https://russjcardiol.elpub.ru
doi:10.15829/1560-4071-2020-4040

ISSN 1560-4071 (print)
ISSN 2618-7620 (online)

Deoxyribonucleic acid methylation in the enhancer region of the *CDKN2A/2B* and *CDKN2B-AS1* genes in blood vessels and cells in patients with carotid atherosclerosis

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Aim. Comparative analysis of the deoxyribonucleic acid (DNA) methylation level in the enhancer region of the *CDKN2A/2B* and *CDKN2B-AS1* genes (9p21.3 locus) in vessels with/without atherosclerotic lesions, as well as in leukocytes of patients with clinically relevant carotid artery (CA) atherosclerosis and healthy individuals.

Material and methods. The group of patients with clinically relevant atherosclerosis included 22 individuals with severe stenosis (>80%) of CA. Samples of atherosclerotic plaques, presenting CA regions, and great saphenous veins, as well as peripheral blood samples (leukocytes) were obtained from patients. The control group consisted of 14 individuals with the mild CA stenosis (≤24%) and without hemodynamically relevant changes; peripheral blood samples were obtained from each of them. DNA methylation level was assessed by targeted bisulfite sequencing of amplicons.

Results. The tissue-specific methylation of 31 CpG-site in the *CDKN2A/2B* and *CDKN2B-AS1* gene enhancer was established: the vascular tissues significantly differed from the peripheral blood leukocytes. At the same time, there was an increase in the methylation level of both certain CpG sites and whole analyzed CA region affected by atherosclerosis (48,6 [34,8; 62,0]%), compared with intact vessels, both arteries (25,2 [23,1; 41,60]%, p=0,0001) and veins (35,0 [31,6; 40,0]%, p=0,0039). Patients had lower methylation levels in all CpG sites in blood leukocytes compared to blood vessel samples (8,7 [6,1; 9,7]%; p<0,05). At the same time, the level of DNA methylation in the blood leukocytes of atherosclerotic patients does not differ from that in healthy individuals (9,3 [8,3; 13,6]%; p>0,8).

Conclusion. In the present study, the relationship between an increase in the DNA methylation in the enhancer of the *CDKN2A/2B* and *CDKN2B-AS1* genes in CA and their atherosclerotic lesions was revealed, as well as the tissue-specific DNA methylation between vessels and peripheral blood leukocytes.

Key words: atherosclerosis, DNA methylation, 9p21.3, *CDKN2A/2B, CDKN2B-AS1*.

Relationships and Activities: none.

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Received: 13.08.2020
Revision Received: 31.08.2020
Accepted: 07.09.2020

For citation: Koroleva Yu. A., Markov A. V., Goncharova I. A., Sleptsov A. A., Babushkina N. P., Valiakhmetov N. R., Sharysh D. V., Zarubin A. A., Kuznetsov M. S., Kozlov B. N., Nazarenko M. S. *Russian Journal of Cardiology*. 2020;25(10):4060. (In Russ.) doi:10.15829/1560-4071-2020-4060

Single nucleotide polymorphisms (SNPs) at the 9p21.3 locus are associated with cardiovascular disease continuum (hypertension, atherosclerosis of coronary and carotid arteries (CA), myocardial infarction, ischemic stroke, dyslipidemia, obesity, type 2 diabetes), as well as significant parameters (blood pressure level, body mass index, blood glucose level) [1].

Most SNPs of this locus are linked to each other in an extended haplotype block (~53 kb), which encompasses a cluster of genes for cyclin-dependent kinase inhibitor: *CDKN2A* (encodes p14/ARF and p16/INK4A) and *CDKN2B* (encodes p15/INK4B). The products of these genes are involved in the regulation of the cell cycle and proliferation [2, 3]. Both genes, *CDKN2A* and *CDKN2B*, are read from deoxyribonucleic acid (DNA) in the reverse direction, while the *CDKN2B-AS1* gene is transcribed in the forward direction, the product of which is the noncoding RNA in the INK4 locus [3].

