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Viral microRNA in HPV16-associated cervical cancer: expression, diagnostic potential, and biological functions

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ABSTRACT

BACKGROUND: Cervical cancer (CC) is the fourth most common cancer among women worldwide in terms of incidence and mortality. High-risk human papillomaviruses (HPVs) are etiologic factor of CC in more than 90% of cases, with type 16 HPV (HPV16) revealed in >50% of cancers. Dysregulation of expression of viral oncogenes *E6* and *E7* is the main cause of malignant transformation in infected cervical epithelial cells. The mechanisms of impaired expression of these genes are still underexplored. The dysfunction of viral microRNAs may be among the underlying factors.

AIM: To analyze the expression of HPV16-associated microRNA-H1 and microRNA-H2 in cervical cancer specimens, evaluate the correlation of their expression to viral load and overall patient survival, and analyze *in silico* their potential viral and cellular targets.

MATERIALS AND METHODS: The expression of HPV16 microRNA-H1 and HPV16 microRNA-H2 was evaluated in the real-time polymerase chain reaction. With this purpose, small RNAs were isolated from 36 specimens of HPV16-positive squamous cell carcinomas of the cervix. Further, the viral load was assessed after calculating the value of HPV16 DNA copies per cell. The association between microRNA expression and the viral load was evaluated using the nonparametric Spearman's correlation coefficient. Kaplan-Meier curves were plotted to analyze the dependence of 5-year overall survival on the level of viral microRNA expression. The miRanda algorithm and online services mirDB, MR-microT and TargetScan Custom 5.2 were used for *in silico* search of theoretical microRNA targets.

RESULTS: MicroRNA-H1 expression was revealed in 33 of 38 specimens (86.8%), microRNA-H2 was detected in 37 of 38 specimens (97.4%) of HPV16-positive cervical cancer. There was a positive correlation between both microRNA-H1 (r=0.36, *p*=0.042) and microRNA-H2 (r=0.51, *p*=0.001) expression and HPV16 viral load. Higher level of expression of viral microRNA-H1 and microRNA-H2 tended to correlate with better overall patient survival. The theoretical microRNA-H1 (*E7*, *E2*, *E5*, *L2* and URR) and microRNA-H2 (*E1*, *E2*, *E5*, *L2*, *L1*, URR) targets in the HPV16 genome were identified *in silico*, as well as theoretical cellular targets indicating possible regulation of cellular signaling pathways by means of viral microRNAs, both controlling normal viral cycle and promoting tumor transformation.

CONCLUSION: The results of this study demonstrate promising further investigation of the functions of viral microRNAs in relation with the infectious process and virus-induced malignant transformation, and their potential importance in the diagnosis of HPV16-associated cancers.

Keywords: HPV16; epigenetics; viral microRNAs; HPV16 microRNA-H1; HPV16 microRNA-H2; cervical cancer.

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Вирусные микроРНК при HPV16-ассоциированном раке шейки матки: анализ экспрессии, диагностического потенциала и биологических функций

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АННОТАЦИЯ

Обоснование. Рак шейки матки (РШМ) — четвёртое по частоте встречаемости и смертности онкозаболевание среди женщин в мире. Вирусы папиллом человека (англ. human papillomaviruses, HPVs) высокого канцерогенного риска являются этиологическим фактором развития РШМ более чем в 90% случаев, при этом на долю HPV типа 16 (HPV16) приходится более 1/2 всех случаев. Дерегуляция экспрессии вирусных онкогенов *E6* и *E7* — основная причина онкотрансформации инфицированных клеток эпителия шейки матки. Механизмы нарушения их экспрессии до сих пор недостаточно изучены. Одной из таких причин может быть нарушение работы вирусных микроРНК.

Цель. Анализ экспрессии кодируемых HPV16 микроРНК-H1 и микроРНК-H2 в образцах РШМ, оценка корреляции их экспрессии с вирусной нагрузкой и общей выживаемостью пациентов, а также *in silico* анализ их потенциальных вирусных и клеточных мишеней.

Материалы и методы. Экспрессию HPV16-микроРНК-H1 и HPV16-микроРНК-H2 оценивали методом полимеразной цепной реакции в реальном времени, для этого выделяли фракцию малых РНК из 36 образцов HPV16-положительных плоскоклеточных карцином шейки матки. После этого определяли вирусную нагрузку, рассчитывая параметр «копии ДНК HPV16 на клетку». Зависимость экспрессии микроРНК от вирусной нагрузки оценивали с помощью непараметрического коэффициента корреляции Спирмена. Кривые Каплана–Майера строили для анализа зависимости 5-летней общей выживаемости от уровня экспрессии вирусных микроРНК. Для *in silico* поиска теоретических мишеней микроРНК использовали алгоритм miRanda и онлайн-сервисы mirDB, MR-microT и TargetScan Custom 5.2.

