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South Russian Journal of Cancer

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The "South Russian Journal of Cancer" is a quarterly scientific and practical peer–reviewed journal. A professional medical publication that reflects the results of current research on the subject of publications: diagnosis and treatment of oncological diseases, issues of carcinogenesis and molecular oncology, new medicines and technologies. It was founded in 2019.

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- To promote the development of oncological medicine in the South of Russia and the implementation of its achievements in practice.
- High-quality published content that includes the latest and trustworthy scientific papers, research or work on oncology issues.

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- Facilitating the exchange of experience and transfer of advanced knowledge between specialists;
- Informing readers about the results of major medical forums;
- Giving scientists the opportunity to publish the results of their research;
- Achieving an international level in scientific publications;
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Журнал входит в рекомендованный ВАК РФ перечень рецензируемых научных журналов и изданий для опубликования основных научных результатов диссертаций на соискание учёной степени кандидата и доктора наук.

«Южно-Российский онкологический журнал» – ежеквартальный научно-практический рецензируемый журнал. Профессиональное медицинское издание, в котором отражаются результаты актуальных исследований по тематике публикаций: диагностика и лечение онкологических заболеваний, вопросы канцерогенеза и молекулярной онкологии, новые лекарственные средства и технологии. Основан в 2019 г.

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- Качественный опубликованный контент, включающий последние и заслуживающие доверия научные труды, исследования или работы по проблемам онкологии.

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- Популяризация современных достижений онкологической службы на Юге России;
- Содействие обмену опытом и передаче передовых знаний между специалистами;
- Информирование читателей о результаты крупных медицинских форумов;
- Предоставление ученым возможности опубликовать результаты своих исследований;
- Достижение международного уровня в научных публикациях;
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- Повышение импакт-фактора журнала.

Журнал принимает к публикации: результаты оригинальных исследований, обзоры литературы, описание клинических случаев.

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CONTENTS

South Russian Journal of Cancer
2024. Vol. 5, No. 3

ORIGINAL ARTICLES

REVIEWS

CONTENTS

СОДЕРЖАНИЕ

ОРИГИНАЛЬНЫЕ СТАТЬИ

СОДЕРЖАНИЕ

South Russian Journal of Cancer. 2024. Vol. 5, No. 3. P. 8-15
https://doi.org/10.37748/2686-9039-2024-5-3-1
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ORIGINAL ARTICLE

The implication of liquid hemostatic matrices to prevent hemorrhages during stereotactic biopsy of brain tumors

E. E. Rostorguev✉, N. S. Kuznetsova, S. E. Kavitskiy, B. V. Matevosyan, G. A. Reznik, V. E. Khatyushin, O. I. Kit

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ABSTRACT

Purpose of the study. Development of a method for preventing hemorrhages during stereotactic biopsy of a brain tumor using liquid hemostatic matrices on the example of the drug "Floseal®".

Patients and methods. The target of the biopsy is the most representative area of tumor tissue according to the data of various modalities of MRI neuroimaging, including contrast-enhanced ones. Out of 133 patients, 60 patients with signs of intraoperative bleeding along the biopsy needle cannula were included in the study group. Further, patients with signs of intraoperative bleeding along the cannula of the biopsy needle were divided into 2 subgroups by independent sequential randomization. Control subgroup (n = 45): cases with signs of intraoperative bleeding of varying severity were operated on, according to the standard technique, without the use of the liquid hemostatic drug Floseal®. The main subgroup (n = 15): in case of intraoperative signs of bleeding, the hemostatic fluid drug Floseal® was injected into the area of tumor material removal.

Results. In 6.7 % of patients of the control subgroup, the formation of massive intracerebral hemorrhages was noted in the postoperative period. In 53.3 % of the observations of the control subgroup according to X-ray computer examinations of the brain, there were signs of minor hemorrhages at the point of tumor material collection, which did not require repeated surgical interventions. Postoperative hemorrhages after injection of the Floseal® liquid hemostatic matrix into the biopsy needle in the study subgroup were not detected according to neuroimaging X-ray CT.

Conclusion. A method of hemostasis has been developed to prevent hemorrhages using liquid hemostatic matrices. If signs of bleeding from the biopsy needle appeare, the introduction of a hemostatic matrix in the volume of 2 ml helps to manage bleeding intraoperatively, as well as to prevent the occurrence of hemorrhage in the early postoperative period.

Keywords: brain tumor, stereotactic biopsy, bleeding prevention, hemorrhagic complications of brain biopsy, hemorrhage, hemostasis

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Compliance with ethical standards: this study adhered to the ethical principles outlined in the World Medical Association Declaration of Helsinki, 1964, ed. 2013. The study was approved by the Committee on Biomedical Ethics at the National Medical Research Centre for Oncology (extract from the protocol of the meeting No. 7 dated 08/08/2022). Informed consents have been obtained from all participants of the study

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ОРИГИНАЛЬНАЯ СТАТЬЯ

Использование жидких гемостатических матриц для предупреждения кровоизлияний при выполнении стереотаксической биопсии опухолей головного мозга

Э. Е. Росторгуев✉, Н. С. Кузнецова, С. Э. Кавицкий, Б. В. Матевосян, Г. А. Резник, В. Е. Хатюшин, О. И. Кит

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РЕЗЮМЕ

Цель исследования. Разработка способа предупреждения кровоизлияний при выполнении стереотаксической биопсии опухоли головного мозга с использованием жидких гемостатических матриц на примере препарата «Floseal®».
Пациенты и методы. Цель биопсии – наиболее репрезентативный участок опухолевой ткани по данным различных модальностей МРТ нейровизуализации, в том числе и с контрастным усилением.
Из 133 пациентов в изучаемую группу включено 60 больных с признаками интраоперационного кровотечения по канюле биопсийной иглы. Далее, методом независимой последовательной рандомизации пациенты с признаками интраоперационного кровотечения по канюле биопсийной иглы разделены на 2 подгруппы. Контрольная подгруппа (n = 45): случаи с признаками интраоперационного кровотечения различной степени выраженности, оперированы по стандартной методике, без использования жидкого гемостатического препарата «Floseal®». Основная подгруппа (n = 15): при интраоперационных признаках кровотечения в зону изъятия опухолевого материала осуществлялось введение гемостатического текучего препарата «Floseal®».
Результаты. У 6,7 % пациентов контрольной подгруппы в послеоперационном периоде отмечено формирование массивных внутримозговых кровоизлияний. В 53,3 % наблюдений контрольной подгруппы по данным рентген компьютерных исследований головного мозга имелись признаки незначительных кровоизлияний в точке забора опухолевого материала, не требовавшие повторных оперативных вмешательств. Послеоперационные кровоизлияния после введения в биопсийную иглу жидкой гемостатической матрицы «Floseal®» в основной подгруппе по данным РКТ нейровизуализации не выявлены.
Заключение. Разработан способ гемостаза для предупреждения кровоизлияний с использованием жидких гемостатических матриц. При появлении признаков кровотечения из биопсийной иглы введение гемостатической матрицы в объеме 2 мл способствует остановке кровотечения интраоперационно, а также профилактике возникновения кровоизлияния в раннем послеоперационном периоде.

Ключевые слова: опухоль головного мозга, стереотаксическая биопсия, профилактика кровотечения, геморрагические осложнения биопсии головного мозга, кровоизлияние, гемостаз

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Соблюдение этических стандартов: в работе соблюдались этические принципы, предъявляемые Хельсинкской декларацией Всемирной медицинской ассоциации (World Medical Association Declaration of Helsinki, 1964, ред. 2013). Исследование одобрено Комитетом по биомедицинской этике при ФГБУ «Национальный медицинский исследовательский центр онкологии» Министерства здравоохранения Российской Федерации (выписка из протокола заседания № 7 от 08.08.2022 г.). Информированное согласие получено от всех участников исследования

Финансирование: финансирование данной работы не проводилось

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INTRODUCTION

Currently, the stereotactic needle biopsy (STB) of various grades brain tumors is performed in anatomically inaccessible areas of the brain and when the tumor is localized in functionally significant areas of the brain [1–4].

Given the lack of visual intraoperative control of the needle trajectory, a serious complication after STB, is hemorrhage in biopsy material withdrawal as well as along the path of access of the biopsy needle, despite the intervention being minimally invasive [5–9].

The purpose of the study was to develop a method for preventing hemorrhages during stereotactic biopsy of a brain tumor using liquid hemostatic matrices using the example of the drug "Floseal®".

PATIENTS AND METHODS

From 2014 to 2020 133 patients with brain tumors were operated on using the method of frameless needle stereotactic biopsy. Informed consent for surgical intervention was obtained from all participants of the study. In our observations, tumor diseases manifested from 5 to 80 years. The average age of the patients was 56 years. 57 % were men and 43 % were women. 75.5 % were operated on with single foci of brain damage, 10.5 % with two foci. 14 % of patients had multiple unverified CNS lesions.

Neoplasms in 28.3 % of cases were localized in subcortical structures of the brain, in 18.3 % – in various parts of the corpus callosum with bilateral distribution, in 53.4 % of cases in periventricular areas under functionally significant areas of the brain.

Neurological symptoms depended on the localization of the lesion in the brain. Upon admission, the functional status on the Karnovsky scale of 100–80 points was noted in 71.7 % of patients, 70–50 points in 21.7 % of patients, and below 40 points was noted in 6.6 % of cases. All patients underwent a comprehensive assessment of the hemostasis system in the preoperative period.

The tissue sampling point was determined by combining MRT T1 BRAVO with intravenous contrast enhancement with data from DTI, 2D-TOF, 3D-TOF, T2, ASL, SWAN modes. If necessary, in the Brainlab® or Medtronic StealthStation® S7® planning software, the digital model was combined with DICOM positron emission tomography (PET CT) data with 11^{C}-methionine. The trajectory of the biopsy needle insertion was constructed taking into account the data of tractography, with the localization of the tumor in functionally significant areas of the brain (Fig. 1).

The purpose of the biopsy is the most representative area of tumor tissue according to various modalities of MRI neuroimaging, including contrast enhancement. Surgical intervention was performed under general anesthesia, according to the operating regulations of manufacturers of systems for frameless stereotactic biopsy Brainlab® or Medtronic StealthStation® S7® using biopsy needles Biopsy Needle Kit (9733068) or Biopsy Needle Kit Tip A (41778C).

Control computed tomography was performed intraoperatively or within 24 hours after surgery.

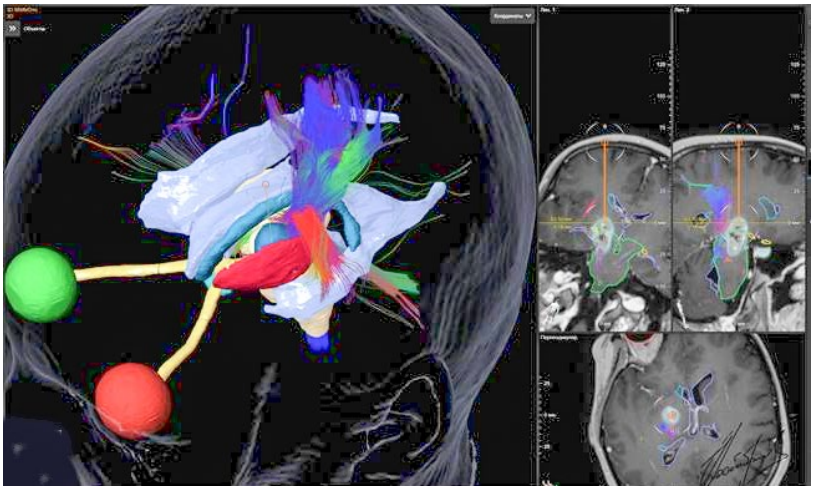

Fig. 1. Planning the access trajectory considering functionally significant areas of the brain

Out of 133 patients, the study group included 60 patients with signs of intraoperative bleeding through the cannula of a biopsy needle (45.1 %). Further, by the method of independent sequential randomization, patients with signs of intraoperative bleeding through the cannula of a biopsy needle were divided into 2 subgroups.

Control subgroup (*n* = 45): cases with signs of intraoperative bleeding of varying severity were operated according to the standard procedure, without the use of liquid hemostatic matrices.

The main subgroup (*n* = 15): with intraoperative signs of bleeding, the hemostatic fluid drug "Floseal®" was injected into the area of removal of tumor material.

The subgroups were comparable in terms of sex, age, localization and histological types of the tumor. We analyzed the complications that arose in these subgroups using the method of X-ray computed tomography performed in the first 24 hours after surgery. All necessary patient data were recorded in the Microsoft Excel electronic database, after

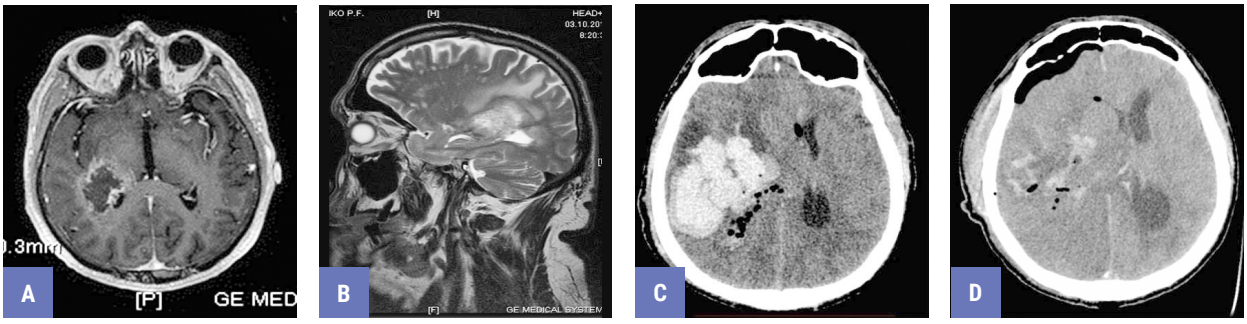

Fig. 2. Patient T., 67 years old. A, B – MRI neuroimaging data of a tumor lesion, hospitalized on 10/07/2016 with a diagnosis of a tumor of the temporal, parietal lobe and islet with a spread to the thalamic tubercle. On 10/10/2016, a stereotactic biopsy was performed. Histological examination: glioblastoma. In the immediate postoperative period, the patient was conscious. 7 hours after the end of the surgery, the patient had a sudden loss of consciousness to coma I, the appearance of right-sided anisocoria, the rapid development of secondary ischemic stem damage in the form of inhibition of pupil photoreaction, loss of oculocephalic reflexes. A brain X-ray computed tomography (CT) scan was performed, extensive hemorrhage (C) in the tumor area with spread to the temporal and parietal lobes of the brain, blood breakthrough into the ventricular system of the brain, pronounced lateral dislocation syndrome to the left, secondary ischemic brain damage was visualized. He was taken to the operating room for vital signs. D – postoperative cerebral CT scan: hematoma and tumor removed. Fatal outcome dated by 10/12/2016

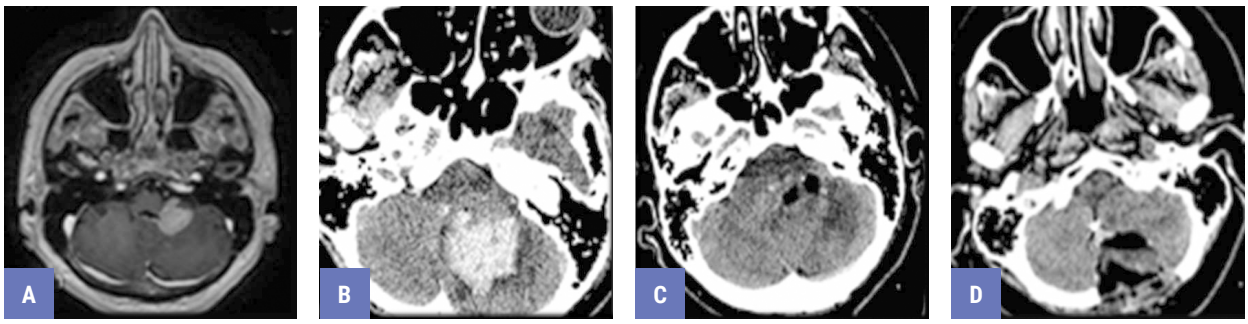

Fig. 3. A–D. Patient Ch., 58 years old, was hospitalized on 11/08/2016 with suspected lymphoma of the left hemisphere of the cerebellum with infiltration of the roof of the 4th ventricle. On 11/09/2016, a stereotactic biopsy of the tumor was performed. Histological examination: non-Hodgkin's lymphoma. Due to the formation of a hematoma in the tumor biopsy area on 11/10/2016, an urgent installation of the Arendt cerebrospinal fluid drainage system into the anterior horn of the right lateral ventricle was performed, 11/10/2016. – subtotal removal of a tumor of the left hemisphere of the cerebellum with growth into the cerebellar bridge angle using neurophysiological monitoring, intraoperative fluorescence microscopy, removal of an intracerebral hematoma in the bed of a removed tumor of the left hemisphere of the cerebellum. In the future, the postoperative period proceeded without complications. Control X-ray CT of the brain dated 11/11/2016: condition after recraniotomy in the suboccipital region. There are areas of reduced density in the surgical area, the postoperative cavity is 3.7 × 3.5 × 3.1 cm. The median structures are not displaced. The patient was discharged in a satisfactory condition

which the data was analyzed in the Statistica 10.0 program. When processing the obtained patient data in the control and main groups, an assessment was carried out based on the nonparametric Pearson's chi-squared test (χ^2).

STUDY RESULTS

In a group of 133 observations, neoplasms were morphologically verified in all cases. In 43.7 % of cases, Grade III–IV gliomas of high malignancy were verified, Grade II – in 40.5 %, CNS lymphomas in 11.4 %, metastases of glandular and squamous cell carcinoma in 4.4 %.

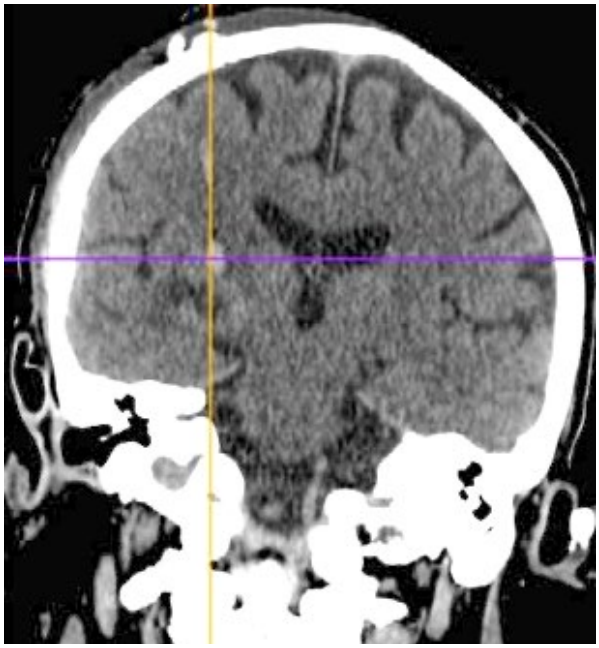

Fig. 4. X-ray CT neuroimaging of postoperative, clinically insignificant microbleeding in the area of biopsy material withdrawal

Mortality after STB in a group of 133 patients was 0.75 % (in one patient, in the immediate postoperative period, the formation of a massive intracerebral hematoma was noted, which required urgent surgical intervention of a tumor localized in a functionally significant area of the brain (Fig. 2)).

We found that in 6.7 % of patients of the control subgroup, the formation of massive intracerebral hemorrhages was noted in the postoperative period (Fig. 3), requiring repeated surgical interventions, removal of both intracerebral and intracerebral hemorrhages with forced cytoreduction of the tumor in a functionally significant area. The mortality rate in the control subgroup was 2.2 %.

In 53.3 % of the observations in the control subgroup, according to X-ray computed studies of the brain performed in the first 24 hours after surgery or intraoperatively, minor hemorrhages were detected at the point of collection of tumor material, requiring a delay in the patient's stay in the hospital and repeated neuroimaging methods (Fig. 4).

The technique of frameless stereotactic needle biopsy in the main subgroup (n = 15) was standard. If signs of bleeding were noted during the withdrawal of tumor material with a biopsy needle, in the form of the release of blood clots, rare, frequent drop or jet bleeding, the hemostatic matrix "Floseal®" was prepared according to the instructions for use of the drug (Fig. 5). The drug is approved for use in the territory of the Russian Federation (RU No. 2019/8305 dated 04/18/2019). "Floseal®" is widely used in abdominal, vascular, and neurological surgical fields as an applicative local hemostatic [10].

