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Variability of Methylation Profiles of CpG Sites in microRNA Genes in Leukocytes and Vascular Tissues of Patients with Atherosclerosis

A. N. Kucher[1,2]*, M. S. Nazarenko[1]*, A. V. Markov[1], I. A. Koroleva[1], and O. L. Barbarash[3]

[1]Research Institute of Medical Genetics, Tomsk National Research Medical Center, Russian Academy of Sciences, 634050 Tomsk, Russia; E-mail: aksana.kucher@medgenetics.ru
[2]National Research Tomsk State University, 634050 Tomsk, Russia
[3]Research Institute for Complex Issues of Cardiovascular Diseases, Siberian Branch of the Russian Academy of Medical Sciences, 650002 Kemerovo, Russia

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Abstract—In this study, we for the first time described the variability of methylation levels of 71 CpG sites in microRNA genes in leukocytes and blood vessels (coronary artery atherosclerotic plaques, intact internal thoracic arteries, and great saphenous veins) in patients with atherosclerosis using the Infinium HumanMethylation27 BeadChip microarray. Most of the analyzed CpG sites were characterized by the low variability, and most of these low-variable sites were hypomethylated in all tissue samples. CpG sites in coronary artery atherosclerotic plaques and leukocytes were similar in their methylation status. The highest variability of CpG methylation levels between different tissues was found for the CpG sites of the *MIR10B* gene; the methylation levels of these sites in leukocytes and atherosclerotic arteries were lower than in intact blood vessels. We also found that several cardiovascular disease risk factors, as well as medications, might affect methylation levels of CpG sites in microRNAs.

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DNA methylation and RNA-mediated regulatory mechanisms play an important role in the functioning of various human organs and systems in both healthy and diseased organisms. Epigenetic mechanisms, such as DNA methylation, histone modification, and gene regulation by small noncoding RNAs, in particular microRNAs, are essential for the regulation of expression of tissue-specific genes during ontogenesis. At the same time, expression of microRNA genes is controlled by methylation of DNA (CpG sites) and histones, whose methylation profiles are affected by the functional state of an organism or by various pathologies. The importance of microRNAs in tissue-specific gene expression [1], organism response to stimuli [2-7], and pathogenesis of various diseases [8-13] has been proven in several studies. This makes evaluation of the methylation profiles of regulatory elements of microRNA genes an important research task; however, such studies have been scarce so far [14, 15]. Thus, examination of epigenetic regulation of microRNA expression in human fibroblasts and mammary epithelial cells revealed that tissue-specific expression of microRNA genes depends on the epigenetic state of their promoters and can be repressed by DNA methylation (38%), H3 histone methylation (H3K27me3) (58%), or a combination of DNA and H3K27 methylation (21%) [14]. Both microRNA expression levels and epigenetic states of promoters showed strong interindividual concordance within a given tissue type.

Most *in vivo* studies on epigenetic regulation of microRNA genes in human tissues have been performed in cancer tumors [16], and only few such studies have accessed other types of pathologies [17]. Variability in DNA methylation is usually evaluated for the same tissue type in different individuals. For this reason, analysis of DNA methylation levels in different tissues from the same individual is of interest, because it might reveal the tissue-specificity of the methylation state in the same patient, as well as interindividual variability for various types of tissues. Data on the methylation state of microRNA genes are of both theoretical and practical significance, since

* To whom correspondence should be addressed.

they can provide a basis for the development of diagnostic approaches for various pathologies, search for new drugs, and optimization of the patient's response to treatment.

The goal of this study was to evaluate the variability of the methylation levels of the CpG sites in microRNA genes in blood and vessel tissues of patients with atherosclerosis.

MATERIALS AND METHODS

The studied cohort included six male patients of the Vascular Surgery Department (average age, 55.5 ± 6.5 years) with coronary artery disease and arterial hypertension. Four of the patients had an acute myocardial infarction in the patient's medical history; three patients were diagnosed with hypercholesterolemia; three patients had type 2 diabetes mellitus. All patients were accessed for angina functional class and heart failure. Two patients were smokers. The intake of prescription drugs (aspirin, ACE inhibitors/calcium channel blockers, loop diuretics, thiazides, spironolactone, nitrates, and insulin) was taken into consideration for all the individuals tested. All patients volunteered and gave signed consent to be in this study.

