ISSN 0026-8933, Molecular Biology, 2023, Vol. 57, No. 3, pp. 457–466. © Pleiades Publishing, Inc., 2023.
Russian Text © The Author(s), 2023, published in Molekulyarnaya Biologiya, 2023, Vol. 57, No. 3, pp. 471–482.

GENOMICS.
TRANSCRIPTOMICS

UDC 577.21+616.13.002.2-004.6

Identification of Functionally Significant Polymorphic Variants in miRNA Genes in Carotid Atherosclerosis

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Received July 19, 2022revised October 5, 2022accepted October 5, 2022

Abstract—miRNAs are vital molecules of gene expression. They are involved in the pathogenesis of various common diseases, including atherosclerosis, its risk factors, and its complications. A detailed characterization of the spectrum of functionally significant polymorphisms of miRNA genes in patients with advanced carotid atherosclerosis is an important research task. We analyzed miRNA expression and exome sequencing data of carotid atherosclerotic plaques of male patients (n = 8, 66–71 years of age, 67–90% degree of carotid artery stenosis). For further study and analysis of the association between the rs2910164 polymorphism of the *MIR146A* gene and advanced carotid atherosclerosis, we recruited 112 patients and 72 relatively healthy Slavic residents of Western Siberia. A total of 321 and 97 single nucleotide variants (SNVs) were detected in the nucleotide sequences of pre- and mature miRNAs in carotid atherosclerotic plaques. These variants were located in 206 and 76 miRNA genes, respectively. Integration of the data of exome sequencing and miRNA expression revealed 24 SNVs of 18 miRNA genes that were processed to mature form in carotid atherosclerotic plaques. SNVs with the greatest potential functional significance for miRNA expression predicted *in silico* were rs2910164:C>G (*MIR146A*), rs2682818:A>C (*MIR618*), rs3746444:A>G (*MIR499A*), rs776722712:C>T (*MIR186*), rs199822597:G>A (*MIR363*). The expression of miR-618 was lower in carotid atherosclerotic plaques of patients with the AC rs2682818 genotype of the *MIR618* gene compared with the CC genotype ($\log_2$FC = 4.8; p = 0.012). We also found an association of rs2910164:C (*MIR146A*) with the risk of advanced carotid atherosclerosis (OR = 2.35; 95% CI: 1.43-3.85; p = 0.001). Integrative analysis of polymorphisms in miRNA genes and miRNA expression is informative for identifying functionally significant polymorphisms in miRNA genes. The rs2682818:A>C (*MIR618*) is a candidate for regulating miRNA expression in carotid atherosclerotic plaques. The rs2910164:C (*MIR146A*) is associated with the risk of advanced carotid atherosclerosis.

Keywords: miRNA, sequencing, carotid atherosclerosis

DOI: 10.1134/S0026893323030172

INTRODUCTION

Atherosclerotic lesions of the carotid arteries often lead to the development of complications such as chronic cerebral ischemia, vascular dementia, transient ischemic attack, and ischemic stroke [1]. The understanding of the pathophysiological processes underlying the formation of atherosclerosis and the mechanisms of its clinical complications has significantly improved and key signaling pathways and molecules involved in the initiation and progression of atherosclerotic plaques have been discovered [2].

Among these molecules, miRNAs are of particular interest; these are small (miRNA; average 22 nucleotides) single-stranded non-coding RNAs that regulate gene expression in the nucleus at the transcriptional level and at the post-transcriptional level in the cytoplasm by binding to the 3'-untranslated region of mRNA. Binding can be either fully complementary with the degradation of the mRNA of the target gene, or partially complementary (more typical for miRNAs) with translation suppression [3]. With partial complementarity, one miRNA can have several hundred target mRNAs at once. The reverse is also true, that is, one mRNA can bind to different miRNAs.

Polymorphism in miRNA genes (regulatory regions, in pri- and pre-miRNA, in the region of the nucleotide sequence of mature miRNA), as well as in the genes of their biogenesis and target genes, may be functionally significant for the formation of atherosclerosis and its complicated course [4]. Variants in miRNA genes can affect the processing and expression level of the corresponding molecules, their bio-

genesis, activity, and the interaction of a mature miRNA with its target mRNA [5]. However, nucleotide variability in the gene region of (pri-/pre-) miRNAs in cardiovascular diseases has not been studied in sufficient detail [5, 6].

Numerous studies have shown that miRNAs are involved in almost all stages of atherogenesis, promoting or suppressing this pathological process [7–9]. The HMDD v3.0: the Human MicroRNA Disease Database (updated March 27, 2019) contains information on 116 experimentally confirmed miRNAs associated with atherosclerosis, of which 26 miRNAs are associated with carotid atherosclerosis and 67 with ischemic stroke [10].

