Short Communications

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Chemotherapy Supports Cancer Cell Dissemination in a Melanoma Preclinical Model

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Abstract

Objective: The aim of the study was to determine how the chemotherapeutic alkylating agent dacarbazine, together with the application of the miR-204-5p mimic in vivo, affects the presence of disseminated melanoma cells in distant organs – the lungs and liver. **Methods:** The study was carried out on B16 melanoma-bearing mice (n = 48). The animals were treated with dacarbazine (50 mg/kg) or dacarbazine combined with the microRNA miR-204-5p mimic (5 nM). Real-time PCR was used to evaluate the expression of miR-204-5p target genes following miR-204-5p mimic application. The presence of melanoma cells in distant organs was assessed by immunovisualization of the melanocyte marker PMEL using an immunohistochemical assay and by evaluating the expression of the melanocyte-specific protein tyrosinase via real-time PCR. **Result:** The level of PMEL expression increased twofold in the lungs of mice treated with dacarbazine ($p = 0.014$) and 4.2-fold in the group of animals treated with a combination of dacarbazine and the miR-204-5p mimic ($p = 0.001$), as compared to the control group. However, tyrosinase expression was detected in the lungs of B16 melanoma-bearing mice treated with dacarbazine and the negative control only, due to the sporadic presence of melanoma cells in distant organs. **Conclusion:** Taken together, dacarbazine and the miR-204-5p mimic favor the dissemination of B16 melanoma cells in the lungs, which may support further metastatic development. Although miR-204-5p has been described as a tumor-suppressive microRNA in melanoma, the application of a synthetic mimic to overexpress it in distant organs promoted tumor cell dissemination.

Keywords: Dacarbazine- disseminated cancer cells- melanoma B16- metastasis- miR-204-5p

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Introduction

Targeted therapy and immune checkpoint inhibitors introduced into clinical practice in recent years for cutaneous melanoma, have significantly increased patient survival rates. However, relatively rapid resistance development to any antitumor agent makes essential further studies to discover mechanisms of melanoma dissemination. Among the drugs used for melanoma treatment, the alkylating cytotoxic agent dacarbazine (DTIC) recognized as baseline and used as a reference tool for new antitumor medicine efficacy evaluation in melanoma [1].

Intensive studies of small non-coding RNAs have allowed to consider miRNA-based agents as perspective tool to reinforce antitumor therapy [2]. MicroRNAs have been shown to act as master regulators of key processes driving melanoma dissemination and drug resistance formation [3]. Previously we have been shown that miR-204-5p expression levels in melanoma are diminished as compared to benign melanocytic nevi that can correspond to miR-204-5p oncosuppressive functioning during melanomagenesis [4]. Several studies pointed out a possible miR-204-5p targeting to modulate tumor progression [5, 6]. Therefore, miR-204-5p overexpression can induce tumor growth inhibition and metastasis development.

Metastasis is originated from disseminated tumor cells that can reside in distant organs in quiescent state corresponding to resting phase, G0 of a cell cycle, for uncertain period of time [7]. Under unknown triggers, disseminated cancer cells re-enter to a cell cycle and start to divide actively, resulting in distant metastasis formation. Therefore, the transition from the G0 phase to a cell cycle is a critical event for disseminated tumor cells located in distant organs that corresponds to tumor progression. As demonstrated previously, miR-204-5p implicated in cancer cell proliferation regulation [8]. But it is undiscovered if miR-204-5p can be involved in a transition of quiescent cancer cells into proliferative state. Therefore, the aim of the present miR-204-5p overexpression study is to determine how miR-204-5p overexpression can affect melanoma cells dissemination in vivo.

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Materials and Methods

Cells and animals

MicroRNA mimic miR-204-5p was purchased by DNA-synthesis (Moscow, Russia). Melanoma B16 cell line was kindly provided by Federal State Budgetary Scientific Institution "Research Institute of Fundamental and Clinical Immunology" (Novosibirsk, Russia).