Tissue samples from the right coronary arteries (CAAP, coronary artery atherosclerotic plaques), morphologically intact internal thoracic arteries (ITA), and great saphenous veins (GSV) were collected during coronary artery bypass surgery performed as a planned procedure for atherosclerosis treatment. Leukocytes (WBC) were isolated from venous blood drawn before the surgery. Samples of solid tissues were thoroughly washed with physiological saline, and all samples were frozen in liquid nitrogen and stored at –80°C.

Genomic DNA from leukocytes and vessel tissues was isolated by phenol/chloroform extraction after standard tissue treatment with proteinase K for 12 h at 37°C.

The methylation states of 71 CpG sites from 22 microRNA genes and one microRNA cluster (Table 1) were analyzed using the Infinium HumanMethylation27 BeadChip (Illumina, USA). Genomic DNA bisulfite modification was performed with EZ DNA Methylation Kit (Zymo Research, USA). Whole genome amplification, enzymatic DNA cleavage, DNA fragment purification, and hybridization were carried out as recommended by the manufacturer (Illumina) [18]. During hybridization, DNA fragments were annealed to the locus-specific DNA probes that corresponded to the methylated and unmethylated cytosine loci, respectively. Hybridization was followed by a single-base DNA extension with labeled ddNTPs. Signal intensity from the microarray was registered with an Illumina BeadArray Reader scanner (Illumina). Raw data were analyzed with the GenomeStudio Methylation Module program suite (Illumina). Methylation status of the interrogated CpG site was calculated as the ratio of signal from a methylated probe relative to the sum of signals from both methylated and unmethylated probes. This value, known as β, ranges continuously from 0 (unmethylated) to 1 (fully methylated). The data were deposited in the Gene Expression Omnibus database (accession number, GSE62867).

Statistical analysis was performed using standard program packages from The R Project for Statistical Computing. Average DNA methylation levels in groups were compared using Student's *t*-test for dependent samples with a correction for multiple testing by the Benjamini–Hochberg method. Correlations between DNA methylation levels and other quantitative parameters were analyzed using Spearman's rank correlation coefficient. The significance (*p*) values for the Spearman's rank correlation coefficient were computed using algorithm AS 89 [19]. The levels of similarity between DNA methylation profiles was estimated by multidimensional scaling and cluster analysis (using Euclidean coordinates as a distance measure and complete-linkage clustering for dendrogram generation) [20].

RESULTS AND DISCUSSION

Variability of methylation of CpG sites in microRNA genes in tissues and studied individuals. Out of 71 CpG sites analyzed, 51 sites (71.8%) displayed low variability in their methylation status: the difference in the methylation levels for these sites was below 0.1 in all tissues tested in all patients studied (Table 1), which could be related to existent DNA methylation conservatism of the corresponding genome regions. Almost all CpG sites with low variability were hypomethylated ($\beta < 0.2$; although in most cases, β did not exceed 0.1), except the only highly methylated cg17723549 in *MIR423* gene ($\beta > 0.90$ in all samples).

Methylation levels of 20 CpG sites (28.2%) varied considerably (difference in the β values was over 0.1) (Tables 1 and 2 and Fig. 1). Eight of these sites were found in WBC; 11 – in GSV, 7 – in ITA, and 11 – in CAAP. CAAP samples displayed the lowest variability in the methylation levels – the difference between the β values in CAAP was 0.01 to 0.20, whereas in other tissues, this difference reached 0.30 and greater.

Analyzed CpG sites were unevenly represented on the Infinium Human Methylation27 BeadChip for different microRNA genes. Thus, eight genes (including miR17/92 cluster) were represented by only one CpG site; 18 genes were represented by two to eight CpG sites (Table 1). All analyzed CpG sites were hypomethylated in eight microRNA genes (*MIR219A1*, *MIR320A*, *MIR564* – two sites; *MIR330*, *MIR92B* – three sites; *MIR632* – four sites; *MIR611* – six sites; *MIR639* – eight

