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Diagnostic potential of miRNA-135A1 in human papillomavirus associated cervical lesions

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Introduction. Human papillomavirus (HPV) infection with high-risk HPVs is an etiological factor in the development of cervical cancer, with HPV type 16 (HPV16) being the most common. The mechanisms leading to disruption of viral oncogene expression and initiation of epithelial cell transformation are poorly understood. Epigenetic regulatory factors, including cellular miRNAs, may play an important role in HPV-induced carcinogenesis, and aberrantly expressed miRNAs may be promising markers for the diagnosis of HPV-associated lesions.

Aim. To search for miRNAs involved in the pathogenesis of HPV16-associated cervical cancer and to evaluate their diagnostic potential for the detection of cervical cancer and precancerous lesions.

Materials and methods. MiRNA expression in clinical samples was assessed by both next generation sequencing and quantitative stem-loop polymerase chain reaction (sl-qPCR). Plasma miRNAs from patients with precancerous and cancerous lesions and healthy donors were analyzed using sl-qPCR. Loss of heterozygosity in cervical cancer samples was assessed by copy number ratio of *MIR135A1* and *ACTB* genes. A total of 67 patients with cervical cancer, 21 with precancerous cervical lesions and 24 healthy donors were included in the study. The effect of DNA methylation on miRNA-135A1 expression was evaluated after treatment with a demethylating agent of the cervical HPV16-positive SiHa cell line. Changes in the expression of the HPV16 *E6* oncogene were analyzed after transfection with synthetic analogues of the mature forms of miRNA-135A1 (miRNA-135a-3p and miRNA-135a-5p).

Results. A significant decrease in the expression of miRNA-135A1 and miRNA-135A2 was detected in tumor tissue samples from HPV16-positive cervical cancer, which was confirmed by sl-qPCR in an independent panel of tumor samples. A decrease in miRNA-135A1 expression was shown to result from both loss of heterozygosity of the gene and aberrant DNA methylation. Transfection of mature forms of miRNA-135A1 into SiHa cells resulted in decreased expression of the *E6* oncogene of HPV16. Blood plasma samples from patients with cervical cancer and precancerous lesions showed lower levels of miRNA-135a-3p than healthy donors, and ROC analysis indicated its high diagnostic potential.

Conclusion. Levels of miRNA-135A1 are significantly reduced in cervical lesions, both in tumor tissue and plasma, and the ability of this miRNA to suppress the expression of the HPV16 *E6* oncogene suggests its oncosuppressive properties. Thus, miRNA-135A1 can be used as a promising new marker for the diagnosis of HPV-associated lesions.

Keywords: cervical precancerous lesion, cervical cancer, human papillomavirus, miRNA-135A1

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Диагностический потенциал микроРНК-135A1 при ассоциированных с вирусом папилломы человека поражениях шейки матки

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Введение. Инфекция вируса папилломы человека (ВПЧ) высокого онкогенного риска является этиологическим фактором развития рака шейки матки, причем наиболее распространенным является ВПЧ 16-го типа (ВПЧ16). Механизмы, приводящие к нарушению экспрессии вирусных онкогенов и запуску процесса онкотрансформации, не-

достаточно изучены. Эпигенетические факторы регуляции, в том числе клеточные микроРНК, могут играть большую роль в ВПЧ-индуцированном канцерогенезе, а аберрантно экспрессированные микроРНК рассматриваться как перспективные маркеры для диагностики ВПЧ-ассоциированных поражений.

Цель исследования – поиск микроРНК, вовлеченных в патогенез ВПЧ16-ассоциированного рака шейки матки, и оценка их диагностического потенциала для выявления рака или предраковых поражений шейки матки.

Материалы и методы. Экспрессию микроРНК в клинических образцах оценивали с помощью секвенирования нового поколения (next generation sequencing, NGS) и количественной stem-loop полимеразной цепной реакции (sl-кПЦР), для анализа микроРНК в плазме крови использовали sl-кПЦР. Потерю гетерозиготности в образцах рака шейки матки оценивали по соотношению числа копий генов *MIR135A1* и *ACTB*. Всего в исследование вошли 67 пациентов с раком шейки матки, 21 с предраковыми поражениями шейки матки и 24 здоровых донора. Влияние метилирования ДНК на экспрессию микроРНК-135A1 оценивали после обработки деметилирующим агентом цервикальной ВПЧ16-положительной клеточной линии SiHa. Изменение экспрессии онкогена *E6* ВПЧ16 оценивали после трансфекции синтетических аналогов зрелых форм микроРНК-135a-3p и микроРНК-135a-5p.

Результаты. В образцах опухолевой ткани ВПЧ16-положительного рака шейки матки выявлено значительное снижение экспрессии микроРНК-135A1 и микроРНК-135A2, что подтверждено на независимой выборке опухолевого материала методом sl-кПЦР. Снижение экспрессии микроРНК-135A1 может быть обусловлено как потерей гетерозиготности соответствующего гена, так и аберрантным метилированием ДНК. Трансфекция зрелых форм микроРНК в клетки SiHa приводила к снижению экспрессии онкогена *E6* ВПЧ16. В образцах плазмы крови пациентов с РШМ и предраковыми поражениями наблюдается более низкий уровень микроРНК-135a-3p, чем у здоровых доноров, а проведенный ROC-анализ указывает на ее высокую диагностическую значимость.

Заключение. Уровень микроРНК-135A1 значительно снижен при цервикальных поражениях как в опухолевой ткани, так и в плазме крови, а способность данной микроРНК подавлять экспрессию онкогена *E6* ВПЧ16 говорит о наличии у нее онкосупрессорных свойств. Таким образом, микроРНК-135A1 является перспективным маркером для диагностики ВПЧ-ассоциированных поражений.