Most studies aimed at searching for associations of polymorphic variants of miRNA genes (miR-let-7, miR-27a, -146a, -149, -196a2, -200b, -423, -499, -618, and -4513) with clinical complications of atherosclerosis mainly analyzed individual single nucleotide variants (SNVs) in Asian populations and obtained conflicting results [11–16].

Changes in miRNA expression in vascular cells affected by atherosclerosis were analyzed using various comparison groups and methods, including real-time PCR and microarray technology [17, 18]. As a result, 31 miRNAs were identified (miR-1, let-7f, -9, -10a, -10b, -16, -19a, -19b, -21, -22, -24, -25, -29b, -29c, -34a, -92a, -100, -106b, -125a, -127, -133a, -133b, -143, -145, -146a, -150, -155, -221, -223, -486, and -497), whose expression in atherosclerotic plaques of the coronary and carotid arteries is statistically significantly different from the expression in intact vascular tissues, or in unstable (symptomatic) and stable (asymptomatic) atherosclerotic plaques in two or more studies [19]. However, up to now, there are no studies that analyze the variability of miRNA genes and miRNA expression in atherosclerotic plaques in the same patients.

In this regard, the study of functionally significant variants in miRNA genes and the analysis of their association with diseases caused by atherosclerosis in European populations is relevant. The study of polymorphic variants in miRNA genes involved in the pathogenesis of atherosclerosis will help to identify new molecular mechanisms of the development of the disease, as well as possible biomarkers and targets for therapy. The data we obtained can be used to identify and stratify individuals and predict the risk of developing clinical complications of atherosclerosis.

In our work, using exome and miRNA sequencing, we characterized the spectrum of functionally significant single nucleotide polymorphic variants in miRNA genes in atherosclerotic plaques of the carotid arteries of the same patients.

EXPERIMENTAL

The material for the study was samples of atherosclerotic carotid arteries obtained during carotid endarterectomy from eight male patients (66–71 years old), who were Slavs and residents of Western Siberia. The degree of carotid stenosis on ultrasound examination ranged from 67 to 90%. All patients smoked, they had a history of arterial hypertension and coronary heart disease; two different patients had experienced an acute cerebrovascular accident (ACVA) and myocardial infarction (MI), and one had type 2 diabetes mellitus. All patients were taking medications from the groups of statins, antihypertensive drugs, and anticoagulants/deaggregants. Carotid biopsy specimens were frozen and stored in liquid nitrogen until the DNA and RNA extraction procedure.

DNA was extracted by the standard phenol–chloroform method. DNA libraries for exome sequencing were obtained using the SureSelect Clinical Research Exome Enrichment Kit (Agilent, United States). The quality of the DNA libraries was assessed using a Bioanalyzer 2100 electrophoresis tool (Agilent). Sequencing was performed on a HiSeq 1500 System (Illumina, United States) in 2 × 150 bp mode. Primary data are stored in the NCBI BioProject repository, PRJNA758796 [20].

Analysis of genetic variants was performed using the Genome Analysis Toolkit (GATK) algorithm for identifying SNVs, short insertions, and deletions [21]. The quality of the reads was assessed using the FastQC v0.11.5 tool [22]. Alignment for the GRCh38 genome assembly was performed using GATK BwaSpark alignment tool, SNV search was performed using GATK HaplotypeCaller tool [21]. Insertions and deletions, as well as single nucleotide variants annotated at several loci of the genome, were excluded from further analysis.

RNA was isolated using the TRIzol reagent (ThermoFisher, United States) and the PureLink RNA Micro Scale Kit (ThermoFisher). Good-quality RNA (RINs > 7.0) was isolated from all carotid tissue samples, except for one sample, which was excluded from further study. The cDNA library was prepared using the NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs, United Kingdom). The quality of the libraries was assessed using a Bioanalyzer 2100 electrophoresis tool (Agilent). Sequencing was performed on a HiSeq 1500 System (Illumina) in 1 × 50 bp mode. miRNA sequencing data were analyzed using the miARma-Seq software package [23]. The quality of reads was assessed using the FastQC v0.11.5 tool. The Trim Galore software package was used to remove adapters [24]. Alignment was performed for the GRCh38 genome assembly using the BWA-MEM software [25]. The number of miRNA reads was counted using the featureCounts algorithm [26]. miRNA expression levels were assessed using the edgeR package in the R software environment. SNV identification in miRNA sequencing data was performed using the GATK software package [21]. Information about precursors (miRNA_pri-

mary_transcript) and mature miRNAs (miRNA) was obtained from the miRBase v22 database [27]. The functional significance of SNV for miRNA expression was predicted using the miRVaS program [28]. The association of miRNAs with atherosclerosis and its complications was assessed using information obtained from the HMDD v3.2 database [29].