Table 1. Analyzed CpG sites of microRNA genes and their location

microRNA gene	Chromosome: CpG island coordinates[1]	CpG site ID[2]
MIRLET7I	12:62996029-62997825	**cg14108394↓**
MIR10A	17:46654923-46656033	cg06760035; **cg08089301↓**; **cg14458834↓**; cg21460081; **cg21546671↓**; cg25145670
MIR10B	2:177014827-177015340	cg00767581; cg14399060
	2:177014251-177014718	cg12127282
MIR17HG	13:91999390-92001609	**cg25308542↓**
MIR196B	7:27203762-27207165	**cg01354473↓;** cg01381846; cg26521404; cg27009703
MIR219A1	6:33175401-33176807	**cg14363494↓; cg18189601↓**
MIR26B	2:219262772-219265676	**cg01264826↓**
MIR320A	8:22101917-22103257	**cg01735903↓; cg09037858↓**
MIR330	19:46142132-46143177	**cg08831348↓; cg20427879↓; cg27301343↓**
MIR345	14:100771314-100773827	**cg09002165↓**
MIR34B	11:111382971-111384054	**cg22879515↓;** cg23211240
MIR378A	5:149109259-149112068	**cg15219393↓**
MIR423	17:28442750-28443335	**cg17723549↑**
	17:28443507-28444244	**cg05141870↓;** cg25346576
MIR564	3:44902534-44904061	**cg23844090↓; cg26889990↓**
MIR611	11:61559702-61561053	**cg01447420↓; cg02005336↓; cg15963971↓; cg18009798↓; cg03114244↓; cg22704775↓**
MIR615	12:54426535-54428864	cg15700739; cg16983211
MIR632	17:30676878-30677782	**cg06168050↓; cg08209724↓; cg21184495↓; cg16007628↓**
MIR636	17:74732156-74734349	**cg00953277↓**
MIR638	19:10828460-10829393	**cg07248407↓; cg08207256↓; cg15392792↓; cg20041257↓; cg15334028↓;** cg17165284
MIR639	19:14639498-14641147	**cg00061059↓; cg23470272↓; cg10007692↓; cg15649193↓; cg17162271↓; cg12846938↓; cg13237829↓; cg22469141↓**
MIR675	11:2017312-2018295	cg13145013; cg15317267
MIR7-3	19:4769438-4769853	**cg01400401↓; cg26005082↓; cg02479575↓;** cg03996793
	19:4769131-4769332	cg19646028; cg20893022; cg21300318
MIR92B	1:155163354-155165347	**cg06420088↓; cg17257175↓; cg24683129↓**

[1] CpG island coordinates are given according to the human genome version GRCh37/hg19.

[2] CpG site identifiers (IDs) are given according to the annotation to the Illumina microchips; sites with the variability in the β values below 0.1 for all studied samples (in all tissues from all individuals) are shown in bold; low-variable CpG sites with $\beta < 0.2$ are indicated with ↓; CpG sites with $\beta > 0.8$ are indicated with ↑.

Table 2. CpG sites with varying methylation levels (β) in the studied samples and interindividual variability of β values for these sites in the studied tissues