Ключевые слова: предраковое поражение шейки матки, рак шейки матки, вирус папилломы человека, микроРНК-135A1

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INTRODUCTION

Cervical cancer (CC) is the fourth most common cancer in women worldwide, with a mortality rate of over 50 % (according to the World Health Organization). The etiological factor of CC is infection with high-risk human papillomaviruses (HPV), of which HPV type 16 (HPV16) is detected in more than 60 % of cases [1]. High-risk HPV infection is the cause not only of CC, but also of some anogenital tumors (vagina, anus, penis and vulva) and head and neck tumors (oropharynx, larynx, nasal cavity and mouth) [2, 3]. Human papillomaviruses are double-stranded DNA viruses that infect the epithelium. Human papillomavirus genomes contain regions encoding early viral genes (*E1*, *E2*, *E4*, *E5* and oncogenes *E6*, *E7*), late genes (*L1* and *L2*) and a non-coding regulatory region, the upstream regulatory region (URR). The products of the early genes are required for viral replication, regulation of its transcription, stimulation of growth and division of infected epithelial cells, while the late genes encode proteins of the viral capsid envelope. The viral oncogenes *E6* and *E7* play a key role in ensuring the normal life cycle of the virus and maintaining the enhanced proliferative potential of the cells. They interact with the tumor growth suppressor proteins p53 and pRB, leading to their degradation and subsequent inhibition of apoptosis and increased cell proliferation [4]. Human papillomavirus infection of the cervical epithelium occurs through its micro-damage, when the virus enters the basal mitotically active cells of the epithelium. This is followed by the lag phase of viral infection, when viral gene expression is at a minimal level and there are no clinical manifestations [5]. In 90 % of cases, high-risk HPV infections are completely cleared by the immune system within 1–2 years [6–8]. However, in 10 % of cases, the infection can become permissive, which is characterized by activation of viral gene expression, active viral replication and the formation of cervical intraepithelial neoplasia grade 1 (CIN1), which is characterized by disturbed differentiation and active proliferation of cells in the lower layers of the epithelium, and transformed cells are found in only half of cases [9, 10]. This infection can last for several years and regression occurs in 90 % of cases, but progression to moderate to severe CIN grade 2 (CIN2) and 3 (CIN3) neoplasia is sometimes observed [11]. These types of lesions are characterized by the development of a transforming HPV infection associated with increased expression of the viral oncogenes *E6* and *E7*, leading to active proliferation of undifferentiated cells of the middle and upper layers of the epithelium, genetic instability and accumulation of mutations that contribute to further progression of the disease [12]. The rate of progression from CIN3 to carcinoma *in situ* and invasive CC is already over 50 %, significantly higher than the CC risk levels for CIN1 and CIN2 [13]. The mechanisms underlying the increased expression of *E6* and *E7* oncogenes during the transition from permissive to transforming infection are currently poorly understood. Until recently, it was thought that the main

mechanism leading to impaired expression of viral oncogenes was the integration of viral DNA into the host cell genome, followed by the loss of expression of the viral protein E2, which negatively regulates the expression of viral oncogenes [14, 15]. However, it has now been shown that HPV DNA integration occurs later in the progression of CIN, while transformed epithelial cells are detected as early as CIN1 [9, 10]. Furthermore, even at the stage of HPV-associated invasive tumors, the viral genome may be present as extrachromosomal circular DNA molecules (episomes) without disruption of E2 protein expression [16, 17].

To date, a large body of data has accumulated indicating an important role for epigenetic regulation in the pathogenesis of virus-associated tumors, including through miRNAs, which can both directly regulate viral transcripts by degrading or stabilizing them, and modulate the function of cellular signaling pathways, thus providing optimal conditions for the viral life cycle [18]. An example of direct interaction between cellular miRNAs and oncoviruses is the suppression of HPV16 *E6* and *E7* oncogene expression under the influence of cellular miRNA-375 and miRNA-187, which is accompanied by restoration of p53 and pRB levels, suppression of cell proliferative activity, migration and invasion, and induction of apoptosis [19–21]. Notably, the expression levels of these miRNAs are significantly reduced in CC tumor tissue compared to normal tissue, which in the case of miRNA-187 is associated with reduced overall and relapse-free survival [19].

In this context, the search for cellular miRNAs that directly regulate viral oncogenes and whose levels are reduced in CC is an urgent task that will allow us to complement the mechanisms of HPV-induced cellular oncotransformation and to characterize new oncosuppressor miRNAs that could form the basis of new diagnostic tests under development.

MATERIALS AND METHODS

Clinical samples. The material used to search for differentially expressed miRNAs by next-generation sequencing (NGS) and to validate the results by the independent method were paraffin sections of CC, from which tumor and adjacent morphologically normal tissue were obtained by microdissection. Fresh frozen cervical squamous cell carcinoma tissue was used to analyze the presence of heterozygosity of the *MIR135A1* gene. Blood plasma from CC patients, CIN patients and healthy donors was used for the analysis of free circulating miRNAs. Cervical swabs collected in BD SurePath™ transport medium (Becton Dickinson, USA) were used for HPV DNA detection and HPV type identification in patients with cervical dysplasia. All samples were collected from patients treated in the Department of Oncogynaecology, clinical diagnosis was confirmed in the Department of Pathological Anatomy of Human Tumors N.N. Blokhin National Medical Research Center of Oncology. The clinical and morphological characteristics of the samples used in the work are shown in table 1.

Cell culture. The SiHa cervical HPV16-positive cell line (ATCC-HTB-35) was used in this study. Cells were

Table 1. *Clinical and morphological characteristics of cervical cancer patients, patients with cervical dysplasia and healthy donors*

Parameter	Cervical cancer patients	Patients with dysplastic lesions of the cervix	Healthy donors
Age, years, median (Q_1–Q_3)	42 (35–49)	37 (32.5–46.5)	27.5 (25.0–33.8)
Primary tumor size, *n* (%): T1 T2 Tis	 48 (72) 14 (21) 5 (7)	–	–
Presence/absence of lymphogenic metastases, *n* (%): N0 N1 Nx	 45 (67) 15 (22) 7 (11)	–	–
Presence/absence of distant metastases, *n* (%): M0	 67 (100)	–	–
Stage of disease (cervical tumor/dysplasia), *n* (%): I II III *in situ*	 37 (55) 12 (18) 14 (21) 4 (6)	–	–
Cervical tumor/dysplasia, *n* (%): CIN1 CIN2 CIN3/*in situ*	–	 4 (19) 4 (19) 13 (62)	–
Total	*67*	*21*	*24*

Note. *Q_1 – 1st quartile; Q_3 – 3rd quartile; CIN1 – cervical intraepithelial neoplasia grade 1; CIN2 – cervical intraepithelial neoplasia grade 2; CIN3 – cervical intraepithelial neoplasia grade 3.*

cultured in DMEM medium supplemented with glutamine and glucose 4.5 g/l (PanEco Ltd., Russia), 10 % fetal bovine serum (Biowest, France) and antibiotics Pen-Strep Solution (Biological Industries®, USA) at 37 °C, 5 % CO_2.

For transfection of cells with synthetic miRNA-135a-3p and miRNA-135a-5p analogues (DNA-Synthesis, Russia), the cationic lipid reagent siLentFect™ Lipid Reagent (Bio-Rad Laboratories, USA) was used according to the manufacturer's instructions. Transfection was performed at an oligonucleotide concentration of 50 nM and results were evaluated after 48 h. SiHa cells were treated with the demethylating agent 5-aza-2'-deoxycytidine (DAC, Sigma-Aldrich, USA) for 96 h, with partial replacement of the culture medium with DAC every 24 h.