The study was approved by the local Ethical Committee of Prof. V.F. Voyno-Yasenetsky Krasnoyarsk State Medical University (No116/2022 issued by 27.12.2022). Experimental studies on laboratory animals were performed in accordance the Rules of Good Laboratory Practice approved by the order of the Ministry of Health of the Russian Federation№. 199 issued by April 1, 2016, and the recommendations in the National Institute of Health's Guide for the Care and Use of Laboratory Animals [9]. C57Bl6 mice female (n=48), 8 weeks old and weighing 16-18 g were obtained from the Federal State Unitary Enterprise "RAPPOLOVO Laboratory Animal Nursery' of the National Research Centre 'Kurchatov Institute".

Experimental therapy by DTIC and miR-204-5p mimic

Animals were divided into groups randomly at the day 7. Control group consisted of animals (n=12) obtaining intraperitoneal injection phosphate buffered saline, PBS, 250 µl (VWR Radnor, USA) on the 8th, 10th, 12th days after tumor cell transplantation. Second group was presented by animals treated by intraperitoneal injections of DTIC, (n=12) at a dosage of 50 mg/kg. Third group of animals (n=12) was treated by intraperitoneal injections of DTIC and 5 nMol Negative Control and NC. Fourth group of animals (n=12) was treated by intraperitoneal injections of DTIC and 5 nMol miR-204-5p mimic. Mice were sacrificed on day 14 after melanoma B16 cells transplantation. Tissue samples were fixed in 10% neutral buffered formaldehyde and embedded in paraffin.

Immunohistochemical study of distant organs of metastasis

For immunohistochemical assay PMEL primary antibodies (Abcam, Waltham, USA) were used at a dilution of 1:100. Horseradish peroxidase (HRP) and AEC chromogen-based labelling was used (Mouse and Rabbit specific HRP/AEC IHC Detection Kit system (Abcam, Waltham, USA). Slides were further stained with hematoxylin and enclosed in mounting medium. PMEL expression level was determined by estimating positively stained cells at a magnification of 400x using an Olympus BX-41 microscope (Olympus, Tokyo, Japan).

Real time PCR

The relative expression level of *TYR*, *SIRT1* and *BCL2* genes in distant organs was determined by real-time PCR on a StepOneTM Real Time PCR-System (AppliedBiosystems, Singapore, Singapore). Total RNA was isolated using DiaGen reagent kit (Dia-M, Moscow, Russia). Reverse transcription reaction was carried out using the MMLV RT kit (Eurogen, Moscow, Russia). Reagent kits containing 20x specific primers were used to assess mRNA expression of *TYR, SIRT1*, *BCL2* in mice: *TYR* (Mm00495817_m1)), *SIRT1* (Mm00490758_m1), *BCL2* (Mm00477631_m1) (Applied Biosystems, USA), ACTB (Mm02619580_g1) and HPRT1 (Mm02800695_m1) (Applied Biosystems, USA). Results were normalized simultaneously using two endogenous controls, ACTB and HPRT1 (AppliedBiosystems, USA). Relative gene expression levels for the tested samples were estimated by ΔΔCT method.

Statistical analysis

Statistical analysis was performed using Statistica 7 (StatSoft, Moscow, Russia). Mann-Whitney U-test was applied. The difference considered as significant if P-level of was <0.05.

Results

In all groups of experimental animals after transplantation of melanoma B16 cells a uniform growth of subcutaneous tumors on the day 7th was determined.

As reported previously, *SIRT1* and *BCL2* were shown as functional gene targets of miR-204-5p. Therefore, *SIRT1* and *BCL2* expression levels in lungs and liver were evaluated to assess miR-204-5p mimic functioning in these organs [10]. Indeed, *SIRT1* was down-regulated in lungs and liver in a group of animals treated by a combination of DTIC and miR-204-5p mimic as compared to a group of animals treated by a combination of DTIC and NC. *BCL2* expression was diminished in the lungs of mice treated by DTIC, whereas it's expression level was increased two times in the livers under DTIC treatment. miR-204-5p mimic application resulted in *BCL2* expression level increase as compared with control, negative control (Figure 1).

The presence of PMEL-positive cells in lungs and liver of melanoma B16-bearing mice was determined by immunohistochemical assay. Besides, *TYR* expression was evaluated in these tissues by real-time PCR. Both *TYR* and PMEL proteins are components of melanosome and pigment granule and expressed in normal melanocytes and melanoma cells [11].