For confirmatory study and association analysis of the rs2910164 gene polymorphism of *MIR146A* with advanced carotid atherosclerosis in the Slavs who were residents of Western Siberia, an expanded sample of patients was used (n = 112, aged 65 (59; 69) years, 78 men and 34 women). The degree of carotid stenosis on ultrasound was ≥70%, which was an indication of carotid endarterectomy. All patients had a history of arterial hypertension; 68 (60.7%) patients smoked, and type 2 diabetes mellitus was found in 2 (1.8%) patients. ACVA and MI were registered in 38 (33.9%) patients both of these events simultaneously were detected in 10 (9%) individuals.

The control group was formed from relatively healthy individuals without a history of cardiovascular pathology and signs of cerebral ischemia during a clinical examination (n = 72, aged 69 (67; 73) years, 45 men and 26 women). All individuals underwent ultrasonography that revealed the initial stages of atherosclerosis of the carotid arteries, but without hemodynamically significant changes (stenosis did not exceed 24%).

Genotyping of peripheral blood leukocyte samples was performed by PCR followed by restriction fragment length polymorphism analysis (PCR-RFLP), according to Yamashita J. et al. [30]. The distribution of genotypes of rs2910164 of the *MIR146A* gene in the control group did not deviate from the Hardy–Weinberg equilibrium.

The obtained data were analyzed using the R software package (The R Foundation). Comparison of quantitative data was carried out using the Wilcoxon test, and qualitative data were compared using the χ^2 test, the Pearson test with Yates' correction for continuity, and/or Fisher's exact test. Numerical values are given below in the format: median [1st quartile, 3rd quartile]. The relative risk for carriers of a disease-associated allele of a polymorphic genetic variant was estimated by the odds ratio (OR), for which a 95% confidence interval (95% CI) was calculated. The threshold significance level is set to $p = 0.05$.

The study was approved by the Biomedical Ethics Committee of the Research Institute of Medical Genetics of the Tomsk National Research Medical Center. All donors signed a voluntary informed consent to participate in the study.

RESULTS AND DISCUSSION

Data from the exome sequencing of DNA isolated from carotid atherosclerotic plaques of eight patients were characterized by 55.9 [55.0; 57.5] millions of pair-ended reads with a length of 150 bp per sample. In the nucleotide sequence of miRNA precursors, 380 SNVs were identified, of which 321 had coverage ≥10× in at least one sample (corresponding to 206 miRNA genes), of which 234 (72.9%) were transitions and 87 (27.1%) were transversions. In the region of the nucleotide sequence of mature miRNA, 113 SNVs were identified, including 97 SNVs (76 genes) with coverage ≥10×, of which 75 (77.3%) were transitions and 22 (22.7%) were transversions. These results are consistent with a higher frequency of transitions in the miRNA gene region than other types of substitutions [31].

As a result of high-throughput sequencing of miRNAs isolated from carotid atherosclerotic plaques of seven patients, we got an average of 7.8 [7.2; 12.3] million single-ended reads of 50 bp long, of which 467 [253; 852] thousand corresponded to miRNA. Expression at a level greater than 10 CPM (counts of reads of a given miRNA per million reads in a sample) in at least one sample was registered for 364 (19%) of 1917 known miRNAs, according to the miRbase v.22 database. Most of the miRNAs (169 out of 278 (61%)) found in atherosclerotic plaques without considering -5p/-3p distinctions have not previously been associated with atherosclerosis, including carotid atherosclerosis and ischemic stroke, according to the HMDD v3.2 database. Moreover, only 28 (8%) of 364 miRNAs with -5p/-3p analysis or 76 (27%) of 278 miRNAs without these details were previously analyzed as biomarkers of coronary artery disease, acute coronary syndrome, MI, and stroke [32].

The highest expression level was found for miR-143-3p: 18.7 [17.3; 18.9], miR-21-5p: 17.0 [16.4; 17.5], and miR-100-5p: 15.9 [15.4; 16.3]. The expression of these miRNAs in atherosclerotic plaques of the carotid arteries reaches 36% of the total expression of all miRNAs, with miR-143-3p accounting for 23%. These results are consistent with the fact that the expression of only a few miRNAs makes a significant proportion of the total miRNA level in the analyzed sample [33].