A liquid hemostatic matrix was injected into the cannula of the inner stylet of the biopsy needle until

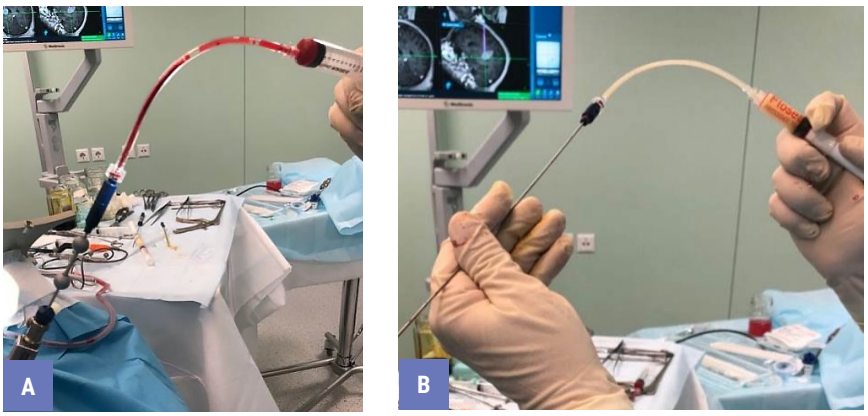

Fig. 5. A. Intraoperative signs of bleeding – the release of blood clots from a biopsy needle. B. After preparing the liquid hemostatic matrix "Floseal®" and filling the inner stylet of the biopsy needle with it, the liquid hemostatic matrix is subsequently injected in a volume of 2 ml

it was completely filled. After the cannula was inserted into the biopsy needle, a hemostatic matrix was additionally injected in a volume of about 2 ml. We have established a satisfactory possibility of injecting this drug into the postoperative cavity through the needle hole for stereotactic biopsy. Next, the biopsy needle was removed, the milling hole was filled with bone chips, ensuring hermetically sealed closure of the bone defect followed by suturing of soft tissues. Intraoperatively, or within a few hours after waking up, the patient underwent CT neuroimaging of the brain to assess postoperative changes and exclude hemorrhagic complications (Fig. 6).

Hemorrhages and postoperative complications were not observed in patients of the main subgroup (n = 15) (χ^2 = 3.99; p = 0.0458).

In the control subgroup (n = 45), the percentage of clinically significant hemorrhages was 6.7 %, which required repeated interventions, removal of both intracerebral and intracerebral hemorrhages. The percentage of clinically insignificant hemorrhages in the control subgroup was 53.3 %. The mortality rate in the control subgroup was 2.2 %.

DISCUSSION

In an international study by Malone Hani et al. [11] 7514 patients after STBs were analyzed. The most common complication of STB was surgically significant intracerebral bleeding, which was diagnosed in 5.8 % of patients. The risk factors for bleeding in this study were associated with age above 40 years, hydrocephalus and cerebral edema. Inpatient mortality according to the study was 2.8 % [11].

In other publications with different series of patients, the risk factors for bleeding after STB are not clearly defined or were associated by the authors with varying degrees of reliability, i. e. with the localization of a pathological focus, e. g. in the brain stem [8, 9], with arterial hypertension, with impaired liver function, with the malignant nature of a central nervous system tumor [12, 13].

According to K. K. Kukanov and co-authors, after performing the control CT neuroimaging, the presence of hemorrhages was noted in 40 % of the observations [14]. Of these, clinically insignificant ones were noted in 25 %, large diffuse hemorrhages with a clinical picture in 5 %, intraventricular hemorrhages with a pronounced clinical picture in 10 % of cases. Researchers see a reduction in the risk of intracranial hemorrhages after the tumor tissue STB procedure in careful preoperative planning of the biopsy trajectory, the use of modern stereotactic devices and biopsy cannulas, as well as the use of preoperative hemostatic therapy in patients with suspected high degree of tumor anaplasia [14].

In the article De Quintana-Schmidt C. with co-authors (2019) [15] published the results of a thrombin-gelatin matrix inmlication in three cases of intense bleeding during the STB procedure. Preliminary results of the work have shown that injection of a thrombin-gelatin matrix is a safe and effective procedure for the treatment of persistent surgical bleeding that cannot be performed by traditional hemostatic methods used in neurosurgery [12].

The presented studies do not provide a clear idea of how to improve the safety of performing stereotactic biopsy. The neurosurgeon faces urgent issues: what to choose as a reliable hemostatic agent, which method of administration to use, how to calculate the administered dose of the drug?

Based on the data of our study conducted from 2014 to 2020, a reliable method of hemostasis was

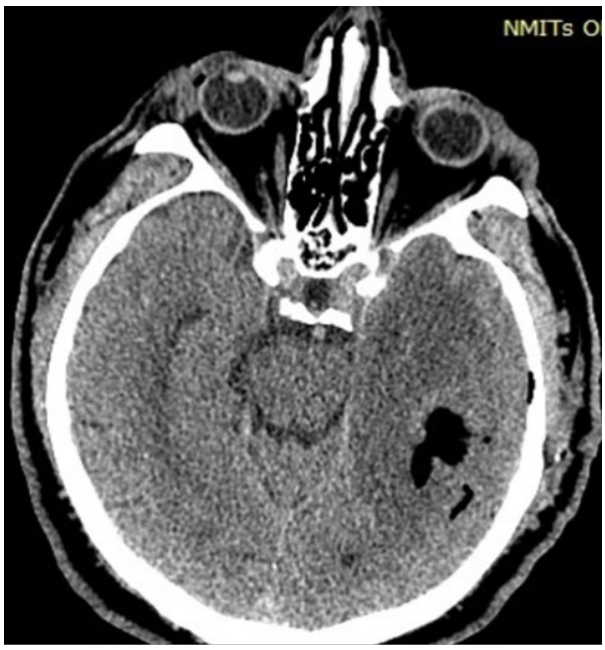

Fig. 6. Example of brain neuroimaging X-ray CT data 40 minutes after surgery in a patient using "Floseal®". A reduced density corresponding to the biopsy point is visualized in the left temporal lobe. No hemorrhage was found in the area of tumor tissue removal

found in a sufficient sample of patients using stereotactic techniques for collecting tumor material. We have proposed a technique for the introduction of a liquid hemostatic matrix as a preventive measure for intraoperative bleeding during STB.

In the course of our study, it was noted that there were no complications, clinically significant hemorrhages or microbleeds in the subgroup of patients with biopsy needle bleeding after the introduction of a liquid hemostatic matrix. On the contrary, in the subgroup of patients with the standard stereotactic biopsy procedure, in cases of intraoperative bleeding on a biopsy needle, macro and micro hemorrhages were observed in 60 % of patients, repeated surgical interventions were performed in 6.7 % of cases, and the mortality rate was 2.2 %.

CONCLUSION

If signs of bleeding from a biopsy needle appear during a stereotactic biopsy of a brain tumor, it is possible to inject a liquid hemostatic matrix in a volume of 2 ml into the point of removal of tumor tissue. The proposed method of preventing hemorrhages demonstrates the potential solution to the only serious type of complications in this minimally invasive diagnostic intervention. A liquid hemostatic matrix as intraoperative hemostasis method at the point of biopsy sampling following the stereotactic intervention helps to prevent the development or even stop bleeding intraoperatively, it also prevents the occurrence of hemorrhages and complications in the early postoperative period.

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Rostorguev E. E. – idea and research design development, analysis of the obtained data, writing the text of the manuscript;
Kuznetsova N. S. – collecting the clinical materials;
Kavitskiy S. E. – review of publications on the topic of the article;
Matevosyan B. V. – collecting the clinical materials;
Reznik G. A. – collecting the clinical materials;
Khatyushin V. E. – collecting the clinical materials, review of publications on the topic of the article;
Kit O. I. – development of the research design, critical revision with the introduction of valuable intellectual content, final approval of the published version of the manuscript.

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ORIGINAL ARTICLE

Experience in creating primary cultures of endometrial cancer and studying cells carrying phenotype of cancer stem cells

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ABSTRACT

Purpose of the study. Was to investigate the possibility of applying the method of spheroid formation in culture for assessment of the endometrial cancer (EC) tumor stem cells (TSC) content in complex samples containing various tumor cells and microenvironment.

Materials and methods. Primary cultures were obtained from fragments of tumors removed during surgery as a first stage of treatment at the Department of Gynecological Oncology, the National Medical Research Center for Oncology. After enzymatic disaggregation of tissue, cell suspension was passaged in DMEM medium containing 10 % fetal bovine serum and 1 % gentamicin to obtain primary two-dimensional cultures. To study the ability of cells to form spheroids, the primary culture was removed from the culture plate and passaged with 2.0×10^4 cells per well of a six-well plate (n = 6) in DMEM medium containing 0.35 % agarose and growth factors EGF (20 ng/ml) and FGF (20 ng/ml). After two weeks of cultivation, the average size, number of formed spheroids, and frequency of spheroid formation were determined. For those cultures that had formed spheroids, immunofluorescent staining of the two-dimensional culture for the marker CD133 was performed, after which the frequency of CD133+ cells was determined.

Results. A total of nine primary cultures of EC were obtained, five of which formed spheroids within two weeks of cultivation under non-adhesive conditions. In these cultures, small polygonal CD133+ cells showed the strongest association with spheroid formation, which were associated with the largest spheroids (98–110 μm in diameter).

Conclusion. There is a large number of microenvironmental cells in mixed cultures of CSC, some of which may express CD133, including healthy stem cells that also form spheroids in soft agar. A more detailed study of CSC subpopulations compared to normal endometrium is required to establish a link between the observed diversity of cells in culture and their ability to form spheroids and other characteristics of tumor stem cells.

Keywords: endometrial cancer, primary cell cultures, cancer stem cells, cell spheroids

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Compliance with ethical standards: the work followed the ethical principles set forth by World Medical Association Declaration of Helsinki, 1964, ed. 2013. The study was approved by the Committee on Biomedical Ethics at the National Medical Research Centre for Oncology (extract from the minutes of the meeting No. 25 dated 09/08/2022). Informed consent has been obtained from all participants of the study

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Conflict of interest: the authors declare that there are no obvious and potential conflicts of interest associated with the publication of this article

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3.1.6. Онкология, лучевая терапия
ОРИГИНАЛЬНАЯ СТАТЬЯ

Опыт создания первичных культур рака эндометрия и исследования в них клеток, обладающих фенотипом опухолевых стволовых клеток

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РЕЗЮМЕ

Цель исследования. Изучить возможность применения метода сфероидообразования в культуре для оценки содержания опухолевых стволовых клеток (ОСК) рака эндометрия (РЭ) в сложных образцах, содержащих различные клетки опухоли и микроокружения.

Материалы и методы. Первичные культуры получали из фрагментов опухолей, удаленных в ходе оперативного вмешательства, проводимого в качестве первого этапа по лечению РЭ в отделении онкогинекологии ФГБУ «Национальный медицинский исследовательский центр онкологии» Министерства здравоохранения Российской Федерации. После ферментативной дезагрегации ткани клеточную суспензию пассировали в среде DMEM, содержащей 10 % фетальной бычьей сыворотки (ФБС) и 1 % гентамицина, для получения первичных двумерных культур. Для изучения способности клеток к сфероидообразованию первичную культуру снимали с культурального пластика и пассировали по 2,0 × 10^4 клеток на лунку 6-тилуночного планшета (n = 6) в среду DMEM, содержащую 0,35 % агарозы и факторы роста EGF (20 нг/мл) и FGF (20 нг/мл). Через две недели культивирования определяли средний размер, количество образующихся сфероидов и частоту сфероидообразования. Для тех культур, которые образовали сфероиды, было проведено иммунофлуоресцентное окрашивание двумерной культуры на маркер CD133, после чего определяли частоту CD133+ клеток.

Результаты. Всего было получено девять первичных культур РЭ, из которых только пять образовали сфероиды спустя две недели культивирования в условиях, не поддерживающих адгезию. В этих культурах наибольшую связь со сфероидообразованием показали небольшие полигональные CD133+ клетки, с которыми ассоциировались наиболее крупные сфероиды (98–110 мкм в диаметре).

Заключение. В смешанных культурах РЭ присутствует большое количество клеток микроокружения, из которого часть клеток может экспрессировать CD133, в том числе нормальные стволовые клетки, также образующие сфероиды в мягком агаре. Требуется более подробное изучение клеточных субпопуляций РЭ в сравнении с нормальным эндометрием для установления связи между наблюдаемым разнообразием клеток в культуре и их способностью к сфероидообразованию и другими характеристиками ОСК.

Ключевые слова: рак эндометрия, первичные культуры, опухолевые стволовые клетки, клеточные сфероиды

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Соблюдение этических стандартов: в работе соблюдались этические принципы, предъявляемые Хельсинкской декларацией Всемирной медицинской ассоциации (World Medical Association Declaration of Helsinki, 1964, ред. 2013). Исследование одобрено Комитетом по биомедицинской этике при ФГБУ «Национальный медицинский исследовательский центр онкологии» Министерства здравоохранения Российской Федерации (выписка из протокола заседания № 25 от 08.09.2022 г.). Информированное согласие получено от всех участников исследования

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INTRODUCTION

Endometrial cancer (EC) is the sixth most common type of cancer in women. Over the past 30 years, the overall incidence of EC has increased by 132 %, reflecting an increase in the prevalence of risk factors, in particular, obesity and aging of the population [1]. In Russia, EC occupies the 2nd rank in the structure of oncological diseases of the female genital organs. The total number of patients with EC reached 195.6 per 100,000 population in 2022, which is 31.8 % more than in 2012 [2]. Thus, there is still a need to develop new therapeutic approaches to significantly improve the prognosis in women with recurrent EC or in the later stages of the disease. In this context, tumor stem cells (TSC) of endometrial cancer, capable of self-renewal and differentiation into mature tumor cells, as well as contributing to tumor recurrence, metastasis, heterogeneity, multidrug and radiation resistance, represent a potential target for drug development [3].

TSC were first identified in patients with acute myeloid leukemia in 1994, and have since been considered potential therapeutic targets in the treatment of oncological diseases, including solid tumors [4]. TSC in EC were first described by Hubbard in 2009 [5]. The discovery of endometrial OSCs has radically changed the views on the study of the biology of EC and the development of approaches to the treatment of this disease. As a rule, EC TSC is identified by the expression of specific antigens, by the weak accumulation of the Hoechst 33342 nuclear dye (the so–called side population – "side population"), by the ability to form colonies under conditions of reduced adhesion and initiate the growth of a tumor containing TSC and differentiated cells originating from them with the same phenotype as "parental", in mice with immunodeficiency [3].

A number of markers associated with the TSC of solid tumors have been studied in EC. Aldehyde dehydrogenase 1 (ALDH1) is one of 19 different enzymes involved in aldehyde oxidation. This enzyme is highly active in the early stages of stem cell differentiation. Atypical EC cells with a high level of ALDH1 expression are more tumorogenic, invasive and resistant to cisplatin than cells with a low level of ALDH1 expression. Also, a high level of ALDH1 expression correlates with a worse prognosis in patients with EC [6]. Receptor tyrosine kinase c-Kit or CD117 is a receptor for the Stem Cell Factor (SCF) and, upon activation, triggers a number of intracellular signaling cascades regulating cell survival, migration and proliferation, including TSC [7]. When studying the cellular composition of EC, it was shown that $CD117^+$ cells isolated from Ishikawa and MFE280 EC cultures exhibit greater proliferative ability, as well as the ability to form colonies in soft agar in the presence of SCF. A high level of CD117 expression was also recognized as an independent prognostic factor correlating with the progression of EC [8]. The CD55 antigen is a complement decay acceleration factor and is expressed at a high level in the TSC of endometrioid ovarian cancer and EC. It has been shown that $CD55^+$ cells are able to regulate cell self-renewal and their resistance to cisplatin to a greater extent than CD55 cells [9]. A link with TSC has also been established for the transmembrane glycoprotein CD44, which plays the role of an adhesion molecule. Cells overexpressing CD44 possess such characteristics of TSC as the ability to self-renew and epithelial-mesenchymal transition (EMF), as well as resistance to chemotherapy and radiation therapy [10]. Probably, this marker is also related to TSC EC, since oncospheres obtained on soft agar from cells of EC cultures with stem properties are CD44 positive [3]. In addition, a number of studies have noted the co-expression of CD44 and another TSC marker CD133 in the EC tissue [11, 12]. The transmembrane glycoprotein of the cell surface prominin-1 or CD133 has attracted considerable attention due to the fact that its expression is often observed in various subpopulations of somatic stem cells. Usually, this glycoprotein is observed in the area of various microvilli and protrusions of the plasma membrane, where CD133 can act as a regulator of the lipid composition of membranes or participate in the mechanisms of cellular polarity and migration [13]. In a study by Rutella et al. (2009) subpopulations of cells with the $CD133^+/CD44^+$ phenotype isolated from permanent endometrial cancer cell lines showed the ability to form tumor spheres, increased chemoresistance and were able to initiate the formation of a tumor with the same phenotype as the original tumor when transplanted to immunodeficient mice [14]. Gao (2012) also investigated the AN 3CA line and showed that $CD133^+$ cells express stem markers, demonstrate greater mobility and invasive ability than CD133 cells [15]. In Friel's work, the expression of CD133 in the cells of primary EC tumors and the mechanism for

controlling the expression of this marker in them were investigated [16]. The authors showed that $CD133^+$ cells accounted for 5.7–27.4 % of the total population of tumor cells in the analyzed primary tumors. These cells had increased tumorigenicity in immunodeficient mice, which suggests that these cells belong to the TSC. Similar results were obtained in the work of Sun (2017), where $CD133^+CD44^+$ cells showed a stronger association with all the classical properties of TSC than for other markers studied in the work, such as CD24, CD47, CD29, CXCR4, SSEA3 and SSEA4. The efficiency of spheroid formation in soft agar was 11.7 % for $CD133^+$ cells and 1.7 % for CD133 cells [17]. In addition, $CD133^+CD44^+$ cells showed an increased expression of stem cell transcription factors Myc, Sox-2, Nanog and Oct4 compared with other subpopulations [16], for which a direct relationship with the degree of malignancy of endometrioid carcinoma was established [18].

Despite the established connection between CD133 and EC TSC in a number of studies, data on the relationship between the expression of this marker and the prognosis of the disease course remain quite contradictory. Thus, the work of Elbasateeny (2016) indicates a more pronounced association of CD133 expression with the early stage of the tumor (I–II) and a decrease in the expression of this marker at later stages of the disease. The authors suggested that CD44 and CD133 may be involved in the development of endometrial cancer in the early stages, and their overexpression may contribute to the early diagnosis of endometrial cancer [11]. These results are supported by the data obtained in Mancebo (2017), in which the authors found that tumors in which CD133 expression was high were less likely to have vascular invasion and more likely to be highly differentiated, and were also associated with higher overall and recurrence-free survival [19]. However, there is also the opposite data. Thus, Nakamura (2010) showed a negative correlation between CD133 expression in tumor tissue and life expectancy without recurrence [20]. The negative prognosis of the course of EC with increased CD133 expression in tumor tissue was confirmed in the work of Park in 2019 [12]. The observed discrepancies in the data of different authors may be related to the fact that CD133 expression is observed not only in the EC TSC, but also in normal cells of the glandular epithelium of the endometrium [21]. Thus, CD133 in endometrial tissue can act simultaneously as a marker of epithelial differentiation and as a marker of OSC.

To study the dynamics, functioning and regulation of stem cells, experimental methods are needed to clearly distinguish between stem cells and their offspring. Due to the lack of unique cell surface markers specific only to stem cells and a distinct morphological phenotype, stem cells are usually identified based on functional criteria. Stem cells from various tissues are usually cultured *in vitro* in the form of spheroids under conditions excluding adhesion [22]. According to the literature data, the study of TSC in EC by the method of counting spheroids in conditions that do not support cell adhesion, both on simple samples obtained after sorting by any marker [14, 17] and in whole samples of the primary culture of EC [23]. In the latter case, the quantitative analysis was reduced to measuring the size of spheroids, while the assessment of the quantitative content of TSC and the establishment of a link with the clinical and pathological characteristics of the sample was not carried out.

The purpose of the study: to study the possibility of using the method of spheroid formation in culture to assess the content of EC TSC in complex samples containing various tumor cells and microenvironments. To achieve this goal, we compared the morphological characteristics of primary EC cultures under conditions of adhesive growth and in soft agar, and also studied the expression of the CD133 marker in two-dimensional cultures.

MATERIALS AND METHODS

Nine primary cultures of EC were obtained from fragments of tumors removed during surgery performed as the first stage of the treatment of EC. Patients with EC were treated in the Oncological Gynecology department of the National Medical Research Centre for Oncology, in 2023. The histological diagnosis was confirmed in the pathoanatomical department of the of the National Medical Research Centre for Oncology. The patients were aware of their participation in the scientific study and signed an informed consent for the collection of biological material. The pathologist isolated a 0.5 cm^3 fragment corresponding to the malignant component of the tumor within 20 minutes after extraction of

the drug and placed it in a Hanks solution (Gibco, USA) containing 1 % gentamicin (Biolot, Russia). Next, the sample was fragmented with a scalpel to a size of 1–2 mm^3, after which 300 u/ml of collagenase I (Thermo Fisher Scientific, USA) was added in a DMEM medium (Biolot, Russia) and incubated for an hour at a temperature of 37 °C with constant stirring. At the end of fermentation, the sample was additionally crushed by pipetting and passed through a sterile nylon filter (d = 70 μm) (Beckton Dickinson, USA). The resulting suspension was washed twice in a phosphate buffer and passed onto a culture vial in a DMEM medium containing 10 % FBS (Biolot, Russia) and 1 % gentamicin and cultured under standard conditions at 37 °C and 5.0 % CO_2.