MiRNA isolation and expression analysis. Material obtained after microdissection of tumor and adjacent normal tissue from HPV16-positive cervical squamous cell carcinoma was subjected to NGS at Genoanalytika (Russia) using an Illumina HiSeq® 1500 sequencer (USA).

MiRNAs were isolated from paraffin sections using the ReliaPrep™ FFPE Total RNA Miniprep System Kit (Promega, USA), from fresh frozen tissue using the PureLink™ miRNA Isolation Kit (Invitrogen, USA), and from blood plasma using the miRNeasy Serum/Plasma Kit (Qiagen, USA). MiRNA levels were measured using the Qubit™ miRNA assay kit (Invitrogen, USA) with a Qubit Fluorimeter 2.0 instrument (Invitrogen, USA).

The expression levels of miRNA-135a-3p and miRNA-135a-5p were analyzed by stem-loop real-time PCR [22] using 'TaqMan MiRNA Assays' kits ID002232 and ID000460 (Applied Biosystems, USA), PCR transcription was performed using 'TaqMan MiRNA Reverse Transcription Kit' (Applied Biosystems, USA), PCR was performed using qPCRmix-HS mix kit (Evrogen, Russia). To assess the expression level of miRNA-16b-5p, we used the gene-specific primer for cDNA synthesis 5'-GTTGGCTCTGG TGGTGCAGGGTCCGAGGTATTCGCACCAGAGCC AACCGCCAA-3', forward (F) and reverse (R) primers for PCR -F: 5'-GTTTGGTGGTAGCAGCAGCACGTAAA TA-3', R: 5'-GTGCAGGGTCCGAGGT-3' and TaqMan assay (TM) 5'-FAM-TTGGCGGTGGTTGGCTCTG-BHQ1-3'. Oligonucleotides were synthesized by DNA-Synthesis LLC (Russia).

Isolation and analysis of mRNA expression. RNA was isolated from SiHa cells using the MagZol reagent kit (Magen, China) according to the manufacturer's instructions. RNA was treated with DNase I (Invitrogen, USA) and cDNA synthesis was performed using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., USA). The following primers were used for real-time PCR: HPV16 *E6* gene F: 5'-GTTACTGC GACGACGTGAGGTATATG-3'; R: 5'-CATTTATCACAT ACAGCATATATGGATTC-3'; *ACTB* gene F: 5'-ATGTGGCCGAGGAGGACTTTGATT-3'; R: 5'-AGT GGGGGGGTGGGCTTTTAGGATG-3'. Real-time PCR was performed using qPCRmix-HS SYBR reagent (Evrogen, Russia).

Evaluation of heterozygosity of the *MIR135A1* gene. The material for analysis was a collection of DNA from squamous cell carcinoma tumor tissue isolated by the standard technique of ultracentrifugation in a cesium chloride density gradient [23] with modifications [24]. Plasmids containing the *MIR135A1* gene locus and *ACTB* were prepared for the construction of calibration curves. Amplification of the DNA fragment containing the *MIR135A1* gene was performed using the following primers and TaqMan probes: forward primer: 5'-TTGCTCAGTGTCAGAACCC-3'; reverse primer: 5'-GGTTGGGGGGTGGAAGAAGAAGTG-3', probe: 5'-FAM-TGCGCCACGGCTCCAATCCC-BHQ1-3'; *ACTB* gene: forward primer: 5'-CAAGTCTTCTTCTGGACTGTGA-3'; reverse primer: 5'-CTCCCTTGAAGGTTGCAGAGAG-3', probe: 5'-HEX-AGCCACTGTGTGCTGGGTGGTGG-BHQ1-3'. To determine the number of copies of each gene, real-time PCR was performed on the DNA matrix of the CC samples and a calibration curve was constructed. The presence of loss of heterozygosity (LOH) was assessed by the ratio of the number of *MIR135A1* and *ACTB* gene copies for each sample (the criterion for the presence of LOH was a value less than 0.6).

Human papillomavirus type identification. To determine the HPV status of patients with CIN, DNA was isolated from cervical smears using the AmpliSens® DNA-Sorb-D kit (Central Research Institute of Epidemiology of Rospotrebnadzor, Russia). The HPV type detection kit AmpliSens® HPV VKR genotype-FL (Central Research Institute of Epidemiology of Rospotrebnadzor, Russia) was also used according to the manufacturer's recommendations.

Statistical analysis. Statistical evaluation of the data obtained was performed using GraphPad Prism 9.5.1 software. The relative change in RNA expression was calculated using the ΔΔCt method, miRNA – normalised ΔΔCt values were compared. The Mann-Whitney U criterion was used to assess the reliability of the change in miRNA expression in tumor tissue compared to normal tissue, as well as the change in *E6* oncogene expression. The Kruskal–Wallis test with Dunn's multiple comparisons was used to statistically process the data from the demethylating agent treatment experiment and to compare miRNA levels in different groups of blood plasma samples. The correlation between miRNA-135a-5p levels and copy number ratio of *MIR135A1* and *ACTB* genes was assessed using Pearson's parametric correlation criterion.

RESULTS

Decreased expression of miRNA-135 in cervical cancer tumor tissue. To search for aberrantly expressed miRNAs in CC, we analyzed the levels of miRNAs in transformed cervical epithelium compared to adjacent morphologically normal epithelium by NGS. Paraffin sections from 9 paired cases of cervical squamous cell carcinoma were used for the study. Only HPV16-positive CC samples were used

in this study because this type of HPV is the most common etiological factor in the development of CC and this selection also allowed the sample to be more homogeneous. Microdissection of tumor tissue and adjacent normal epithelium was performed to enrich the study material and minimize the presence of stromal components in the samples examined.

Analysis of the NGS results showed that tumor tissue and adjacent normal cervical tissue have different miRNA expression patterns; 65 miRNAs showed significant differences, of which 25 miRNAs were overexpressed and 40 miRNAs showed reduced levels in tumor tissue (fig. 1, *a*). In this study, we primarily focused on those differentially expressed miRNAs that met the following selection criteria. First, these miRNAs should be characterized by a low level of expression in tumor tissue compared to the adjacent normal cervical epithelium, i. e. they should be potential oncosuppressor miRNAs. Second, the investigated miRNAs should be able to suppress viral gene expression and thus have direct antiviral effects. Among all differentially expressed miRNAs, miRNA-135A1 and miRNA-135A2, whose expression is significantly reduced in tumor tissue (see fig. 1, *a*), met the selection criteria and these miRNAs have 4 potential interaction sites with HPV16 transcripts according to bioinformatic analysis using the RegRNA 2.0 service [25].