It has been shown that ANRIL is a component of many gene pathways involved in cell proliferation, adhesion, senescence, and apoptosis — the key mechanisms of atherosclerotic arterial lesions [4, 5]. There is evidence that SNPs at the 9p21.3 locus cause an increase in the expression of ANRIL in vascular cells, which suppresses *CDKN2A/2B*, which, in turn, enhances cell proliferation and promotes the development of atherosclerosis [2]. The association of polymorphisms of the 9p21.3 locus with atherosclerosis is also explained by the presence in this region of long-range enhancers altering the functional activity of genes of this locus, including CDKN2A/2B. However, the detailed mechanism of the relationship between genetic variants and the pathological phenotype remains unknown [3].

At the same time, the development of atherosclerosis as a multifactorial disease can be caused not only by genetic variants, but also by epigenetic modifications that regulate gene expression without disrupting the primary DNA nucleotide sequence [6]. One of the most studied epigenetic mechanisms of regulation of gene function is the methylation of CpG dinucleotides, the so-called CpG islands. Basically, the study of DNA methylation is carried out in relation to gene promoters, and methylation of CpG islands in their promoter regions correlates with the silencing of gene transcription. However, there is evidence that hypomethylation of enhancer DNA, allowing the binding of transcription factors, also leads to the activation of transcription of corresponding genes [7].

There are low number of studies devoted to the analysis of DNA methylation of the 9p21.3 locus in blood vessels and cells in cardiovascular disease continuum [8-10]. In these studies, the level of DNA methylation in the region of CpG islands of the *CDKN2A/2B* gene promoters is analyzed in only one tissue in the vascular wall, or peripheral blood leukocytes in patients with atherosclerosis, including those complicated by acute vascular events.

The aim of this study was a comparative analysis of DNA methylation in the enhancer region of *CDKN2A/2B* and *CDKN2B-AS1* genes in the tissues of vascular wall, as well as in the leukocytes of patients with clinically relevant CA atherosclerosis and relatively healthy individuals.

Material and methods

Creation of samples and examination of patients with clinically relevant CA atherosclerosis was carried out on the basis of the Cardiology Research Institute (Tomsk National Research Medical Center). The general inclusion criteria of individuals in the study were the absence of family ties between individuals, Caucasian race, and the absence of cancer. The study was approved by the ethics committee of the Research Institute of Medical Genetics (Tomsk National Research Medical Center). All participants signed informed consent.

The group of patients with clinically relevant atherosclerosis consisted of 22 people aged 53-77 years (men, 17; women, 5) (Table 1). In all patients, ultrasound revealed severe CA stenosis (>80%), which is an indication for carotid endarterectomy. Prior to surgery, peripheral blood samples (leukocytes) were taken from each patient.

By surgery, samples of atherosclerotic plaques (ASP), macroscopically unchanged presenting areas of CA and great saphenous veins were obtained from all patients. Each vessel sample was examined and cleaned from calcification masses, lipid deposits and blood clots, washed in sterile saline in order to remove blood clots. Tissue samples were frozen in liquid nitrogen and stored at -80° C until the examination.

Histological examination of 20 ASP samples with hematoxylin-eosin staining was carried out (Table 1). Diapedesis of erythrocytes in the arterial wall was observed in 15% of cases, as well as the pronounced mononuclear infiltration, cap defect — in 10%, calcification — in 50%. Most (59,1%) of the analyzed ASP samples were classified as type V (fibroatheroma) [18]. The remaining 7 samples were classified as type VI due to signs of ASP destabilization (surface defects, hemorrhage, mononuclear cells in ASP).

The control group consisted of 14 individuals without cardiovascular disease —10 men and 4 women aged 53 to 78 years (Table 1). All individuals underwent an ultrasound, which revealed the initial stages of CA atherosclerosis (⩽24%) without hemo-

