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Profiling miRNAs in Exosomes for the Development of a Diagnostic Panel for Melanoma Metastasis in Melanocyte and Melanoma Cell Line Models*

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Профилирование miRNAs в аспекте разработки диагностикой панели метастазирования меланомы кожи в модели клеточных линий меланомы

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Introduction. One possible mechanism of metastasis underlying melanoma oncogenesis involves changes in the expression profile of miRNAs. The expression pattern of miRNAs in exosomes of melanoma cell lines can be evaluated as potential biological markers for melanoma metastasis stages.

Aim. In cutaneous melanoma cell lines to determine the profile of miRNAs as potential biomarkers of melanoma metastatic stage using a panel of five miRNAs (miR-21-5p, miR-149-3p, miR-150-5p, miR-155-5p and miR-193a-5p).

Materials and Methods. The expression level of miRNAs was analysed in a skin melanoma cell line using real-time qRT-PCR. The relative expression level was calculated using the ΔΔCt method with determination of the relative quantification (RQ) compared to the exogenous control. Parameters were determined depending on the passage of cultivation.

Results. Analysis of the miRNA profile of a superficial melanoma cell line as a function of cultivation time showed that the expression of miR-149-3p, miR-21-5p increased in earlier stages of cultivation and that miR-155-5p, followed by miR-150-5p, miR-193-5p, that were the main miRNAs associated with melanoma progression in later stages.

Conclusion. The analysed profile of miRNAs of a superficial spreading skin melanoma cell line as associated with melanoma progression can be proposed as diagnostic and/or prognostic biomarkers and can also be considered as potential therapeutic targets.

Keywords: melanoma; miRNA; melanocytes in vitro; diagnosis; qRT-PCR; metastasis

Введение. Один из возможных механизмов метастазирования, лежащий в основе онкогенеза меланомы представляет собой изменение экспрессии профиля miRNAs. Характер экспрессии miRNAs в клеточных линиях меланоцитов меланомы оцениваются как потенциальные биологические маркеры этапов метастазирования меланомы.

Цель. Определить в клеточных линиях меланомы кожи профиль miRNAs как потенциальных биомаркеров этапов метастазирования меланомы, используя панель из пяти miRNAs (miR-21-5p - miR-149-3p - miR-150-5p - miR-155-5p - miR-193a-5p).

Материалы и методы. В клеточной линии меланомы кожи методом qRT-ПЦР в режиме реального времени анализировали уровень экспрессии miRNAs, проводили расчет относительного уровня по методу ΔΔCt с определением относительной концентрация (RQ) относительно экзогенного контроля. Параметры определялись в зависимости от пассажа культивирования.

Результаты. Анализируемый профиль miRNAs клеточной линии поверхностно распространяющейся меланомы кожи, в зависимости от сроков культивирования выявил, что на более ранних сроках культивирования повышается экспрессия miR-149-3p, miR-21-5p и на более поздних в первую очередь miR-155-5p, далее miR-150-5p, miR-193-5p, как ассоциированные с прогрессированием меланомы.

Выводы. Анализируемый профиль miRNAs клеточной линии поверхностно распространяющейся меланомы кожи как ассоциированные с прогрессированием меланомы могут быть предложены в качестве диагностических и/или прогностических биомаркеров, а также могут рассматриваться в качестве потенциальных терапевтических мишеней.

Ключевые слова: меланома; miRNA; меланоциты in vitro; диагностика; qRT-ПЦР; метастазирование

* The full text of the article in Russian can be found on the issue's website
Полный текст статьи на русском языке доступен по ссылке https://voprosyonkologii.ru/index.php/journal/article/view/4-24-Comparative/2032

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Introduction

Skin melanoma is clinically diverse, the most aggressive and molecularly heterogeneous disease, characterized by early metastasis and its incidence is increasing worldwide. The 10-year survival rates from stage IA to IV range from 95 % to 10–15 %, respectively [1–3].