The human miRNA-135 family is represented by 3 genes that encode the formation of 3 miRNA precursors, which are processed to form 5 unique sequences of the mature forms of miRNA-135 (fig. 1, *b*). The miRNA-135 genes are located on three different chromosomes and encode the formation of three pre-miRNAs: the *MIR135A1* gene on chromosome 3 (3p.21.2), the *MIR135A2* gene on chromosome 12 (12q23.1) and the *MIR135B* gene on chromosome 1 (1q32.1). The processing of miRNA-135 precursors results in the formation of 5 unique mature forms of miRNAs – miRNA-135a-5p, miRNA-135a-3p, miRNA-135a-2-3p, miRNA-135b-3p and miRNA-135b-5p. Furthermore, the mature 5p forms of miRNA-135 are characterized by a high degree of homology (96 % or more), whereas the 3p forms are 60 % identical (fig. 1, *c*). In this study, we also focused on the expression of the mature forms of miRNA-135a-5p and miRNA-135a-3p, which are the products of the *MIR135A1* gene, whose expression level is most reduced in CC according to NGS data.

High-throughput NGS data were validated by an independent method on a large sample set of paired clinical cases of CC. The tumor material sample included 23 paired samples of HPV16-positive cervical squamous cell carcinoma and adjacent morphologically normal epithelium. Mature forms of miRNA-135A1 were detected by reverse transcription and quantitative real-time polymerase chain reaction in a stem-loop PCR modification [22]. As shown in fig. 2, *a*, 12 out of 23 tumor tissue samples show a more than 2-fold decrease in miRNA-135a-5p expression, while 10 samples show no change in miRNA-135a-5p level or up to a 2-fold increase compared to normal epithelium. The expression level of miRNA-135a-3p was decreased more than 2-fold in 7 out of 23 tumor tissue samples, in 15 samples there was a less pronounced suppression

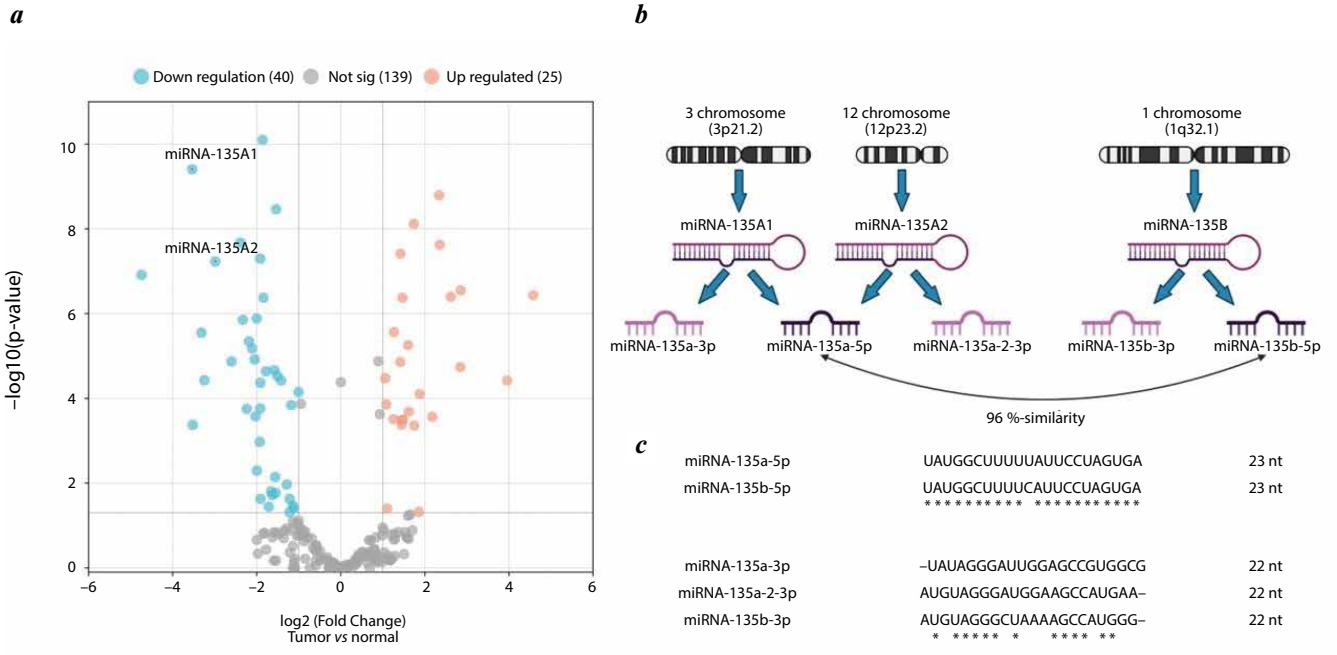


Fig. 1. *Differential miRNA expression in HPV16-positive (HPV16 – human papillomavirus type 16) cervical cancer and representation of selected miRNA-135 family: a – volcano plot showing the differential expression of miRNA in HPV16-positive cervical cancer samples as a function of significance level. The x-axis shows the log2 of the ratio of miRNA levels in tumor tissue to normal tissue, the y-axis shows the negative log10 of the p-value significance level; b – schematic representation of genes, precursors and mature forms of miRNA-135; c – alignment of nucleotide sequences of mature forms of miRNA-135. Asterisks indicate nucleotides that match in the compared miRNA sequences*