Table 1

Clinical characteristics of the groups included in the study

Parameter	Patients with clinically relevant CA atherosclerosis (N=22)	Control group of healthy individuals (N=14)
Clinical parameters		
Sex (men: women)	17:5	10:4
Age, years (Q2 [Q1; Q3])	64 [60; 69]	66 [59; 71]
BMI kg/m^2 (Q2 [Q1; Q3])	27 [26; 31]	29 [26; 32]
History of myocardial infarction, abs. (%)	14 (63,6)	0 (0,0)
History of coronary artery disease, abs. (%)	17 (77,3)	0 (0,0)
History of TIA, stroke, abs. (%)	4 (18,2)	0 (0,0)
Hypertension, abs. (%)	21 (95,5)	8 (56,1)
Smoking, abs. (%)	17 (77,3)	4 (28,6)
Diabetes, abs. (%)	6 (27,3)	2 (14,2)
CA ultrasound (Q2 [Q1; Q3])		
Stenosis degree, %	80 [75; 81]	24 [19; 26]
Histological type of ASP (AHA classification, 1995) [18]		
Type V: fibroateroma with possible calcification	13 (59,1%)	–
Type VI: mixed ASP with possible surface defects, hemorrhage or blood clots	7 (31,8%)	–
Not classified	2 (9,1%)	–
Laboratory data (Q2 [Q1; Q3])		
Glucose, mmol/l	5,5 [5,2; 5,8]	5,6 [5,2; 6,3]
Total cholesterol, mmol/l	4,2 [3,8; 5,2]	5,2 [4,7; 5,3]
Triglycerides, mmol/l	1,5 [1,3; 1,6]	1,3 [1,0; 1,6]
LDL, mmol/l	2,0 [1,8; 2,1]	3,0 [2,8; 3,6]
HDL, mmol/l	1,1 [1,1; 1,4]	1,3 [1,1; 1,7]
Atherogenic index	2,4 [1,8; 2,4]	2,6 [2,3; 3,0]
Medications, abs. (%)		
Anticoagulants/antiplatelet agents	20 (90,8)	2 (14,2)
Antihypertensive agents	16 (72,7)	4 (28,6)
Statins	13 (59,09)	1 (7,1)
Hypoglycemic drugs	5 (22,7)	1 (7,1)

Abbreviations: abs. — number of individuals in the group, ASP — atherosclerotic plaque, CAD — coronary artery disease, BMI — body mass index, HDL — high density lipoproteins, LDL — low density lipoproteins, CVA — cerebrovascular accident, CA — carotid artery, TIA — transient ischemic attack, Q1 — 25% quartile, Q2 — 50% quartile (median), Q3 — 75% quartile.

dynamically related changes. Samples of peripheral blood (leukocytes) were obtained from each individual included in the control group.

The material was DNA isolated from the vascular wall using the QIAamp DNA Mini Kit (Qiagen, USA) and from leukocytes of peripheral blood by the standard phenol-chloroform method. The DNA was then treated with bisulfite using the EZ DNA Methylation Kit (Zymo Research).

To study the DNA methylation level, a region was selected that is localized in exon 2 of *CDKN2B* gene and in intron 1 of *CDKN2B-AS1* gene, which contains the GH09J022005 enhancer of *CDKN2B/2A* and *CDKN2B-AS1* genes, according to the GeneHancer database (Figure 1) [11]. DNA methylation assay was performed by targeted bisulfite sequencing of amplicons using high throughput parallel sequencing.

Primers (F: 5'-TAAAATTAAAAAGTAGTAAGTTATAAGGGG-3' и R: 5'-AACCTACAAACCTATCTAAAACTCACAAAA-3) were used to carry out the polymerase chain reaction of the DNA region containing the enhancer fragment GH09J022005. The selection of primers was performed using the MethPrimer 2 [12]. The studied fragment (chr9:22,005,065-22,005,876, according to genome

Figure 1. Localization and epigenetic context of the GH09J022005 enhancer of *CDKN2A/2B* and *CDKN2B-AS1* genes: the studied region is highlighted in blue. Deciphering of human cell lines studied in the ENCODE project: GM12878 — B-lymphocytes, H1-hESC — embryonic stem cells, K562 — blood cells from a patient with chronic myeloid leukemia, HepG2 — hepatocellular carcinoma cells, HUVEC — umbilical vein endothelial cells.
Note: the color image is available in the electronic version of the issue.

assembly GRCh37/hg19) included 31 CpG sites and SNP — rs3217986, for which an association with severe coronary atherosclerosis and myocardial infarction was previously shown [13, 14].