microRNA gene	CpG site ID	All analyzed tissues			WBC		GSV		ITA		CAAP	
		min-max β values for all groups	Δ	min-max Δ between β values for tissues in individuals	min-max β values	Δ	min-max β values	Δ	min-max β values	Δ	min-max β values	Δ
MIR10A	cg06760035	0.01-0.26	0.25	0.00-0.16	0.01-0.02	0.01	0.09-0.26	0.17	0.01-0.01	0.01	0.05-0.19	0.14
	cg21460081	0.03-0.16	0.14	0.00-0.08	0.03-0.04	0.01	0.05-0.06	0.02	0.09-0.16	0.08	0.03-0.08	0.05
	cg25145670	0.03-0.25	0.22	0.00-0.12	0.03-0.04	0.01	0.08-0.25	0.17	0.03-0.04	0.01	0.08-0.14	0.06
MIR10B	cg00767581	0.19-0.92	0.73	0.00-0.56	0.21-0.45	0.23	0.69-0.92	0.23	0.79-0.88	0.09	0.19-0.38	0.18
	cg12127282	0.13-0.88	0.74	0.03-0.67	0.13-0.21	0.08	0.74-0.87	0.12	0.78-0.88	0.10	0.26-0.36	0.10
	cg14399060	0.04-0.65	0.60	0.01-0.49	0.04-0.13	0.09	0.39-0.55	0.16	0.45-0.65	0.20	0.06-0.11	0.05
MIR196B	cg01381846	0.09-0.20	0.12	0.01-0.04	0.14-0.15	0.02	0.09-0.12	0.03	0.09-0.13	0.04	0.10-0.20	0.10
	cg26521404	0.01-0.27	0.27	0.00-0.12	0.03-0.27	0.24	0.01-0.04	0.03	0.02-0.05	0.03	0.04-0.24	0.20
	cg27009703	0.01-0.37	0.36	0.01-0.23	0.13-0.31	0.18	0.01-0.13	0.11	0.14-0.28	0.14	0.19-0.37	0.18
MIR34B	cg23211240	0.03-0.18	0.15	0.01-0.08	0.07-0.18	0.12	0.04-0.06	0.02	0.03-0.06	0.03	0.07-0.11	0.05
MIR423	cg25346576	0.02-0.20	0.18	0.01-0.04	0.02-0.14	0.12	0.02-0.16	0.14	0.02-0.20	0.18	0.02-0.09	0.07
MIR615	cg15700739	0.10-0.53	0.44	0.03-0.29	0.12-0.16	0.04	0.10-0.40	0.31	0.19-0.53	0.35	0.10-0.25	0.15
	cg16983211	0.05-0.35	0.31	0.02-0.20	0.05-0.07	0.02	0.07-0.17	0.10	0.11-0.35	0.25	0.05-0.09	0.04
MIR638	cg17165284	0.05-0.19	0.14	0.01-0.04	0.07-0.11	0.04	0.06-0.14	0.08	0.07-0.09	0.02	0.05-0.19	0.14
MIR675	cg13145013	0.81-0.94	0.13	0.01-0.07	0.93-0.94	0.01	0.84-0.93	0.09	0.84-0.90	0.06	0.81-0.92	0.11
	cg15317267	0.63-0.92	0.29	0.02-0.23	0.86-0.92	0.06	0.63-0.71	0.08	0.66-0.68	0.03	0.64-0.73	0.09
MIR7-3	cg03996793	0.05-0.16	0.11	0.00-0.02	0.05-0.12	0.07	0.05-0.14	0.08	0.05-0.16	0.11	0.05-0.10	0.05
	cg19646028	0.19-0.38	0.19	0.01-0.06	0.25-0.37	0.12	0.21-0.28	0.07	0.21-0.29	0.08	0.19-0.38	0.19
	cg20893022	0.08-0.30	0.22	0.02-0.06	0.13-0.30	0.17	0.08-0.22	0.14	0.14-0.21	0.08	0.14-0.27	0.13
	cg21300318	0.29-0.62	0.33	0.02-0.07	0.30-0.62	0.32	0.29-0.42	0.12	0.34-0.48	0.14	0.38-0.49	0.11

Note: min-max, minimal and maximal registered β values, respectively; Δ, difference between the maximal and minimal β values of the index.

sites) in all tissues. For two microRNA genes (*MIR10B* – three sites; *MIR615* – two sites), the levels of CpG methylation varied in different samples. The highest variability for the methylation levels in different tissues was observed for the CpG sites of the *MIR10B* gene. Only a single site (cg13145013 in the *MIR675* gene) was hypermethylated in all samples. Both low-variable (hypo- or hypermethylated) and variable CpG sites were found in six microRNA genes (Tables 1 and 2). In several cases (*MIR10A*, *MIR196B*, *MIR34B*, *MIR423*, *MIR638*, and *MIR7-3*), the same CpG islands contained CpG sites that differed in their methylation status, although the methylation patterns were similar in tested patients (Fig. 1).

Aavik et al. [21] observed decreased DNA methylation levels in tissues of atherosclerotic femoral arteries as compared to intact ITA. In addition, DNA hypomethylation in the imprinted 14q32 locus induced transcription of a microRNA gene cluster in this locus [21]. In our study, we found that several CpG sites in the *MIR10B* gene were less methylated in CAAP than in intact vessels (similar to the results of [21]); the methylation levels of CpG sites in atherosclerotic arteries were similar to the DNA methylation levels in WBC. This was typical for cg12127282 located close to the 5′-UTR (difference between mean β values in the compared groups, $\Delta\beta = 0.55$; $p < 0.001$), as well as for cg00767581 ($\Delta\beta = 0.56$; $p = 0.001$) and cg14399060 ($\Delta\beta = 0.48$; $p < 0.001$), located in the coding region and close to the 3′-UTR, respectively.