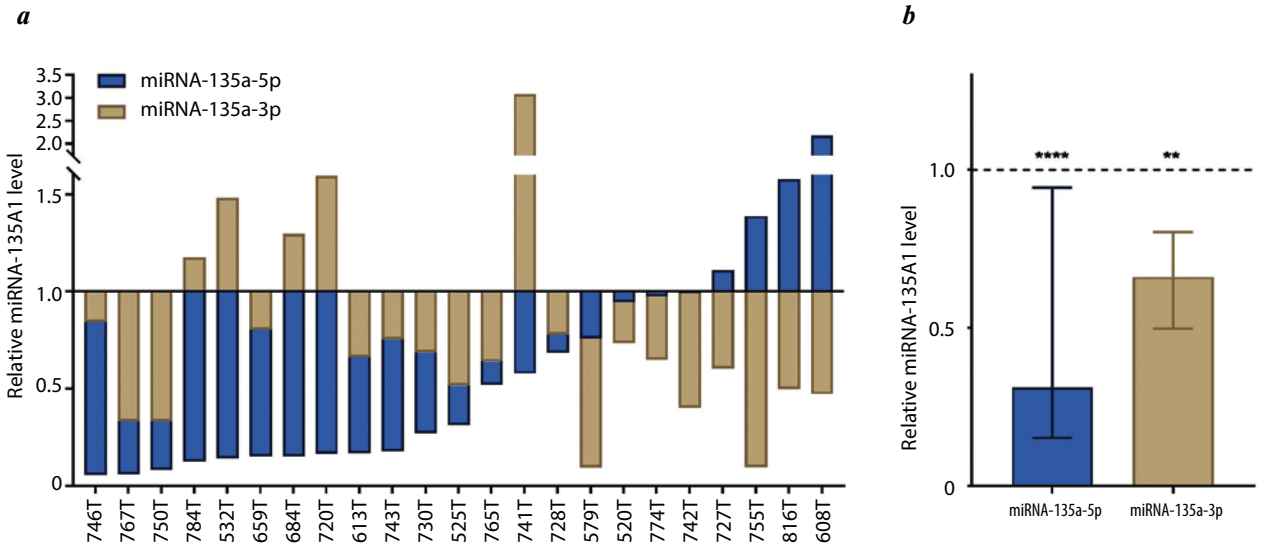


Fig. 2. *Relative expression levels of miRNA-135a-5p and miRNA-135a-3p in cervical tissue: a – relative expression levels of miRNA-135a-5p and miRNA-135a-3p in paired samples of cervical squamous cell carcinoma compared to adjacent normal epithelium. The solid line indicates the level of miRNA expression in the morphologically normal epithelium from which the calculations were made; b – graph showing the total change in the relative expression level of miRNA-135a-5p and miRNA-135a-3p in tumor tissue compared to the adjacent normal epithelium in all cervical cancer samples analyzed. The dotted line indicates the expression level in the normal epithelium from which the calculations were made. **p <0.01; ****p <0.0001.*

of expression or its increase up to 2-fold. It is worth noting that in 17 out of 23 samples (more than 70 %) the expression of at least one of the mature forms of miRNA-135A1 was significantly decreased by less than 2-fold, and in only two out of 23 samples (less than 10 %) was its expression increased by more than 2-fold. Thus, the majority of CC samples were indeed characterized by low levels of mature miRNA-135a-5p and miRNA-135a-3p expression (fig. 2, *b*).

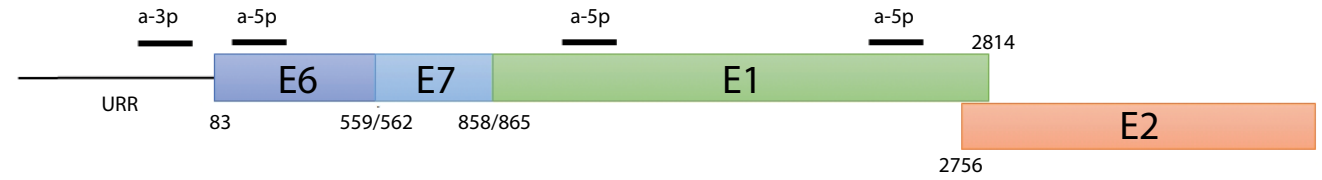
Thus, the expression of miRNA-135A1 is significantly reduced in the majority of CC tumor tissue samples compared to the conditionally normal adjacent epithelium, indicating its potential oncosuppressive properties. However, a low level of expression of the gene under investigation in tumor tissue does not necessarily indicate that it can be characterized as an oncosuppressor. As mentioned above, the selected miRNAs should also be able to inhibit the expression of viral transcripts, including viral oncogenes, whose increased expression is required for both the initiation and maintenance of HPV-associated oncotransformation of cells.

Changes in the expression of viral oncogenes under the influence of miRNA-135A1. A bioinformatic search for potential interaction sites of miRNA-135a-5p and miRNA-135a-3p isoforms using the RegRNA2.0 service [25] with HPV16 transcripts revealed 4 potential interaction sites, one of which is located in the reading frame of the viral oncogene *E6* and the others are located in the reading frame of the viral helicase *E1* and in the non-coding regulatory region URR (fig. 3, *a*). The positions of miRNA 135a-5p and miRNA-135a-3p interaction sites and their level of significance are listed in the table in fig. 3, *b*. It should be noted that in addition to the presence of a miRNA-135a-5p binding site in the reading frame of the *E6* oncogene, the URR regulatory region also contains a miRNA-135a-3p interaction site (see fig. 3, *a*), the effect of which may be mediated by non-coding regulatory RNA located in this region [26]; therefore, a role for miRNA-135a-3p in the regulation of *E6* oncogene expression cannot be excluded.

To evaluate the effect of the miRNAs on the expression of the *E6* viral oncogene, synthetic analogues of the mature forms of miRNA-135a-3p and miRNA-135a-5p were transfected into the cervical HPV16-positive SiHa cell line, containing 1–2 copies of the viral genome per cell (fig. 3, *c*). It was shown that transfection of miRNA-135a-3p and miRNA-135a-5p oligonucleotides into SiHa cells resulted in a decrease in the level of *E6* oncogene mRNA, suggesting a potential oncosuppressive role of this miRNA through suppression of *E6* oncogene expression and, consequently, HPV-associated cell transformation.

Mechanisms responsible for the reduced expression of miRNA-135A1. The processes leading to changes in miRNA expression in tumor cells are extremely diverse and include many genetic and epigenetic mechanisms. One of the mechanisms leading to decreased miRNA-135A1 levels in tumor tissue compared to normal tissue may be LOH of the gene encoding the miRNA-135A1 precursor. The *MIR135A1* gene in the human genome is located on the short arm of chromosome 3 (3p21.2), which is characterized by a high frequency of LOH in CC [27]. To test this hypothesis, the ratio of *MIR135A1* gene copy number to β-actin (*ACTB*) gene copy number was analyzed in 41 HPV16-positive CC samples using quantitative real-time PCR. As shown in fig. 4, *a*, more than 30 % (13 of 41)

a



b

Position in HPV16 (AF125673.1)	Score	Energy (kCal/Mol)	Interaction scheme
7544-7567 (URR)	150	–29.00	135–3p: 3'gcGGUGC–CGAGG–UUAGGGAUAu 5' HPV16: 5'tqCCATGCGTGCCAAATCCCTGTt 3'
260-282 (*E6*)	145	-18.92	135–5p: 3' agUGUAUCCUUAUUUUUCGGUAu 5' HPV16: 5' gtATATAGAGATGGGAATCCATa 3'
1183-1203 (*E1*)	145	–11.59	135–5p: 3' aguGUAUCCUUAUUUUUCGGUAu 5' HPV16: 5' aqtCCTAG--ATTAAAAGCTATa 3'
2364-2386 (*E1*)	150	–10.92	135–5p: 3' aguguauccuuauUUUUCGGUAu 5' HPV16: 5' ttttgtaaattctAAAAGCCATt 3'