Результаты. Экспрессия микроРНК-H1 выявлена в 33 из 38 образцов (86,8%), а микроРНК-H2 детектировалась в 37 из 38 образцов (97,4%) HPV16-положительного РШМ. Получена положительная корреляция экспрессии как микроРНК-H1 (r=0,36, p=0,042), так и микроРНК-H2 (r=0,51, p=0,001) с вирусной нагрузкой HPV16. Прослеживается тенденция к лучшей общей выживаемости пациентов при более высокой экспрессии вирусных микроРНК-H1 и микроРНК-H2. *In silico* определены теоретические мишени в геноме HPV16 для микроРНК-H1 (*E7*, *E2*, *E5*, *L2* и URR) и микроРНК-H2 (*E1*, *E2*, *E5*, *L2*, *L1*, URR), а также теоретические клеточные мишени, указывающие на возможную регуляцию клеточных сигнальных путей с помощью вирусных микроРНК, — как поддерживающих нормальный вирусный цикл, так и способствующих опухолевой трансформации.

Заключение. Результаты исследования указывают на перспективность дальнейшего изучения функций вирусных микроРНК при инфекции и вирус-индуцированной онкотрансформации, и их потенциала для диагностики HPV16-ассоциированных онкопатологий.

Ключевые слова: HPV16; эпигенетика; вирусные микроРНК; HPV16-микроРНК-H1; HPV16-микроРНК-H2; рак шейки матки.

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BACKGROUND

According to the International Agency for Research on Cancer (IARC), approximately 12% of all human cancers (2 300 000 cases) are attributable to diseases associated with infectious agents, including bacteria, viruses, and parasites [1]. Among these diseases, oncogenic viruses account for approximately 65% (1 490 000 cases). To date, human oncogenic viruses include DNA-containing hepatitis B virus (HBV), high-risk human papillomaviruses (HR-HPVs), Epstein–Barr virus (EBV), Kaposi sarcoma-associated herpesvirus (KSHV), Merkel cell polyomavirus (MCPyV), and RNA-containing hepatitis C virus and human T-cell lymphotropic virus type 1 (HTLV-1). There are several strategies that oncogenic viruses use to maintain the normal viral cycle, which, in the presence of viral persistence and additional risk factors, promote malignant transformation of infected cells [2]. One of such strategies is to alter epigenetic mechanisms regulating cellular and viral gene expression [3]. Oncogenic viruses are able to modulate cellular epigenetic processes; moreover, they use their own tools, namely viral microRNAs, which are currently considered to be key infectious factors along with viral oncoproteins.

MicroRNAs are short RNA oligonucleotides of 17–25 bases in length; they are among the most significant epigenetic regulators of gene expression [4, 5]. Viral microRNAs, first discovered in EBV in 2004 [6], play an important role in the regulation of the viral cycle by modulating viral and cellular gene expression. According to the miRBase microRNA database [7], 320 microRNA precursors were annotated and experimentally confirmed for 34 different viruses, resulting in more than 500 mature forms of viral microRNAs. According to the miRBase database, three viruses such as EBV, KSHV, and MCPyV are considered oncogenic as they possess microRNAs. EBV encodes 44 mature microRNAs from 25 precursors, whereas KSHV encodes 25 mature microRNAs from 13 precursors, and MCPyV encodes 2 mature microRNAs from 1 precursor. In addition to these three viruses, there is experimental evidence for the functional microRNA in HBV, with confirmed biological functions in *in vitro* experiments [8]. Viral microRNAs were shown to regulate the expression of cellular and viral genes, as well as to modulate proliferation, apoptosis, migration, and immune response. These microRNAs are capable of maintaining both the normal viral cycle and causing disruption of cell signaling pathways associated with carcinogenesis, which contributes to the initiation of irreversible malignant transformations [9, 10].

HR-HPVs are responsible for more than 730 000 cases of cancers of various locations (31.7% of all cancers associated with infectious agents) [11]. Cervical cancer (CC) is the fourth most common cancer among women worldwide in terms of incidence and mortality [12]. Notably, CC is the most common type of cancer associated with HR-HPVs, accounting for 80% of all HPV-associated cancers, which totals to 570 000 cases [13]. HR-HPVs are an etiologic factor of cervical cancer (CC) in over 90% of cases, with HPV16 being the most common HPV type in CC, accounting for over 50% of all HPV-positive CC cases [11]. The key cause of malignant transformation is the dysregulation of expression of the major viral oncogenes *E6* and *E7*, which inhibit the function of the major cellular oncosuppressor proteins p53 and Rb. However, the mechanisms of such dysregulation are not yet fully understood. One potential reason for this may be the disruption of recently discovered HPV16 viral microRNAs.

Currently, nine microRNAs encoded by various types of HPV have been identified, including HPV6, HPV16, HPV38, HPV45, and HPV68 [14]. For HPV16, four microRNAs (HPV16-microRNA-H1, HPV16-microRNA-H3, HPV16-microRNA-H5, and HPV16-microRNA-H6) were experimentally validated [15]. However, these microRNAs exhibited remarkably low expression levels, as assessed in a limited and heterogeneous sample of cervical lesion specimens exhibiting varying degrees of infection with different types of HR-HPVs. A comprehensive analysis of HPV16-microRNAs on a wide and homogeneous sample of clinical specimens in terms of disease staging and HPV infection will allow revealing regularities in their expression pattern, associations with their viral load levels, and expression of viral oncogenes. This analysis will provide a more reliable approach to the search for potential biological targets of viral microRNAs. This, in turn, will expand the current knowledge about the regulation of the normal viral cycle and the exploration of new mechanisms of dysregulation of HPV16 viral oncogene expression and associated malignant transformations.