DNA amplification was carried out on a ProFlex PCR System (ThermoFisher Scientific) according to the program: 95° C — 5 min, 40x (95° C — 30 sec, 62° C — 30 sec, 72° C — 60 sec), 72° C — 5 min. Bisulfite DNA sequencing was performed on a MiSeq system (Illumina). Statistical analysis was performed using R software (version 3.6.2). To assess the methylation levels of CpG sites, nonparametric distribution estimates were used as follows: M [Q1; Q3], where M is the median, Q1 — 1st quartile (25th percentile), Q3 — 3rd quartile (75th percentile). The similarity of the samples in methylation profiles of CpG sites in the analyzed genome region was visualized using the t-distributed Stochastic Neighbor Embedding (t-SNE). Comparison of methylation levels was also carried out using nonparametric criteria. Differences were considered significant at $p<0,05$. Since the methylation levels of all CpG sites were highly correlated (minimal Spearman's Rho value, 0,76), it was inappropriate to use statistical corrections, such as the Bonferroni or Benjamini-Hochberg corrections.

Results and discussion

The analysis of the methylation level of 31 CpG sites in the enhancer region of the *CDKN2A/2B* and *CDKN2B-AS1* genes revealed that the samples of blood vessels (arteries and veins) are clustered separately from the DNA samples of blood leukocytes (Figure 2). Moreover, according to the DNA methylation profile, CA with atherosclerotic lesions are located in the same group with intact blood vessels, and the patients' leukocytes are grouped with those of healthy individuals. Thus, there is a pronounced tissue specificity of methylation of this region for blood vessels and leukocytes, which is consistent with the literature data [15].

An increase in the methylation level of both individual CpG sites and the mean DNA methylation level as a whole in CA and atherosclerotic areas (48,6 [34,8; 62,0]%) in comparison with both arteries (25,2 [23,1; 41,60]%, $p=0,0001$) and veins (35,0 [31,6; 40,0]%, $p=0,0039$; Figures 3A and 3B) was revealed. Differences in DNA methylation level between intact arteries and veins did not reach significance ($p>0,05$). Patients had lower methylation levels in all CpG sites in blood leukocytes compared to blood vessel samples (8,7 [6,1; 9,7]%; $p<0,05$). However, the DNA methylation level in the studied region in leukocytes of patients with atherosclerosis does not differ from those of healthy individuals (9,3 [8,3; 13,6]%; $p>0,8$, Figures 3A and 3B).

The enhancer region of *CDKN2A/2B* and *CDKN2B-AS1* genes contains separate CpG sites (chr9: 22,005,288 and chr9: 22,005,564), which are included in the Illumina microarrays cg19481686 and cg08390209, respectively. Table 2 provides information on the methylation level of these CpG