To study the ability of cells to spheroid formation, a two-dimensional culture was removed from culture plastic using a standard technique using a 0.1 % trypsin solution (Biolot, Russia). The resulting cell suspension was mixed with 0.35 % agarose solution in a serum-free DMEM medium with the addition of insulin-transferin and growth factors EGF (20 ng/ml) and FGF (20 ng/ml) and layered on a base of 0.7 % agarose in the same medium. In total, 2.0 × 104 cells were added in this way to the well of a 6-hole tablet. There were 6 repetitions for each culture. The plates with cells were cultured for 2 weeks, during which time the spheroids were photographed. At the end of cultivation, the average size was determined and the spheroids in the well were counted using a Lionheart FX imager (BioTek, USA) using embedded software. The frequency of spheroid formation was calculated as the ratio of the number of spheroids larger than 40 μm in diameter to the total number of cells passioned into the well of the tablet.

To carry out immunophenotyping on CD133, primary cultures were planted on cover glasses. After the formation of the cellular monolayer, the glasses were fixed in a 4 % paraformaldehyde solution for 15 minutes at room temperature, after which permeabilization was carried out in a 0.5 % Triton X-100 solution on a phosphate buffer for 10 minutes. After washing, the glass was blocked for an hour in a solution of 5 % normal goat serum (Gibco, USA) in a phosphate buffer, after which it was kept overnight at 4 °C in a solution of primary polyclonal rabbit antibody to CD133 (ab19898, Abcam, USA) in a phosphate buffer (1/100 dilution) with the addition of 1 % goat serum. After washing the glass three times, it was kept at room temperature for an hour in a solution of secondary goat antibody conjugated with Alexa Flour®594 (ab150080, Abcam, USA) (1/500 dilution), after which they were washed with a phosphate buffer, repainted in a solution of the nuclear dye DAPI (ab228549, Abcam, USA) and mounted on slides in an Anti-Fade Fluorescence Mounting Medium (ab104135, Abcam, USA). The proportion of CD133 positive cells was determined on the Lionheart FX imager (BioTek, USA) using embedded software. 3 glasses were examined for each culture.

The values of the diameter of the spheroids, the frequency of spheroid formation and the proportion of $CD133^+$ cells are given as a sample mean ± standard deviation.

STUDY RESULTS

We received 9 primary cultures of EC from 9 patients who were treated in the Department of Oncological Gynecology at the of the National Medical Research Centre for Oncology in 2023. The results of the pathological examination showed that 2 tumors belonged to the histological type of highly differentiated (G1), 6 to moderately differentiated (G2) and one tumor to the low–differentiated type G3 of endometrioid adenocarcinomas. At the same time, lympho-vascular invasion was detected in three cases, but none of the patients had metastases in regional lymph nodes. All cases were attributed to stage I–II of the disease.

All primary cell cultures formed a monolayer on day 3 of cultivation under conditions that support adhesion. The EC cells in the monolayer culture were relatively small in size and polygonal in shape, often forming "islands" and "rosettes" that were located among the more elongated stroma cells, presumably fibroblasts. When cultured in conditions that do not support cell adhesion on agarose in the medium for TSCs, the formation of spheroids was noted in 5 out of 9 cultures, starting from the 5th day of cultivation. In four other cultures, spheroids did not form even after 2 weeks of cultivation. 1 culture obtained from a highly differentiated tumor and 3 cultures from moderately differentiated tumors did not form spheroids. Thus, there was no obvious relationship between the frequency of spheroid formation and the degree of tumor differentiation. For cultures that formed spheroids in soft agar, additional staining of

the two-dimensional culture on the CD133 marker was performed.

EC cell culture No. 1. Highly differentiated G1 endometrioid adenocarcinoma without signs of vascular invasion. In the primary culture, single large cells of polygonal or rounded shape were observed among numerous elongated cells, presumably fibroblasts (Fig. 1A).

CD133 expression is weak, and rare marker granules are observed in all cells (Fig. 1b). The frequency of $CD133^+$ cells ranged from 0.2 to 1.0 % (0.8 ± 0.15 %). In the culture in agarose, a very slow growth of cellular spheroids from single large cells was observed. On the fifth day of cultivation, spheroids had not yet been detected (Fig. 1B), and two weeks later small spheroids of 4–8 cells were formed (Fig. 1G). The frequency of spheroid formation on the 14th day ranged from 0.1 to 0.7 % (0.5 ± 0.1 %).

EC cell culture No. 2. Low-grade G3 endometrioid adenocarcinoma without signs of vascular invasion. Under cultivation conditions supporting cell adhesion, the cells of the primary culture formed a monolayer of two types of cells, similar in appearance to the culture of EC No. 3 (Fig. 3A): small polygonal cells united in islands among larger and elongated cells similar to fibroblasts (Fig. 2A).

At the same time, islands of polygonal cells could form spheroid-like structures, within which increased CD133 expression was observed. Also, among the monolayer, there were individual large cells expressing CD133 above the general level (Fig. 2B). The frequency of $CD133^+$ cells ranged from 2.7 to 8.0 % (4.8 ± 1.5 %). On the fifth day of cultivation in conditions that do not support adhesion, in the medium

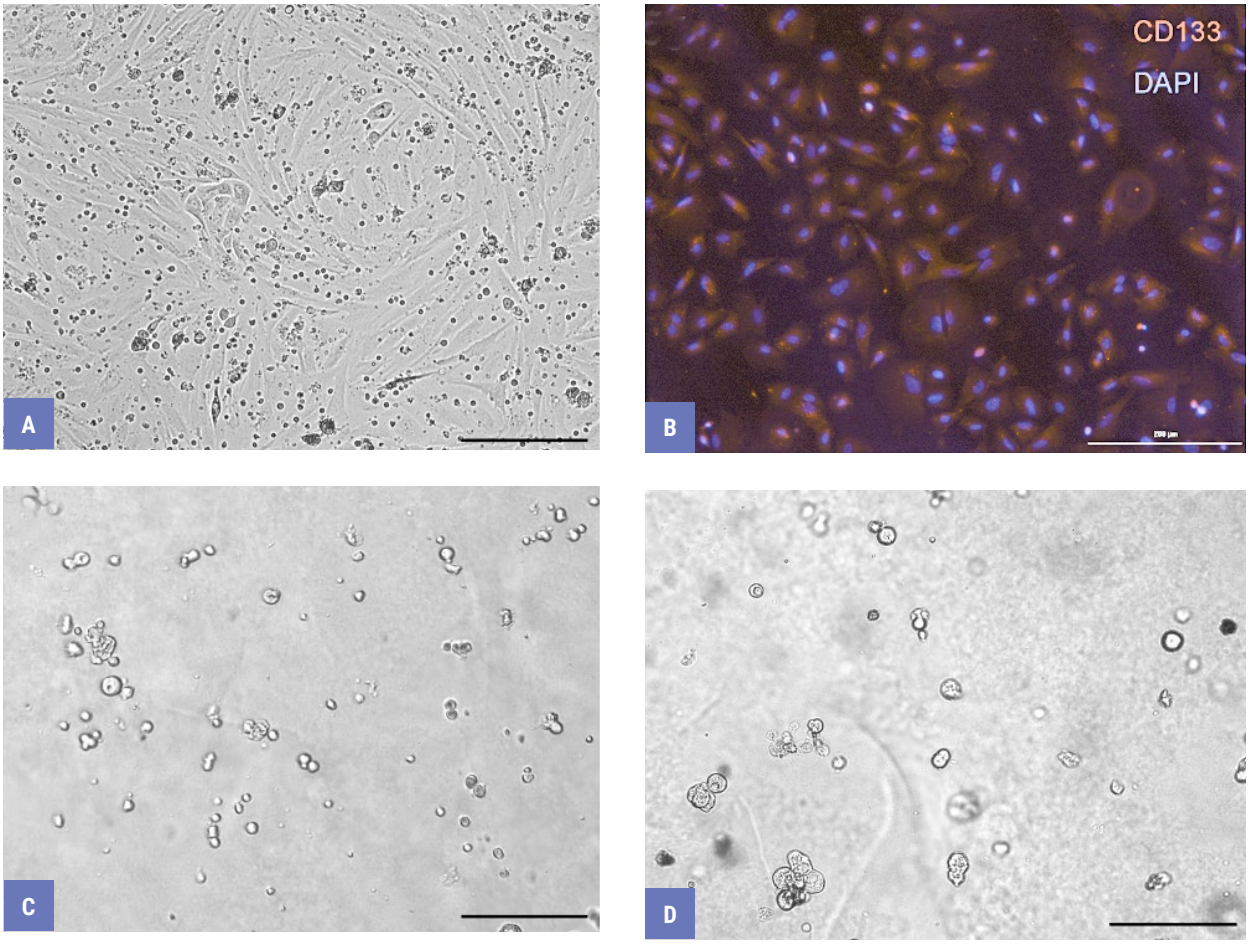

Fig. 1. Primary culture of endometrial cancer No. 1. A – general view of monolayer culture; B – staining of monolayer culture on CD133; C – view of cellular spheroids in agarose on the 5th day of cultivation. G – a type of cellular spheroids in agarose on the 14th day of cultivation. The size of the scale ruler is 200 μm

for TSC, the culture of EC No. 3 formed small cellular spheroids of 4–8 cells with a frequency of about 15 % (Fig. 2B). Further, some of the cell spheroids showed rapid growth and after two weeks of cultivation reached 80–150 μm in diameter (110.3 ± 32.7 μm). The frequency of spheroid formation on the 14th day ranged from 0.3 to 2.5 % (1.5 ± 0.8 %) (Fig. 2G).

EC cell culture No. 3. Moderately differentiated G2 endometrioid adenocarcinoma with signs of vascular invasion. The culture in the monolayer had a pronounced division into small polygonal cells, united into islands among larger and elongated cells similar to fibroblasts (Fig. 3A).

Immunofluorescence staining showed an increased content of the CD133 marker in small polygonal cells, while individual cells showed a particularly bright label (Fig. 3B). The frequency of $CD133^+$ cells ranged from 5.8 to 16.9 % (11.2 ± 5.2 %). When grown on agarose in a medium for TSC on the 5th day in culture, about 15 % of the cells formed spheroids 5–30 μm in diameter (Fig. 3C). After two weeks of cultivation in agarose, individual cellular spheroids increased in size to 30–150 μm in diameter, the remaining spheroids degraded (98.3 ± 51.4 μm). The frequency of spheroid formation on day 14 ranged from 0.3 to 10.1 % (5.7 ± 4.1 %) (Fig. 3D).

EC cell culture No. 4. Moderately differentiated G2 endometrioid adenocarcinoma without signs of vascular invasion. In the monolayer culture, large polygonal cells were observed separately or assembled in small groups against the background of elongated cells of various sizes (Fig. 4A).

When stained with the CD133 EC TSC marker, a positive reaction was shown not only by large po-

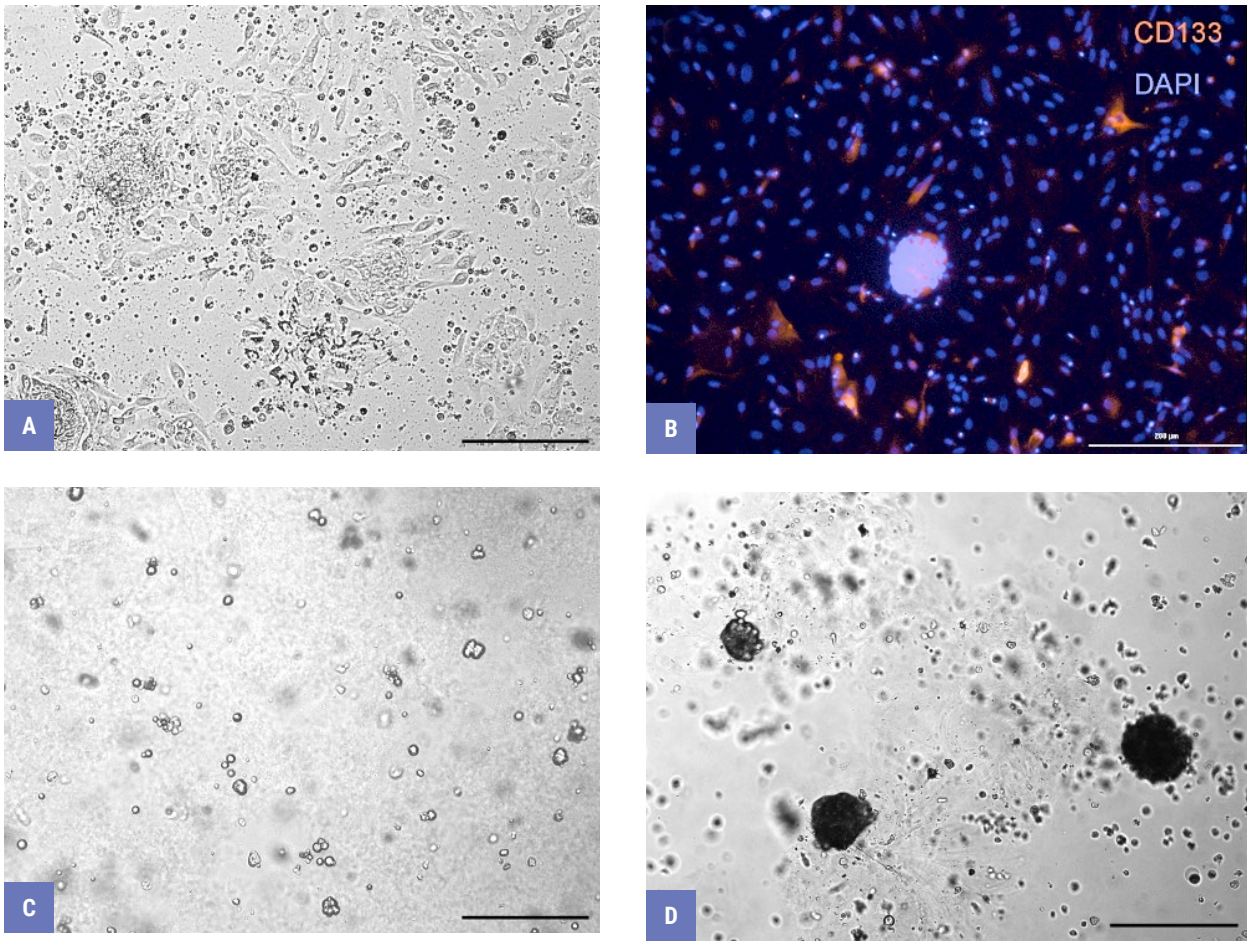

Fig. 2. Primary culture of endometrial cancer No. 2. A – general view of the monolayer culture; B – staining of the monolayer culture on CD133; C – type of cellular spheroids in agarose on the 5th day of cultivation; D – type of cellular spheroids in agarose on the 14th day of cultivation. The size of the scale ruler is 200 μm

lygonal cells and their clusters, but also by numerous small elongated cells. Large multinucleated cells positive for CD133 were also found in the preparation (Fig. 4B). The frequency of CD133⁺ cells ranged from 40.1 to 65.2 % (average 52.2 ± 10.2 %). After five days of cultivation in agarose, about 5–10 % of the culture cells formed small loose spheroids of 4–16 cells (Fig. 4C), two weeks later the spheroids increased to 20–80 μm in diameter (54.7 ± 28.4 μm), the frequency of spheroid formation on the 14th day ranged from 2.1 to 8.4 % (5.1 ± 2.7 %) (Fig. 4D)

EC Culture No. 5. Moderately differentiated G2 endometrioid adenocarcinoma without signs of vascular invasion. In the monolayer culture, islands of rather large polygonal cells of epithelial morphology were found among elongated cells of stromal origin (Fig. 5A).

The reaction to CD133 was similar to the EC culture No. 2, namely, positive staining was demonstrated by individual large cells of elongated or epithelial morphology with a certain group of small elongated cells, which, due to staining, stand out against the background of larger elongated cells negative for CD133 (Fig. 5B). The frequency of CD133⁺ cells ranged from 22.4 to 51.2 % (35.5 ± 12.7 %). On the fifth day of cultivation in conditions that do not support adhesion, EC culture No. 5 formed small cellular spheroids of 2–4 cells with a frequency of about 5 % (Fig. 5C). Further, some of the cell spheroids showed rapid growth and reached 30–80 μm in diameter after two weeks of cultivation (average 55.4 ± 25.1 μm). The frequency of spheroid formation on day 14 ranged from 0.5 to 2.8 % (1.8 ± 0.9 %) (Fig. 5D).

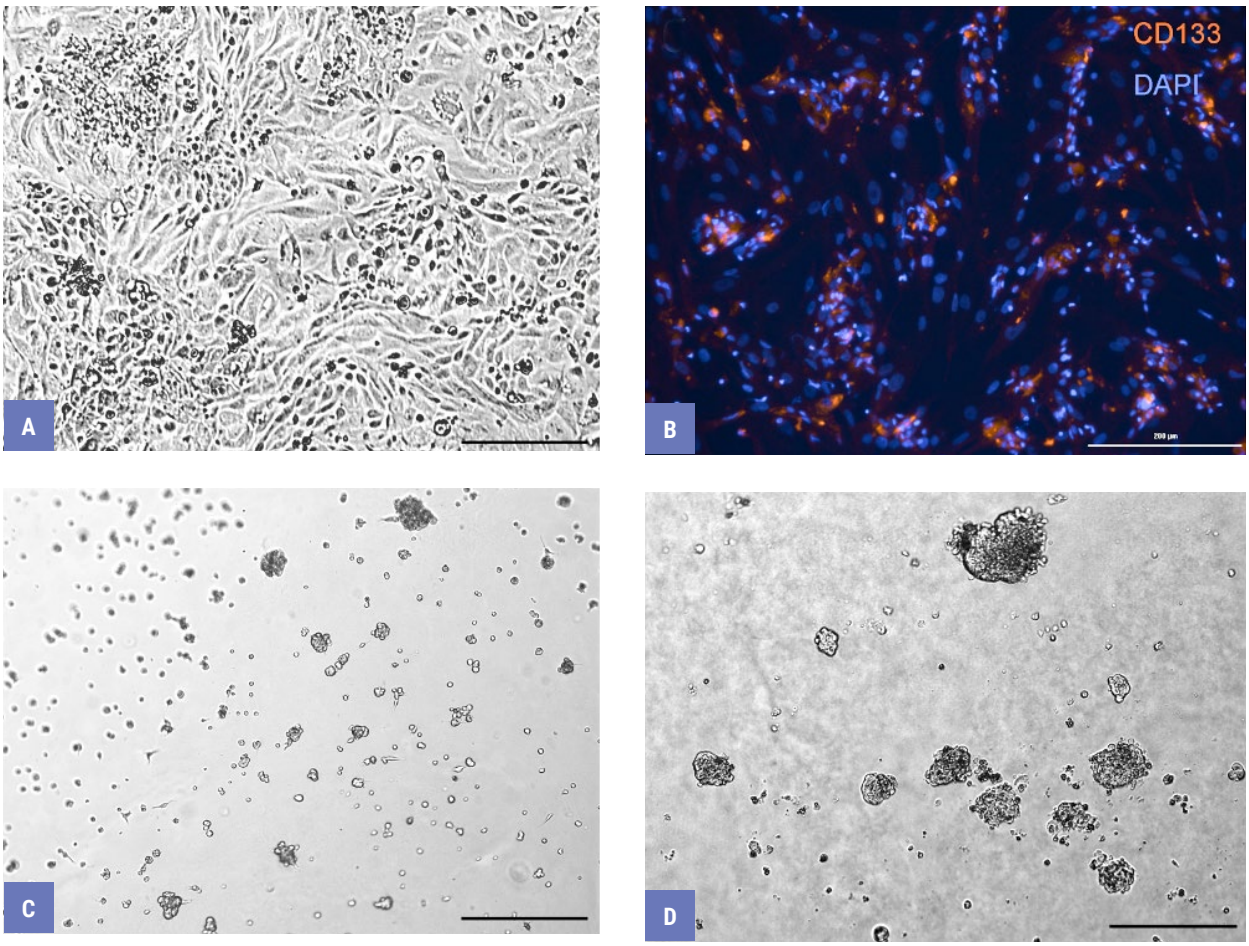

Fig. 3. Primary culture of endometrial cancer No. 3. A – general view of the monolayer culture; B – staining of the monolayer culture on CD133; C – type of cellular spheroids in agarose on the 5th day of cultivation; D – type of cellular spheroids in agarose on the 14th day of cultivation. The size of the scale ruler is 200 μm

The characteristics of primary EC cultures are combined in the table (Table 1).

The lowest frequency of spheroid formation, as well as the lowest frequency of $CD133^+$ cells, were observed in a culture obtained from a highly differentiated tumor.

DISCUSSION

In all the obtained primary cultures, the presence of a stromal component is noted, represented by elongated cells of different sizes, demonstrating a negative (No. 1, No. 2, No. 3) up to medium and high CD133 expression (No. 4, No. 5). In addition, polygonal cells are noted in cultures, which occur as islands among stromal cells. Their sizes vary between cultures, and on this basis, cultures can be divided into two groups – including small polygonal cells that assemble into dense colonies-"domes" (cultures No. 1 and No. 2), and including larger polygonal cells that can form flat islands of different sizes (cultures No. 1, No. 4 and No. 5). The expression of the CD133 marker in these cells is quite pronounced, especially against the background of a weakly colored stromal component consisting of large cells (cultures No. 4 and No. 5).