c

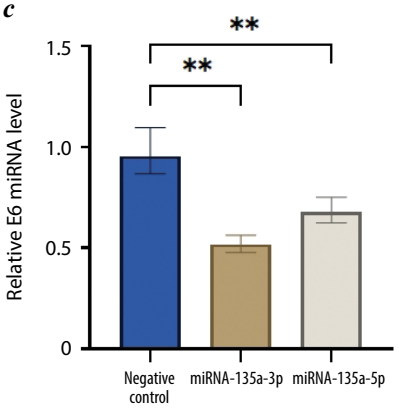


Fig. 3. *Effect of miRNA-135A1 on human papillomavirus type 16 (HPV16) oncogene mRNA levels: a — potential interaction sites of miRNA-135a-3p (a-3p) and miRNA-135a-5p (a-5p) in the HPV16 genomic region containing early genes. Upstream regulatory region (URR), non-coding regulatory region of HPV16; E6, E7, E1 and E2, early genes of HPV16; b — table showing the positions of potential miRNA-135a-5p and miRNA-135a-3p interaction sites in the HPV16 genome obtained using the RegRNA2.0 service with the features describing the significance of the identified sites; c — relative mRNA expression level of HPV16 oncogene E6 after transfection of SiHa cell line with synthetic analogues of miRNA-135a-3p and miRNA-135a-5p. **p <0.01*

of the squamous cell carcinoma samples analyzed show LOH in the genomic region containing the *MIR135A1* gene, which is consistent with literature data showing LOH at this locus in 33–48 % of cases [27, 28]. The results support the hypothesis that the decrease in miRNA-135A1 levels in a proportion of CC cases may be due to the loss of one of the copies of the *MIR135A1* gene, as confirmed by correlation analysis performed on a group of 17 CC samples (fig. 4, *b*).

In addition to chromosomal aberrations resulting from general tumor genetic instability, disruption of epigenetic regulation, such as aberrant DNA methylation, alteration of the histone code and others, can lead to decreased gene expression, including miRNA. To investigate the effect of DNA methylation on miRNA-135A1 levels, we treated cervical HPV16-positive SiHa cells with the demethylating agent 5-aza-2'-deoxycytidine (DAC) and then evaluated the change in expression of miRNA-135a-3p and miRNA-135a-5p (fig. 4 *c*, *d*). The use of the SiHa cell line as a model in this experiment was due to the fact that the expression level of mature forms of miRNA-135a-5p and miRNA-135a-3p was significantly reduced in this cell line compared to another HPV16-positive CaSki cell line (data not shown). If the low level of miRNA-135A1 expression in the SiHa cell line is due to DNA hypermethylation, then treatment of the cells with the demethylating agent DAC would be expected to restore miRNA-135A1 expression. As shown in fig. 4, *c*, the expression level of miRNA-135a-3p did not change upon treatment of SiHa cells with DAC, whereas the expression of miRNA-135a-5p increased 2-3-fold (fig. 4, *d*); moreover, there is a direct correlation between the expression level of miRNA-135a-5p and the concentration of DAC demethylating agent. It should be emphasized that the results obtained are only indirect evidence that DNA methylation regulates the expression level of miRNA-135A1, since the treatment of cells with demethylating agents represents a non-specific epigenetic effect in which genome-wide demethylation occurs. Furthermore, in this case it is not possible to unambiguously assess the effect of general DNA demethylation on the expression of each of the three miRNA-135 genes, due to the high degree of homology of the 5p isoforms of miRNA-135 and the inability to selectively detect each of them. In addition, it is not possible to assess the effect of DNA methylation on miRNA-135A1 levels in clinical samples of tumor material, as there is no known region of the genome whose methylation uniquely regulates miRNA-135A1 expression. A more thorough search for genomic loci with CpG-containing regulatory sequences whose methylation level directly affects miRNA-135A1 expression is required in the future.

This suggests that miRNA-135A1 is a potential oncosuppressor miRNA in cervical cancer, with significantly reduced levels in tumor tissue compared to normal tissue. The suppression of miRNA-135A1 expression in a proportion of samples may be due to LOH of the miRNA-135A1 gene and may also depend on the level of DNA methylation. Importantly, both isoforms of this miRNA can suppress