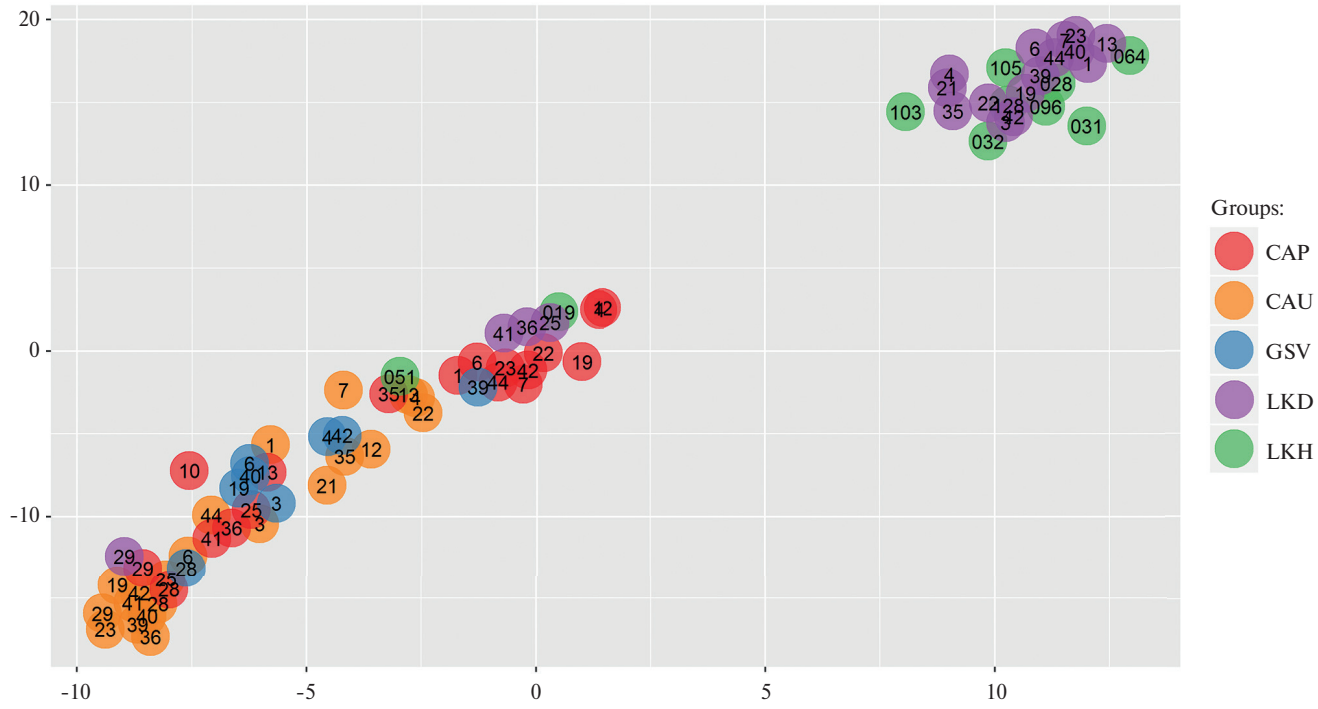


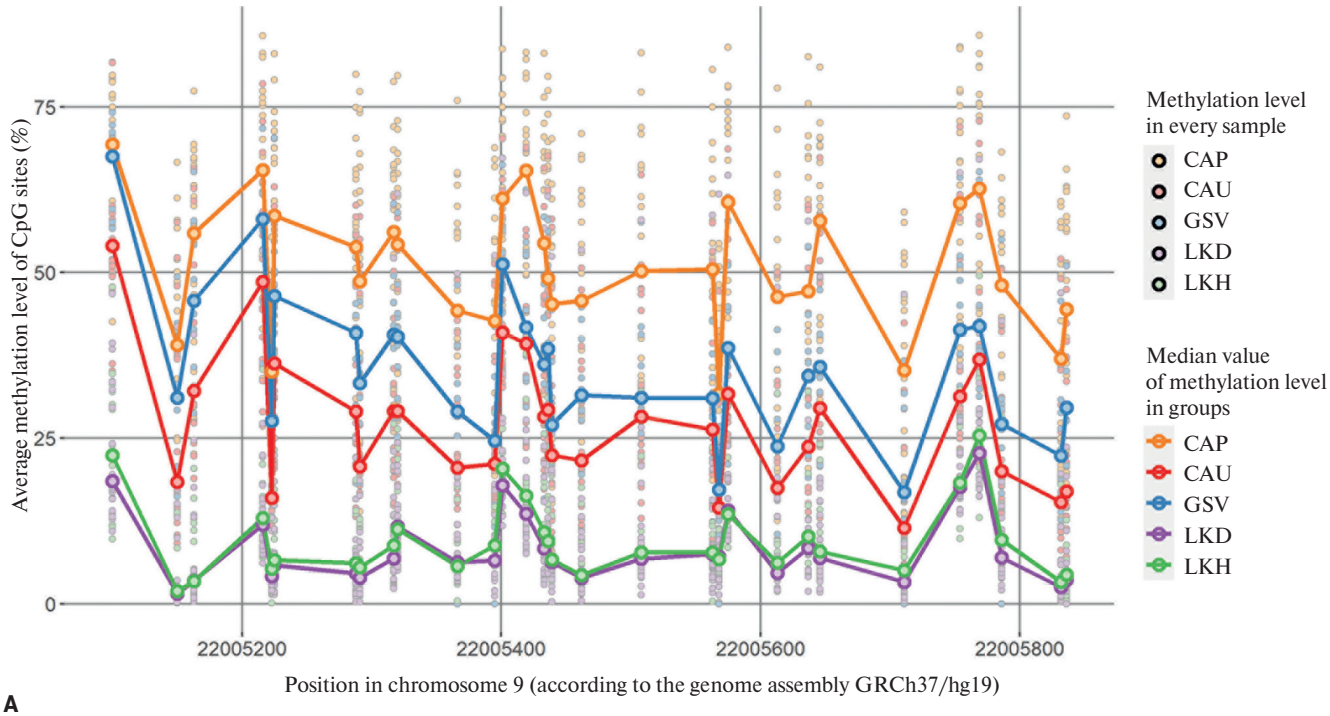
Figure 2. Mutual arrangement and clustering of blood vessels and leukocytes (according to the t-SNE method) depending on the methylation profiles of CpG sites in the enhancer region of *CDKN2A/2B* and *CDKN2B-AS1* genes.
Note: the color image is available in the electronic version of the issue.
Abbreviations: GSV — samples of great saphenous veins, LKD — blood leukocytes of patients with atherosclerosis, LKH — blood leukocytes of the control group, CAP — samples of carotid atherosclerotic plaques, CAU — samples of macroscopically unchanged carotid arteries.