Skin melanoma is more common in the geriatric population, with a median age at diagnosis of 65 years, and is also diagnosed in adolescents and young adults between the ages of 25 and 50 years [4]. The global incidence by sex is 3.5 % in men and 2.9 % in women, with a reverse trend after the age of 40 years. Molecular alterations are characterised by hyperactivation of the PI3K/AKT and MAPK pathways, inactivation of p53 and alterations of the CDK4/CDKN2A axis [5]. UV radiation, melanocytic and dysplastic nevi are two major risk factors for skin melanoma.

One of the possible mechanisms underlying melanoma oncogenesis is the alteration of miRNA profile expression, including under the influence of UV radiation [6–8]. Present in exosomes, miRNAs are involved in intercellular cyto-communication, modulate the characteristics of melanoma neighbouring cells or directly affect the tumour niche by modifying the extracellular matrix [5, 9]. Individual miRNAs allow the differentiation of melanoma into metastatic and non-metastatic melanoma, while manifesting as oncogenes or tumor suppressors with key functions in carcinogenesis and cancer progression [7, 8, 10]. Studies indicate the existence of a unique specific expression profile of miRNAs in different tumor types and at different stages of tumor development [8]. The expression pattern of miRNAs in melanoma cell lines and clinical samples was evaluated as a biological marker for early stage melanoma in 2006 [11].

miRNAs, 22-26 nucleotides in size, belong to a class of non-coding RNAs that can regulate gene expression by recognising and specifically binding to complementary sequences in the 3‘-untranslated region (3’-UTR) of miRNAs and subsequently leading to degradation and repression of translation of the target transcript and proteins. Less frequently, they interact with the 5’UTR of the coding or promoter region [6, 7, 12]. Exosome miRNAs act as regulators of cellular processes by simultaneously modulating the expression levels of hundreds of miRNAs, thereby exhibiting pleiotropic modulation of a wide range of biological processes, including proliferation, differentiation, apoptosis and development. The ability of miRNAs to inhibit multiple targets simultaneously is being considered as a new pharmacological strategy for cancer treatment [7].

Exosomes are membrane nano-vesicles that are secreted by most cell types, including tumor cells. The biochemical composition of exosomes including miRNAs appears to be the ‘molecular profile’ of the cells secreting them. Exosomes of tumor origin can be considered as natural and biologically stable complexes of molecular tumor markers [13]. In particular, the role of the miRNA miR-193a-3p in cutaneous melanoma has only been partially investigated [7]. During RNA biogenesis, two mature miRNAs are formed from pre-miR-193a: miR-193a-3p and miR-193a-5p. Both are expressed in all tissues under physiological conditions [13] and their regulation is disrupted in various types of cancer, where they manifest as tumor suppressors or oncogenes. miRNA miR-21-5p is involved in the regulation of PTEN, PDCD4, BTG2 levels and is associated with various pathological conditions, including neo-oncogenesis [14–16]. High level of miR-21-5p expression is a negative predictor of survival in various forms of cancer [12]. An important role of miR-21-5p in cell proliferation, tumor progression [17], and functionally increases the invasiveness of melanoma cells [18] has been noted. miR-155 play an important role in various physiological and pathological processes, in the control of angiogenesis in melanoma [19, 20] inhibiting glycolysis and ATP formation [5, 9]. The p53-sensitive miR-149-3p plays a major role in the process of cell migration [21].

In vitro 3D models of melanoma melanocytes allow us to study tumor oncogenesis in a microenvironment closer to that in vivo. Analysis of melanoma melanocytes provides an opportunity to directly analyze the miRNA spectrum directly isolated from tumors and thus simulate different stages of the metastatic process. The data obtained will optimize the development of therapies targeting specific stages of metastasis to improve clinical response rates and overall survival of patients. The miRNA panel analyzed in this work is considered as potential diagnostic markers or targets for future

therapies. Understanding the important role of miRNAs in melanoma progression will facilitate the development of targeted therapy using miRNAs that interact with the most important regulatory pathways of melanoma development and progression — MAPK/ERK and PI3K/PTEN/Akt [22, 23].