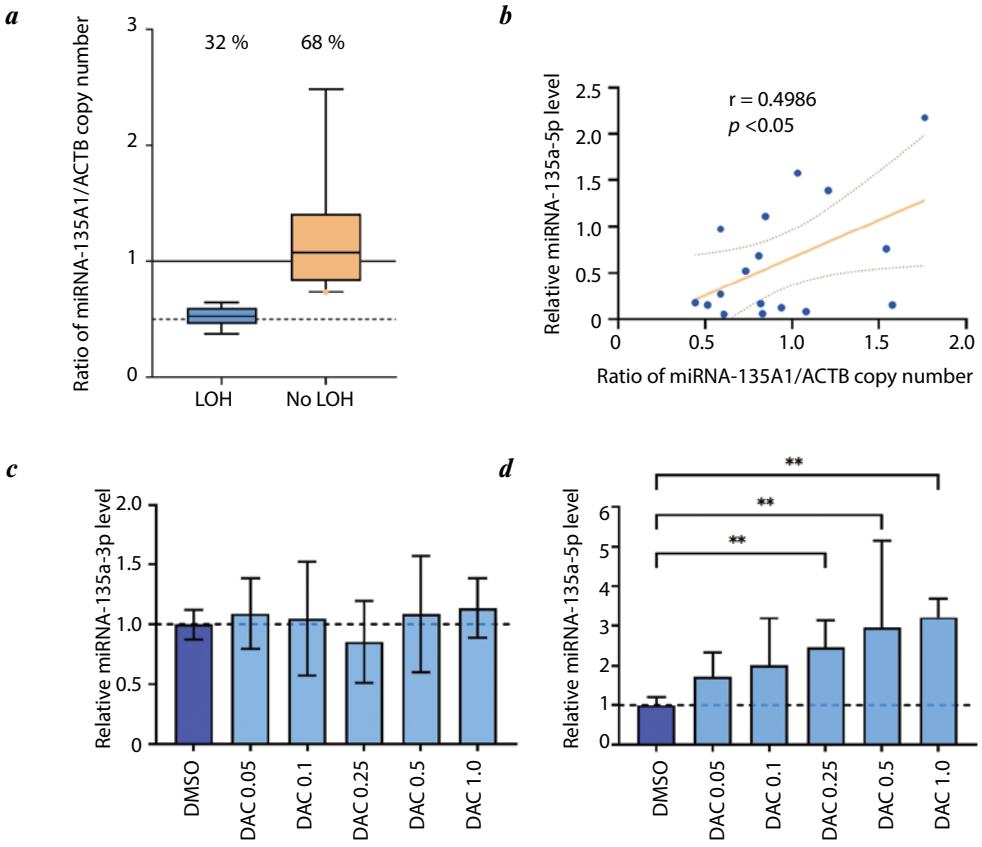


Fig. 4. *Analysis of the mechanisms involved in the suppression of miRNA-135A1 expression: a – distribution of squamous cell carcinoma samples according to the presence of loss of heterozygosity (LOH) in the MIR135A1 gene. The graph shows two groups of cell carcinoma – with LOH in the region of MIR135A1 gene (LOH) and without LOH of MIR135A1 gene (No LOH). The copy number ratio of MIR135A1 and β-actin (ACTB) genes is plotted on the y-axis. The number of samples falling into each group is given as a percentage; b – correlation analysis of the dependence of miRNA-135a-5p expression level on the copy number ratio of MIR135A1 and ACTB genes. Pearson correlation coefficient and p-value significance level are shown; c – diagram of the dependence of miRNA-135a-3p expression levels on the concentration of the demethylating agent DAC in SiHa cells; d – diagram of the dependence of miRNA-135a-5p expression levels on the concentration of the demethylating agent DAC in SiHa cells. The y-axis indicates the relative expression value of miRNA-135a-3p and miRNA-135a-5p, the x-axis indicates different concentrations of DAC (0.05 μM to 1 μM), DMSO is control samples treated with solvent without demethylating agent. **p <0.01*

the expression of the viral oncogene *E6*, thereby reducing the oncogenic effect of HPV16 in tumor cells.

MiRNA-135A1 levels in plasma of patients with HPV-associated cervical lesions. In the previous phase of our work, we showed that miRNA-135A1 is a potential oncosuppressor miRNA in CC, the levels of which are significantly reduced in tumor tissue compared to adjacent morphologically normal epithelium. It is therefore of great interest to evaluate its diagnostic potential in the plasma of patients with HPV-associated cervical lesions.

To this end, we evaluated the changes in miRNA-135A1 levels in the plasma of patients with CC and patients with cervical precancerous lesions compared to healthy donors. A total of 20 blood plasma samples from patients with CC, 21 blood plasma samples from patients with CIN of varying severity and 24 blood plasma samples from healthy donors were analyzed.

As shown in fig. 5, *a*, the level of miRNA-135a-3p is significantly reduced in the plasma of patients with CC compared to healthy donors, and the level of miRNA-135a-5p is not different in all compared groups (data not shown). A decrease in the level of miRNA-135a-3p in blood plasma is also observed in the group of patients with CIN, with the level of this miRNA being intermediate position between the group of CC patients and healthy donors (see fig. 5, *a*). This dependence of the severity of HPV-induced lesions on the degree of reduction of miRNA-135a-3p levels in the plasma of patients may suggest that its low plasma level may reflect the presence of a predisposition to the development of CC.

The absence of differences in miRNA-135a-5p levels in the groups studied against the background of pronounced changes in another mature form of miRNA-135a-3p may suggest that there is selection for certain forms of miRNA-135A1 during their secretion into the extracellular environment, including in the composition of extracellular vesicles, as has been shown for many miRNAs [29]. Such extracellular vesicles of dysplastic and tumor cells, whose composition is characterized by the directed secretion of miRNA-135a-3p, may form the main pool of this miRNA in plasma, changes in which are detected in patients with CIN and CC.

We further analyzed the change in plasma levels of miRNA-135a-3p in patients with CIN according to the severity of dysplastic changes in the cervical epithelium, comparing the groups of precancerous lesions of low severity (CIN I/II) and high severity (CIN3/*in situ*), 8 and 13 patients, respectively (fig. 5, *b*). In addition, we evaluated the dependence of plasma miRNA-135a-3p levels on the type of HPV found in the patients. For this purpose, all samples with CIN were divided into those associated with HPV16 – 15 patients, and those showing infection with other highly oncogenic HPV types (31, 33, 68, 56, 59) – 6 patients (fig. 5, *c*).

As shown in fig. 5, *b* and *c*, the decrease in plasma miRNA-135a-3p levels in patients with CIN is independent of factors such as the severity of cervical dysplasia and the type of papillomavirus infection detected in cervical smears. These data suggest that reduced levels of miRNA-135a-3p do not reflect the severity of cervical epithelial lesions and are not determined by infection with a particular type of HPV, but probably only demonstrate a general pattern that CIN and CC occur in a group of individuals with initially reduced levels of this miRNA.

We then evaluated the diagnostic potential of miRNA-135a-3p in the plasma of patients with CC and precancerous cervical lesions using ROC analysis (fig. 5, *d*).

As shown in fig. 5, *d*, miRNA-135a-3p has a high diagnostic potential in both patients with cervical squamous cell carcinoma (sensitivity 0.96; specificity 0.95; 95 % confidence interval 0.8–0.99; AUC 0.99) and CIN (sensitivity 0.83; specificity 0.81; 95 % confidence interval 0.64–0.93; AUC 0.87).

Comparing the results obtained by ROC analysis of miRNA-135a-3p levels in plasma compared to healthy donors with the diagnostic parameters obtained by using a combination of cervical smear cytology and HPV typing (AUC 0.837; sensitivity 0.886; specificity 0.656), it can be concluded that the proposed diagnostic test based on the determination of miRNA-135a-3p in blood plasma will have similar values of sensitivity and specificity compared to standard cytological methods [30].