The aim of study was to analyze the expression of HPV16-encoded microRNA-H1 and microRNA-H2 in CC specimens, assess the correlation of their expression with viral load and overall patient survival, and analyze *in silico* their potential viral and cellular targets.

MATERIALS AND METHODS

Characteristics of cervical cancer specimens

A total of 36 postoperative specimens of HPV16-positive squamous cell carcinoma from patients treated at the Department of Gynecologic Oncology of the Research Institute of Clinical Oncology of the Blokhin Scientific Medical Research Center of Oncology between 2000 and 2006 (Table 1) were used for the analysis of viral microRNA-H1 and microRNA-H2 expression.

Description of research methodology

Cell lines

The HPV16-positive cervical cell lines SiHa and Ca Ski and the HPV-negative cell lines C33a (cervical line, American Type Culture Collection, USA) and HaCaT (spontaneously immortalized skin keratinocytes, CLS, Cell Line Service, DKFZ, Heidelberg, Germany) were used in the study. The cells were cultivated in the Dulbecco's Modified Eagle's Medium (#C410E, PanEco, Russia) spiked with antibiotics Pen-Strep Solution × 10 (#03-031-5B, Biological Industries Israel Beit Haemek, LTD, Israel) at final concentrations of 100 units/mL penicillin and 100 *μg*/mL streptomycin, and 10% fetal bovine serum (#S1810-500, Biowest, France). Cultivation was performed in N-BIOTEK ND-203 CO_2 incubator (Republic of Korea) at 37°C and 5% CO_2.

DNA extraction

DNA was isolated from fresh frozen tissues using the ExtractDNA Blood & Cells Kit (#BC111M, Eurogen, Russia) according to the manufacturer's instructions and stored at -20°C. The concentration was determined using a NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific, USA).

Determination of HPV16 viral load in cervical cancer specimens

A kit for the qualitative and quantitative detection and differentiation of high-risk HPV (HR-HPV) DNA was used to assess viral load. The kit, designated as AmpliSense® HR-HPV Genotype-Titer-FL (#H-2261-1-13, Central Research Institute of Epidemiology, Russia), employs real-time polymerase chain reaction (RT-PCR) for this purpose. A total of 50 ng of genomic DNA extracted from freshly frozen squamous cell carcinoma tissues were used as a matrix for RT-PCR. The PCR mixtures were prepared and RT-PCR was performed according to the manufacturer's instructions. RT-PCR was performed using a CFX96 Touch amplifier (BioRad Laboratories, Inc., USA). The calibration curves obtained by RT-PCR using the kit were utilized to calculate the number of HPV16 DNA copies and beta-globin copies. The viral load was calculated using the formula: (HPV16 DNA copy number) / ([β -globin copy number]×0.5). The resulting viral load value was thus expressed in units of HPV16 DNA copies per cell.

Table 1. Main characteristics of cervical squamous cell carcinoma clinical specimens used in the work

Characteristics	Values
Age, years	Me 42 [35–50]
Primary focus size, *n* (%)	T1: 27 (75%) T2: 9 (25%)
Metastases to the regional lymph nodes, *n* (%)	N0: 23 (64%) N1: 13 (36%)
Distant metastases, *n*	M0: 36 (100%)
Disease stage, *n* (%)	I: 15 (42%) II: 8 (22%) III: 13 (36%)
Five-year overall survival, *n* (%)	>5 лет: 16 (44%) <5 лет: 10 (28%) Withdrawn from the study: 10 (28%)
Total number of specimens, *n* (%)	36 (100%)

MicroRNA extraction

The small RNA fraction was isolated from fresh frozen squamous cell carcinoma tissue samples using the Ambion™ PureLink™ RNA Isolation Kit (#K157001, Invitrogen™, USA) and TRIzol™ Reagent (#15596018, Invitrogen™, USA). Tissue homogenization and lysis were first performed using TRIzol™ Reagent to improve the quality of microRNAs. Lysates were processed according to the established protocol to yield an aqueous phase, which was subsequently purified from DNA and RNA impurities using the PureLink™ microRNA Isolation Kit in accordance with the manufacturer's guidelines. The isolated microRNA was stored at -70°C. The concentration of microRNA was subsequently quantified using the Qubit™ 2.0 fluorimeter (Invitrogen™, USA) and the Qubit™ microRNA Assay Kit (#Q32881, Invitrogen™, USA).

Reverse transcription and RT-PCR of viral microRNA-H1 and microRNA-H2

The reverse transcription (RT) was first performed to assess the expression level of viral microRNA-H1 and microRNA-H2 using the stem-loop technique described by Chen et al. [16]. Primers were designed for RT followed by real-time amplification with TaqMan probes. The sequences of these probes are available upon request.

The Dialat reagent kit (Dialat Ltd., Russia) was used for RT. The final reaction mixture of 25 μL contained 25 ng of purified microRNA without DNA/RNA impurities, 1 pmol of RT primers for microRNA-H1 or microRNA-H2, 1 pmol of an RT primer for U6, 50 units of reverse transcriptase (#RT-10), and a single RT buffer (#RTM-100). The RT reaction was performed under the following conditions: 25°C for 10 min, 42°C for 30 min, and 85°C for 5 min. The resulting cDNA was stored at -20°C.