Therefore, the aim of our work was to determine the profile of exosome miRNAs as potential biomarkers of melanoma metastasis stages in melanoma melanocyte cell lines using a panel of five miRNAs: miR-21-5p, miR-149-3p, miR-150-5p, miR-155-5p, miR-193a-5p.

Materials and Methods

Comparative analyses were performed in an in vitro model. The biological material was human skin melanoma cell lines obtained from six patients with superficial spreading melanoma (SSM). Primary culture was obtained using a 2.0 × 5.0 cm skin flap explant method with the informed consent of each patient during routine surgery (VM-Clinic Multidisciplinary Hospital, Ulyanovsk). All works were performed according to the Freshney's protocol [24]. To obtain primary culture the explant was mechanically and chemically dispersed, then the cell suspension was placed in Petri dishes (TRR, Switzerland) and 25 cm^2 vials (TRR, Switzerland) with nutrient medium (RPMI-1640 (Paneco, Russia) with 10 % calf embryo serum (NuClone, USA), gentamicin (Paneco, Russia). Incubation was performed in a CB-53 multi-gas incubator (BINDER GmbH, Germany), at +37 °C, CO_2 5 %. Cell sedimentation was performed on an Allegra X-30R centrifuge (Beckman Coulter, Germany). For subculturing, monolayer cells were transferred into suspension with trypsin-EDTA solution (PanEco) and dispersed into new vials and Petri dishes at a concentration of 200 ths/ml per 1 vial/Petri dish. Cell counting was performed using an automatic cell counter (BioRad, USA) with preliminary staining with trypan blue. The growth of cell lines was monitored using an inverted fluorescence microscope 'Axio Vert. A1 FL' with digital color video camera Axiocam 105 (Carl Zeiss, Germany). Cell lines were obtained from six patients with a confirmed diagnosis of superficial spreading melanoma (SSM). The cell lines of melanocytes from pigmented nevus served as controls.

For a single PCR assay, the required number of cells is 104–10^6/1 ml. The mirVana miRNA Isolation Kit (Ambion/Thermo Scientific, Germany) was used to isolate total cell culture miRNA. For this purpose, melanocyte cell lines were pre-eluted from the vial with 0.25 % trypsin-Versen solution, washed with chilled 1xPBS solution. Before adding lysis buffer to the cell suspension, 1 µl. 0.05 µM of cel-miR-39-3p solution was added for each 10^6 cells/ml as an internal control. The resulting total miRNA preparation was further extracted into 1.5 ml Eppendorf tubes and stored at -80 °C.

TaqMan Advanced miRNA cDNA Synthesis Kit (Thermo Scientific, Germany) was used for reverse transcription. TaqMan Fast Advanced Master Mix (Thermo Scientific, Germany), TaqMan™ Advanced miRNA Assay Kit (Thermo Scientific, Germany) miR-21-5p, miR-149-3p, miR-150-5p, miR-155-5p, miR-193a-5p were used for real-time qRT-PCR. A single Ct value of 0.1 (qPCRsoft 3.0 software) was chosen for all samples according to the manufacturer's instructions as an indicator of the expression level of the analysed miRNAs. The value of ΔCt corresponding to the number of cycles at which the fluorescence curve crossed the given background level was determined. Each experiment was performed in two process repeats with further calculation of the average value of ΔCtsr. The relative level was calculated using the ΔΔCt method with determination of the relative concentration of miRNA (RQ) relative to the exogenous control [25]. The nucleotide sequences of the analysed miRNAs (tab. 1) were obtained from the miRBase database (www.mirbase.org).