According to immunohistochemical study, PMEL-positive cells were found in lungs of animals from all experimental groups and in livers of control group animals treated by PBS as well as in the group of animals treated by a combination of DTIC and miR-204-5p mimic (Figure 2).

PMEL expression in lungs was 2-fold higher in the group of animals treated by DTIC ($p = 0.014$) versus PMEL expression in lungs of animals treated by PBS. Besides, PMEL expression increased 4.2-fold in the group of animals treated by a combination of DTIC and miR-204-5p mimic versus PMEL expression levels in a group of animals obtaining PBS ($p = 0.001$), as well as in comparison with the group of animals that were treated by a combination of DTIC and negative control ($p = 0.00003$) (Figure 3).

Surprisingly, PMEL expression was not observed in livers of animals treated by DTIC, DTIC and NC. PMEL-positive cells were found in the liver of animals treated with PBS and a combination of DTIC and miR-204-5p mimic.

Tyr showed increased expression in the lungs of

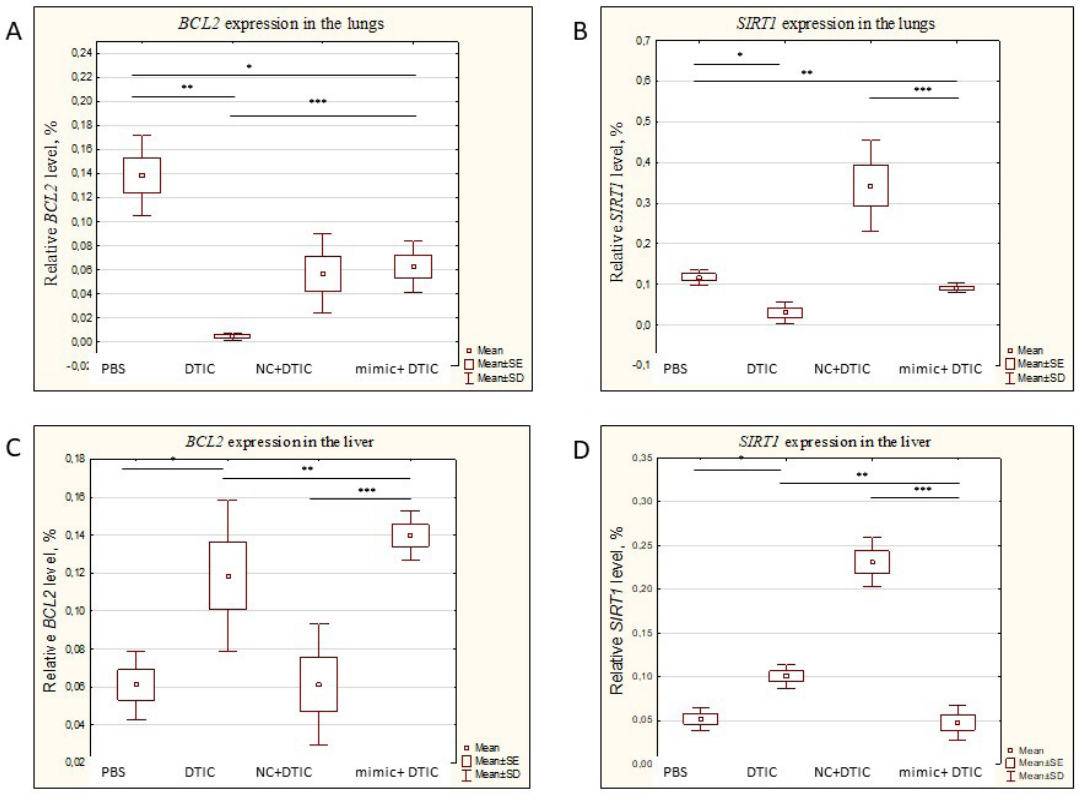

Figure 1. *BCL2* expression in the lungs (A), liver (C) of melanoma B16-bearing mice under DTIC (50 mg/kg), miR-204-5p mimic (5nmol) treatment. SIRT1 expression in the lungs (B), liver (D) of C57Bl6 melanoma B16-bearing mice under DTIC (50 mg/kg), miR-204-5p mimic (5nmol) treatment ($p<0.05$). SIRT1 down-regulation in lungs of melanoma B16-bearing mice treated by a combination of DTIC and miR-204-5p mimic in comparison to a group of animals treated by DTIC and NC that confirms efficient delivery and action of miR-204-5p mimic