RT-PCR was performed using a 5X qPCRmix-HS ready-to-use mixture (#PK145L, Eurogen, Russia). The final 20 μL reaction mixture for viral microRNA amplification contained a single qPCRmix-HS mixture, 2 pmol forward (microRNA-specific) primer, 1 pmol reverse (universal) primer, 0.2 pmol

Taqman probe, and 1 µL RT-derived cDNA. The RT-PCR mode for microRNA-H1 and microRNA-H2 detection was as follows: 95°C for 5 min; 45 cycles including the steps of 95°C for 15 s, 52°C for 1 min, and signal detection. The final reaction mixture for the amplification of the internal control and reference gene for the assessment of relative microRNA expression—small nuclear 20 µL RNA U6—contained a single qPCR-mix-HS mixture, 1 pmol forward and reverse primers, 0.2 pmol TaqMan probe, and 1 µL of 10-fold diluted RT-derived cDNA. The RT-PCR mode for U6 detection consisted of the following steps: 95°C for 5 min, 35 cycles including the steps of 95° for 15 s, 60°C for 1 min, and signal detection. The CFX96 Touch amplifier (BioRad Laboratories, Inc., USA) was used for RT-PCR.

Bioinformatic search for microRNA-H1 and microRNA-H2 targets

The miRanda algorithm [17] was utilized to identify potential interaction sites of viral microRNA-H1 and microRNA-H2 within the HPV16 genome (GenBank: AF125673.1). The following inclusion criteria were met by sites to be deemed significant: a threshold score of at least 120 and a negative interaction energy (kcal/mol) of no more than –10. The algorithm was executed using the SRplot online platform [18].

A search for potential cellular targets of viral microRNA-H1 and microRNA-H2 was conducted using three online services: mirDB [19, 20], TargetScan Custom 5.2 [21], and MR-microT [22, 23]. These services utilize distinct algorithms to identify interaction sites with target sequences, thereby facilitating a more reliable identification of theoretical target genes. The identification of significant target genes for mirDB was determined by a threshold score of ⩾50, whereas for MR-microT, it was ⩾0.5. For TargetScan Custom 5.2, thresholds were determined by the algorithm. The resulting targets were then compared with each other using Venn Diagramm (https://bioinformatics.psb.ugent.be/webtools/Venn/). The target genes identified by at least two services were subsequently analyzed for category enrichment using the Metascape online service (v3.5.20240901) [24] with standard settings. This analysis yielded the 20 most significant categories with a logP-value <-5, which were identified as being theoretically regulated by microRNA-H1 and microRNA-H2. The Venn unions and category enrichments were then visualized using SRplot [18].

Ethical review

The study protocol was approved by the Local Ethical Committee of the Blokhin National Medical Research Center of Oncology (Protocol No. 7 dated July 25, 2024).

Data analysis and statistical processing

The RT-PCR results were then subjected to evaluation, and the number of HPV16 DNA copies per cell was calculated using Microsoft Excel 2016 to analyze viral load. Subsequently, the expression level of viral microRNA-H1 and microRNA-H2 was determined by Ct method ΔΔCt [25], calculating the value of –ΔΔCt for each sample using Microsoft Excel 2016 by the formula: –([Ct microRNA]–[Ct U6]). A thermal map was generated to illustrate the relationship between viral microRNA expression and viral load, employing the SRplot online program [18]. The correlation between viral microRNA-H1 and microRNA-H2 expression and viral load were analyzed using the nonparametric Spearman's correlation coefficient in the GraphPad Prism 8.4.3 (GraphPad Software, LLC, USA) program. The correlation was considered significant at p <0.05. The groups of patients with low and high expression levels of microRNA-H1 and microRNA-H2 were determined using the R software package, which allows the identification of thresholds for the separation of different data sets using the MaxSpSe parameter [26]. Kaplan–Meier plots for 5-year overall survival were constructed and analyzed using the SRplot online service [18]. Differences in survival curves were considered significant at p <0.05.

RESULTS

Primary findings

Analysis of viral microRNA-H1 and microRNA-H2 expression in HPV16-positive cervical cancer tissues

According to the literature, the expression of five viral microRNAs, including HPV16-microRNA-H1, -H2, -H3, -H5, and -H6, was demonstrated using high-throughput next-generation sequencing (NGS) for HPV16. These microRNAs were encoded by different regions of the HPV16 genome. Specifically, the *E1* gene was identified as the source of microRNA-H1 and microRNA-H5, whereas the *L1* gene was identified as the source of microRNA-H6. The untranslated regulatory region (URR) was identified as the source of microRNA-H2 and microRNA-H3. The authors further validated the results of NGS on viral microRNA expression by using RT-PCR and *in situ* hybridization methods [14, 15]. For this purpose, a variety of clinical materials were utilized, including approximately 60 samples of cervical lesions of varying degrees of severity, including cervical intraepithelial neoplasia grades 1–3, cervical squamous cell carcinoma and adenocarcinoma, as well as normal smears. The clinical samples exhibited significant variation in terms of sample preparation, encompassing both formalin-fixed and paraffin-embedded samples and cervical smears collected using various transport media. The presence of both HPV16-positive and non-HPV16-positive samples resulted in a high degree of heterogeneity among the samples. Consequently, the analysis revealed the expression of only four viral microRNAs in a limited number of samples (up to 20% for

different HPV16-microRNAs), with an exceptionally low level of expression.