The observed differences between the DNA methylation levels in the atherosclerotic and intact arteries in the same individual, on one hand, and in the same type of tissues in different subjects, on the other hand, might reflect different functional states of these tissues and organisms. In particular, low DNA methylation levels might result in transcription activation of the corresponding microRNAs, which would affect the posttranslation-

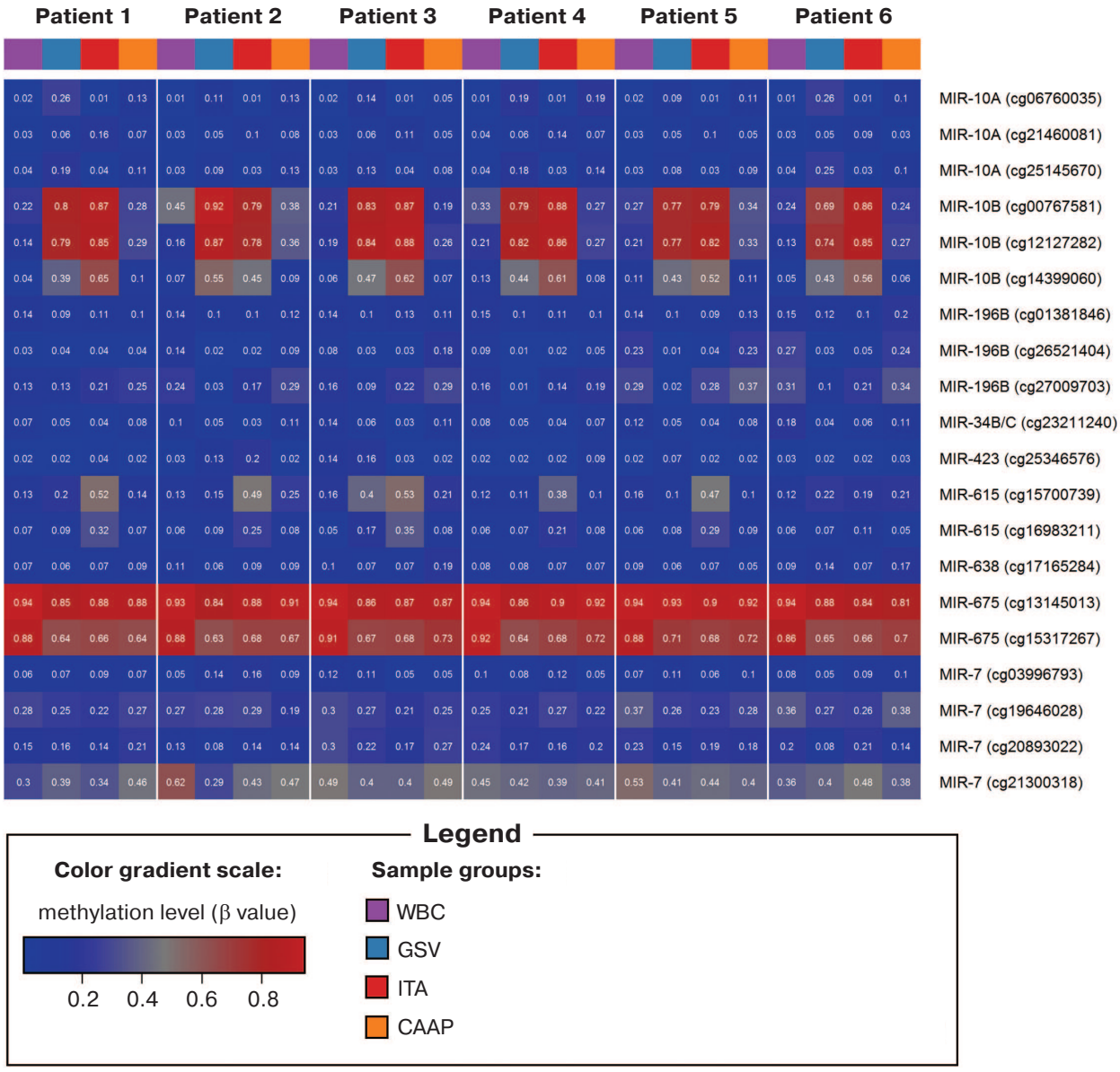

Fig. 1. DNA methylation heatmap for CpG sites with varying methylation levels in the studied tissues.

al regulation (protein synthesis suppression) of the target genes.