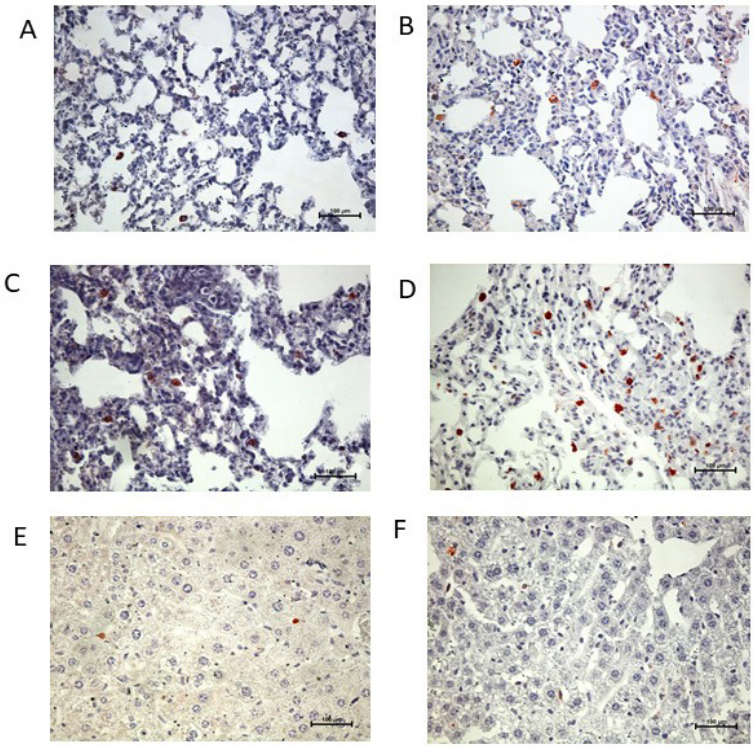

Figure 2. Immunostaining for Visualization of the Melanocyte Marker PMEL in the Lungs of Melanoma B16-bearing Mice Treated by PBS (A), DTIC (B), NC+DTIC (C), miR-204-5p mimic + DTIC (D) in liver tissues of mice treated by PBS (E) and DTIC+MIMIC (F) and. (×400). Lung and liver tissue sections were stained for the melanocyte marker PMEL and hematoxylin. Micrographs represent positive staining of single tumor cells (red) among the mouse lung and liver cells (hematoxylin, blue). The combination of the miR-204-5p mimic and DTIC led to a marked increase in PMEL-positive cells compared to other treatments.

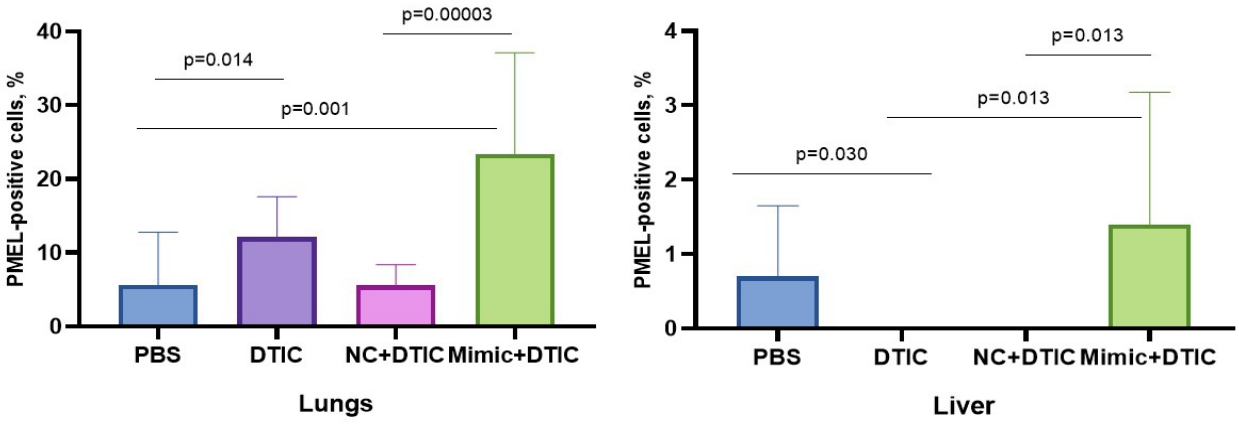

Figure 3. *PMEL* Expression in Lungs and Liver of Melanoma B16-Bearing Mice under Treatment with PBS, DTIC, NC+DTIC, miR-204-5p mimic+DTIC. The combination of the miR-204-5p mimic and DTIC led to a marked increase in a percentage of PMEL-positive cells compared to other treatments ($p<0.05$).