sites according to current study and previously published papers. The methylation level of cg19481686 and cg08390209 in atherosclerotic CA in the present study (53,85 [30,18; 63,57]% and 50,45 [32,24; 60,88]%, respectively) coincides with those in CA obtained from both patients with stroke (55,03 [50,39; 61,24]% and 47,31 [43,50; 56,06]%, respectively) and without a history of stroke (55,27 [49,70; 59,63]% and 50,04 [42,04; 53,88]%, respectively).

However, in the coronary arteries (69,13 [56,37; 78,95]% and 67,11 [57,87; 72,87]%) and aorta (69,28 [66,95; 70,93]% and 66,52 [60,41; 71,18]%) with atherosclerotic lesions, the methylation level of cg19481686 and cg08390209 is significantly higher than in the CA. At the same time, the methylation level of cg19481686 and cg08390209 in the aorta with atherosclerotic lesions was significantly lower by 9-15% compared to the intact aorta, and the coronary arteries with atherosclerotic lesions had a lower methylation level of these CpG sites by 2-4% than intact internal thoracic arteries, but a higher compared to the great saphenous veins by 4%.

In general, in the vessels of patients with atherosclerosis of various localization, the methylation level of the analyzed CpG sites was significantly higher than that in leukocytes of patients with CA atherosclerosis (4,60 [2,84; 7,76]% and 7,47 [5,44; 8,77]%, respectively), including in stroke (11,38 [9,65; 13,64]% and 12,50 [10,63; 14,23]%, respectively), as well as healthy individuals (6,11 [3,61; 12,25]% and 7,77 [5,92; 11,41]%). At the same time, the methylation level in cg19481686 and cg08390209 in blood leukocytes in patients with atherosclerosis complicated by stroke was significantly 5% higher than in healthy individuals. At the same time, the methylation level of cg19481686 and cg08390209 in blood leukocytes between patients with CA atherosclerosis does not significantly differ from healthy individuals. Thus, in the present study, the relationship between an increase in DNA methylation level of the enhancer of *CDKN2A/2B* and *CDKN2B-AS1* genes in CA and their atherosclerotic lesions was revealed. To resolve the issue of relationship between the DNA methylation level of the analyzed genome region with coronary and aortic atherosclerosis, as well as with the risk of acute vascular events, an additional study is needed with large samples.

It should be noted that the literature contains data on changes in DNA methylation level in 9p21.3 locus in blood vessels and leukocytes, as well as its relationship with atherosclerotic lesions of the arteries. The region of CpG islands of *CDKN2A/2B* and *CDKN2B-AS1* gene promoters is most often analyzed in the studies. In particular, in our previous