Based on the methylation levels of the analyzed CpG sites, all studied microRNAs could be distributed into two major clusters (Fig. 2), one of them including WBC and CAAP samples, and the other – GSV and ITA samples. Interestingly, samples most similar in the CpG methylation levels were from CAAP and WBC and could be subdivided into three subclusters: only CAAP, only WBC, and a mixed group. The cluster that included samples from GSV and ITA contained two distinct subclusters corresponding to the types of blood vessels (Fig. 2).

Pronounced difference in the methylation levels of CpG sites in intact vessels and WBC (the difference in the mean β values for each tissue type, >0.2) was observed for three CpG sites in the *MIR10B* gene and cg15317267 in the *MIR675* gene; in CAAP and GSV – for the same sites in the *MIR10B* gene and cg15700739 in the *MIR615* gene (for these sites, the CpG methylation levels in CAAP were close to those in WBC) (Table 2).

The differences in the methylation levels of CpG sites in intact vessels (GSV and ITA) correlate with existing concepts on the tissue-specificity of epigenetic genome modifications [22]. The observed differences between the CpG methylation states in atherosclerotic and intact vessels were not completely unexpected, because some of the analyzed microRNA genes with variable CpG sites have been found to participate in various

stages of atherogenesis (see reviews [23, 24]). Similar methylation levels of CpG sites in CAAP and WBC could be explained by the inflammation of atherosclerotic tissues.

Factors associated with methylation levels of CpG sites in microRNA genes. Clusterization of the analyzed CpG sites according to their methylation levels in the studied individuals was different in different tissues, which might reflect the effects of individual factors and/or functional states (e.g. pathologies) of the patients tested. For example, the WBC + CAAP cluster could be subdivided into three groups: CAAP only, WBC only, and a mixed group (Fig. 2). In this last mixed group, CAAP samples that clustered with WBC samples, were obtained from the smokers. Samples from the same patients clustered "incorrectly" in the GSV + ITA cluster: the ITA sample from one of the patients fell into the GSV group, while the GSV sample from the other patient fell into the ITA group. The highest correlation between the methylation status and smoking was observed for cg17165284 in the *MIR638* gene – the methylation level of this site was 10% higher in the CAAP samples from smokers than from nonsmokers (Table 3). This microRNA plays an important role in the development of emphysematous lung destruction in smokers with chronic obstructive bronchitis [25]. It should be noted that not one but multiple factors can affect methylation status of CpG sites in microRNA genes, and combined action of such factors in individuals will complicate elucidation of the contribution of factors to the regulation of DNA methylation.

It is known that expression of some microRNAs is determined not only by the tissue type, but also by the functional state of cells and the organism as a whole. It might also be affected by drug treatment of the patients [2-7]. We found that in WBC, methylation level of the *MIR10A* gene cg06760035 site correlated with the low-density lipoprotein cholesterol content, and methylation of the *MIR10B* gene cg00767581 site correlated with the triglyceride content and body mass index (Table 4). *MIR10A* and *MIR10B* are located within the *HOX* gene cluster (*HOXB3/4* and *HOXD3/4*, respectively) and display pleiotropic effects that are not limited to the cross-regulation of their expression [26]. The content of circulating miR-10a is associated with hyperlipidemia [27]. mir-10b is involved in lipid metabolism regulation and can presumably regulate *in vitro* levels of triglycerides via interacting with PPAR-α [28, 29].

In CAAP samples, methylation levels of cg25346576 in the *MIR423* gene positively correlated with the total blood cholesterol content, and methylation levels of cg19646028 in the *MIR7-3* gene negatively correlated with the glucose content. Prabu et al. [30] revealed weak negative correlation between the levels of circulating miR-423-5p and high-density lipoprotein cholesterol concentration in the blood of patients with impaired glucose tolerance and type 2 diabetes mellitus. However, we failed to identify any association between the methylation levels of *MIR423* CpG sites and these pathologies.