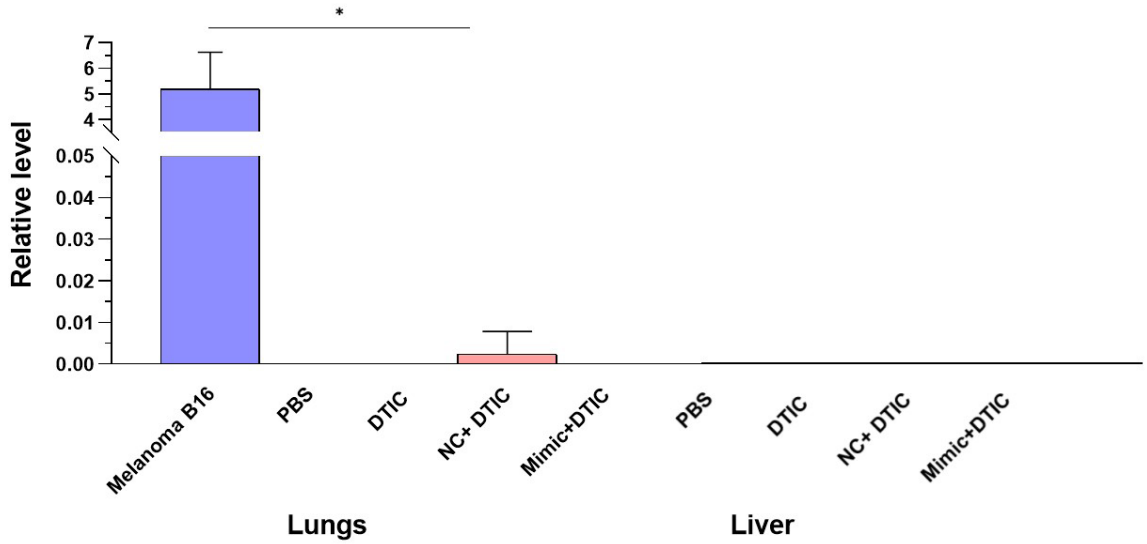

Figure 4. Relative Tyrosinase Expression in Melanoma B16 Tumor Cells and Distant Organs of Melanoma B16-Bearing Mice under Treatment with PBS, DTIC, NC+DTIC, miR-204-5p mimic+DTIC. Tyrosinase expression was detected in lungs of melanoma B16-bearing mice treated by dacarbazine and negative control (*$p<0.05$).

animals treated with NC+DTIC. However, most prominent *TYR* expression was showed in melanoma B16 cells obtained from mice tumors that were used as positive control (Figure 4).

Discussion

Previous studies revealed that miR-204-5p is implicated in melanoma development and progression. miR-204-5p was shown to be downregulated in melanoma as compared with melanocytic nevi. Besides, miR 204-5p inhibits apoptosis in DTIC-treated melanoma cells [5]. miR-204-5p mimic hindered invasion, whereas miR-204-5p inhibitors stimulated cancer cell migration [8].

At the beginning of the present study we determined the efficiency of miR-204-5p mimic delivery to distant organs by miR-204-5p target genes expression [12]. Our previous studies as well as other reports showed that *SIRT1* and *BCL2* considered as functional gene targets of the miR [5, 13].

Indeed, we observed *SIRT1* down-regulation in lungs of animals treated by a combination of DTIC and miR-204-5p mimic in comparison to a group of animals treated by DTIC and NC that confirms efficient delivery and action of miR-204-5p mimic. However, *BCL2* expression levels were increased in the livers of melanoma B16-bearing mice treated by a combination of DTIC and miR-204-5p mimic as compared to a group of animals treated by DTIC and NC. Previously, target gene overexpression as microRNAs effect was described referred to their non-canonical effects [14].