The presence of elongated or process-shaped cells resembling fibroblasts in primary EC cultures and capable of forming spheroids under conditions that do not support cell adhesion was also noted in the work of Helweg (2022) [23]. The picture of the composition of cell cultures obtained by us is also similar in cell morphology to the results obtained in the work of Chan et al. (2004) [24]. In this study, the

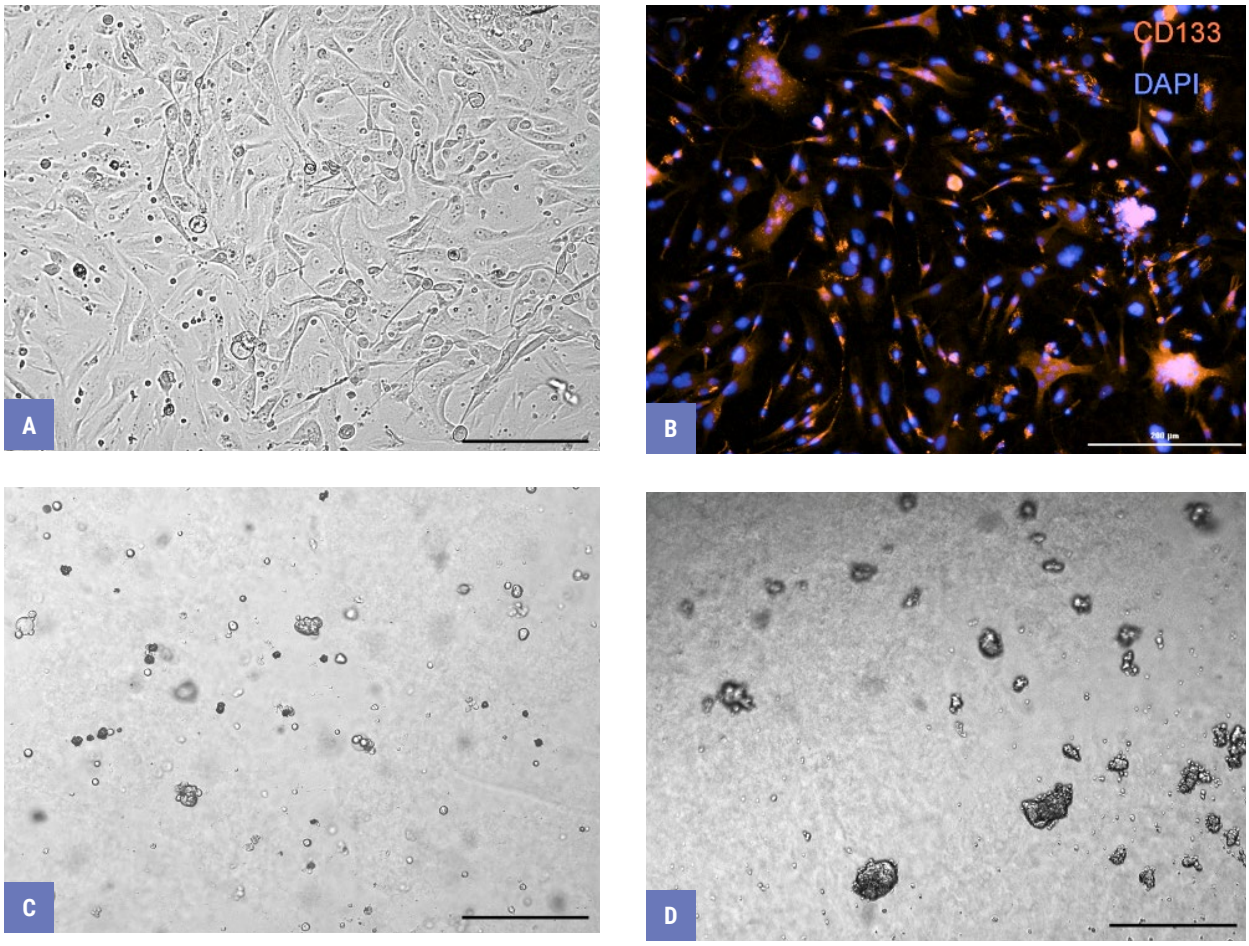

Fig. 4. Primary culture of endometrial cancer No. 4. A – general view of the monolayer culture; B – staining of the monolayer culture on CD133; C – type of cellular spheroids in agarose on the 5th day of cultivation; D – type of cellular spheroids in agarose on the 14th day of cultivation. The size of the scale ruler is 200 μm

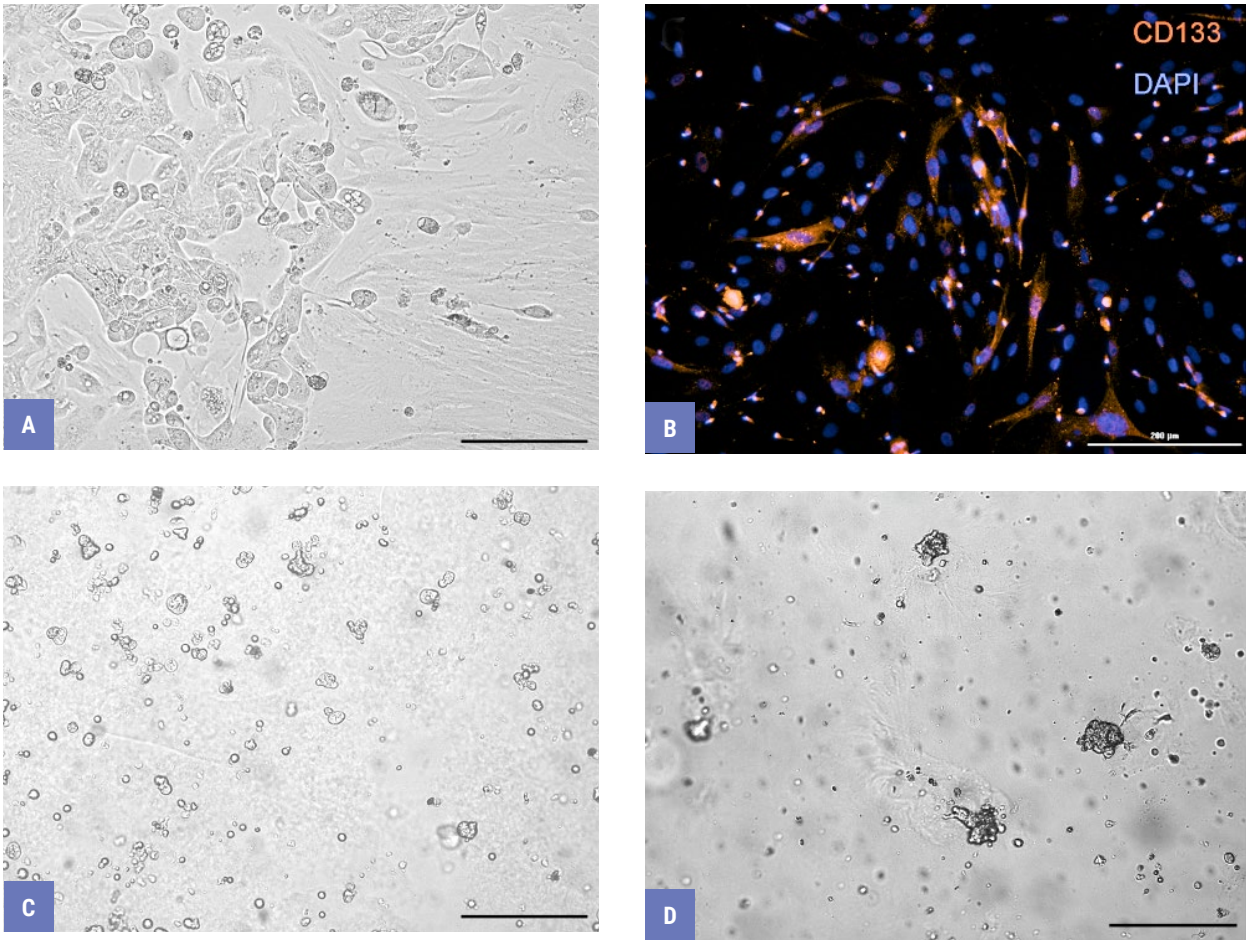

Fig. 5. Primary culture of endometrial cancer No. 5. A – general view of monolayer culture; B – staining of monolayer culture on CD133; C – type of cellular spheroids in agarose on the 5th day of cultivation; D – type of cellular spheroids in agarose on the 14th day of cultivation. The size of the scale ruler is 200 μm

Table 1. Summary characteristics of primary endometrial cancer cultures

Culture No.	Grade	Lympho-vascular invasion	Reaction to CD133, proportion of CD133+ cells, mean ± SD %	The frequency of spheroids ≥ 40 μm per 14 days, average ± SD, %	The diameter of the spheroids for 14 days, average ± SD, μm
No. 1	G1	n/p	0.8 ± 0.15 %, weak reaction in some large stromal cells, single polygonal cells with bright coloration	0.5 ± 0.1	20 ± 10
No. 2	G3	n/p	4.8 ± 1.5 %, small polygonal cells in dense colonies, individual stromal cells	1.5 ± 0.8 %	110.3 ± 32.7
No. 3	G2	p	11.2 ± 5.2 %, small polygonal cells in dense colonies	5.7 ± 4.1 %	98.3 ± 51.4
No. 4	G2	n/p	52.2 ± 10.2 %, polygonal process large multinucleated cells, stromal small elongated cells	5.1 ± 2.7 %	54.7 ± 28.4
No. 5	G2	n/p	35.5 ± 12.7 %, separate large and small elongated stromal cells, separate large polygonal cells	1.8 ± 0.9 %	55.4 ± 25.1

Note: not present – n/p, present – p

authors studied the behavior in culture of various cells of the normal human endometrium isolated from the epithelial and basal layers [24]. According to the authors, the epithelial layer gave two groups of polygonal cells – small cells that gather in close colonies with a high ability to proliferate, and larger cells that form looser colonies on cultural plastic. The stromal component of endometrial tissue consisted of two types of elongated cells: large loosely lying cells and small closely lying cells, giving curls in a monolayer. In addition, human endometrial stromal cells were positively stained for fibroblast markers (CD90, 5B5, type I collagen), which confirms the relationship of these cells to fibroblasts and, possibly, myofibroblasts, which express markers of both fibroblasts and smooth muscle cells. Thus, based on the picture of the two-dimensional culture obtained in our work, it is impossible to unambiguously identify which cells belong to the TSC, since externally they are little distinguishable from normal endometrial cells.

The expression of the TSC CD133 marker in the cultures obtained by us ranged from 0.8 to 52.2 % on average. The data obtained go beyond the range of 5.7–27.4 % indicated by other authors [16, 17]. A nonlinear relationship between CD133 expression and the degree of tumor differentiation was also revealed – both high and low differentiation were associated with reduced immunoreactivity on CD133 compared with average differentiation. No connection was found between the level of CD133 expression, the degree of differentiation and other clinical and pathological characteristics of the tumor also in Nakamura [20]. CD133 is known to occur in normal differentiated endometrial cells. Thus, based on existing data, it is impossible to determine to what extent CD133 expression reflects the degree of cell malignancy in primary EC cultures and the level of their differentiation. To solve this problem, more extensive studies are required, including a comparison of normal endometrial tissue and RE.

The frequency of spheroid formation in our cultures ranged from 0.5 to 5.7 % on average. It is known that the frequency of cells with SC properties in the normal endometrium is 0.02–0.1 % [24]. It would not be too much of an assumption to accept these levels as indicative for estimating the frequency of spheroid formation in cultures of normal endometrium. In the scientific literature, we have not found data on the frequency of spheroid formation in conditions that do not support cell adhesion in whole EC cultures, since individual subpopulations purified by marker expression or nuclear dye retention are usually the object of research. However, taking into account the fact that the frequency of spheroid formation is the highest among $CD133^+$ EC cells and amounts to 11.7 % in this subpopulation [17], as well as the fact that the total content of these cells in EC ranges from 5.7 to 27.4 % [16], we can multiply these indicators to obtain an approximate the frequency of spheroid formation in a mixed culture of EC, which in this case will be 0.7–3.2 %. Thus, our results lie in the range corresponding to the known data on EC, therefore, the main part of the spheroids in our cultures is presumably formed by malignant cells with the TSC phenotype.

Our data show that in EC cultures, the overall response to CD133 does not show a clear relationship with the frequency of spheroid formation. Thus, in cultures No. 4 and No. 5, the frequency of spheroid formation is an order of magnitude lower than the content of $CD133^+$ cells. In this regard, the question arises as to which cells are the sources of spheroids in EC cultures? Comparison with the morphological features and phenotype of the normal endometrium [21, 24, 25] indicates a number of patterns. Thus, in culture No. 1, obtained from a highly differentiated tumor, in a monolayer we see large cells similar to the limited dividing progenitor cells of the stromal and epithelial components of the normal endometrium [24]. The expression of the CD133 marker in these cells is very weak, while this culture formed small lymphoid (loose) spheroids on soft agar with a frequency of less than 1 %. Lymphoid colonies in conditions that do not support adhesion can form immune cells, namely, T lymphocytes and NK in the presence of specific cytokines such as IL-2, IL-15 or IL-7 [26]. Nevertheless, their characteristic appearance can be considered a reliable difference between lymphocytes and other cells – even in a stimulated state, these are small cells (6–15 μm) with a high nuclear-cytoplasmic ratio [27]. In our case, the cells in the colonies have larger sizes (about 40 μm in diameter), which means that they are not lymphocytes with a high probability. At the same time, the spheroids of culture No. 1 are similar to the lymphoid colonies that were obtained from the EC tissue in Tabuchi's work [28]. Culture No. 2, obtained from a low-grade tumor (G3), also contains cd133 fibro-

blast-like cells, however, a subpopulation of small polygonal cells stands out well against their background, demonstrating bright immunoreactivity on CD133, which morphologically most resembles cells with stem properties, in the work of Chan et al. [24]. Also in this culture, when grown on soft agar, large (about 90 μm in diameter) spheroids were observed, albeit with a small frequency (about 1.5 %). Despite the fact that no special marker selection was carried out, it would be a small assumption to assume that these CD133⁺ cells form spheroids, since large fibroblast-like cd133 cells did not produce similar large spheroids in culture No. 1. Confirmation of this assumption may also be the fact that culture No. 3, similar in phenotype to culture No. 2, with a subpopulation of small CD133⁺ cells forming dense colonies clearly standing out against the background of a weakly colored stromal component, also formed large (about 115 μm in diameter) spheroids in soft agar with a frequency of about 10 %. Another source of spheroids may be small spindle-shaped cells brightly colored on CD133, found in cultures No. 5 and No. 4. The morphological features of these cells correspond to tissue stem cells, of which CD133 express, for example, epithelial or endothelial SC, which are normally present in the endometrium [30, 31]. According to the literature data, the size and appearance of colonies formed on soft agar by such normal stem cells do not differ from spheroids obtained from TSC [31]. Finally, for a subpopulation of relatively large polygonal CD133⁺ cells that are present in cultures No. 4 and No. 5, the connection with spheroids cannot be traced, although these cells can be confidently attributed to the malignant component, since they sometimes show signs of multinucleation. It can also not be argued that CD133-negative cells do not produce spheroids on soft agar, since in the work of Ding [29], in particular, they showed that both CD133⁺ and CD133 cells sorted from endometrial tumor tissue by this marker can form spheroids, but in the second case less effectively.

Thus, it is impossible to unambiguously determine which of the cells of the primary EC cultures obtained by us took part in the formation of spheroids. Some presumptive relationship can be established only for small epithelial and fibroblast-like CD133⁺ cells, of which only the first can be conditionally classified as malignant. To identify exactly TSC EC, it is necessary to conduct a comparative study of cell cultures on soft agar obtained from tumor tissue and the corresponding normal tissue, in combination with cell sorting using markers CD133, CD44, CD117, CD24, CD47 and others, for which a connection with TSC and normal stromal stem cells has been established [32].

CONCLUSION

We were able to obtain and characterize the culture of cellular spheroids from the postoperative EC material. However, the indicators of the frequency of spheroid formation and the average size of spheroids in this culture cannot serve as a marker of the amount of TSC in tumor tissue without comparing these data for tumor tissue and normal endometrium. A more detailed study of the cellular subpopulations of EC in comparison with normal endometrium is required to establish a link between the observed diversity of cells in culture and their ability to spheroid formation and other characteristics of TSCs.

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ORIGINAL ARTICLE

Prognostic factors influencing survival rates in elderly patients with metastatic renal cell carcinoma

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ABSTRACT

Purpose of the study. To determine the influence of prognostic factors on survival rates in patients with mRCC aged ≥ 75 years.
Materials and methods. A retrospective study included 77 mRCC patients aged ≥ 75 years who received systemic therapy at the Municipal Oncologic Hospital No. 62 in Moscow and the Municipal Oncologic Dispensary in St. Petersburg from 2006 to 2019. Clinical data from medical records were obtained and analyzed retrospectively, all patients underwent clinical, laboratory, and pathomorphological examination. Patients' survival rates were evaluated using the statistical method of survival time analysis (Survival Analysis). Descriptive characteristics of survival time were calculated in the form of life tables, and Kaplan-Meier curves were constructed.
Results. In the present study, a favorable prognosis according to International Metastatic Renal Cell Carcinoma Database Consortium (IMDC)was noted in 20.8 % of patients with mRCC aged ≥ 75 years; 6.5 % had solitary metastases. The 3- and 5-year survival rates were 35.8 % and 21.2 %.
In single-factor analysis in mRCC patients ≥ 75 years of age, it was found that ECOG status ($p < 0.001$), histological subtype ($p = 0,01$), Fuhrman grade of tumour differentiation ($p = 0.003$), type of metastases ($p = 0.045$), liver metastases ($p < 0.001$), IMDC prognosis ($p = 0.042$) and nephrectomy ($p = 0.014$).
Conclusion. In a multivariate analysis, factors affecting survival in patients with mRCC aged ≥ 75 years included sex, histologic subtype, number of metastases, bone and lymph node metastases, IMDC prognosis, and radiation therapy and nephrectomy. Further studies are needed to identify additional personalized prognostic factors in elderly patients with metastatic renal cell carcinoma (mRCC).

Keywords: metastatic renal cell carcinoma, overall survival rate, elderly patients

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Compliance with ethical standards: the study adhered to the ethical principles outlined in the World Medical Association Declaration of Helsinki (1964, revised in 2013). The research was approved by the Ethics Committee of the Saint Petersburg City Clinical Oncological Dispensary (protocol No. 2356 dated May 20, 2022). Informed consent was obtained from all study participants

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ОРИГИНАЛЬНАЯ СТАТЬЯ

Прогностические факторы, влияющие на показатели выживаемости у больных старческого возраста с метастатическим почечноклеточным раком

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РЕЗЮМЕ

Цель исследования. Определить влияние прогностических факторов на показатели выживаемости у пациентов с метастатическим почечно-клеточным раком (мПКР) в возрасте ≥ 75 лет.
Материалы и методы. В ретроспективное исследование были включены 77 пациентов с мПКР в возрасте ≥ 75 лет, которые получали системную терапию на базе Городской онкологической больнице № 62 г. Москвы и Городском онкологическом диспансере г. Санкт-Петербурга с 2006 по 2019 гг. Клинические данные из медицинских карт были получены и проанализированы ретроспективно, всем пациентам было проведено клинико-лабораторное, патоморфологическое исследование. Показатели выживаемости пациентов оценивали с помощью статистического метода анализа времени жизни (Survival Analysis) с расчетом описательных характеристик времени жизни в форме таблицы жизни и построения кривых Каплана-Мейера.
Результаты. В настоящем исследовании благоприятный прогноз по IMDC у больных с мПКР ≥ 75 лет отмечен у 20,8 % пациентов, солитарные метастазы у 6,5 %. Показатели 3 и 5-тилетней выживаемости составили 35,8 % и 21,2 %.
При однофакторном анализе у больных мПКР ≥ 75 лет, выявлено, что отрицательное влияние на показатели выживаемости оказывали ECOG статус (p < 0,001), гистологический подтип (p = 0,01), степень дифференцировки опухоли по Fuhrman (p = 0,003), тип метастазов (p = 0,045), метастазы в печень (p < 0,001), прогноз по IMDC (p = 0,042) и проведение нефрэктомии (p = 0,014).
Заключение. Факторами, влияющими на показатели выживаемости у пациентов с мПКР в возрасте ≥ 75 лет, при многофакторном анализе являлись пол, гистологический подтип, количество метастазов, метастазы в кости и лимфатические узлы, прогноз по IMDC, а также проведение лучевой терапии и нефрэктомии.
Для определения дополнительных персонализированных факторов прогноза у больных старческого возраста с мПКР необходимы дальнейшие исследования.