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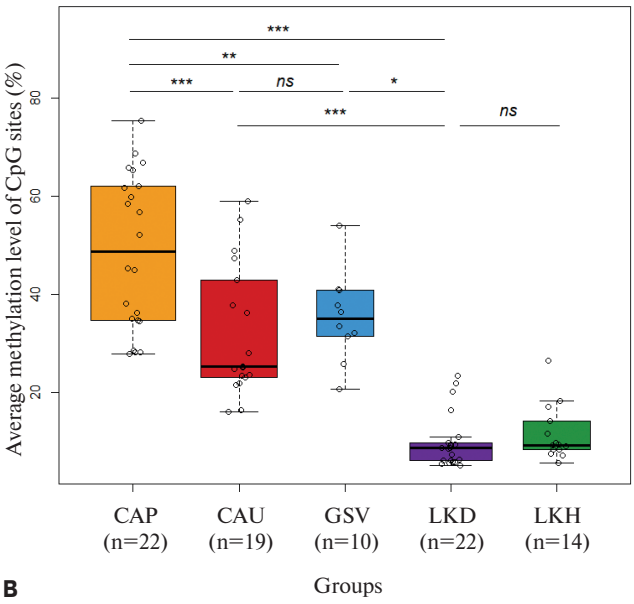

B

Figure 3. Methylation profiles (**A**) and average methylation level (**B**) of CpG sites in the enhancer of *CDKN2A/2B* and *CDKN2B-AS1* genes in blood vessels and leukocytes.
Notes: ns — not significant, * — p<0,05, ** — p<0,001, *** — p<0,001.
Abbreviations: GSV — samples of great saphenous veins, LKD — blood leukocytes of patients with atherosclerosis, LKH — blood leukocytes of the control group, CAP — samples of carotid atherosclerotic plaques, CAU — samples of macroscopically unchanged carotid arteries.

study, no differences were found in the methylation level of the CpG islands of *CDKN2A/2B* gene promoters in CA ASP and the adjacent macroscopically intact vascular wall in the same patients. However, DNA hypermethylation was noted in the enhancer region of *CDKN2A/2B* and *CDKN2B-AS1* genes, especially in CA ASP [8].

The genome-wide study revealed hypomethylation of the *CDKN2B-AS1* promoter in the chr9: 21,993,116-21,994,101 region and exon 7 of this gene (chr9: 22,056,255-22,056,627), but hypermethylation of exon 8 (chr9: 22,056,256-22,056,628) in the femoral arteries compared with unaffected internal thoracic arteries [16]. At the same time, in a study using bisulfite DNA pyrosequencing, a high methylation level (>55%) of 9 CpG sites (region of *CDKN2B-AS1* promoter, chr9: 21,993,583-21,993,721) in umbilical vein endothelial cells taken from newborns correlated with increased pulse wave velocity in the same children aged 9 years, which is considered a risk factor for future cardiovascular diseases [17].

Zhou S, et al. (2016, 2017) studied the methylation level of 24 CpG sites of *CDKN2A* gene (chr9: 21.993.993-21.995.909) and 12 CpG sites of *CDKN2B* gene (chr9: 22,008,804-22,009,259) in peripheral blood leukocytes of patients with ischemic stroke, depending on concomitant calcification of large vessels. In the first study, an increased methylation level of 1 CpG site of *CDKN2A* gene and 7 CpG sites of *CDKN2B* gene, as well as of the analyzed region as a whole, was found in patients with CA calcification [9]. In the second study, an increased methylation level of 4 CpG sites of *CDKN2A* gene and 11 CpG sites of *CDKN2B* gene in patients with aortic arch calcification was revealed [10]. At the same time, according to both studies,

Table 2

Methylation level of individual CpG sites in the enhancer region of *CDKN2A/2B* and *CDKN2B-AS1* genes in blood vessels and leukocytes in atherosclerotic lesions of the arteries

