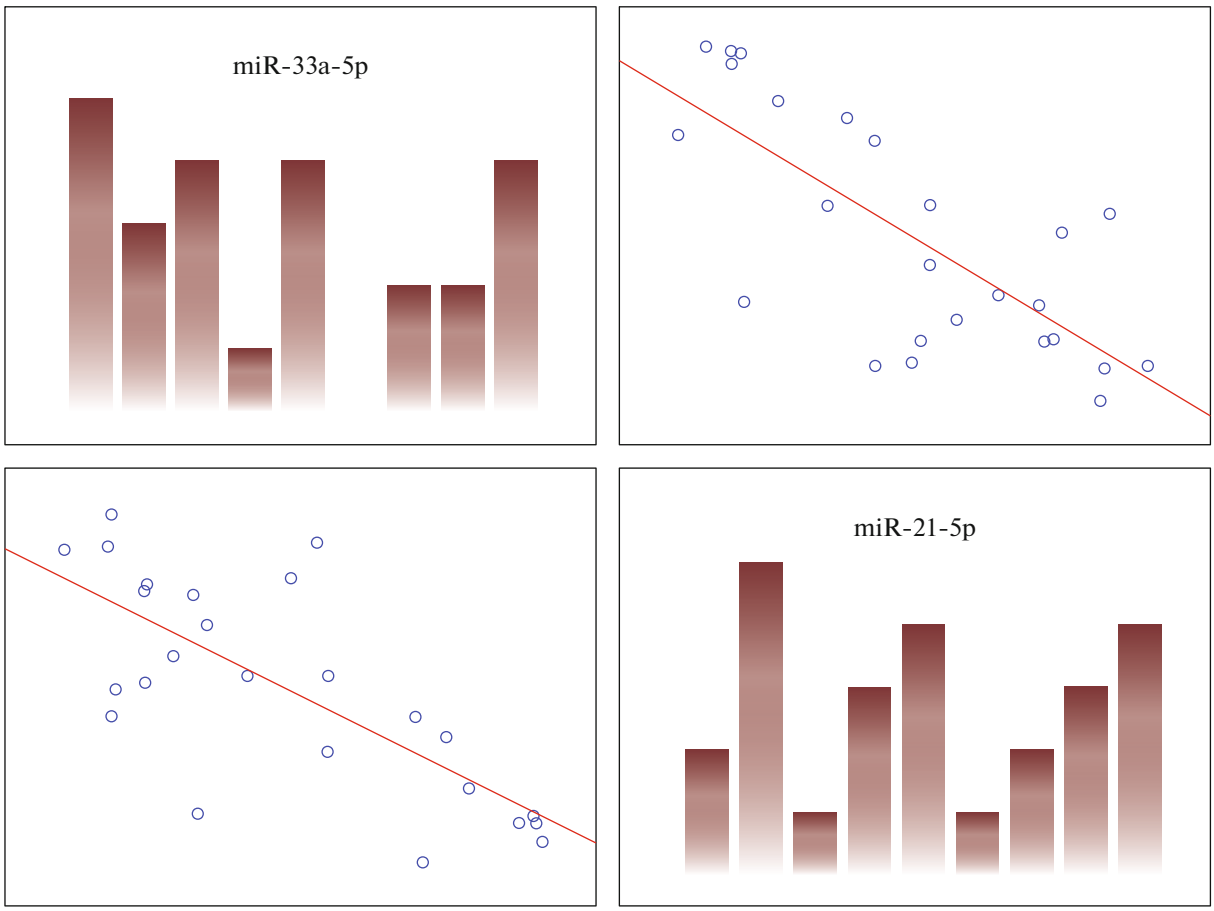

Fig. 3. Relationship between miR-33a-5p and miR-21-5p expression in patients with carotid atherosclerosis (Spearman coefficient −0.71, $p < 0.05$).

Committee (Minutes of meeting no. 11-4/19 dated 20/11/19); all patients provided written voluntary informed consent.

AUTHOR CONTRIBUTIONS

Raskurazhev A.A.—grant executor, study initiator, concept, plan and methodology development, statistical processing, writing the initial version of the manuscript.

Tanashyan M.M.—creating the research concept, monitoring the execution, final review of the manuscript.

Shabalina A.A.—preparing the laboratory protocol for microRNA study, performing the tests, statistical data processing.

Kuznetsova P.I.—patient recruitment for the study.

Kornilova A.A.—grant co-executor, patient recruitment for the study, database management, manuscript preparation.

Burmak A.G.—performing laboratory tests, taking blood samples for the study.

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