Ключевые слова: метастатический почечно-клеточный рак, общая выживаемость, пожилые пациенты

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Соблюдение этических стандартов: в работе соблюдались этические принципы, предъявляемые Хельсинкской декларацией Всемирной медицинской ассоциации (World Medical Association Declaration of Helsinki, 1964, ред. 2013). Исследование одобрено Этическим комитетом СПбГБУЗ «Городской Онкологический Диспансер» (протокол № 2356 от 20.05.2022 г.). Информированное согласие получено от всех участников исследования

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INRODUCTION

Over the past decade, the incidence of RCC has been increasing annually. RCC occurs in 25 % of patients with newly diagnosed RCC over the age of 75 ([1]. Currently, the majority of patients with metastatic renal cell carcinoma (mRCC) receive various options for systemic therapy, due to which the median overall survival (s) increased to 4 years [2, 3]. It is known that in elderly patients, the activity of the tumor process is lower due to a slowdown in metabolic processes in the body. The implementation of modern drug therapy contributes to an increase in the indicators of OS in patients with mRCC, including in elderly patients [4]. But aggressive systemic therapy in patients over 75 years of age is associated with an increased risk of undesirable side effects that worsen the quality of life. In our study, we studied prognostic factors in patients with mRCC aged ≥ 75 years, affecting survival rates.

MATERIALS AND METHODS

A retrospective analysis of 77 patients aged ≥ 75 years who received systemic therapy at the Moscow City Oncological Hospital No. 62 and

Table 1. Characteristics of patients

Characteristic	≥ 75 лет (*n* = 77) *n* (%)
Gender: male female	 51 (66.2) 26 (33.8)
ECOG status: 0 1 2 3	 1 (1.3) 29 (37.7) 35 (45.5) 12 (15.6)
Histological type: clear-cell carcinoma non-clear-carcinoma	 64 (83.1) 13 (16.9)
Differentiation grade: G1 G2 G3	 18 (23.4) 27 (35.1) 32 (41.6)
Metastasis type: metachronous synchronous	 53 (68.8) 24 (31.2)
Number of metastases: solitary single multiple	 5 (6.5) 27 (35.1) 45 (58.4)
IMDC prognosis: favorable intermediate poor	 16 (20.8) 32 (41.6) 29 (37.7)
Metastatic site: lungs bones liver lymph nodes	 48 (62.3) 26 (33.8) 6 (7.8) 21 (27.3)
Prior nephrectomy:	71 (92.2)
Metastasectomy:	11 (14.3)
Radiation therapy:	10 (13)

the St. Petersburg City Oncological Dispensary from 2006 to 2019 was carried out. All patients received systemic antitumor therapy. The influence of clinical and morphological factors on overall survival(s) was assessed. Detailed characteristics of patients are given in Table 1.

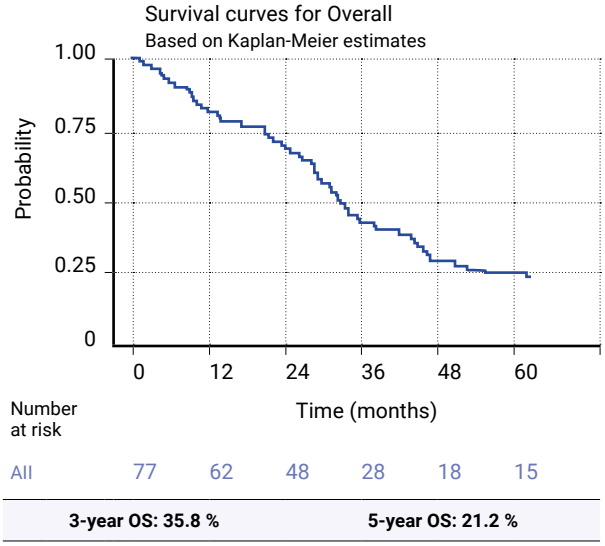

Fig. 1. Overall survival (OS) of patients ≥ 75 years of mRCC (*n* = 77)

Patient data was consolidated in the form of spreadsheets and analyzed using the Statistica 12 for Windows program. Life expectancy was calculated from the date of diagnosis to the date of last observation or death. Survival was assessed using the Kaplan-Mayer method, survival differences were determined using a log-rank test; Cox regression analysis was used to exclude factors that do not have independent prognostic significance. An analysis of the S of patients with mRCC ≥ 75 years old was performed, depending on the histological variant of the tumor, the degree of tumor differentiation according to Fuhrman, the IMDC prognosis group, the number, type and localization of metastases, cytoreductive surgery and radiation therapy.

STUDY RESULTS

The clinical and morphological characteristics of 77 patients are presented in Table 1. The average age was 79 (75–95) years. Clear cell renal cell carcinoma was detected in 64 (83.1 %) patients. Favorable, intermediate and unfavorable forecast for IMDC in 16 (20,8 %), 32 (41,6 %) and 29 (37.7 %) patients. Solitary, single and multiple metastases

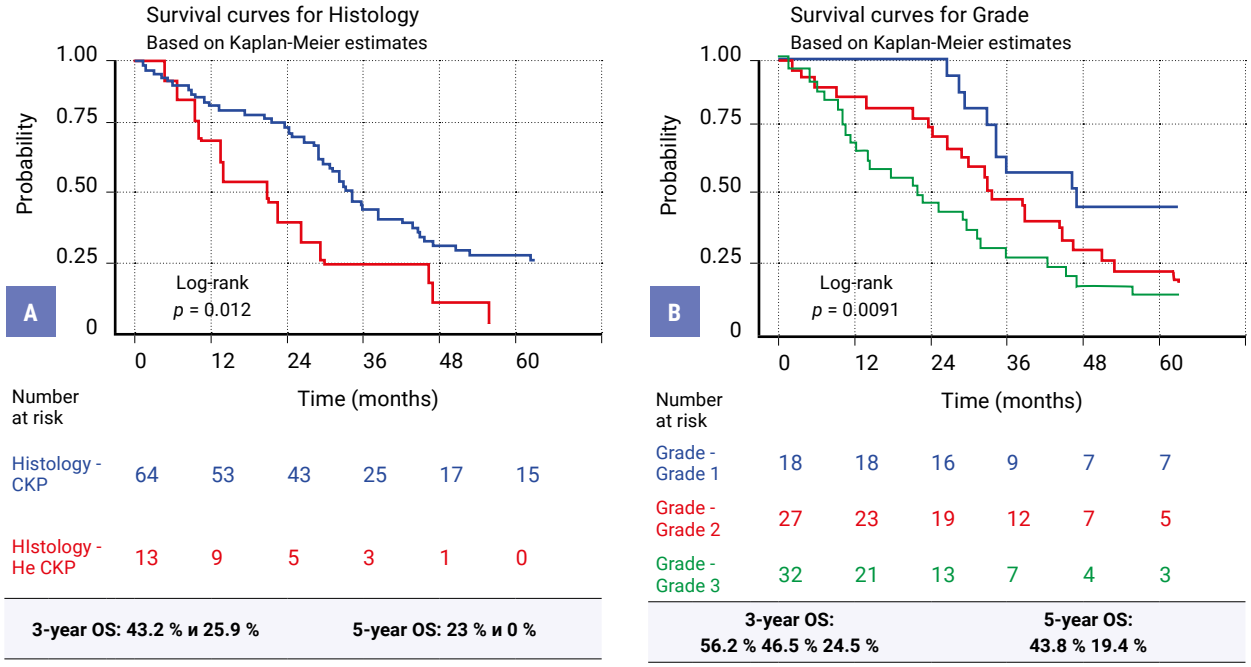

Fig. 2. Overall survival (OS) in patients ≥ 75 years of mRCC depending on histologic subtype (A) and tumor differentiation according to Fuhrman (B) (*n* = 77)

were detected in 5 (6,5 %), 27 (35,1 %) and 45 (58.4 %) patients, respectively.

The indicators of 3- and 5-year OV in patients with mRCC were 39.7 % [29.8–52.76 %; 95 % CI] and 21.2 % [13.6–33.24 %; 95 % CI], respectively, in patients, while the median OS was 32.4 [28.9–38.6 %; 5 % CI] months (Fig. 1).

A single-factor analysis in patients with mRCC ≥ 75 years old revealed that ECOG status ($p < 0.001$), histological subtype ($p = 0.01$), degree

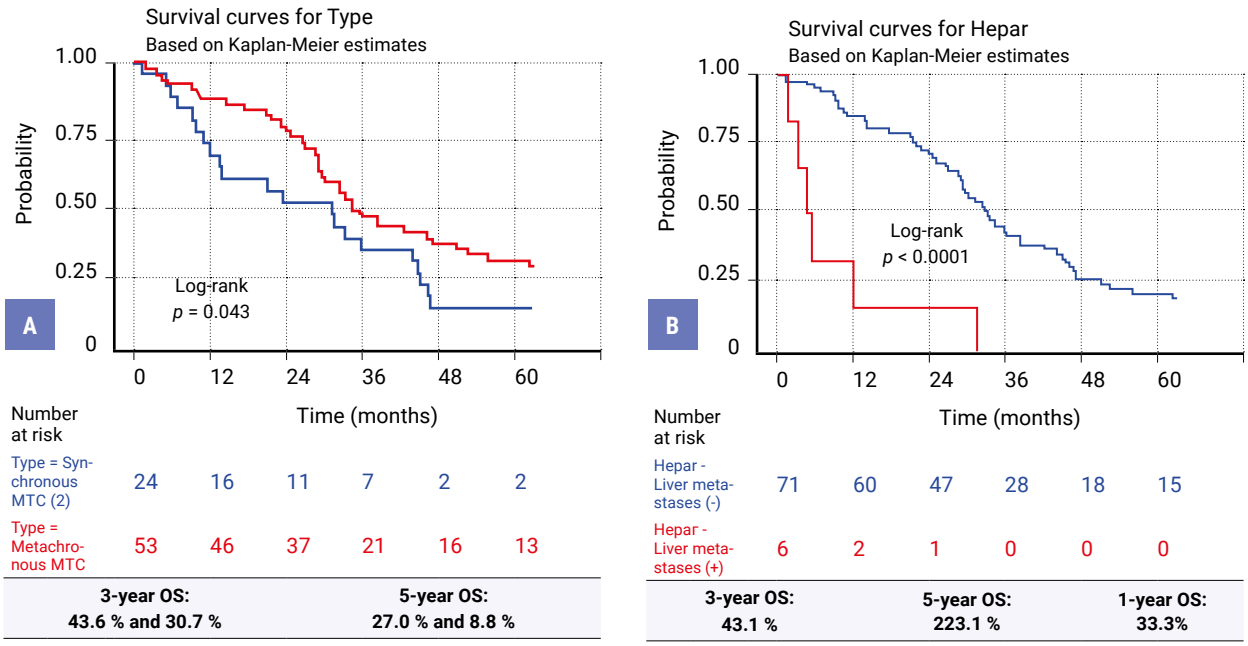

Fig. 3. Overall survival (OS) in patients ≥ 75 years of mRCC according to type of metastases (A) and liver metastases (B) ($n = 77$)

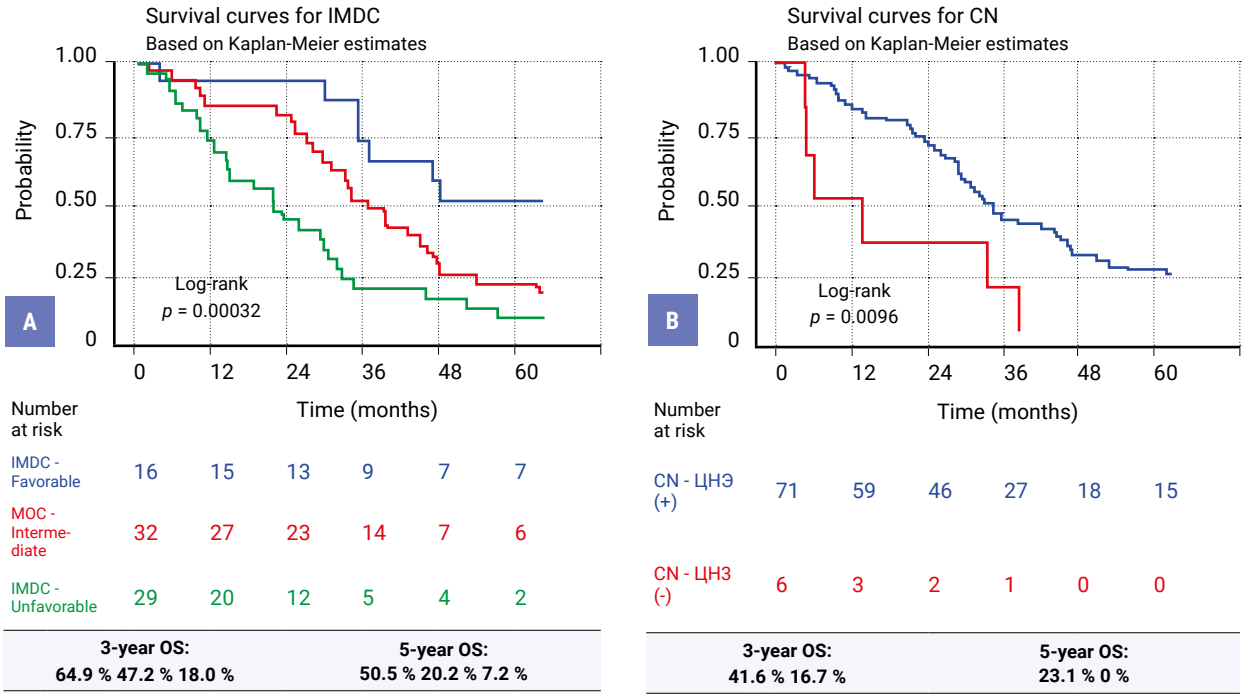

Fig. 4. Overall survival (OS) in patients ≥ 75 years depending on prognosis IMDC (A) and nephrectomy performance (B) ($n = 77$)

Table 2. Prognostic factors for overall survival in patients ≥ 75 years of age with mRCC (*n* = 77) (single-factor and multivariate analysis)*

Factor	Hazard rario (95 % confidence interval)	
	Univariate test	Multivariate test
Gender: male female	– 0.62 (0.37–1.05, *p* = 0.077)	– 0.27 (0.16–0.46, *p* < 0.001)
ECOG status: 0 1 2 3	– 20379783.9 (0.00-Inf, *p* = 0.997) 38320352.5 (0.00-Inf, *p* = 0.997) 82331370.5 (0.00-Inf, *p* = 0.996)	– 41895.8 (24218–1572477.23, *p* < 0.001) 69920.5 (41671.28–117320.25, *p* < 0.001) 112174.0(53540.28-235019.58, *p* < 0.001)
Histological type: clear-cell carcinoma non-clear-carcinoma	– 2.19 (1.17–4.09, *p* = 0.014)	– 1.92 (1.02–3.60, *p* = 0.043)
Differentiation grade: G1 G2 G3	– 1.74 (0.90–3.36, *p* = 0.100) 2.71 (1.40–5.25, *p* = 0.003)	– 1.43 (0.86–2.36, *p* = 0.169) 1.38 (0.83–2.32, *p* = 0.218)
Metastasis type: metachronous synchronous	– 0.59 (0.35–0.99, *p* = 0.045)	– 1.22 (0.71–2.08, *p* = 0.478)
Number of metastases: solitary single multiple	– 0.64 (0.24–1.71, *p* = 0.372) 1.33 (0.52–3.42, *p* = 0.553)	– 0.59 (0.35–1.00, *p* = 0.050) 1.66 (1.00–2.73, *p* = 0.049)
Lung metastases: present absent	– 1.58 (0.38-6.64, *p* = 0.532)	– 1.29 (0.81–2.08, *p* = 0.139)
Bone metastases: present absent	1.17 (0.69–1.98, *p* = 0.557)	3.09 (1.82–5.23, *p* < 0.001)
Liver metastases: present absent	– 6.68 (2.74–16.28, *p* < 0.001)	– 1.86 (0.74–4.69, *p* = 0.186)
Lymph nodes metastases: present absent	– 1.16 (0.68–1.97, *p* = 0.595)	– 0.54 (0.31–0.93, *p* = 0.026)
IMDC prognosis: favorable intermediate poor	– 4.00 (1.93-8.29, *p* < 0.001) 2.07 (1.03–4.18, *p* = 0.042)	– 1.94 (1.13–3.33, *p* = 0.016) 1.93 (1.17–3.20, *p* = 0.010)
Radiation therapy: no yes	– 0.99 (0.44-2.20, *p* = 0.979)	– 0.28 (0.12-0.64, *p* = 0.002)
Prior nephrectomy: yes no	– 2.95 (1.25-6.99, *p* = 0.014)	– 6.08 (2.54–14.58, *p* < 0.001)
Metastasectomy	0.41 (0.05–3.53, *p* = 0.419)	0.65 (0.28–1.46, *p* = 0.323)

Note: the table only presents factors with prognostic significance

of tumor differentiation according to Fuhrman (p = 0.003), type of metastases (p = 0.045), liver metastases had a negative effect on survival rates (p < 0.001), IMDC prognosis (p = 0.042) and nephrectomy (p = 0.014) (Table 2, Fig. 2–4).

The median OS in clear cell and non-light cell cancers was 34.4 [30.0–44.6; 95 % CI] and 21.2 [10.4–NA; 95 % CI] months, respectively, and in G1, G2 and G3 was 46.4 [34.4–NA; 95 % CI], 33,4 [26,9–51; 95 % CI] and 21.7 [13.9–31.6; 95 % CI] months, respectively.

The median S in metachronous and synchronous metastases was 33 [29.1–46.9; 95 % CI] and 23.7 [12–44.6; 95 % CI] months, respectively, and in the absence and presence of liver metastases was 33.4 [29.1–44.6; 95 % CI] and 5.1 [3.5–NA; 95 % CI] months, respectively.

The median OS with favorable, intermediate and unfavorable forecasts was 65.2 [35.9–NA; 95 % CI], 35,8 [29–46,3; 95 % CI] and 21.2 [13.7–31.1; 95 % CI] months, respectively, and in the presence or absence of nephrectomy was 32.6 [29–44.6; 95 % CI] and 10 [5–NA; 95 % CI] months, respectively.

In multivariate analysis, gender [HR = 0.27 (95 % CI = 0.16–0.46], ECOG status [HR = 112174.0 (95 % CI = 53540.28–235019.58], histological subtype [HR = 1.92 (95 % CI = 1.02–3.60], number of metastases [HR = 1.66 (95 % CI = 1.00–2.73], bone metastases [HR = 3.09 (95 % CI = 1.82–5.23] and lymph nodes [HR = 0.54 (95 % CI = 0.31–0.93], IMDC prognosis [HR = 1.93 (95 % CI = 1.17–3.20], as well as radiation therapy [HR = 0.28 (95 % CI = 0.12–0.64] and nephrectomy [HR = 6.08 (95 % CI = 2.54–14.58] were additional factors that had an independent negative effect on the indicators of OS in elderly patients with mRCC (Table 2).

DISCUSSION

Currently, due to an increase in life expectancy, the number of senile patients with renal cell carcinoma is growing in the world, 25 % of patients over 75 years of age are diagnosed with RCC for the first time ([1]. In a retrospective study by Kanesvaran R et al. old age does not affect the indicators of OS in mRCC [5]. In our study, the rates of 3- and 5-year-olds in patients with mRCC ≥ 75 years were 35.8 % and 21.2 %. In the work of Ryuichi Mizuno et al. A decrease in OS in senile patients is possible due to an unbalanced distribution of patients in the IMDC prognosis groups, a higher incidence of anemia, as well as a higher percentage of patients receiving symptomatic therapy instead of second-line systemic therapy [6]. In our study, anemia was noted in 35.1 % of patients, 1/5 of patients in the group with a favorable prognosis according to IMDC, one third of patients had bone metastases.

The decrease in OS indicators may occur due to ineffective therapy of senile patients and early transfer of this category to symptomatic treatment [7]. Modern drug therapy over the past few decades has led to a significant increase in OS rates in patients with mRCC. Elderly patients after the first line of systemic therapy are not always transferred to the second line due to the high risk of adverse events. This is due to a lack of evidence that elderly patients can tolerate systemic therapy of two or more lines in order to increase the indicators of OS. Currently, in addition to prognostic prognostic factors, personalized therapeutic strategies are being developed [8, 9]. In our study, we identified additional prognostic factors affecting survival rates in senile patients with mRCC.

The limitations of this study are the size of the studied group of patients and its retrospective nature. Further research is needed to develop personalized approaches in elderly patients with mRCC to improve the quality of life and increase the indicators of OS.

CONCLUSION

The factors influencing survival rates in patients with mRCC aged ≥ 75 years in multivariate analysis were gender, histological subtype, number of metastases, bone and lymph node metastases, IMDC prognosis, as well as radiation therapy and nephrectomy.

Further studies are needed to determine additional personalized prognostic factors in senile patients with mRCC.

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Shirokorad V. I. – development of the study design, obtaining data for analysis, editing of the manuscript;
Kostritsky S. V. – collection of material, analysis of the obtained data.

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ORIGINAL ARTICLE

Endovascular surgery in patients with coronary artery disease in combination with cancer

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ABSTRACT

Purpose of the study. To analyze the long-term results from various strategies of endovascular treatment for coronary artery disease (CAD) in patients concomitant with cancer.

Patients and methods. 74 patients with both CAD disease and cancer were treated in A. V. Vishnevskiy Center from 01/01/2018 to 12/31/2022. By a multidisciplinary council, patients were divided into three groups: group 1 (*n* = 39) – staged treatment: percutaneous coronary intervention (PCI) is the first stage, the second is surgical treatment of cancer; group 2 (*n* = 14) – staged treatment: the first stage was surgical treatment of cancer, and the second stage was PCI; group 3 (*n* = 21) – PCI and open surgery were performed on the same day.