Tissue	CpG site methylation level, %, Q2 [Q1;Q3]		Source
	chr9:22,005,288 (cg19481686)	chr9:22,005,564 (cg08390209)	
Vessels			
Atherosclerotic CA (n=22)	53,85 [30,18; 63,57]	50,45 [32,24; 60,88]	Current study
Intact CA (n=18)	28,98 [24,19; 46,30]	26,31 [22,87; 36,78]	
Great saphenous veins (n=10)	40,90 [31,65; 43,00]	31,00 [28,57; 36,79]	
Atherosclerotic CA in patients with stroke (n=19)	55,03 [50,39; 61,24]	47,31 [43,50; 56,06]	GSE66500 [19]
Atherosclerotic CA in patients without stroke (n=19)	55,27 [49,70; 59,63]	50,04 [42,04; 53,88]	
Atherosclerotic coronary arteries (n=6)	69,13 [56,37; 78,95]	67,11 [57,87; 72,87]	GSE62867 [20]
Intact internal thoracic arteries (n=6)	73,20 [68,76; 73,57]	69,21 [67,30; 70,02]	
Intact great saphenous veins (n=6)	64,93 [59,79; 69,18]	63,18 [62,70; 65,00]	
Atherosclerotic aorta (n=15)	69,28 [66,95; 70,93]	66,52 [60,41; 71,18]	GSE46401 [21]
Intact aorta (n=15)	78,54 [77,12; 80,39]	81,34 [78,13; 85,01]	
Blood leukocytes			
Patients with severe CA atherosclerosis (n=22)	4,60 [2,84; 7,76]	7,47 [5,44; 8,77]	Current study
Healthy individuals (n=14)	6,11 [3,61; 12,25]	7,77 [5,92; 11,41]	
Patients with severe coronary atherosclerosis (n=8)	14,50 [9,04; 20,88]	15,54 [10,31; 18,84]	GSE107143 [22]
Healthy individuals (n=8)	12,85 [9,82; 14,58]	13,55 [11,24; 15,54]	
Patients with stroke due to atherosclerosis (n=132)	11,38 [9,65; 13,64]	12,50 [10,63; 14,23]	GSE69138 [23]
Patients with lacunar stroke (n=141)	11,51 [9,96;13,60]	12,98 [10,93; 14,27]	
Patients with cardioembolic stroke (n=127)	11,47 [11,51; 11,38]	12,20 [12,98; 12,50]	

Abbreviations: CA — carotid artery, Q1 — 25% quartile, Q2 — 50% quartile (median), Q3 — 75% quartile.

the average level of *CDKN2A* and *CDKN2B* methylation in peripheral blood leukocytes of patients with ischemic stroke did not exceed 6% [9, 10], which is generally consistent with this study.

Analysis of targeted bisulfite sequencing of amplicons using high-throughput parallel sequencing makes it possible to estimate the frequency of genotypes and their relationship with the DNA methylation level. In the region of GH09J022005 enhancer of *CDKN2A/2B* and *CDKN2B-AS1* genes, rs3217986:T>G is located (Figure 1). The G allele rs3217986 is associated with the risk of severe coronary atherosclerosis and myocardial infarction in two different populations [13, 14]. The distribution of rs3217986 genotypes in the patient group was as follows: 21 (95,5%) individuals with the TT genotype and 1 (4,5%) with the TG genotype, and in the control group — 13 (92,9%) individuals with the TT genotype and 1 (7,1%) with the TG genotype.

The relatively small sample size did not make it possible to accurately determine the relationship between the DNA methylation level in blood vessels and leukocytes, and SNP rs3217986:T>G and significant signs of atherosclerotic lesions of CA. The revealed difference in the DNA methylation level in the region of the enhancer of *CDKN2A/2B* and *CDKN2B-AS1* genes in CA may be associated not so much with their atherosclerotic lesion, but with the cellular heterogeneity of the vessels. To determine the functional significance of changes in DNA methylation level in cells and tissues, it is necessary to analyze the functional activity of genes. On the other hand, this work used modern technologies for analyzing the DNA methylation level in several types of tissues (vascular wall and peripheral blood leukocytes) in the same individuals. This approach takes into account the individual variability of DNA methylation. In addition, this study was the first to analyze the enhancer region of *CDKN2A/2B* and

CDKN2B-AS1 genes in vascular tissues and peripheral blood leukocytes of patients with clinically relevant atherosclerosis and healthy individuals.

Conclusion

In the present study, the relationship between an increase in the DNA methylation in the enhancer of the *CDKN2A/2B* and *CDKN2B-AS1* genes in CA and their atherosclerotic lesions was revealed, as well as the tissue-specific DNA methylation between vessels and peripheral blood leukocytes. Thus, the molecular genetic mechanisms of atherosclerotic arterial lesions should be studied using tissues and cells of arteries, and the search for biomarkers of this pathology, using peripheral blood leukocytes.

Relationships and Activities: none.

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