All the studied patients had arterial hypertension. The methylation levels of six CpG sites in CAAP and

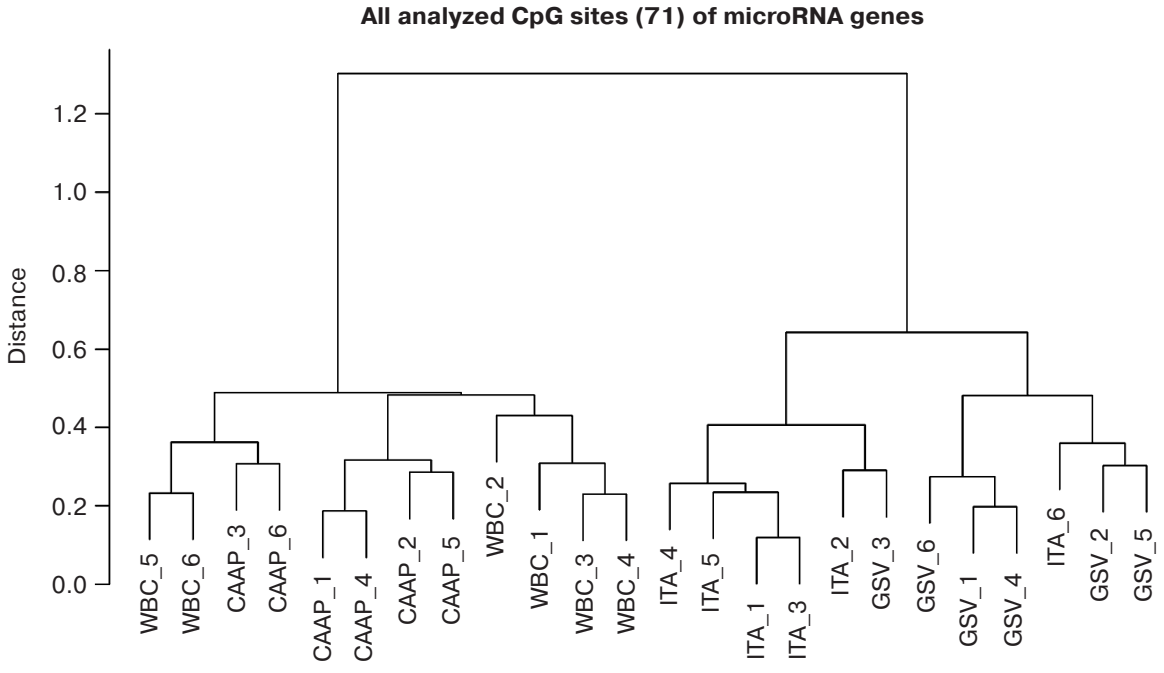

Fig. 2. Dendrogram reflecting tissue sample clusterization based on methylation levels of 71 CpG sites of microRNA genes (numbers correspond to individual patients).

Table 3. Factors associated with the methylation level of studied variable CpG sites in microRNA genes

Factor	microRNA gene	CpG site ID	Tissue*
Smoking	*MIR638*	cg17165284	CAAP
Angina functional class	*MIR7-3*	cg20893022	CAAP, GSV
	MIR196B	cg01381846	CAAP
	MIR196B	cg26521404	WBC
	MIR675	cg15317267	WBC
Heart failure functional class	*MIR196B*	cg01381846	CAAP
	MIR675	cg15317267	WBC
Myocardial infarction in patient's medical history	*MIR196B*	cg26521404	CAAP, WBC
	MIR196B	cg27009703	CAAP, WBC
Hypercholesterolemia in patient's medical history	*MIR615*	cg15700739	CAAP
Intake of statins	*MIR615*	cg16983211	ITA
Intake of metformin	*MIR423*	cg25346576	GSV

* Only tissues, in which the studied factor affected methylation levels, are shown.

Table 4. Clinical factors that correlate with the methylation levels of analyzed variable CpG sites in microRNA genes

Factor	microRNA gene	CpG site ID	Tissue*	Spearman correlation coefficient (ρ)	*p*
Age	*MIR423*	cg25346576	WBC	–0.943	0.005
	MIR638	cg17165284	WBC	–0.886	0.019
Duration of arterial hypertension	*MIR10B*	cg00767581	CAAP	0.880	0.021
	MIR10B	cg12127282	CAAP	0.880	0.021
	MIR675	cg15317267	CAAP	–0.820	0.046
	MIR615	cg16983211	WBC	0.880	0.021
	MIR7-3	cg03996793	WBC	–0.941	0.005
	MIR7-3	cg20893022	WBC	–0.820	0.046
Body mass index	*MIR10B*	cg14399060	WBC	0.883	0.020
Glucose level	*MIR7-3*	cg19646028	CAAP	–0.830	0.042
Total cholesterol level	*MIR423*	cg25346576	CAAP	0.829	0.042
LDL-cholesterol level	*MIR10A*	cg06760035	WBC	–0.943	0.005
TG level	*MIR10B*	cg00767581	WBC	0.943	0.005

Note: LDL-cholesterol, low-density lipoprotein cholesterol; TG, triglycerides.
* Only tissues, in which studied factor affected DNA methylation levels, are shown.