Results. In the immediate period, 3 (4.0 %) deaths were observed: 2 (5.1 %) in group 1, 1 (4.8 %) in group 3, the cause of which was complications arising after oncological surgical interventions. One (2.6 %) patient from group 1 had acute myocardial infarction (AMI) due to acute stent thrombosis in the left anterior descending artery (LAD). The patient underwent successful emergency PCI. In the long-term period, 15 (25.4 %) patients died, out of which 11 (18.7 %) from progression of cancer, and 4 (6.7 %) from other causes. Among the major cardiovascular complications, the following were observed: 1 (3.2 %) AMI in group 1 and 1 (7.1 %) in group 2.

Conclusion. In the long-term follow-up period, the leading cause of death (73,3 %) was progression of cancer. There were no detected from deaths AMI, which confirms the importance and feasibility of myocardial revascularization in this severe group of patients. PCI in patients with coronary artery disease in combination with cancer allows for effective and safe surgical treatment of malignant pathology without cardiac mortality both in the immediate and long-term follow-up periods.

Keywords: coronary heart disease, oncological disease, percutaneous coronary intervention, malignant neoplasm, cancer, surgical treatment, coronary artery stenting

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Compliance with ethical standards: this research has been carried out in compliance with the ethical principles set forth by the World Medical Association Declaration of Helsinki, 1964, ed. 2013. The study was approved by the Research Ethics Committee of the A. V. Vishnevskiy National Medical Research Center of Surgery (extract from the protocol of the meeting No. 009–2021 dated 11/26/2021). Information consent was obtained from all study participants

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3.1.6. Онкология, лучевая терапия
ОРИГИНАЛЬНАЯ СТАТЬЯ

Рентгенэндоваскулярная хирургия в лечении пациентов с ишемической болезнью сердца в сочетании со злокачественными новообразованиями

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РЕЗЮМЕ

Цель исследования. Провести анализ отдаленных результатов различных стратегий рентгенэндоваскулярного лечения ишемической болезни сердца (ИБС) у пациентов с сопутствующими злокачественными новообразованиями (ЗНО).
Пациенты и методы. В ФГБУ «Национальный медицинский исследовательский центр хирургии им. А. В. Вишневского» Министерства здравоохранения Российской Федерации в период с 01.01.2018 по 31.12.2022 гг. проходили лечение 74 пациента с ИБС в сочетании с ЗНО. Мультидисциплинарным консилиумом пациенты были распределены на три группы: группа 1 (*n* = 39) – этапное лечение: первым этапом – выполнение чрескожного коронарного вмешательства (ЧКВ), вторым – хирургическое лечение ЗНО; группа 2 (*n* = 14) – этапное лечение: первым этапом выполнялось хирургическое лечение ЗНО, а вторым – ЧКВ; группа 3 (*n* = 21) – выполнение ЧКВ и открытого хирургического вмешательства проводилось в один день.
Результаты. На госпитальном этапе отмечено 3 (4,0 %) летальных исхода: 2 (5,1 %) – в группе 1, 1 (4,8 %) – в группе 3, причиной которых были осложнения, возникшие после онкологических хирургических вмешательств. У 1 (2,6 %) пациента из группы 1 отмечен инфаркт миокарда (ИМ) вследствие острого тромбоза стента в передней межжелудочковой ветви (ПМЖВ). Пациенту было выполнено успешное экстренное ЧКВ. В отдаленном периоде умерло 15 (25,4 %) пациентов, из которых 11 (18,7 %) – от прогрессирования ЗНО, а 4 (6,7 %) – от других причин. Среди больших сердечно-сосудистых осложнений наблюдались: 1 (3,2 %) ИМ в группе 1 и 1 (7,1 %) – в группе 2.
Заключение. В отдаленном периоде наблюдения ведущей причиной смерти (73,3 %) было прогрессирование ЗНО. Не было зафиксировано ни одного летального исхода от ИМ, что подтверждает важность и целесообразность выполнения реваскуляризации миокарда у данной группы пациентов. ЧКВ у пациентов с ИБС в сочетании с ЗНО позволяет эффективно и безопасно выполнить хирургическое лечение злокачественной патологии без кардиальной смертности как на госпитальном, так и в отдаленном периодах наблюдения.

Ключевые слова: ишемическая болезнь сердца, онкологическое заболевание, чрескожное коронарное вмешательство, злокачественное новообразование, рак, хирургическое лечение, стентирование коронарных артерий

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Соблюдение этических стандартов: в работе соблюдались этические принципы, предъявляемые Хельсинкской декларацией Всемирной медицинской ассоциации (World Medical Association Declaration of Helsinki, 1964, ред. 2013). Исследование одобрено Комитетом по этике научных исследований ФГБУ «Национальный медицинский исследовательский центр хирургии им. А. В. Вишневского» Министерства здравоохранения Российской Федерации (выписка из протокола заседания № 009–2021 от 26.11.2021 г.). Информированное согласие получено от всех участников исследования

Финансирование: финансирование данной работы не проводилось

Конфликт интересов: все авторы заявляют об отсутствии явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи

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INDRODUCTION

In the Russian Federation, primary cancers were detected in 580,415 patients in 2021: 265,039 men and 315,376 women.

The most common cancers in men were: tumors of the trachea, bronchi, lung (16.4 %), prostate (15.1 %), skin (except melanoma) (9.8 %), stomach (7.0 %), colon (7.0 %), rectum, rectosigmoid, anus (5.8 %). The main neoplasms in the females were: breast cancer (22.1 %), skin neoplasms (except melanoma) (13.4 %), uterine body (8.1 %), colon (7.2 %), cervix (4.9 %), rectum, rectosigmoid junction, anus (4.6 %), lymphatic and hematopoietic tissue (4.4 %), stomach (4.2 %), ovary (4.2 %), trachea, bronchi, lung (4.1 %). Compared with 2020, the growth of primary detected malignant neoplasms in the Russian Federation amounted to 4.4 % [1].

Morbidity and mortality from both oncological and cardiovascular diseases, including coronary artery disease (CAD), increases with age [2]. At the same time, a combination of both nosologies is often found. Thus, according to S. G. Al-Kindi et al., the incidence of coronary artery disease in patients with lung cancer is 21 %, with breast cancer – 6 %, with colorectal cancer – 12 %, and with kidney cancer – 17 %. [3].

The survival results of 3,234,256 cancer patients showed that in the long-term period 1,228,328 (33 %) patients died directly from cancer, while from cardiovascular diseases – 365,689 (11.3 %), while 76.3 % of them were occupied by heart disease [4].

50.6 % of patients underwent radical treatment among all malignant neoplasms in oncological institutions of the Russian Federation. At the same time, the surgical method was predominant and amounted to 59.5 %, and the share of combined treatment was 28.6 % [5].

Complications arising after surgical interventions, including oncological ones, lead to an increase in inpatient care, in the cost of treatment, and an increase in mortality [6].

A major paper by Ramamoorthy et al. presents the incidence of major cardiovascular complications (MACCE) in 2,854,810 patients over 40 years of age who underwent extensive surgical interventions for malignant neoplasms. The overall incidence of major cardiovascular events in the perioperative period was 2.4 % (67,316). At the same time, they accounted for 0.7–0.8 %. The highest incidence of MACCE was in patients who underwent surgery on the esophagus (6,706 per 100,000 oncological operations), followed by surgery on the liver (5,284 per 100,000 oncological operations), pancreas (4,820 per 100,000 oncological operations), colon (4,038 per 100,000 oncological operations). Of the 2,854,810 patients treated, 400,063 (14 %) had coronary artery disease, of which 31,423 (7.8 %) had a major cardiovascular event in the preoperative period [7].

The purpose of the study: to analyze the long-term results of various strategies for endovascular treatment of coronary artery disease (CAD) in patients with concomitant cancers.

PATIENTS AND METHODS

In the period from 01/01/2018 to 12/31/2022, 74 patients with severe coronary artery disease in combination with cancers were treated at the A. V. Vishnevskiy National Medical Research Center of Surgery, the Russian Federation Ministry of Health. The cardiovascular council determined PCI by revascularization in connection with direct indications for endovascular interventions, or the refusal of cardiac surgeons to perform coronary bypass surgery (CABG) due to the impossibility of myocardial revascularization or concomitant pathology. All patients were discussed at a multidisciplinary consultation with oncologists, surgeons, cardiovascular surgeons, endovascular surgeons, cardiologists, anesthesiologists and intensive care specialists. Based on the stage and degree of prevalence of the oncological process, its manifestations and complications, as well as the severity of coronary artery lesions and the clinical picture of coronary artery disease, the stages and sequence of surgical interventions were determined.

The criteria for inclusion in the study were the following: a combination of active cancer and angiographically significant (more than 75 %) coronary artery damage; consent of the patient or his legal representative to participate in the study after receiving relevant information about the study.

The criteria for exclusion from the study were: the presence of contraindications to receiving double disaggregated therapy; acute renal and hepatic

insufficiency, acute coronary syndrome (ACS) at the time of hospitalization.

Based on the chosen treatment strategy, the patients were divided into three groups:

— group 1 (39–52.7 % of patients) – stage-by-stage treatment: PCI was performed in the first place, surgical treatment of oncological disease in the second;

— group 2 (14–18.9 % of patients) – staged treatment: the first stage was surgical treatment of cancer, and the second stage – PCI;

— group 3 (21–28.4 % of patients) – PCI and open surgery were performed on the same day.

The expediency of performing an early oncological operation on the same day with PCI (group 3) was due to severe damage to the coronary arteries and a malignant process complicated by bleeding, or in a situation where it is impossible to postpone the oncological stage of treatment even for one month, since a delay in treatment could lead to the unresectability of malignant formation. On the scheduled day, patients in the catheterization laboratory first underwent endovascular myocardial revascularization, after which they were immediately transferred to the surgical operating unit to perform oncological intervention.

As a preoperative examination, all patients underwent computed tomography, electrocardiography, echocardiography, ultrasound, MRI, and selective coronary angiography.

There were 63 men (85 %) and 11 women (15 %) out of the 74 patients. The average age of the patients was 68.8 ± 7.2 years (95 % confidence interval – 95 % CI 67.1–70.5). MI in the anamnesis occurred in 29 (39.1 %) patients, angina of functional class III–IV – in 20 (27 %) patients.

As shown in Table 1, the patients of the three groups did not differ statistically in the main clinical characteristics.

All 74 patients had angiographically significant coronary artery lesions: at the same time, a single-vessel lesion occurred in 25 (33.8 %), a two-vessel lesion in 25 (33.8 %), and a three-vessel lesion in 24 (32.4 %) patients. Lesion of the left main cor-

Table 1. Clinical characteristics of patients

Parameters	Group 1 (PCI + ONCO stages) (*n* = 39)		Group 2 (ONCO + PCI stages) (*n* = 14)		Group 3 (simultaneous PCI and ONCO) (*n* = 21)		
	abs.	%	abs.	%	abs.	%	*p*
Male sex	35	89.7	11	78.6	17	81.0	0.457
Female sex	4	10.3	3	21.4	4	19.0	
Age. years old	68.08 ± 6.86	–	67.5 ± 6.51	–	71.00 ± 8.10	–	0.25
BMI	26.57 (23.59–28.56)	–	27.20 (25.90–32.81)	–	25.00 (23.25–31.38)	–	0.239
Arterial hypertension	30	76.9	12	85.7	19	90.5	0.434
Angina functional classes							
Classes III–IV	12	30.8	3	21.4	5	23.8	0.827
Painless form	19	48.7	5	35.7	13	61.9	0.329
Postinfarction cardiosclerosis	17	43.6	8	57.1	4	19.0	0.059
Arrythmia	7	18.4	0	0.0	6	28.6	0.076
Diabetes mellitus	9	23.1	5	35.7	7	33.3	0.610
Chronic Kidney Disease. GFR ≤ 59.9 ml/min/1.73 m^2	8	20.5	5	35.7	6	28.6	0.493

onary artery (LMCA) was observed in 8 (10.8 %), chronic occlusion of the coronary arteries was detected in 18 (23.3 %) patients.

The most common oncological diseases were: kidney cancer in 12 (16.2 %) patients, stomach cancer in 12 (16.2 %), prostate cancer in 11 (14.9 %), colorectal cancer in 10 (13.5 %), lung cancer in 9 (12.2 %) (Fig. 1). The distribution of patients by stages of the tumor process was as follows: stage I was detected in 18 (24.3 %) patients, stage II in 19 (25.7 %), stage III in 29 (39.2 %), stage IV in 8 (10.8 %) (Fig. 2). Distant metastases They were observed in 7 (9.5 %) patients.

In 74 patients, 150 primary surgical interventions were performed: 75 – PCI (one patient in group 1, due to the severity of the clinical condition, PCI was performed in two stages: stenting of the LAD and Circumflex Artery (CA), and then stenting of the Right Coronary Artery (RCA)) and 75 – surgical operations for cancer (one patient was treated with primary multiple cancer in two stages: the first is mastectomy, the second is kidney resection).

From the table. 2 it can be seen that in groups 1 and 3 there were patients with more severe coronary artery lesion. In group 1, 48.7 % of patients underwent two– and three-vessel PCI, and 2.6 % underwent stenting of the LMCA. While in group 3, 47.6 % of patients underwent two- and three-vessel PCI and in 9.5 % of cases PCI of the LMCA.

Of the 75 oncological surgical interventions, the

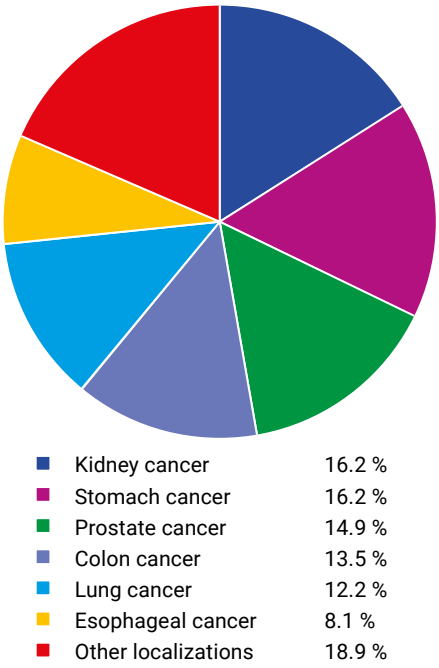

Fig. 1. Cancer localization

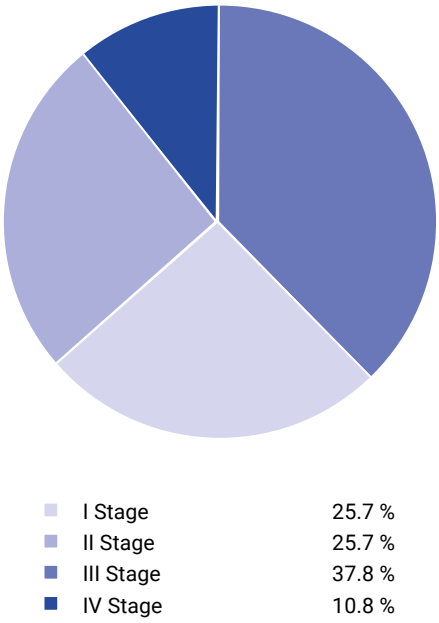

Fig. 2. Cancer stages

Table 2. Distribution of completed PCI

PCI	Group 1 (PCI + ONCO stages) (*n* = 39)		Group 2 (ONCO + PCI stages) (*n* = 14)		Group 3 (Simultaneous PCI and ONCO) (*n* = 21)	
	n	%	*n*	%	*n*	%
One vessel	20	51.3	12	85.7	11	52.4
Two vessel	17	43.6	2	14.3	8	38.1
Three vessel	2	5.1	0	0	2	9.5
LMCA	1	2.6	0	0	3	14.3

most frequent were: prostatectomy – 11 (14.9 %), gastric resection – 8 (10.8 %), lung lobectomy – 8 (10.8 %), hemicolectomy – 6 (8.1 %), gastrectomy – 6 (8.1 %), nephrectomy – 6 (8.1 %), resection kidneys – 6 (8.1 %). (Fig. 3).

The median time interval between the stage of myocardial revascularization and surgical oncological intervention was 56.0 (41.5–107.0) days in group 1 and 42.5 (29.0–76.0) days in group 2 (p = 0.338).

To describe quantitative variables, the data were combined into a series of variations and the arithmetic averages (M) and standard deviations (SD) or medians (Me) and confidence interval (CI) were calculated depending on the normality of the distribution. The normality of the distribution was calculated using the Shapiro-Wilk criterion. Nominal data were marked using absolute values and percentages. In comparing independent samples in the presence of a normal distribution, one-factor analysis of variance (ANOVA) was used, in the absence of a normal distribution, the Kruskal-Wallis criterion was used. The nominal data were compared using the χ^2-Pearson criterion or the exact Fisher criterion, depending on the number of observed phenomena less than 5. The results were evaluated as statistically significant at $p < 0.05$.

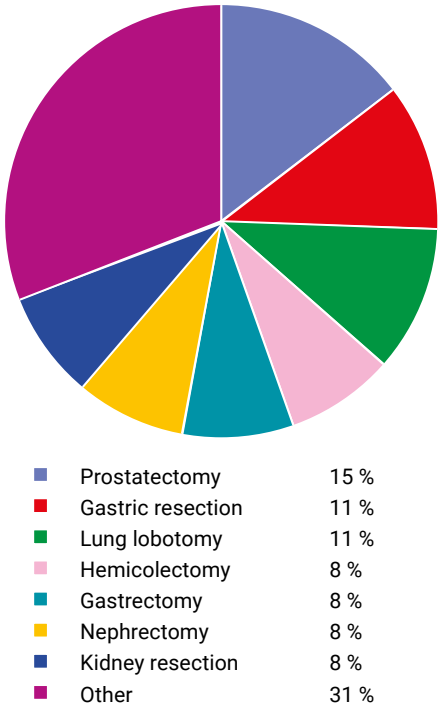

Fig. 3. The structure of oncological interventions

STUDY RESULTS

During the hospital period, when 74 patients underwent two-stage treatment (150 interventions), 3 (4.0 %) deaths were noted as follows: 2 (5.1 %) in group 1, 1 (4.8 %) in group 3, caused by complications arising after oncological surgical interventions (Table 3).

1 (2.6 %) patient of group 1 had acute coronary syndrome (ACS) with ST segment elevation on the 10th day after gastrectomy. This patient was initially scheduled for simultaneous PCI and oncological surgery. However, during the stenting of permanent residence with a bare-metal stent, pulmonary edema arose, which required the transfer of the patient to the intensive care unit. After stabilization of the condition, after 3 days, he underwent a gastrectomy on the background of dual antiplatelet therapy. After the onset of ACS, the patient was immediately transported to the catheterization laboratory, a coronary angiography was performed, the results of which revealed thrombosis of the stent of the LAD. Successful recanalization and repeated stenting of the artery was performed to achieve an optimal angiographic result [8].

Long-term treatment results were evaluated in 59 (83 %) of 71 patients discharged from the Center. The analysis was carried out on the basis of outpatient examinations, repeated hospitalizations, and survey data. The duration of the follow–up period in group 1 averaged 36.9 ± 18.7 months (median 29.3 [20.6–54.8]; 95 % CI 30.8–43.7), in group 2–42.5 ± 12.8 months (median 41.6 [34.8–53.3]; 95 % CI 35.1–49.9), in group 3–36.7 ± 14.1 months (median 31.1 [25.3–46.0]; 95 % CI 28.6–44.9), (p = 0.387).

In total, 15 (25.4 %) patients died in the long-term period (Table 4), of which 11 (18.7 %) died from the oncological process, and 4 (6.7 %) from other causes. In group 1, there were 8 (25.8 %) deaths, 5 (16.1 %) of which were from the cancer progression, 1 (3.2 %) – from acute cerebral circulatory disorders of hemorrhagic type against the background of a prolonged hypertensive crisis, 1 (3.2 %) – from complications of a new coronavirus infection, to establish the cause of death is still one patient failed.

In group 2, 2 (14.3 %) deaths from the progression of cancers were recorded. In group 3, 5 (25.4 %) deaths occurred: 4 (28.6 %) – from the progression of cancer, 1 (1.7 %) – from bleeding due to complications after surgery for urolithiasis.