Using GATK, we searched for genetic variants of miRNAs in sequencing data. As a result, 427 SNVs were identified, of which coverage ≥10× in at least one sample was reached in 207 (48%), but only three of these SNVs (1.5%) (rs4534339, rs771605638, and rs775920236) were located in the region of the nucleotide sequence of mature miRNA (miR-1843 and miR-100, respectively). Moreover, rs4534339 was also confirmed by exome sequencing data: all patients had the TT genotype (the minor allele frequency (MAF) in Europeans was 0.99 according to gnomAD).

A comparison of the results of exome sequencing and miRNA expression revealed 24 SNVs located in 18 miRNA genes that are of interest regarding their potential influence on the regulation of the expression of the corresponding miRNAs in atherosclerotic plaques of the carotid arteries (Table 1). Of these 24 SNVs, 14 (58%) were frequent according to the gnomAD database (MAF gnomAD ≥ 5%). Frequent

Table 1. The spectrum of SNV in miRNA genes expressed in carotid atherosclerotic plaques

No.	dbSNP:ref./alt.	miRNA gene	Genotype, number of patients	The level of miRNA expression in carotid atherosclerotic plaques ($n = 7$)	MAF alt (gnomAD)	Localization of SNV relative to miRNA	MFE	MFE deltaG ref./alt.	Association with carotid and coronary atherosclerosis and its complications (HMDD, NCBI)
1	rs776722712:C>T	*MIR186*	CC, 7; CT, 1	**miR-186-5p = 10.3 [9.4; 10.4]**	0.0002	**Mature 3p**/miR-186	**Mature**	**−40.6/−36.9**	AT [43, 44]
2	rs4534339:C>T	*MIR1843*	TT, 8	miR-1843 = 5 [4.8; 5.7]	**0.9397**	NA	NA	NA	–
3	rs71428439:A>G	*MIR149*	AA, 7; AG, 1	miR-149-5p = 4.5 [4.1; 6]	**0.1333**	Arm 3p/miR-149	No change	−56.1/−56.1	MI [42]
4	rs2292832:T>C	*MIR149*	TC, 5; CC, 3	miR-149-5p = 4.5 [4.1; 6]	**0.6889**	Arm 3p/miR-149	No change	−56.1/−58.3	IS [34, 35], MI [42], AT [45]
5	rs28645567:G>A	*MIR378D1*	GG, 7; G.A., 1	miR-378d = 3.8 [3.6; 4.4]	**0.0766**	Arm 5p/miR-378d-1	No change	−19.6/−17.3	–
6	rs2910164:C>G	*MIR146A*	CC, 1; CG, 2; GG.5	**miR-146a-5p = 12.1 [11.8; 12.7]**	**0.7018**	**Mature 3p, seed**/miR-146a	**Seed**	**−38.8/−41.8**	IS [12, 13, 36], CAD [12, 15], MI [42], AT [46, 47]
7	rs76481776:C>T	*MIR182*	CC, 7; CT, 1	miR-182-5p = 5.9 [5.1; 7.8]	**0.05786**	Arm 3p/miR-182	Arm	−47.3/−47.9	AT [48]
8	rs7911488:A>G	*MIR1307*	AA, 4; AG, 4	miR-1307-3p = 8.1 [7.5; 8.4]; miR-1307-5p = 6.3 [6.1; 6.7]	**0.2697**	Loop/miR-1307	No change	−55.4/−55.4	–
9	rs17091403:C>T	*MIR2110*	CC, 7; CT, 1	miR-2110 = 5.9 [5.4; 6.1]	**0.0751**	Arm 5p/miR-2110	Mature	−34.6/−35.4	–
10	rs796224492:T>C	*MIR10527*	TT, 3; TC, 5	miR-10527-5p = 3.2 [3.0; 3.6]	0.0467	NA	NA	NA	–
11	rs796361602:C>T	*MIR10527*	CC, 3; CT, 5	miR-10527-5p = 3.2 [3.0; 3.6]	0.0547	NA	NA	NA	–
12	rs796973621:C>T	*MIR10527*	CC, 3; CT, 5	miR-10527-5p = 3.2 [3.0; 3.6]	0.0428	NA	NA	NA	–
13	rs796153424:A>T	*MIR10527*	AA, 3; AT, 5	miR-10527-5p = 3.2 [3.0; 3.6]	0.0212	NA	NA	NA	–
14	rs878994369:T>C	*MIR10527*	TT, 4; TC, 4	miR-10527-5p = 3.2 [3.0; 3.6]	0.0139	NA	NA	NA	–

Table 1. (Contd.)