Nevertheless, the analysis of HPV-encoded microRNAs remains highly relevant and requires further investigation. In this study, the expression of two microRNAs, HPV16 microRNA-H1 and HPV16 microRNA-H2 (hereafter referred to as microRNA-H1 and microRNA-H2), which have the highest number of readings according to NGS results [14], was analyzed. A homogeneous sample of 36 fresh frozen HPV16-positive cervical squamous cell carcinoma tissue samples was selected to evaluate their expression. Furthermore, a detection system for microRNA-H1 and microRNA-H2 based on the stem-loop RT-PCR method described by Chen et al. was developed [16], using locked nucleic acid TaqMan probes to increase the amplification specificity. The analysis of viral microRNA expression in HPV16-positive cell lines revealed the expression of only microRNA-H2 in SiHa, whereas the expression of both microRNA-H1 and microRNA-H2 was detected in Ca Ski. The absence of microRNA-H1 expression in SiHa was associated with the loss of the *E1* gene coding region as a result of HPV16 integration into the cell DNA. In addition, HPV-negative cell lines C33a and HaCaT were analyzed to assess the specificity of the developed method. The absence of microRNA-H1 and microRNA-H2 expression in these cell lines during RT-PCR further substantiates the high specificity of the developed systems for detecting viral microRNAs.

In the analysis of microRNA-H1 and microRNA-H2 expression in clinical materials, HPV16-positive cervical carcinoma samples were conditionally divided into three groups based on their HPV16 DNA load: low (0–1 copy per cell), medium (1–10 copies per cell), and high (10–450 copies per cell). This division was conducted to ascertain the correlation between viral load and microRNA levels. The presence of microRNA-H1 was identified in 33 of the 38 (86.8%) samples, with a median ΔCt value of -18.58 (-19.59; -17.44). Conversely, microRNA-H2 expression was detected in 37 of 38 (97.4%) samples, with a median ΔCt value of -17.93 (–18.86; –16.02). A schematic representation of microRNA expression levels and their correlation with viral load is shown as a thermal map in Fig. 1.

Therefore, the presence of two viral microRNAs in more than 85% of CC specimens was shown to be significantly superior to the results of Auvinen et al. [15]. This high percentage of positive samples of invasive cervical carcinomas suggests a possible role of viral microRNAs in oncogenesis and the further need for their additional study in other cervical lesions, as well as a more detailed analysis of their biological functions.

Evaluation of the correlation of viral microRNA-H1 and microRNA-H2 expression with HPV16 viral load and overall survival rates

In the majority of cervical lesions, the viral load of HR-HPV is known to be positively correlated with more severe precancerous lesions, the severity of the CC course, and lower patient survival [27, 28]. In addition, the expression of early viral genes, particularly the oncogenes *E6* and *E7*, is positively correlated with viral load [29, 30]. Given the capacity of viral microRNAs to modulate both viral gene expression and various cellular processes, an analysis was conducted to ascertain the dependence of microRNA-H1 and microRNA-H2 expression on HPV16 viral load. The results demonstrated a positive correlation between the expression levels of both microRNA-H1 (r=0.36, p=0.04) (Fig. 2a) and microRNA-H2 (r=0.51, p=0.001) (Fig. 2b) and the HPV16 viral load. This finding suggests a potential indirect indication of their involvement in the regulation of viral gene expression.

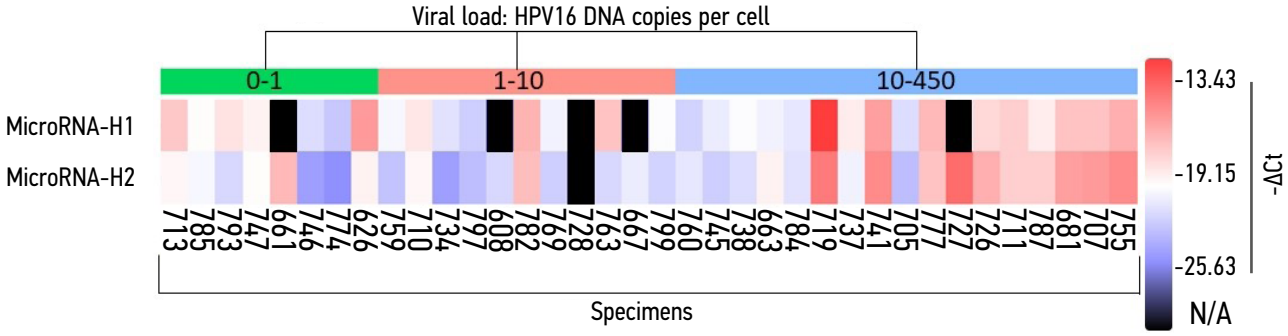

Fig. 1. Heat map of viral microRNA-H1 and microRNA-H2 expression in clinical samples of HPV16-positive squamous cell carcinoma. The microRNA level is represented by a color scheme from red (maximum expression level) to blue (minimum expression level), black rectangles indicate samples without amplification of the corresponding microRNA (N/A). Samples in the scheme are grouped according to the viral load of HPV16 DNA: 0–1 — samples with low viral load; 1–10 — samples with medium viral load; 10–450 — samples with high viral load. The lower part shows the labels of the CC samples.