Among the major cardiovascular complications, there were: 1 (3.2 %) AMI in group 1 and 1 (7.1 %) AMI in group 2. In both cases, the patients were admitted to the hospital, where they underwent successful PCI. It is worth noting that in the long-

Table 3. Hospital complications after two stages of treatment

Parameter	Group 1 (PCI + ONCO stages) (*n* = 39)		Group 2 (ONCO + PCI stages) (*n* = 14)		Group 3 (simultaneous PCI and ONCO) (*n* = 21)		*p*
	n	%	*n*	%	*n*	%	
Cardiovascular complications							
MACCE	1	2.56	0	0.0	0	0.0	–
AMI	1	2.56	0	0.0	0	0.0	–
Stroke	0	0.0	0	0.0	0	0.0	–
Surgical complications							
Bleedings	1	2.56	1	7.1	4	19.0	0.073
Organ dysfunction	2	5.1	0	0.0	3	14.3	–
Infectious complications	3	7.7	1	7.1	2	9.5	1.0
Repeated surgical intervention	2	5.1	2	14.3	3	14.3	0.365
Minor complications: arrhythmia, hypotension, bradycardia, anemia	10	25.6	2	14.3	9	42.9	0.177
Lethal outcomes	2	5.1	0	0.0	1	4.8	–

Table 4. Long-term treatment results

	Group 1 (PCI + ONCO stages) (*n* = 39)		Group 2 (ONCO + PCI stages) (*n* = 14)		Group 3 (simultaneous PCI and ONCO) (*n* = 21)		Overall *n* = 59		*p*
	n	%	*n*	%	*n*	%	*n*	%	
All-cause deaths:	**8**	25.8	**2**	14.3	**5**	35.7	**15**	25.4	0.387
AMI	**–**	–	–	–	–	–	–	–	–
Stroke	**1**	**3.2**	**–**	–	–	–	**1**	**1.7**	–
Cancer	5	16.1	2	14.3	4	28.6	**11**	**18.7**	0.667
COVID-19	**1**	**3.2**	**–**	–	**–**	–	**1**	**1.7**	–
Bleeding	**–**	–	–	–	**1**	**7.1**	**1**	**1.7**	–
Other causes	**1**	**3.2**	**–**	–	–	–	**1**	**1.7**	–
MACCE:	**2**	6.5	**1**	7.1	**1**	7.1	**4**	6.8	1.0
AMI	1	3.2	1	7.1	–	–	2	3.4	–
Stroke	1	3.2	–	–	1	7.1	2	3.4	–

term period, not a single fatal outcome from AMI was recorded.

There were no significant differences detected between the groups, when analyzing the overall survival rate with the Kaplan-Meier method (Fig. 4) and when conducting a Log-rank test (p = 0.366).

DISCUSSION

As a rule, patients with malignant neoplasms were most often excluded from most large randomized cardiac trials and registries. In view of this, there is currently insufficient information about the effect of coronary artery disease on cancer patients. Therefore, the treatment of this group of patients is based on the experience of individual specialists and clinics.

Early detection, followed by the prescribed special treatment of cancer, is a fundamental factor in increasing the survival rate of these patients [9].

However, in patients with combined severe coronary artery disease, performing surgical oncological intervention in the first stage is associated with the risk of developing cardiovascular complications. At the same time, performing early oncological surgery after percutaneous coronary intervention against the background of patients receiving dual antiplatelet therapy (DAPT) is associated with the risks of intra- and postoperative bleeding. Recent generations of stents have made it possible to solve this problem to a greater extent by safely reducing the intake of DAPT to 1 month [10, 11].

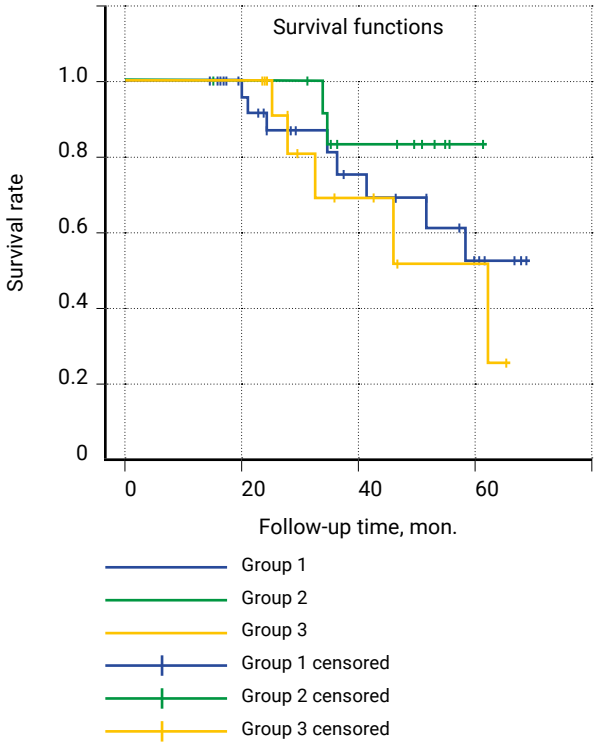

Fig. 4. Kaplan-Meier patient survival curves

A recently published study by Yun T., et al., compared the results of stage-by-stage treatment of patients with lung malignancy and combined coronary artery disease. The patients were divided into two groups according to the timing of the interventions performed: group 1 – patients who underwent PCI in the first stage, and lobectomy in the second stage for up to 3 months; group 2 – patients who underwent PCI in the first stage, and lobectomy in the second stage after 3 months. The analysis of hospital results showed that the time of surgery, hospital stay and blood loss did not significantly differ between the groups (p > 0.05). However, evaluating the long-term results, the authors found that survival for 5 years was significantly higher in patients who underwent lobectomy up to 3 months after PCI (p < 0.05). The authors concluded that lobectomy is more appropriate to perform in the early period after PCI [12].

Indeed, untimely treatment of cancer can reduce long-term survival. The results of a meta-analysis by T. P. Hanna et al., confirmed that a delay in the treatment of malignant neoplasms, even by 4 weeks, increases mortality with surgical, systemic and radiological methods of treatment [13].

In our study, the start of the planned special treatment, including surgical treatment, was carried out as early as possible. In patients with mildly aggressive forms of cancers and without complications (bleeding), who had the opportunity to delay radical treatment for 4 weeks, PCI was performed using bare-metal stents (at the initial stage of our research), and subsequently polymer-free stents of the latest generation (CRE8, CID, Italy) and stents with a bioresorbable polymer (Synergy, Boston Scientific, USA) (group 1). This made it possible to safely reduce the intake of DAPT to 1 month and shorten the time before performing oncological surgery.

In the case when a delay in performing oncological surgery was associated with the risk of rapid progression and metastasis of the tumor process,

or there was a cancer with recurrent bleeding, which in turn did not allow the appointment of DAPT, PCI and surgical treatment of cancers were performed on the same day (group 3). These patients were predominantly (84 %) implanted with standard drug-coated stents (Resolute Integrity, Xience Xpedition, Promus Premier).

Despite the differences in the timing of oncological interventions in each group for deaths from all causes, including from the progression of malignant neoplasms, there were no significant differences between the groups (p = 0.387 and p = 0.667, respectively).

In the treatment of patients with cancer in combination with coronary artery disease, it is important not only to carefully perioperative management of such patients, but also their subsequent rehabilitation and curation after surgical interventions, especially in the immediate postoperative period. In the work of Guo W. et al., the development of large cardiovascular events in the long-term period in oncological and non-oncological patients who underwent PCI was investigated. The analysis showed that AMI was more common in oncological patients over 5 years (16.1 % vs. 8.0 %; $p < 0.001$), stent thrombosis (6.0 % vs. 2.3 %; $p < 0.001$), repeated revascularization (21.2 % vs. 10.0 %; $p < 0.001$). It should be noted that late stent thrombosis occurred most often (52 %), which determines the significance of the first year after PCI in this group of patients [14].

In our study, there were two cases of AMI: the first – a patient 601 days after PCI had ACS with ST segment elevation due to thrombosis of a previously implanted drug-coated stent of the LAD against the background of a new coronavirus infection (COVID-19). As an emergency, the patient underwent recanalization and stenting of the LAD. The second patient had ACS without ST segment elevation after 867 days. This patient underwent balloon angioplasty and stenting of stenosis "de novo" of the LAD. It is important to emphasize that in our study, not a single patient died from AMI in the long term, which once again emphasizes the need for myocardial revascularization in this group of patients.

CONCLUSION

1. In the long-term follow-up, 15 (25.4 %) deaths were detected, 11 (73.3 %) of which were associated with the progression of cancer

2. In the long-term follow-up, only 1 (1.7 %) of 71 patients had a fatal outcome associated with a cardiovascular event (acute cerebral circulatory disorder of the hemorrhagic type). Acute myocardial infarction occurred in only 2 (3.4 %) patients and was successfully treated with stenting of the infarct-related artery.

3. There was no significant difference between the three groups in terms of such indicators as death from all causes (p = 0.387) and oncological mortality (p = 0.667).

4. In the long-term follow-up, no deaths from myocardial infarction were recorded, which confirms the importance and expediency of performing myocardial revascularization in patients with significant damage to the coronary arteries and concomitant cancer.

5. Percutaneous coronary intervention in patients with oncological diseases makes it possible to perform surgical treatment of cancer effectively and safely without cardiac mortality both at the hospital and in the long-term follow-ups.

6. The stage-by-stage treatment of patients with coronary artery disease in combination with cancers should be determined by the decision of a multidisciplinary team consultation.

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Gritskevich A. A. – scientific leadership, conductiong the surgical stage of treatment, contribution to the concept and design of the study, correction of the article, approval of the final version for publication;
Karapetyan N. G. – performing the endovascular stage of treatment, contribution to the concept and design of the study, obtaining and interpreting study data, correction of the article;
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Tokmakov E. V. – provision of anesthesia at the endovascular stage of treatment, correction of the article;
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Revishvili A. Sh. – contribution to the concept and design of the study, correction of the article, approval of the final version for publication.

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ORIGINAL ARTICLE

Cellular, genomic and transcriptomic effects of secondary metabolites of the Hybrid Butterbur on the HeLa cell line

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ABSTRACT

Purpose of the study. To evaluate the cellular, genomic (gene copy number) and transcriptomic (gene expression) effects of *P.hybridus* (L.) secondary metabolites when they affect the HeLa cell line.
Materials and methods. The isolation of secondary metabolites from plant material and its identification were carried out by preparative chromatography. The composition was determined using mass spectrometric analysis, and the final verification of structural formulas was carried out by nuclear magnetic resonance at the Department of Natural Compounds, the Faculty of Chemistry of the Southern Federal University. The subsequent phase of the study was conducted using both cultural and molecular methods. HeLa cells were cultivated under standard conditions in a MEM medium. Once the confluence level was reached 75–80 %, the nutrient medium was replaced with the introduction of the studied compounds (at a concentration of 4 micrograms/ml) and cultivated for 72 hours. Cell mortality was determined using a NanoEnTek JuliFl counter (Korea) in the presence of 0.4 % trypan blue. The assessment of apoptosis following secondary metabolite exposure was conducted on a BD FACSCanto II flow cytometer using the FITC Annexin V Apoptosis Detection Kit I. The level of replication and expression of the genes responsible for apoptosis was assessed by digital droplet PCR (ddPCR).
Results. The following compounds were isolated and verified, and were assigned the following sequence numbers to facilitate their use in the experiment: No. 2 – 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one, No. 3 – 5-(hydroxymethyl) furan-2-carbaldehyde, No. 5.3 – 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde, *P. hybridus* (L.) At the stage of cell death assessment, it was found that the greatest effect was achieved in the compound under ordinal No. 2. However, the evaluation of the copy number and expression of the *CASP8, CASP9, CASP3, BAX, BCL2, TP53, MDM2, CDKN1B, CDK1, CCND1, CCND3*, and *RB1* genes by DD-PCR revealed the presence of apoptosis initiation in tumor cells at the molecular level under the action of compounds No. 2 and No. 5.3 obtained from *P. hybridus* (L.).
Conclusion. The outcomes were multifeatured. Only compound 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one exhibited a pronounced cytostatic effect out of all compounds utilized in the experiment. Concurrently, the compound 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde was found to induce an increase in the expression of the *CASP3, CASP8, TP53*, and *BAX* genes.

Keywords: secondary plant metabolites, apoptosis, gene expression, copy number variation, HeLa cell line, digital droplet PCR

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3.1.6. Онкология, лучевая терапия
ОРИГИНАЛЬНАЯ СТАТЬЯ

Клеточные, геномные и транскриптомные эффекты вторичных метаболитов Белокопытника гибридного на клеточную линию HeLa

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РЕЗЮМЕ

Цель исследования. Оценить клеточные, геномные (копийность генов) и транскриптомные (экспрессия генов) эффекты вторичных метаболитов *P. hybridus* (L.) при их воздействии на клеточную линию HeLa.
Материалы и методы. Выделение вторичных метаболитов из растительного материала и его идентификацию проводили методом препаративной хроматографии, определение состава – с помощью масс-спектрометрического анализа, окончательную верификацию структурных формул – методом ядерно-магнитного резонанса на кафедре природных соединений химического факультета ФГАОУ ВО «Южный федеральный университет». Следующую часть исследования выполняли с использованием культуральных и молекулярных методов. Культивирование HeLa проводили в стандартных условиях в среде MEM. При достижении 75–80 % уровня конфлюэнтности заменяли питательную среду с внесением исследуемых соединений (в концентрации 4 мкг/мл) и культивировали 72 ч. Смертность клеток определяли на счетчике NanoEnTek JuliFl (Корея) в присутствии 0,4 % трипанового синего. Оценку апоптоза после воздействия вторичных метаболитов проводили на проточном цитофлюориметре BD FACSCanto II с помощью FITC Annexin V Apoptosis Detection Kit I. Оценку уровня копийности и экспрессии генов, ответственных за апоптоз, выполняли методом цифровой капельной полимеразной цепной реакции (ПЦР) (DD-PCR).
Результаты. Были выделены и верифицированы следующие соединения, которым для упрощения использования в эксперименте были присвоены следующие порядковые номера: № 2 – 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one, № 3 – 5-(hydroxymethyl)furan-2-carbaldehyde, № 5.3 – 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde *P. hibridus* (L.). На этапе оценки клеточной гибели было обнаружено, что наибольший эффект достигается у соединения под порядковым № 2. Однако оценка показателей копийности и экспрессии генов *CASP8, CASP9, CASP3, BAX, BCL2, TP53, MDM2, CDKN1B, CDK1, CCND1, CCND3* и *RB1* методом DD-PCR выявила наличие инициации апоптоза в опухолевых клетках на молекулярном уровне под действием соединений под № 2 и № 5.3, полученных из *P. hibridus* (L.).
Заключение. Получены результаты разнонаправленного характера. Из всех использованных в эксперименте соединений выраженный цитостатический эффект выявлен только у соединения 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one. В то же время при использовании соединения 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde выявлено увеличение экспрессии генов *CASP3, CASP8, TP53, BAX*.

Ключевые слова: вторичные метаболиты растений, апоптоз, экспрессия генов, копийность генов, клеточная линия HeLa, цифровая капельная ПЦР

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INTRODUCTION

Cervical cancer is one of the main causes of female mortality. Every year, more than 528,000 new cases of breast cancer and more than 266,000 deaths from this disease are detected [1, 2]. The HeLa cell line is a very convenient and simple object for conducting model experiments *in vitro*. This cell line was obtained on February 8, 1951 from a cervical tumor of a patient named Henrietta Lacks at the Baltimore hospital [3]. In our study, we used this cell line to evaluate the cytotoxic effect of the organic compounds of plant origin that we obtained.

Plants synthesize a huge number of secondary metabolites, and in fact it is these metabolites that form the basis of many commercial pharmaceuticals, as well as herbal medicines. Many secondary metabolites, such as alkaloids, terpenoids and phenylpropanoids, are being considered for drug development [4].

Secondary metabolites of plants are structurally diverse compounds that do not directly participate in the growth, development and reproduction of plants, but more often perform a protective function. These compounds with different chemical structures can act as potential multi-purpose anticancer agents [5]. For the first time in history, the term secondary metabolite was proposed by the German biologist Albrecht Kessel in 1891. when he gave a lecture "On the chemical composition of cells" for the Berlin Society of Physiologists, in which he said: "I propose to call compounds that are important for each cell primary, and compounds that are not present in any plant cell secondary" [6]. Currently, the secondary metabolites of plants are divided into several large groups. Terpenoids (isoprenoids) cover more than 40,000 structures and form the largest class of all known plant metabolites. They represent a class of hydrocarbons, i. e. products of biosynthesis of the general formula (C_5H_8) n, with a carbon skeleton that is a derivative of isoprene $CH_2=C(CH_3)–CH=CH_2$. Alkaloids are characterized as heterocyclic compounds containing a nitrogen molecule in a heterocycle and count about 21,000 compounds. Phenolic compounds are aromatic compounds with a benzene ring containing at least one hydroxyl group [7].

The species selected in our work for the isolation of secondary metabolites is the hybrid *Petasites hibridus* (L.) Gaertn., B. Mey. & Scherb is a herbaceous perennial plant of the *Asteraceae* family, found in the European territory of Russia, and, in particular, in the Krasnodar Territory and the Republic of Adygea. The reasons for the interest in this species are that various representatives of the genus *Petasites*, including *P. hibridus* (L.) itself, contain compounds with cytotoxic effects on tumor cells of various nosologies [8]. So in the Japanese White-collar *Petasites japonicus* (Siebold & Zucc.) Maxim. sesquiterpene I and sesquiterpene II were detected, which showed cytotoxic effect against both human astrocytoma U-251MG tumor cells, as well as against the MDA-MB-231 breast cancer cell line [9].

Various methodological approaches are used to study the effect of secondary plant metabolites on tumor cells, including cytometry and flow cytofluorometry, model experiments on cell cultures and molecular genetic studies. The latter include the assessment of the level of replication and gene expression. CNV (copy number variation) is a type of genetic polymorphism that leads to a change in the number of certain genetic loci and, as a result, a change in the expression of these genes and their products – proteins and non-coding RNAs [10]. Studies of the effect of secondary plant metabolites on the expression and replication of genetic loci regulating apoptosis and the cell cycle in cervical cancer are currently few, so this aspect requires additional study. This is what this work is dedicated to.

The study purpose was to evaluate the cellular, genomic (gene replication) and transcriptomic (gene expression) effects of secondary metabolites of *P. hibridus* (L.) when they are exposed to the HeLa cell line.

MATERIALS AND METHODS

Extraction of metabolites. The primary plant material was collected and determined with the participation of the staff of the Department of Botany of the Academy of Biology and Biotechnology of the D. I. Ivanovsky Southern Federal University. Isolation and verification of secondary metabolites of *P. hibridus* (L.) were carried out by employees of the Department of Natural and High Molecular Weight Compounds of the Faculty of Chemistry of the Southern Federal University. Tetrachloroethylene was used as a solvent for primary extraction, which was poured into mechanically purified and crushed rhizomes.

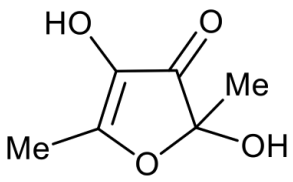
The primary extraction process lasted for four months. To extract tetrachloroethylene from vegetable raw materials, the decantation method was used, followed by concentration of the solution by distillation of the solvent in a distillation unit. Tetrachloroethylene was used as a solvent to reduce the amount of polar compounds (mono- and disaccharides, amino sugars, etc.). The next step was the separation of the resulting concentrated solution using column chromatography using silica gel as a sorbent on column 20*2. Various eluents were used: first, tetrachloroethylene, which allowed to obtain 10 fractions of various colors, from colorless to light yellow. Then methylene chloride was used, which gave 10 more fractions. After that, the eluent was changed, and the column was filled with a mixture of methylene chloride and alcohol in a ratio of 10/1, which led to the production of two more fractions. All fractions were concentrated by evaporation on a rotary evaporator.

The method of high-performance liquid chromatography with mass detection was used to identify the isolated compounds. The mass spectra were analyzed using NIST 2011 biotechnology, which confirms the results of studies with alkaloids and other biologically active compounds.

Fractions containing higher fatty acids, nitrogenous bases of nucleic acids and their glycosides were excluded from further work. In addition, the previously isolated fractions were further purified using column chromatography, and the purified compounds were identified using nuclear magnetic resonance (^{1}H NMR). The identification of purified fractions using NMR made it possible to determine the purity and confirm the structure of compounds previously assumed using mass detector chromatography. The following main names have been identified for experimental use: No. 2 – 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-oh, No. 3 – 5-(hydroxymethyl)furan-2-carbaldehyde, No. 5.3 – 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde (Fig. 1).

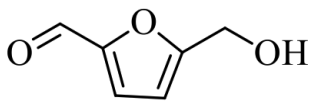
Assessment of biological effects

The biological effect of the isolated compounds was evaluated on the HeLa CCL2 cell line. The cell line was obtained from the biobank of the National Medical Research Centre for Oncology, which works in accordance with the recommendations on the organization of the structure of biorepositories and the ethical requirements of the latest edition of the ISBER Best Practices and based on the ISO 9001 standard [11]. The cells were cultured at 37 °C and 5 % CO_2 in a nutrient medium Igla MEM (BioloT) containing 10 % fetal serum from cows (HyClone, USA), up to a number of 1 × 10^6 cells. When 80 % confluence was achieved, the nutrient medium was replaced with a similar one with the addition of 4 micrograms / ml of furfural and azulene derivatives to the test samples, and without the addition of the studied substances in the negative control. The exposure time was 72 hours. After that, the cells were removed from culture vials with 0.1 % trypsin solution. The number of living and dead cells was determined using an automatic NanoEnTek JuliFl counter (Korea) with 0.4 % trypan blue staining. Cells removed from culture vials were preserved in an RNA



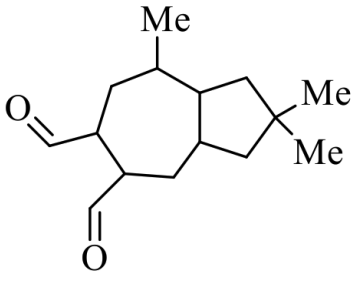
2,4-dihydroxy-2,5-dimethylfuran-3(2*H*)-one (**2**)

Chemical Formula: $C_6H_8O_4$
Molecular Weight: 144,13



5-(hydroxymethyl)furan- -2-carbaldehyde (**3**)

Chemical Formula: $C_6H_6O_3$
Molecular Weight: 126,11



2,2,8-trimethy|decahydroazulene-5,6-dicarbaldehyde (**5.3**)

Chemical Formula: $C_{15}H_{24}O_2$
Molecular Weight: 236,3499

Fig. 1. Structural formulas of three compounds isolated from the hybrid *P. hibridus* (L.) protein

medium (IntactRNA Eurogene). Cellular apoptosis was assessed on a BD FACSCanto II flow cytofluorometer using the FITC Annexin V Apoptosis Detection Kit I. Cells stored in an RNA medium were divided into two equal aliquots, from which total DNA/RNA preparations were extracted using the commercial DNA-sorb-B and Trizol kit, respectively.