No.	dbSNP:ref./alt.	miRNA gene	Genotype, number of patients	The level of miRNA expression in carotid atherosclerotic plaques ($n = 7$)	MAF alt (gnomAD)	Localization of SNV relative to miRNA	MFE	MFE deltaG ref./alt.	Association with carotid and coronary atherosclerosis and its complications (HMDD, NCBI)
15	rs796405877:G>A	*MIR10527*	GG, 6; GA, 2	miR-10527-5p = 3.2 [3.0; 3.6]	0.0071	NA	NA	NA	–
16	**rs2682818:A>C**	*MIR618*	AA, 1; AC, 2; CC, 5	miR-618 = 2.7 [1.6; 3.1]	**0.8077**	**Arm 3p**/miR-618	**Seed**	**–34.5/–38.3**	IS [37]
17	rs75330474:C>T	*MIR323B*	CC, 7; CT, 1	miR-323b-3p = 3.4 [2.1; 3.7]	0.0249	Mature 5p/miR-323b	No change	–40.2/–40.2	–
18	rs41280052:G>T	*MIR184*	GG 8	miR-184 = 2.3 [1.6; 4.0]	0.0110	Arm 5p/miR-184	Arms	–37.2/–37	–
19	rs6505162:A>C	*MIR423*	AA, 3; AC, 3; CC, 2	**miR-423-5p = 12.1 [10.4; 12.3]; miR-423-3p = 11.6 [10.8; 11.7];**	**0.4244**	Arm 3p/miR-423	No change	–48.6/–48.6	CAD [39]
20	rs745666:G>C	*MIR3615*	GG, 4; GC, 3; CC, 1	miR-3615 = 6.1 [6.0; 7.1]	**0.4086**	Arm 3p/miR-3615	No change	–45.4/–45.4	–
21	rs895819:T>C	*MIR27A*	TT, 1; TC, 6; CC, 1	**miR-27a-3p = 12.9 [12.0; 13.3];** miR-27a-5p = 7.9 [6.4; 8.0]	**0.3732**	Loop/miR-27a	No change	–37.8/–37.8	MI [40], AT [49]
22	rs3746444:A>G	*MIR499A*	AA, 5; AG, 2; GG, 1	miR-499a-5p = 4.5 [3.9; 4.9]	**0.1862**	**Mature 5p**/miR-499b	**Seed**	**–25.4/–21**	IS [13]; CAD [15]; MI [41, 42]
23	rs199822597:G>A	*MIR363*	GG, 6; AA, 2	miR-363-3p = 6.5 [5.7; 8.2]	0.0022	**Mature 5p**/miR-363	**Mature**	**–26.4/–23.7**	AT [50]
24	rs5907732:G>T	*MIR320D2*	GG, 2; TT, 6	miR-320d = 3.4 [2.6; 5.3]	**0.7043**	Upstream/miR-320d-2	NA	NA	–

dbSNP, according to the NCBI database; ref./alt., reference/alternative allele; MAF, minor allele frequency; gnomAD, Genome Aggregation Database; MFE is the minimum free energy; CAD, coronary artery disease; IS, ischemic stroke; MI, myocardial infarction; AT, atherosclerosis; mature, mature miRNA; arm, miRNA arm; seed, miRNA "seed" region; loop, a miRNA loop, upstream, located higher in relation to miRNA; NA, no data. Bold type in column 5 indicates miRNAs whose level is higher than 10 $\log_2$(CPM); column 6, frequent genetic variants in the population (frequency of MAF gnomAD ≥ 5%); columns 7 and 8, indicators important for prediction in silico SNVs with the highest potential functional significance for miRNA expression.