The present study sought to evaluate the association of viral microRNA expression with overall survival of patients with CC. The R programming environment package was utilized to define thresholds to separate different data sets, with samples thus divided into two groups: those with low and high expression of microRNA-H1 (threshold value -ΔCt=-18.41) and microRNA-H2 (threshold value -ΔCt=-17.56). The 5-year overall survival curves were subsequently delineated (Fig. 3).

There is no statistically significant difference between the survival rates of patients exhibiting high and low expression levels of microRNA-H1 and microRNA-H2. However, a tendency toward enhanced survival was observed in patients with elevated viral microRNA expression. This observation, together with viral load, may serve as a prognostic indicator for disease progression. Consequently, further investigation is necessary to thoroughly examine HPV16 viral microRNA expression and its correlation with various clinical parameters.

Bioinformatic analysis of viral microRNA-H1 and microRNA-H2 functions

The expression of viral microRNA-H1 and microRNA-H2 in more than 85% of the examined samples indicates a potential role of these microRNAs in the regulation of cellular and viral gene expression, modulating various pathways of carcinogenesis. The potential viral and cellular targets of microRNA-H1 and microRNA-H2 were bioinformatically searched to elucidate the possible biological functions of viral microRNAs. *In silico* analysis revealed a variety of interaction sites of viral microRNAs with early and late genes and the non-coding region of HPV16. Therefore, ten potential interaction sites were identified for microRNA-H1, including sites within the *E7*, *E2*/*E4*, *E5*, and

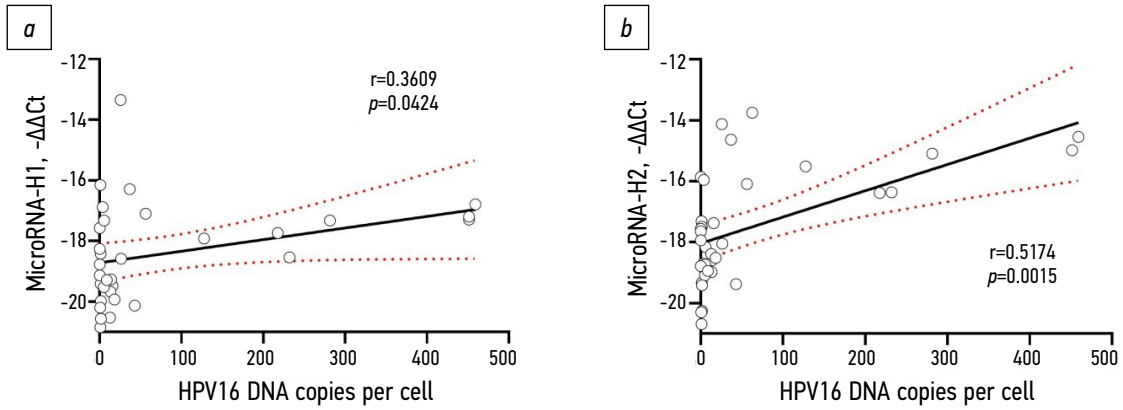

Fig. 2. Correlation analysis of viral microRNA-H1 (*a*) and microRNA-H2 (*b*) expression from HPV16 viral load. r — correlation coefficient, *p* — p-value.

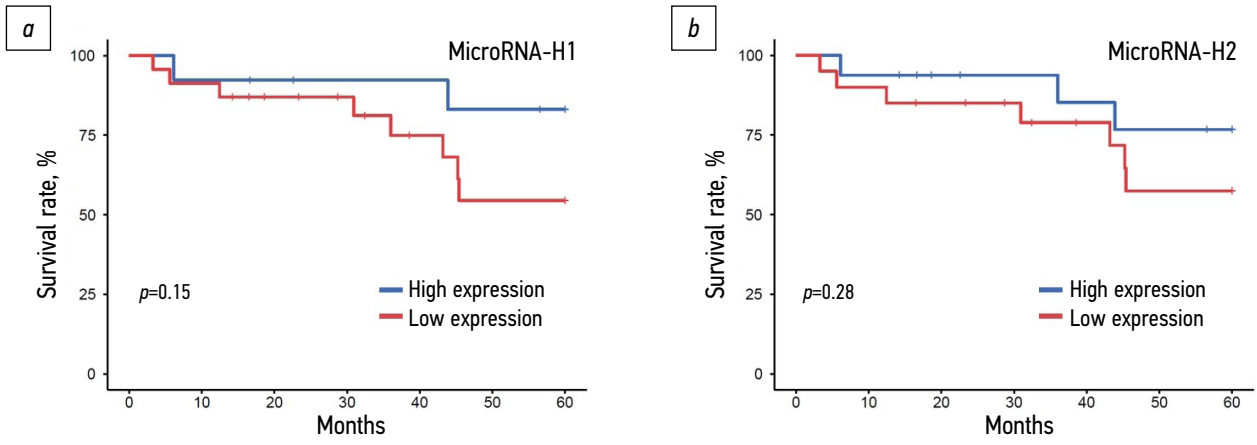

Fig. 3. Kaplan–Meier curves of the 5-year overall survival of patients with squamous cell carcinoma depending on the expression level of microRNA-H1 (*a*) and microRNA-H2 (*b*), *p* — значение p-value.