Table 1. Nucleotide sequences of the analyzed miRNAs

miRNAs	Nucleotide sequence
miR-155-5p	UUAAUGCUAAUCGUGAUAGGGGUU
miR-149-3p	AGGGAGGGACGGGGCUGUGC
miR-150-5p	UCUCCCAACCCUUGUACCAGUG
miR-193a-5p	AACUGGCCUACAAAGUCCCAGU
miR-21-5p	AUGCUUAUCAGACUGAUGUUGA

qRT-PCR analysis was performed by real-time endpoint on a CFX96 Touch amplifier (Bio-Rad Laboratories, USA).

Statistical processing. Prism 8.0.1 (Graphpad, USA) was used to process the obtained data. The Shapiro-Wilk (SW) method was used to determine the normality of data distribution. Since the p value (SW) is greater than 0.05, the null hypothesis of normal data distribution was accepted and parametric methods were used for statistical processing. At the initial stage of analyzing the obtained quantitative data, descriptive statistics methods were used; the mean (Mean) and standard deviation (Sd) were calculated for each sample. The statistical significance of differences in values was assessed using Student's t-test. Multiple pairwise comparisons of ΔCtsr of each investigated miRNA from 1st to 6th passages were performed using Tukey's test. When testing statistical hypotheses, differences at the critical significance level of $p < 0.05$ were considered reliable.