WBC correlated either positively (three CpG sites in *MIR10B* and *MIR615*) or negatively (three CpG sites in *MIR675* and *MIR7-3*) with the disease duration (Table 4). Among the products of these genes, only miR-615-5p was found in increased concentrations in the blood of patients with essential hypertension [31]. This microRNA also promotes the phagocytic capacity of splenic macrophages in cirrhosis-related portal hypertension [32].

Recent studies in human epigenetics have focused on the age dynamics of DNA methylation and its association with aging [33]. We found that methylation levels of CpG sites in *MIR423* (cg25346576) and *MIR638* (cg17165284) correlate with the patient's age (48 to 67 years in our study) (Table 4). It is interesting to note that there were interactions between *MIR423* rs6505162 and age group among the patients with esophageal cancer [34]. The cg25346576

site is located in the CpG island in the *MIR423* gene promoter and, therefore, can be involved in the gene expression regulation. At the same time, it has been found before [35] that miR-423-5p expression decreases in aging human epithelial cells, which contradicts the results of our study. However, it should be remembered that the methylation status of a gene can differ in different tissues and cells. The *MIR638* gene, which contains a CpG site whose methylation level negatively correlates with the patient's age, is a part of the CpG island, and according to [36], expression of miR-638 is activated during replicative senescence of human fibroblasts.

Other factors that could affect the methylation status of some CpG sites are pathologies and drug intake (Table 3). In our study, these were the CpG sites of the *MIR196B* gene: differences in the methylation levels of these sites were observed in WBC and CAAP in patients with different functional classes of angina and patients that had suffered myocardium infarction in the past. In case of CAAP, these differences were also found in patients with functional classes of heart failure. Similarly, the difference in the methylation levels of cg15317267 site of the *MIR675* gene was found in WBC of patients with angina and heart failure. It is interesting to note that in mice, the development of hyperhomocysteinemia (an independent factor in pathogenesis of cardiovascular disorders) was accompanied by a decrease in the methylation levels of the differentially methylated domain of the *H19* gene (host gene for *MIR675*) in the liver, but caused an increase in the methylation levels of the same gene in the brain and aorta [37]. This allowed the authors to suggest that methylation of the differentially methylated domain of the *H19* gene in hyperhomocysteinemia is a tissue-specific process. The effects of hyperhomocysteinemia and folic acid on DNA methylation were demonstrated in humans as well [38].

Based on these results, we suggest that intake of certain drugs might affect the methylation levels of CpG sites in microRNA genes. Thus, we found that intake of statins affected CpG methylation level in *MIR615* in intact arteries, and intake of metformin affected CpG methylation in *MIR423* in GSV. The fact that expression of microRNA genes might be modified by various supplements and drugs has been confirmed in other studies. However, CpG methylation levels changed differently in different tissues in response to the drug treatment, which corresponds well to the studies of other research groups [37]. Although our results were obtained using a relatively small cohort of patients, the observed effect of drugs and pathologies on the methylation of CpG sites in microRNA genes has been corroborated in other studies, which makes the problem of the effect of clinical factors on DNA methylation worth further study.

In conclusion, we demonstrated that out of 71 studied CpG sites in microRNA genes, 51 sites (71.8%) exhibited low variability of their methylation state in all analyzed samples: 50 of these sites were hypomethylated, and one site was hypermethylated. Twenty CpG sites (28.2%) varied in their methylation levels. The number of variable CpG sites in CAAP and GSV was higher than in WBC and ITA. Only two CpG sites were highly variable in all sample tissues, but they displayed different methylation levels. The highest variability between tissue samples was observed for the CpG sites in the *MIR10B* gene (the methylation levels of these sites in WBC and CAAP were lower than in intact ITA). We also identified several clinical factors that might affect methylation of CpG sites in microRNA genes.

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