L2 readings and in the URR. For microRNA-H2, 17 potential interaction sites were recorded, including sites within the *E1*, *E2*, *E2*/*E4*, *E5*, *L1*, and *L2* gene readings and in the URR. *In vitro* and *in vivo* experiments are necessary to confirm the interaction of viral microRNAs with early and late transcripts and with the non-coding region of HPV16. These experiments will allow for the assessment of their influence on the regulation of the normal viral cycle and participation in the dysregulation of viral oncogene expression, initiating malignant transformations.

In addition, an *in silico* search was conducted for potential cellular targets of viral microRNA-H1 and microRNA-H2. Three resources were utilized: mirDB, TargetScan Custom 5.2, and MR-microT, which employ distinct algorithms to identify potential cellular targets of microRNAs. Consequently, numerous theoretical targets were obtained, which were subsequently analyzed for common genes to enhance their significance. Genes identified by at least two algorithms were then used to further explore category enrichment (Fig. 4a; 4b), resulting in a total of 426 potential targets for microRNA-H1 and 518 for microRNA-H2.

When assessing category enrichment, various cellular processes associated with carcinogenesis were analyzed (Fig. 4c; 4d).

For microRNA-H1, these include categories such as response to growth stimuli, factors and pathways affecting insulin-like growth factor 1 and Akt-signaling pathways, cascades regulating stem cell pluripotency, negative regulation of cell proliferation, and the Rho GTPase cycle. MicroRNA-H2 includes processes such as protein phosphorylation, methyl-CpG-binding protein 2 transcriptional regulation, actin filament-related processes, TP53 transcriptional regulation, growth factor response, and nuclear receptor signaling pathways. The presence of potential targets of viral microRNAs associated with these biological processes suggests their possible regulatory function aimed at maintaining both the normal viral cycle and tumor transformation processes.

The results of bioinformatic analysis of potential viral and cellular targets of microRNA-H1 and microRNA-H2 suggest the need for a more detailed analysis of their biological functions *in vitro* and *in vivo* to identify their regulatory role in the HPV16 life cycle and in malignant transformations.

DISCUSSION

Viral microRNAs are one of the important tools of oncoviruses to maintain the normal viral cycle by modulating various cellular processes such as cell proliferation, differentiation, and survival. On the one hand, viral microRNAs are able to maintain the normal viral

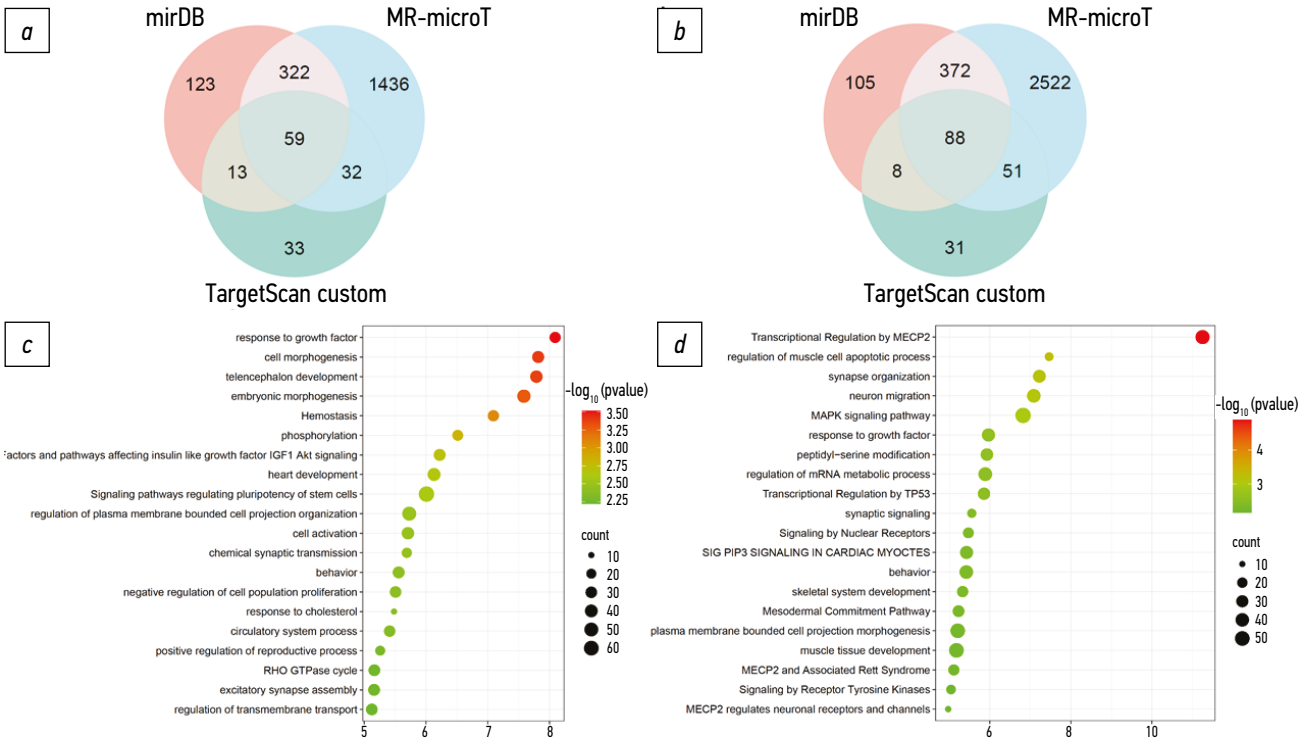

Fig. 4. Results of bioinformatic search for potential cellular target genes of viral microRNA-H1 and microRNA-H2, *a*, *b* — Venn diagrams of target association for microRNA-H1 (*a*) and microRNA-H2 (*b*), *c*, *d* — Top 20 enriched categories for microRNA-H1 (*c*) and microRNA-H2 (*d*) target genes, ranked by significance level (-log10 *p*-value).