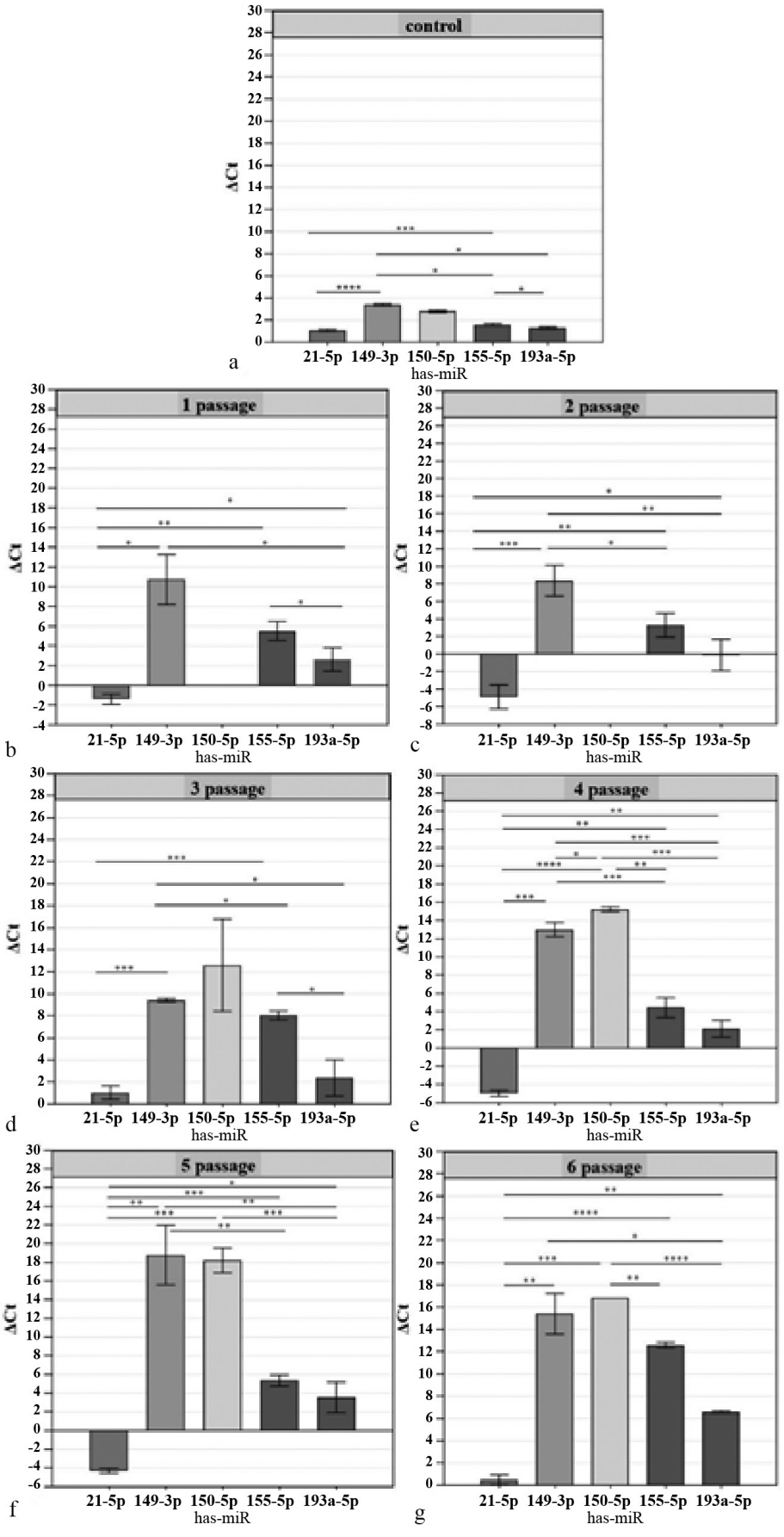

Fig. 1. ΔCtsr values of miRNAs expression level in melanoma melanocyte cell line from the first to the sixth passage of cultivation by real-time qRT-PCR

Results

First Passage. The profile of the analyzed miRNA species revealed that miR-149-3p had the highest ΔCtsr in the analyzed miRNA panel (fig. 1), while miR-193a-5p had the lowest. However, no expression of miR-150-5p was detected.

Second passage. The nature (fig. 1) of the indicators of the analyzed panel of miRNA exosomes of second and first passage melanoma melanocytes show similar ΔCtsr values, with the only difference that the expression level of miR-149-3p and miR-155-5p is slightly lower, in contrast to the expression level of miR-21-5p, the expression level of which is increased.

Third passage. A distinctive feature is the registration of miR-150-5p expression (fig. 1), which exhibits the highest levels among all analyzed miRNAs in this passage.

The ΔCtsr values of miR-149-3p and miR-155-5p are slightly lower (in contrast to the previous passages), with the lowest ΔCtsr values noted for miR-193a-5p.

Fourth passage. Also, as in the third passage (fig. 1), a high level of miRNA expression of miR-150-5p and miR-149-3p is observed, with the lowest ΔCtsr values of miR-193a-5p.

Fifth passage. The highest expression levels (fig. 1) were still observed for miR-150-5p and miR-149-3p. Lower expression of ΔCtsr miR-155-5p. Within this passage, miR-21-5p and miR-193a-5p showed the lowest expression level.

Sixth passage. In contrast to the fifth passage, in addition to high expression levels (fig. 1) of miR-150-5p, miR-149-3p, high expression levels of miR-155-5p were observed. Also, a distinctive feature of this passage is a pronounced increase in the expression of miR-193a-5p in comparison with other passages.

In relation to the control parameters (melanocytes of pigmented nevus), the intergroup comparison revealed (fig. 2) a significant increase in the