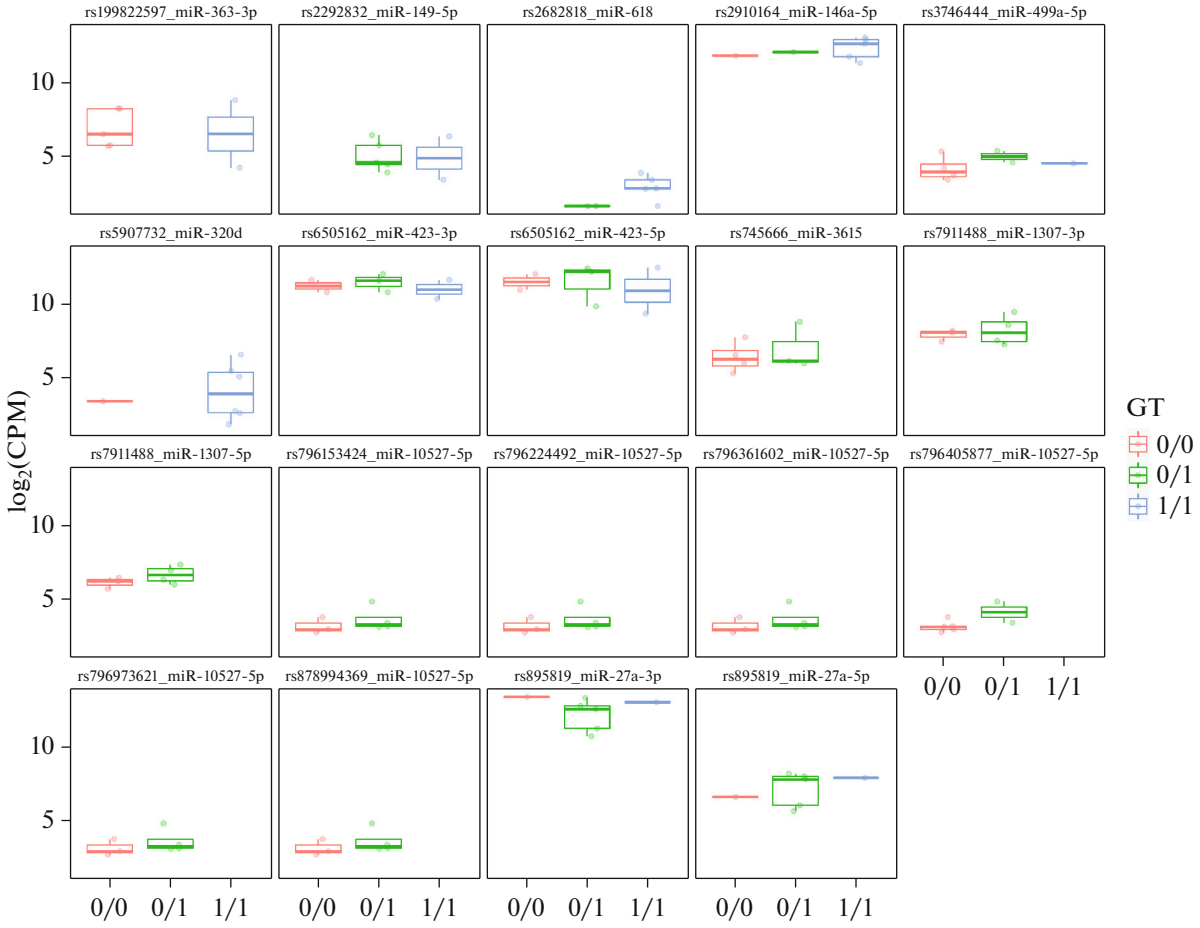

Fig. 1. The expression levels of 19 miRNAs in carotid atherosclerotic plaques depending on the SNV genotype of miRNA gene. GT, genotypes; 0/0, homozygous for the reference allele; 1/1, homozygotes for the alternative allele; 0/1, heterozygotes. Above each cell is the name of the SNV and the corresponding miRNA; the *X* axis shows genotypes (0/0, 0/1, and 1/1), and the *Y* axis shows the level of miRNA expression.

variants rs71428439 and rs2292832 are located in the region of the *MIR149* gene, and rare rs796224492, rs796361602, rs796973621, rs796153424, rs878994369 and rs796405877 variants are located in the *MIR10527* gene. Potentially, rs776722712:C>T (*MIR186*), rs2910164:C>G (*MIR146A*), rs2682818:A>C (*MIR618*), rs3746444:A>G (*MIR499A*), and rs199822597:G>A (*MIR363*) have the greatest potential functional significance for miRNA expression predicted in silico.

We identified five miRNAs with the highest level of expression in atherosclerotic plaques of the carotid arteries: miR-27a-3p—12.9 [12.0; 13.3], miR-146a-5p—15.9 [15.4; 16.2], miR-423-5p—12.1 [10.4; 12.3], miR-423-3p—11.6 [10.8; 11.7] and miR-186-5p—10.3 [9.4; 10.4] (Table 1).

Genotypes of miRNAs at risk for ischemic stroke—a complication of atherosclerosis of the carotid arteries—included the previously associated C allele/CC genotype of rs2292832 (*MIR149*) in Asian populations [34, 35], G allele/GG genotype of rs2910164 (*MIR146A*) in South Koreans [12, 13, 36], and in the Chinese population, GT+TT genotypes of rs2682818 (*MIR618*) [37] and GG genotype of rs3746444 (*MIR499A*; Table 1) [13].