The development of drug resistance in cutaneous melanoma is facilitated by the transition of tumor cells to a dormant state corresponding resting phase (G0) of a cell cycle. Chemoresistant tumor cells can survive and become reactivated causing metastatic disease when disseminated [15]. Distant organs form a plausible environment for their colonization by disseminated cancer cells. It is well documented now that disseminated cancer cells achieve distant organs at early stages of tumor development [16],

where they can persist without forming visible metastasis for uncertain period of time [17]. It is assumed that disseminated cancer cells preferably exist in quiescent state in distant organs [18]. Disseminated quiescent cancer cells reactivation is forced by unknown yet stimuli. Therefore triggering disseminated cancer cell reactivation is a key target to regulate metastatic process.

Alkylating anticancer agents induce genome damage and reactive oxygen species production activation [19]. Although chemotherapy is well-recognized cancer treatment, in some cases tumor growth and metastasis are rapidly enhanced by chemotherapy, leading to treatment failure. The biological mechanism mediating the development of tumor cell resistance is explained by several processes including the ability of proliferating cancer cells reversely exit from cell cycle to G0 phase [20]. Targeted agents are disrupting mostly cell proliferation signal pathways leading to intracellular signaling blockade followed by apoptosis development. However, due to the heterogeneity of tumor cells, the efficacy of targeted therapeutics is limited because of reactivation mechanisms or signal pathways activation with similar functional affect and the presence of cancer cells with diverse molecular alterations [21, 22]. Besides, slowly cycling cells are escaping apoptosis-inducible stimuli provided by anticancer drugs [23].

In our study, we used alkylating agent DTIC, which induced an increase of disseminated tumor cells presence in the lungs of melanoma B16-bearing mice. Although chemotherapy has a suppressive effect on tumors, it can affect host tissues and enhance cancer progression leading to drug resistance via several mechanisms: supression of apoptosis-related proteins, drug target modification, drug efflux, activation of DNA repair, signal pathway reactivation, etc. [24]. According to the report of Zenitani et al., liver metastasis in B16F10 melanoma bearing mice was enhanced by the administration of cisplatin. The authors explained it by profibrotic microenvironment development under cisplatin treatment and MMP-2-mediated periostin production leading to fibrosis [25]. In our study, microRNA mimic miR-204-5p, when co-administered with DTIC, increased the level of disseminated tumor cells in distant organs - lungs and liver. A "pre-metastatic" niche formation and favorable microenvironment in distant organs is crucial for disseminated cancer cells survival favoring further progression of melanoma and metastasis [15, 26]. Our findings may indicate the ability of tumor cells to evade apoptosis when exposed to DTIC in distant organs.

In the present study we observed an increase of PMEL-positive cells percentage in lungs of melanoma B16-bearing mice under treatment by DTIC in combination with miR-204-5p mimic. We suggest that it can be explained by miR-204-5p-mediated melanoma cells phenotype switch from proliferative to invasive. Indeed, there are several studies reported an ability of miR-204-5p to modulate invasive capacities of cancer cells [27, 28]. Disseminated cancer cells enhancement in target organs followed by metastasis development can be associated with miR-204-5p-mediated down-regulation of it's target genes such as SMAD4 [29], that was identified as cancer cell invasion promoter [30].

DTIC administration in combination with miR-204-5p mimic enhances PMEL-positive cells presence both in lungs and liver. Such alterations can be due to ability of cancer cells to alter their phenotype under injurious stimuli. Further investigation of cancer cell plasticity is needed to develop more effective cancer treatment approaches.

Author Contribution Statement

Ekaterina Lapkina: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. Tatiana Ruksha: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Supervision, Writing – review & editing.

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Ethical Declaration

The study was carried out according to the recommendations in the National Institute of Health's Guide for the Care and Use of Laboratory Animals (website ncbi.nlm.nih.gov/books/NBK54050) and was approved by the Local Ethics Committee of the Krasnoyarsk State Medical University (approval no 116/2022, date issued: 27.12.2022).

Data Availability

The authors declare that the data supporting the findings of this study are available within the paper. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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