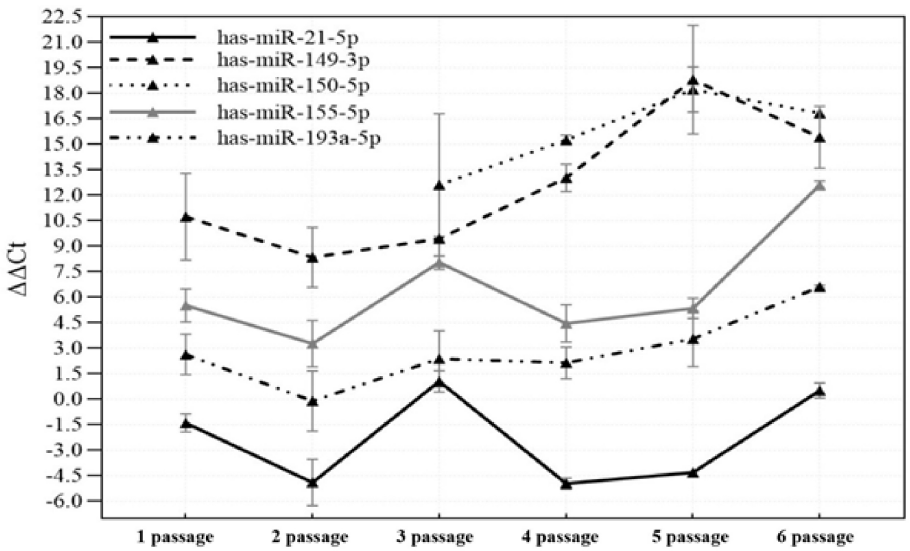

Fig. 2. Intergroup analysis of the relative expression level (ΔΔCtsr) of the panel of miRNAs analyzed, from the first to the sixth passage

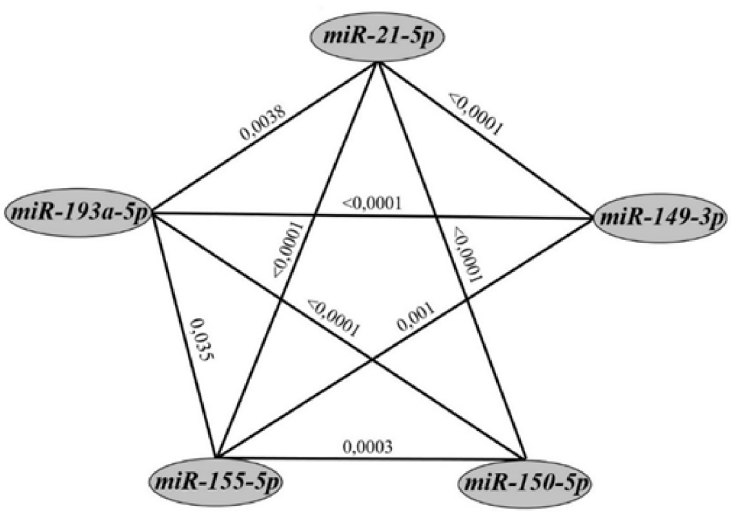

Fig. 3. Multiple pairwise comparison of the ΔCt of the miRNAs studied from passages 1 to 6 (Tukey's test)

expression level (ΔΔΔCtsr) of the analysed miRNA panel. Thus, in particular the expression of miR-21-5p significantly increases on the first passage by 27 %, on the other passages more than 4-fold. The expression of miR-149-3p is significantly increased at the first passage by 3.2-fold, at the second and third passages by more than 2-fold, at the fourth passage by 3.8-fold, and at the fifth and sixth passages by more than 5-fold. The expression of miR-150-5p significantly increases from the fourth to the sixth passage more than 5-fold. The expression of miR-155-5p significantly increases at the first, fourth passage more than 3-fold, the most pronounced increase in expression was observed at the fifth passage (5-fold) and at the sixth passage more than 7-fold. The expression of miR-193a-5p relative to the control is significantly increased only at the sixth passage by 5-fold.

Multiple pairwise comparison (fig. 3) of ΔCtsr from the first to the sixth passage revealed that all analyzed miRNAs types differed significantly in their degree of expression except in the pairing of miR-149-3p and miR-150-5p.

Discussion

Taking into account the presence of a unique specific expression profile of miRNAs at different stages of skin melanoma development [8], we have revealed the peculiarities of expression of the analyzed panel of miRNAs.