Alleles associated with an increased risk of coronary artery disease (CAD) include rs2292832 (*MIR149*) in South Korea [38], A allele of rs6505162 (*MIR423*) in India [39], while in Asian populations, CC genotype of rs2910164 (*MIR146A*) [12, 15] and GG genotype of rs3746444 (*MIR499A*; Table 1) [15]. The risk of MI is increased for carriers of CC genotype of rs895819 (*MIR27A*) [40] and GG genotype of rs3746444 (*MIR499A*; Table 1) [41]. However, a recent meta-analysis (eight studies, 2507 patients with MI and 3796 healthy Asians, 11 miRNA gene polymorphisms) showed that, in contrast, the risk of MI is increased in carriers of the GG genotype of rs2910164 (*MIR146A*), as well as AA+AG genotypes of rs3746444 (*MIR499A*) (Table 1) [42].

In vitro studies on cell cultures showed the involvement of miR-186-5p, miR-149-5p, miR-146a-5p, miR-182-5p, miR-27a-3p, miR-363-3p in the development of atherosclerotic lesions of the arteries (Table 1) [43–50].

Table 2. The distribution of alleles and genotypes of rs2910164 (G>C) in the *MIR146A* gene in patients with advanced carotid atherosclerosis and in relatively healthy residents of Western Siberia of Slavic origin

Genotype/allele	Healthy individuals, abs. (%)	Patients with clinically pronounced atherosclerosis, abs. (%)	χ^2; OR (95%CI); p
GG	46 (63.9)	42 (37.5)	$\chi^2 = 11.20$; OR = 2.95 (95% CI: 1.60–5.45); $p = 0.001$
GC + CC	26 (36.1)	70 (62.5)	
G	116 (80.5)	143 (63.8)	$\chi^2 = 10.96$ OR = 2.35 (95% CI: 1.43–3.85) $p = 0.001$
C	28 (19.5)	81 (36.2)	

Figure 1 shows the expression levels of 19 miRNAs in atherosclerotic plaques of the carotid arteries, depending on the SNV genotype located in the miRNA gene region. However, the expression of only one miR-618, in atherosclerotic plaques of the carotid arteries of patients with the AC genotype of rs2682818 in the *MIR618* gene was 27 times lower than in carriers of the CC genotype ($\log_2$FC = 4.8; $p = 0.012$; Fig. 1). The rs2682818 polymorphism can affect the expression of miR-618, preventing the formation of the secondary hairpin structure and the processing of the miR-618 precursor to its mature form. It has been shown that the A allele of rs2682818 is associated with a decrease of mature miR-618 level in HeLa cells compared to the C allele [51]. In addition, the AC/AA genotypes of rs2682818 act as a negative predictor of ischemic stroke recurrence [37].

Of all miRNAs expressed in atherosclerotic plaques of the carotid arteries, miR-146a is the best studied (Table 1). miR-146a expression is upregulated in human and mouse atherosclerotic plaques, while it inhibits NF-κB signaling in endothelial cells and macrophages by interfering with TRAF6 and IRAK1/2, thereby reducing the expression of pro-inflammatory cytokines [7, 46, 52–54]. Thus, miR-146a is an anti-inflammatory miRNA that confers atheroprotective properties to the vessel wall.

Genetic variants in pre-miRNAs affect the processing of individual miRNAs and, accordingly, reduce the level of mature miRNAs, in particular, the rs2910164:C>G variant of the *MIR146A* gene. However, associations of this polymorphism with carotid and coronary artery atherosclerosis phenotypes have been analyzed mainly in Asian populations. Only one study showed the association of the CC genotype of rs2910164 in the *MIR146A* gene with the risk of restenosis of the coronary arteries in Europeans in the German population [55]. According to gnomAD data, the frequencies of the minor allele C of rs2910164 differ in the populations of East Asia and Europe, 63 and 23%, respectively.

In this regard, within the framework of this study, we perform the genotyping of rs2910164 in the *MIR146A* gene in leukocytes of patients with advanced carotid atherosclerosis and relatively healthy residents of Western Siberia who are ethnic Slavs (Table 2). Comparison of the frequencies of alleles and genotypes of this polymorphism in these two groups revealed an association of the C allele and genotypes (CC+GC) of rs2910164 in the *MIR146A* gene with the risk of advanced carotid atherosclerosis: OR = 2.35 (95% CI: 1.43–3.85), $p = 0.001$, OR = 2.95 (95% CI: 1.60–5.45), $p = 0.001$.