cycle by regulating viral gene expression. For example, KSHV microRNA-K12-7, by inhibiting the expression of the RTA protein of KSHV, reduces the expression of early viral proteins and contributes to the maintenance of latent viral infection [31]. MCPyV microRNA-M1 inhibits the expression of the large T antigen, thereby reducing viral replication and maintaining a constant level of MCPyV DNA in infected cells, contributing to the establishment of persistent infection [32, 33]. On the other hand, in virus-associated cancers, many viral microRNAs fulfill the functions of oncogenic microRNAs by altering the expression of key genes that regulate pathways associated with carcinogenesis. For example, EBV microRNA-BART1 was shown to inhibit PTEN expression in nasopharyngeal carcinoma, thereby stimulating cellular processes involved in migration, invasion, and metastasis [29]. Similarly, KSHV microRNA-K12-1-5p was observed to inhibit SOCS6 expression in Kaposi sarcoma cells, inhibiting apoptosis and promoting cell proliferation, migration, and invasion [34]. The study of recently discovered HPV16 viral microRNAs is of great scientific and practical interest due to the integral role of viral microRNAs in the regulation of various biological processes. This study focused on two viral microRNAs, HPV16 microRNA-H1 and HPV16 microRNA-H2 [15].

The results of the experiments demonstrated that viral microRNA-H1 was expressed in 33 out of 38 samples (86.8%) of HPV16-positive cervical squamous cell carcinoma, whereas microRNA-H2 was expressed in 37 out of 38 samples (97.4%). These findings suggest that viral microRNAs may play a regulatory role in various signaling pathways associated with malignant transformations. The absence of microRNA-H1 expression in certain samples may be associated with the status of the HPV16 genome within the cell. During the prolonged persistence of the virus, there is a possibility for integration of the HPV16 genome into the host cell chromosomes. This integration occurs with the breakage of the virus's DNA, specifically in the region of the *E1/E2* genes and the late *L1* gene. This process is accompanied by the loss of a portion of the virus genome, particularly the region of the *E1* gene responsible for the expression of microRNA-H1. The loss of viral microRNA expression due to such integration has the potential to contribute to the dysregulation of viral oncogene expression. The findings reveal a positive correlation between microRNA-H1 and microRNA-H2 expression and viral load, as well as the identification of theoretical interaction sites in oncogene *E7* and early genes *E1* and *E2*. These genes are the primary regulators of viral replication and transcription. The results suggest a potential role for microRNAs in regulating viral gene expression during the normal viral cycle and malignant transformations. Furthermore, the theoretical cellular targets of viral microRNAs predicted *in silico* suggest the potential for regulation of viral and various cellular signaling pathways, including cell growth and differentiation processes.

Further study of microRNA-H1 and microRNA-H2, as well as a more detailed analysis of other HPV16 microRNAs, will allow for further elucidation of the mechanisms underlying viral cycle regulation and their potential involvement in the dysregulation of viral oncogenes *E6* and *E7*, which is the primary trigger mechanism of malignant transformation of infected cells.

CONCLUSION

The study demonstrated the expression of two viral microRNAs, HPV16 microRNA-H1 and HPV16 microRNA-H2, in the majority of cervical squamous cell carcinoma specimens. A positive correlation of microRNA-H1 and microRNA-H2 expression with HPV16 viral load was shown for the first time, and a trend toward better survival was identified in patients with higher levels of viral microRNAs. *In silico* analysis revealed numerous potential sites of interaction between microRNAs and the HPV16 genome and cellular genes, suggesting a probable regulation of their expression through viral microRNAs. This regulation may be responsible for the increased expression of the viral oncogenes *E6* and *E7* and the dysregulation of cellular genes involved in various signaling cascades, including oncogenic ones. Further study of viral microRNAs will allow for the complementation of existing mechanisms of malignant transformation during HPV16 persistence, as well as for the evaluation of their potential in the diagnosis of HPV16-associated oncopathologies.

ADDITIONAL INFORMATION

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Authors' contribution. N.V. Elkina — experiments on estimation of microRNA expression levels, analysis of their correlation with viral load and overall survival, article writing; M.D. Fedorova — DNA isolation, HPV typing and estimation of viral load in cervical carcinoma samples; R.S. Faskhutdinov — bioinformatic search of viral and cellular targets of microRNA-H1 and microRNA-H2; Yu.O. Iurchenko — isolation of small RNAs; K.I. Zhordania — coordination of the clinical unit for material collection; E.A. Mustafina — tissue collection of cervical squamous cell carcinoma; L.S. Pavlova — selection of clinical material samples for analysis; S.V. Vinokurova — idea, research design development, project management, scientific editing.

Compliance with the principles of ethics. The study protocol was approved by the local ethics committee (Blokhin National Medical Research Center of Oncology, protokol N 7, 25.07.2024). Approval and protocol procedure was obtained according to the principles of the Declaration of Helsinki.

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