In particular, the expression of miRNA miR-155-5p from the first passages of cultivation and by the sixth increased 7-fold, which is the highest expression level among the analyzed miRNAs. It is known that miR-155-5p play an important role in various physiological and pathological processes; they are part of exosomes involved in the control of angiogenesis in melanoma [19], play a crucial role in the reprogramming of the tumor microenvironment, inhibition of proliferation processes and induction of apoptosis [9]. Although tumor angiogenesis is modulated by various factors, exosomal miR-155-5p could be a potential target for controlling melanoma angiogenesis and could be used to develop new treatment strategies.

A distinctive feature of miR-150-5p in our experiment is the absence of expression from the first to the third passage and high expression rates from the fourth to the sixth passage. Notably, miR-150-5p ranks second after miR-155 in terms of expression upregulation. miR-150-5p is one of the most studied miRNAs and plays an important role in the processes of melanoma oncogenesis - it suppresses proliferation, determines cytoskeleton restructuring, cell invasion, inhibits SIX1 gene expression by targeting its 3'-UTR, inhibiting glycolysis, ATP formation and determines the development of mechanisms of decreased sensitivity to some drugs [20]. Glycolysis regulated by miR-150-5p/SIX1 is crucial for the regulation of tumor growth, apoptosis, metastasis prediction and cell survival both in vitro and in vivo [5]. miR-150-5p targets a wide range of oncogenes or tumor suppressor genes, transcription factors such as ZEB1, HMGA2, FOXO4 and c-MYB, and key signaling pathways including wnt/β-catenin and TGFβ, affecting multiple effectors including matrix metalloproteinases (MMP14 and MMP13), cell adhesion molecules (ITGA3, ITGA6). TGFβ is thought to have a tumor suppressive effect in the early stages of cancer by preventing cell proliferation and promoting apoptosis, but TGFβ stimulates tumor metastasis in late stage tumors [26], which we observed in our study. It was also noted that miR-150-5p regulates the transcription factor KLF2, which is actively involved in the modelling of inflammatory chemokines in cells of the immune system, and, can be used as prognostic and diagnostic markers of regulators of differentiation and activation of immune cells that are involved in innate and adaptive immune responses. It has been observed that high expression of miR-150-5p in patient tumor samples indicates better prognosis and better response to adjuvant chemotherapy.

The expression level of miR-149-3p increased smoothly from the first to the sixth passage, it is also noteworthy that miR-149-3p ranked third after miR-150-5p and miR-155-5p in terms of expression level increase. The expression level of miR-149-3p plays a major role in the process of cell migration and tumor progression [21]. It has been observed that P53-sensitive miR-149*, is overexpressed in human metastatic melanoma isolates and targets GLYCOGEN SYNTHASE KINASE-3 ALPHA (GSK3A), inducing melanoma cell resistance to apoptosis by increasing MCL-1 expression, regulate cyclin-independent cell cycle proteins [27]. Multiple primary melanomas have been found to have higher expression of miR-149-3p family compared to single skin melanomas and nevi, which was also observed in our study. TGF-B2, WNT/B-Catenin, HIPPO, TWEAK/EGFR and IL-6/STAT3 pathways are the major signaling pathways regulating miR-149-3p expression. According to the results of TARGET SCAN [28], it is suggested that miR-149-3p exhibits anti-OC abilities by inactivating the PI3K/AKT pathway.

The smooth steady increase in miRNA miR-21-5p expression, which we observed, reflects a disturbance in the regulation of signaling pathways of tumor suppressor proteins PTEN, PDCD4, BTG2, the development of the pathological state of the organism, including neo-oncogenesis, may contribute to tumor growth, metastasis and invasion, and decreased sensitivity to chemotherapy. High expression level of miR-21-5p is a negative

predictor of survival in various forms of cancer [12], which increases depending on the duration of cultivation. It is important to note that the role of miR-21-5p in cell proliferation, tumor progression due to overexpression in melanoma tissues is significantly higher compared to benign tumors, in our case compared to pigmented nevus; therefore it can act as a prognostic biomarker [17].

miR-21-5p functionally increases the invasiveness of melanoma cells by inhibiting tissue inhibitor of metalloproteinases 3 (MMP-3) [18]. A large number of studies indicate a disruption of cell cycle regulation at the G1/S, S and G2/M phase restriction point for virtually all human tumor types, including melanoma. At the same time, the regulatory role of miR-21-5p in this case likely to be through its effect on the skin melanoma CDKN2C gene with the subsequent effect of enhancing proliferation at the G1/S transition level of the cell cycle [12].