It should be noted that in Asian populations, the CC genotype of rs2910164 (*MIR146A*) is associated with the risk of CAD, but the G allele/GG genotype is associated with the risk of complications of atherosclerosis of the carotid and coronary arteries — stroke and MI [12, 13, 36]. In this work, we show the association of the C allele and genotypes (CC+GC) of rs2910164 in the *MIR146A* gene with the risk of advanced carotid atherosclerosis in the inhabitants of Western Siberia of Slavic origin. However, the association with ACVA remains unclear, since acute vascular accidents were registered in 38 people from the total sample, which is not a large enough sample for analysis.

The rs2910164 polymorphism results in a G>C nucleotide substitution in the pre-miRNA sequence of the *MIR146A* gene and further in the "passenger chain" of the mature miR-146a-3p. As a result, the processing of pre-miR-146a, the conformation of its secondary structure, and stability are disrupted, and the production of mature miR-146a in the U2OS cell line decreases [56]. Furthermore, it was shown that the CC genotype and the C allele of rs2910164 were associated with a decrease in the expression of miR-146a in cells, which increases the content of its target mRNAs (IRAK1 and TRAF6), contributing to the formation of the pro-inflammatory profile and, therefore, the risk of the disease.

However, in peripheral blood mononuclear cells of patients with CAD and the CC genotype of rs2910164 in the *MIR146A* gene, an increase in the level of miR-146a

and a decrease in the level of IRAK-1, TRAF-6, NF-κB, and C-reactive protein were found compared to carriers of the GG genotype [52]. In addition, Xiong et al. showed an association of GC/CC genotypes of rs2910164 with the risk of CAD and increased expression of miR-146a in mononuclear blood cells of patients [57]. At the same time, in type 2 diabetes mellitus, which is often recorded in patients with atherosclerotic lesions, a decrease in miR-146a levels in blood mononuclear cells is associated with the CC genotype of rs2910164 [58]. However, we have not found an association between the expression of miR-146a-5p in carotid atherosclerotic plaques and genotype of rs2910164 in the *MIR146A* gene. Possibly, there are cell- and tissue-specific differences in miR-146a expression in carriers of different genotypes of rs2910164 of the *MIR146A* gene depending on the stage and distribution of atherosclerotic lesions as well as comorbid status of the patients.

On the other hand, the relatively small size of the samples used to analyze the association between the gene polymorphism and miRNA expression in carotid atherosclerotic plaques may be the reason for the differences in the results obtained in our study and other studies. In addition, it is necessary to replicate the results of the association of rs2910164 of the *MIR146A* gene with disease in large and ethnically different groups, including patients of different sexes, with atherosclerotic lesions of different distribution and complications. It is possible that the cumulative effect of not one, but many genetic variants, including those localized in the miRNA gene region, and environmental factors play a significant role in the predisposition to atherosclerosis.

The results of the association analysis of miRNA gene polymorphisms with atherosclerosis should be interpreted in the context of assessing the expression of pre- and mature miRNAs and their targets in cells/tissues of target organs. In order to establish causal relationships and detailed molecular genetic mechanisms, these works should be supplemented by functional studies using cell cultures and model animals. It has been shown that miR-146a contributes to the formation and stability of atherosclerotic plaques by regulating the proliferation of smooth muscle cells through the Notch signaling pathway and the inflammation process in IRAK1/TRAF6/NF-κB macrophages [59, 60]. However, the functional significance of miR-618 in atherosclerosis remains unclear.

CONCLUSIONS

The present study for the first time characterizes the spectrum of SNVs in miRNA genes in Slavs living in Western Siberia with advanced carotid atherosclerosis by exome sequencing. Expression of mature miRNAs in carotid atherosclerotic plaques was assessed using miRNA sequencing. We found twenty-four SNVs located in 18 miRNA genes that are expressed in carotid atherosclerotic plaques. The expression of miR-618 in carotid atherosclerotic plaques of the patients with the AC genotype of rs2682818 in the *MIR618* gene was decreased compared with the CC genotype ($\log_2FC = 4.8$; $p = 0.012$). We established an association of the C allele and genotypes (GC+CC) of rs2910164 in the *MIR146A* gene with the risk of advanced carotid atherosclerosis in the Slavs living in Western Siberia (OR = 2.35; 95% CI: 1.43–3.85; $p = 0.001$, OR = 2.95; 95%CI: 1.60–5.45; $p = 0.001$, respectively).

FUNDING

The work was carried out within the framework of the State task of the Ministry of Science and Higher Education of the Russian Federation.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflicts of interest.

Statement of compliance with standards of research involving humans as subjects. This work was approved by the Biomedical Ethics Committee of the Research Institute of Medical Genetics of the Tomsk National Research Medical Center. All material donors signed a voluntary informed consent to participate in the study.

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