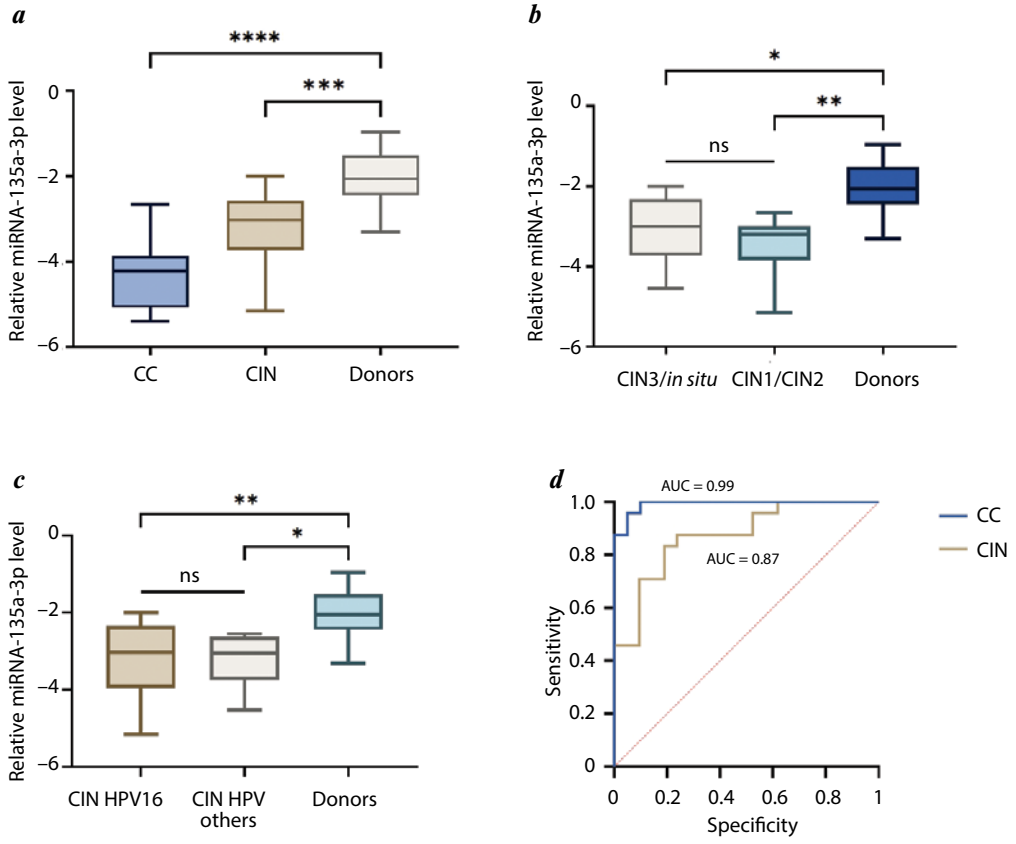


Fig. 5. *MiRNA-135A1 levels in plasma of patients with HPV-associated (HPV – human papillomavirus) cervical lesions: a – levels of miRNA-135a-3p in plasma of patients with cell carcinoma (CC), cervical intraepithelial neoplasia (CIN) compared to healthy donors. The negative difference of miRNA-135a-3p and miRNA-16-5p expression is plotted on the ordinate axis, which is the normalizer of the miRNA expression level in plasma; b – diagram of the dependence of miRNA-135a-3p levels in plasma of CIN patients and healthy donors on the severity of cervical epithelial lesions; ns – not significant; c – comparison of miRNA-135a-3p levels in plasma of CIN patients according to HPV type 16 (HPV16) status. HPV16 CIN group – patients have HPV16 infection of the cervical epithelium, HPV other CIN group – HPV other types are detected in the samples; ns – not significant; d – ROC curves reflecting the diagnostic significance of miRNA-135a-3p when comparing groups of patients with cell carcinoma, and CIN compared to conditionally healthy donors (p <0.0001). *p <0.05; **p <0.01; ***p <0.001; ****p <0.0001*

DISCUSSION

In conclusion, miRNA-135A1 is a potential oncosuppressor miRNA, capable of suppressing the expression of the viral oncogene *E6*, and its expression level is significantly reduced in cervical tumor tissue compared to the morphologically normal adjacent epithelium. In tumor tissue, the reduced expression of this miRNA in a proportion of samples can be explained by the loss of one of the copies of the gene encoding the miRNA-135A1 precursor and probably by the presence of aberrant DNA methylation.

Analysis of plasma miRNA-135A1 levels revealed low levels of miRNA-135a-3p in patients with cervical precancer and squamous cell carcinoma compared to healthy donors, consistent with suppression of expression of this miRNA in transformed cervical epithelium. Since changes in the miRNA expression profile in cervical neoplasia, where epithelial lesions are not as extensive as in invasive cervical cancer, are also reflected in plasma, we can infer a likely predisposition to progression of HPV-associated lesions in individuals with lower miRNA-135a-3p levels. These findings are consistent with the fact that a significant proportion of people infected with high-risk HPV clear the virus and that progression to invasive carcinoma occurs in a small number of patients [13, 31]. In addition, a decrease in the mRNA level of the HPV16 *E6* oncogene has been shown under the influence of miRNA-135A1, which may explain the relationship between miRNA-135A1 expression and progression to cervical neoplasia and invasive cancer.

Today, various serologic markers are mainly used for the minimally invasive diagnosis of CC, of which squamous cell carcinoma antigen (SCC-Ag) is the most widely used. However, the use of this marker is limited due to insufficient sensitivity to detect cervical tumor pathology at early stages [32]. Differentially expressed circulating miRNAs in blood plasma are currently being actively investigated for their potential use as diagnostic markers that may improve existing protocols for the diagnosis of cancer and precancerous lesions, particularly CC and CIN. For example, the levels of miRNA-7d-3p, -30d-5p, -143 and -4636 are reduced in the plasma of patients with CC and CIN and have a high diagnostic potential for these lesions [33, 34]. In addition to circulating miRNAs whose levels are reduced in CC, hyperexpressed plasma miRNAs (miRNA-26b-5p, -146b-5p, 191-5p, -484, -574-3p, and -625-3p) have also been identified, and their use in the diagnosis of CC and precancerous lesions seems promising [35].

MiRNA-135 exhibits diverse properties in the pathogenesis of tumor diseases. The oncosuppressive properties of miRNA-135 have been described in tumors such as prostate [36–38], kidney [39, 40], and glioblastoma [41, 42], whereas the oncogenic properties have been described in colorectal cancer [43, 44] and hepatocellular carcinoma [45, 46]. In addition, colorectal, lung and ovarian tumors are characterized by changes in miRNA-135 levels also in blood plasma, making this miRNA a potential diagnostic marker in these diseases [47–49]. However, there are currently no studies in CC that clearly characterize the role of miRNA-135 in tumor progression and its potential application in diagnosis.

CONCLUSION

The present study demonstrated that miRNA-135A1 is decreased in both cervical tumor tissue and plasma of patients with precancerous lesions and CC. Such changes in the level of miRNA-135a in blood plasma allow its use as a diagnostic marker and can serve as a basis for the development of new test systems. Diagnostic miRNA panels can significantly improve diagnosis, especially when cytologic (morphologic) results are unclear and serologic markers are negative. To assess the prognostic potential of this marker, further clinical studies with a significant follow-up period are required.

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Authors' contributions
D.S. Elkin: analysis of heterozygosity loss, assessment of the effect of DNA demethylation, cell culture cultivation, article writing, analysis of the obtained data;
M.I. Taubinskaya: miRNA extraction from blood plasma, qPCR, DNA extraction from cervical smears, HPV typing of cervical smears;
N.V. Elkina: HPV typing of CC tissue samples, primer design for miRNA analysis, transfection of cells with synthetic analogs of miRNAs, qPCR performance;
R.S. Faskhutdinov: transfection of cells with synthetic miRNA analogues, qPCR performance;
M.D. Fedorova: qPCR on miRNA matrix from FFPE sections;
A.N. Katargin: isolation of miRNA from FFPE sections;
K.I. Zhordania: idea, coordination of the work of the clinical unit;
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