Suppression of miR-21-5p expression inhibited the growth and metastatic potential of melanoma through activation of apoptosis, and increased the chemo- or radiosensitivity of human skin melanoma, indicating the potential application of miR-21-5p for therapeutic purposes.

miR-21-5p ranked fourth after miR-155-5p, miR-150-5p, and miR-149-3p in terms of increased expression.

In our study, no increase in miR-193-5p expression was detected in the first passages of cultivation (early stages), which has also been reported in other studies [7, 15]. At the same time, a sharp increase in miR-193-5p expression at the sixth passage of cultivation determines a significant decrease in cell viability, expression of genes involved in proliferation (ERBB2, KRAS, PIK3R3 and MTOR) and apoptosis (MCL1 and NUSAP1), suppression of Akt and Erk pathways, inhibition of anti-apoptotic factors and expression of apoptosis ligand PD-L1. This creates the potential for melanoma to 'escape' immune system factors [29]. The obtained results of miR-193a-5p expression may represent a new method of treatment for patients with skin melanoma, taking into account the fact that miR-193 expression is associated with the mutational status of the BRAF gene in melanoma tissues [14].

We observed that a panel of analysed miRNAs of melanoma melanocytes may provide novel biomarkers predicting patient response to immune checkpoint inhibition, and it is reasonable to speculate that combining miRNAs with different immune checkpoint targets may mimic and possibly improve the effect of combination therapy of immune checkpoint blockade. Targeted therapy and immunotherapy have significantly improved the outcome of advanced melanoma patients, but resistance and toxicity as well as incomplete therapeutic response often occur. Moreover, analysis of miRNAs profile as clinical biomarkers is more correct to be performed directly from tumor cells from the position of analysis of metastasis mechanisms, rather than circulating in the blood for reasons such as - the presence of cellular detritus, hemolysis and other factors that can potentially affect reproducibility and sensitivity.

Conclusion

The study examined a model of melanoma metastasis development using the duration of melanocyte culture of superficially spreading melanoma in correlation with the expression level of a panel of five analyzed miRNAs. The profile of miRNAs analyzed according to the duration of cultivation showed that the expression of miR-149-3p, miR-21-5p increased in earlier periods of cultivation, and at later periods, first of all miR-155-5p, then miR-150-5p, miR-193-5p, as associated with melanoma progression and can be proposed as diagnostic and/or prognostic biomarkers, as well as being considered as potential therapeutic targets.

Conflict of interest

The authors declare no conflict of interest.

Compliance with the rules of bioethics

The study was carried out in accordance with the WMA. Declaration of Helsinki as amended in 2013. The meeting of the Ethics Committee of the Ulyanovsk «VM-Clinic» approved the study and the informed consent form on 11 September 2023, Protocol No. 21. The research material was obtained from the multiprofile hospital «VM-Clinic», Ulyanovsk. All patients gave written informed consent to participate in the study.

Financing

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Authors' contributions

Antonova E.I. — suggested the idea for the publication, wrote the manuscript;
Antonova E.I., Kunitsyna A.V. — chose material for the study;
Kunitsyna A.V., Achilov A.B., Koroleva A.K. — introduced information about the study and analyzed the data;
Baldueva I.A., Nekhaeva T.L., Firsova N.V., Sikharulidze S.V. — edited the article.
All authors have approved the final version of the article prior to publication and have agreed to take responsibility for all aspects of the work, including the proper investigation and resolution of issues relating to the accuracy or integrity of any part of the paper.

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