Molecular methods

The evaluation of copy number variations and gene expression was performed by digital drip PCR using the QX200™ ddPCR™ EvaGreen Supermix kit (Bio-Rad, USA). The Droplet Digital polymerase chain reaction system (ddPCR™) was developed for high-precision absolute quantitative analysis of target sequences of nucleic acids encapsulated in discrete droplets of water-oil emulsion determined by volumetric method. Using a droplet generator, each sample of the studied locus was divided into 20,000 droplets in three repeats. Amplification was carried out to the end point (40 cycles) on the C1000 Touch Thermal Cycler Bio-Rad.

After the amplification was completed, a QX200 Bio-Rad reader was placed on the sample plate, which counted droplets giving fluorescent positive and negative signals to calculate the concentration of target DNA and ctDNA. The principle of measuring the level of copyness and expression indicators using digital drip PCR technology was to directly count events via the FAM channel. In positive droplets containing at least one copy of the target DNA, the droplet reader shows fluorescence, unlike negative droplets in which amplification did not occur. QuantaSoft v1 software.7.4 measures the number of positive and negative droplets in each sample, and then applies an algorithm for calculating the Poisson distribution function to determine the initial concentration of target DNA molecules in units of "copies/μl" (Fig. 2).

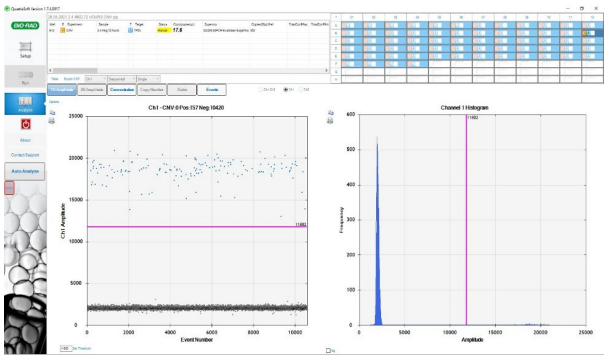


Fig. 2. Screenshot of the QuantaSoft v1 software.7.4 during the result processing

The level of CNV and gene expression was calculated as follows. According to the formula, the concentration of each studied locus / concentration of the reference locus × the number of copies of the reference loci in the genome (as a rule 2).

Statistical data processing

Statistical data processing was carried out using the Statistica 19.0 program (StatSoft Inc., USA). To assess the significance of the differences, a single-factor analysis of variance was used (the critical level of statistical significance was $p < 0.05$).

STUDY RESULTS

At the first stage of the study, the purification and verification of compounds that can exhibit cytotoxic effects on tumor cells of various nosologies was carried out. The identification of the isolated compounds was performed by mass spectometry and nuclear magnetic resonance (NMR); during it, 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one was verified, which was assigned the serial number 2,5-(hydroxymethyl)furan-2-carbaldehyde with the serial number 3, as well as 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde with the serial number 5.3. All three compounds are isolated from the rhizomes of *P. hibridus* (L.). The data obtained by evaluating the cytotoxic effect of the compounds involved in the experiment on the NanoEnTek JuliFl cell counter are presented in Table 1.

As can be seen from the data presented in Table 1, all compounds according to the results of the trepan blue test had an approximately equivalent effect on HeLa tumor cells; in experimental samples, the number of dead cells exceeded the control by 1.97–2.44 times. The images below, obtained using an inverted microscope (Leica DM IL LED), show a comparison of a control sample of the HeLa cell line with a sample treated with compound No. 2. After exposure, a violation of the monolayer in the experimental sample is seen associated with weaker cell attachment or lysis, and a large number of cells are "scalded" (Fig. 3).

The following images show the effect achieved when exposed to compound No. 3 in comparison with the control, a dense monolayer of cells is observed, at the same time a large number of scalding cells (Fig. 4).

Figure 5 shows a comparison of the control of Hela cells with cells that were exposed to compound 5.3, in the experimental sample there is a dense monolayer of cells and an increase in the number of scalding cells that exceeds that observed in the control.

The results of the evaluation of the antitumor effect of the metabolites used by us are also confirmed by the data of flow cytofluorometry presented below.

The most pronounced cytotoxic effect was shown by (2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one) at No. 2 at a concentration of 4 micrograms /ml at exposure for 72 hours. The remaining compounds used did not have such an effect according to flow cytofluorometry (Fig. 6–8, table 2).

As can be seen from Figure 6, 72-hour incubation with (2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one) had a cytostatic effect on HeLa cells, expressed in an increase in the number of cells in the state of early apoptosis from 7.2 to 13.3 %, and late apoptosis from 5.8 to 8.3 %. The total number of cells in the state of apoptosis after exposure (2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one) increases 1.6 times.

Based on the data in Fig.7, it follows that a 72-hour incubation with 5-(hydroxymethyl)furan-2-carbaldehyde did not have a cytotoxic effect on HeLa cells, the difference with the control in early apoptosis changed from 7.2 to 7.3 %, and late apoptosis – from 5.8 to 6.2 %. The total number of cells in the state of apoptosis after exposure to 5-(hydroxymethyl) furan-2-carbaldehyde increases by 0.5 times.

Figure 8 demonstrates that 72-hour incubation with 5-(hydroxymethyl)furan-2-carbaldehyde also did not have a cytotoxic effect on HeLa cells, the difference with the control in early apoptosis changed from 7.2 to 7.5 %, and late apoptosis – from 5.8 to 6.8 %. The total number of culture cells in the state of apoptosis after exposure to 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde increased 1.3 times.

Digital drip PCR was used to evaluate changes in CNV and expression (CNV/EXP) indices under the influence of secondary metabolites from *P. hibridus* (L.) isolated by us. When exposed to 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde at a concentration of 4 micrograms/ml exposure for 72 hours, there were increases in the level of expression of *CASP3* in relation to the control by 28.28 times ($p < 0.05$), and *CASP8* by 46.71 times ($p < 0.05$). At the same time, the expression of the *CASP9* locus increased by 3.43 times ($p < 0.05$). Exposure to 5-(hydroxymethyl) furan-2-carbaldehyde at a concentration of 4 micrograms/l and an exposure of 72 hours had the following effect: the expression level of *CASP3* increased by 4.57 times relative to the control ($p < 0.05$), it also increased the expression level of *CASP8* by 10.48 times ($p < 0.05$). When using 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one at a concentration of 4 micrograms/ml exposure for 72 hours, the expression of the *CASP3* locus relative to the control increased by 3.95 times ($p < 0.05$), and *CASP8* by 3.38 times ($p < 0.05$). At the same time, the indicators of the copy level (CNV) of the *CASP8*, *CASP9*, and *CASP3* loci did not undergo major changes (Fig. 9).

At the same time, the assessment of changes in the levels of copy number variability (CNV) and expression (EXP) at the *TP53* and *MDM2* loci showed the following results. The compound 2,2,8-trimeth-

Table 1. The number of living and dead HeLa cells after exposure to isolated secondary metabolites after staining with trypan blue

Compounds, concentrations	72 hours, living cells	72 hours, dead cells
Control	93.52 %	6.48 %
№ 2, 4 μg /ml	87.23 %	12.77 %
№ 3, 4 μg /ml	86.66 %	13.34 %
№ 5.3, 4 μg /ml	84.16 %	15.84 %

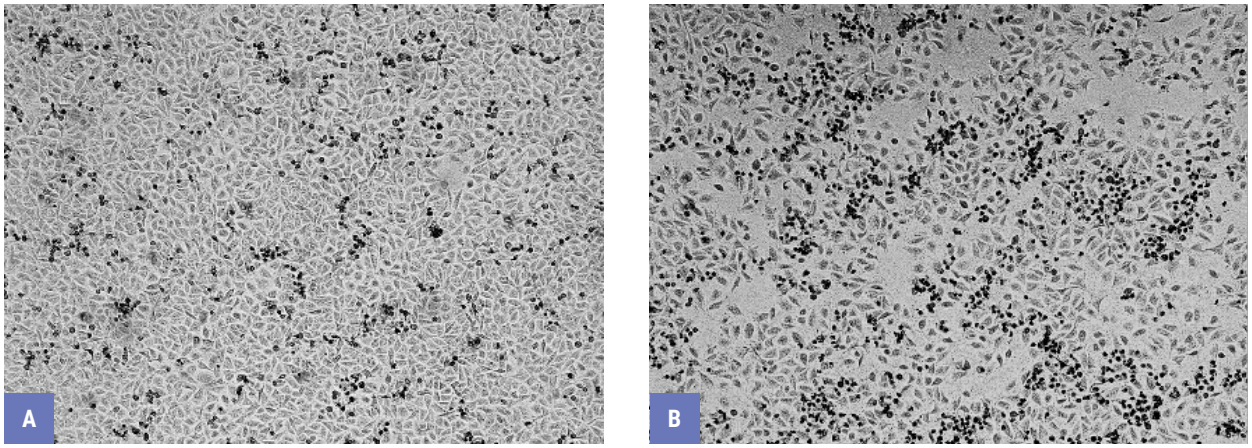


Fig. 3. HeLa cells after exposure to 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one. A – control specimen; B – experimental specimen

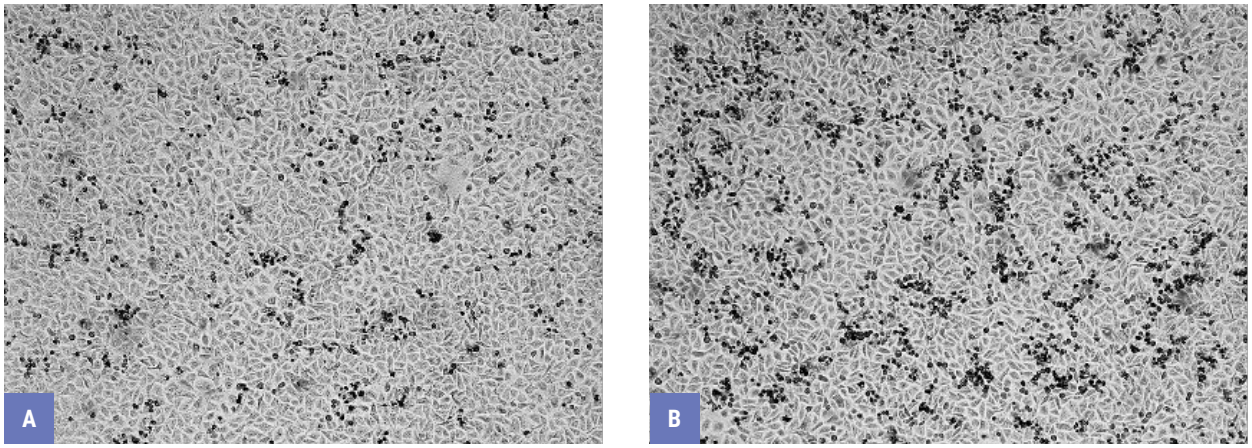


Fig. 4. HeLa cells after exposure to terpenoid 5-(hydroxymethyl)furan-2-carbaldehyde. A – control specimen; B – experimental specimen

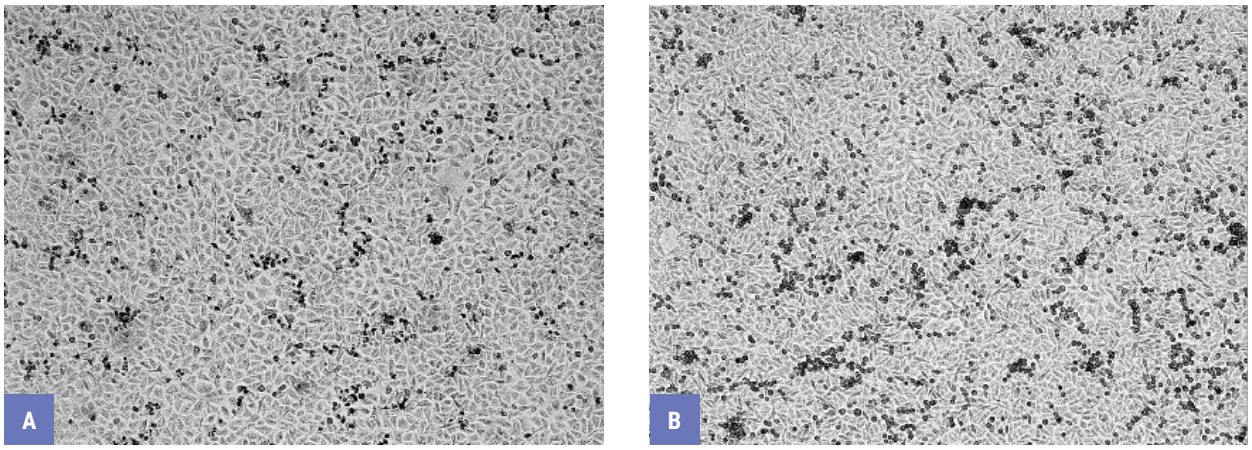


Fig. 5. HeLa cells after incubation with the terpenoid 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde. A – control specimen; B – experimental specimen

yldecahydroazulene-5,6-dicarbaldehyde at an exposure of 72 hours and a concentration of 4 micrograms/ml increased the *TP53* copy level by 1.05 times ($p < 0.05$), and *MDM2* decreased by 0.26 times ($p < 0.05$) relative to the control. The difference between them was 4 times. In addition, 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one increased *TP53* expression level by 1.46 times ($p < 0.05$) at exposure of 72 hours and concentration of 4 micrograms/ml, while *MDM2* decreased by 0.88 times ($p < 0.05$). The difference was 1.66 times (Fig. 10).

The following data were obtained when evaluating changes in the level of replication and expression of the *BAX* and *BCL2* loci. The terpenoid 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde increased the level of CNV of the I locus by 0.9 times relative to the control ($p < 0.05$), the level of *BCL2* decreased by 0.13 times ($p < 0.05$). The difference between them

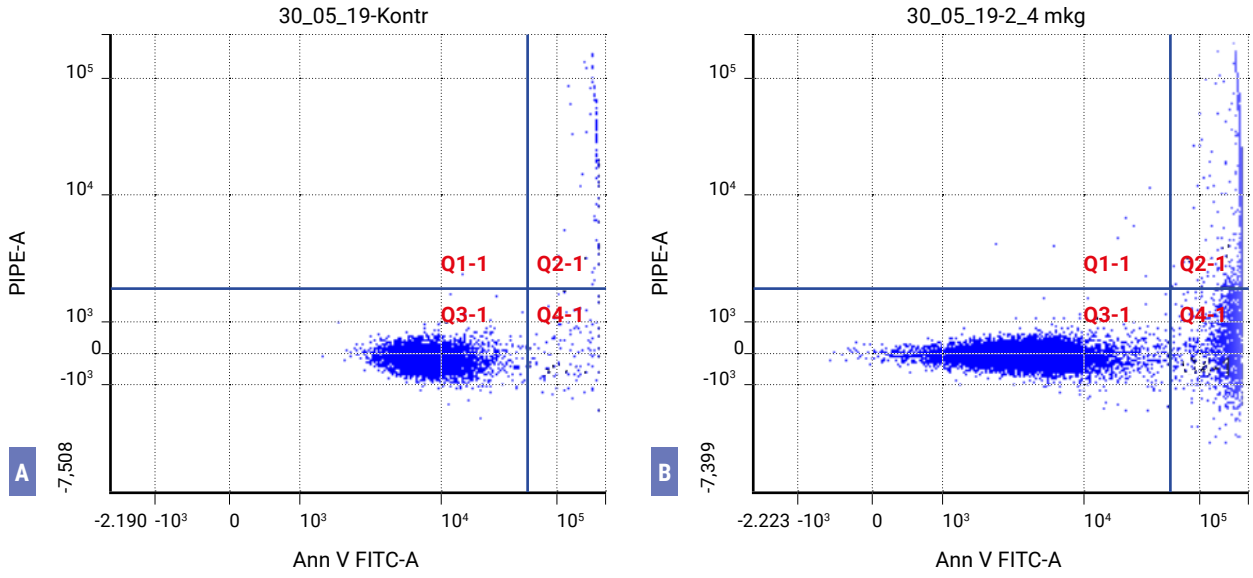


Fig. 6. Effect of (2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one) on necrosis/apoptosis of the HeLa cell line: A – control specimen; B – experimental specimen, (Q3-1 – living cells, Q4-1 – early apoptosis, Q2-1 – late apoptosis/necrosis, Q1-1 – dead cells)

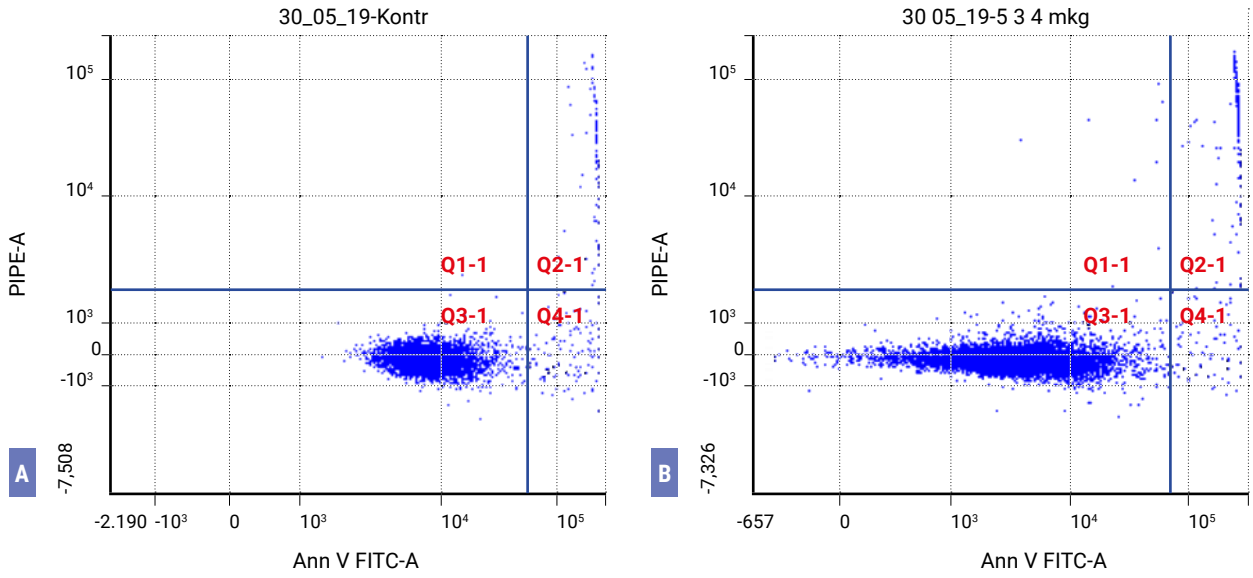


Fig. 7. Exposure to 5-(hydroxymethyl)furan-2-carbaldehyde on necrosis/apoptosis of the HeLa cell line: A – control specimen; B – experimental specimen, (Q3-1 – living cells, Q4-1 – early apoptosis, Q2-1 – late apoptosis/necrosis, Q1-1 – dead cells)

was 6.92 times. Also, when exposed to the same compound, the expression level of *BAX* increased by 1.73 times ($p < 0.05$), and *BCL2* decreased by 1.19 times ($p < 0.05$). The difference between them was 1.45 times in favor of an increase in *BAX* (Fig. 11).

The furan and azulene derivatives of *P. hibridus (L.)* metabolites used in our study changed the level of replication and expression of *CDKN1B*, *CDK1*, *CCND1*, *CCND3* and *RB1* loci as follows. Thus, 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde increased *CCND3* expression by 20.66 times relative to the control ($p < 0.05$), RB1 expression increased by 7.35 times ($p < 0.05$) at an exposure of 72 hours and a concentration of 4 micrograms/ml. In turn 5-(hydroxymethyl) furan-2-carbaldehyde with an exposure of 72 hours and a concentration of 4 micrograms/ml also increased the expression level of *CCND3* by 5.23 times ($p < 0.05$). At the same time, 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one at similar concentrations and exposures increased the *CCND3* copy level by 3.48 times ($p < 0.05$), and the expression level increased by 2.42 times relative to the control ($p < 0.05$). At the same time, 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one at the point of 4 micrograms/ml with an exposure of 72 hours increased the expression level of the *RB1* locus relative to the control by 4.51 times ($p < 0.05$) (Fig. 12).

DISCUSSION

Since the early 2000s, many works have been published worldwide on the search for new compounds of natural origin, including plant origin ones, with cytostatic or cytotoxic effects on tumor cells of various diseases [12]. In our study, we conducted not only a model experiment to assess the level of cytotoxic

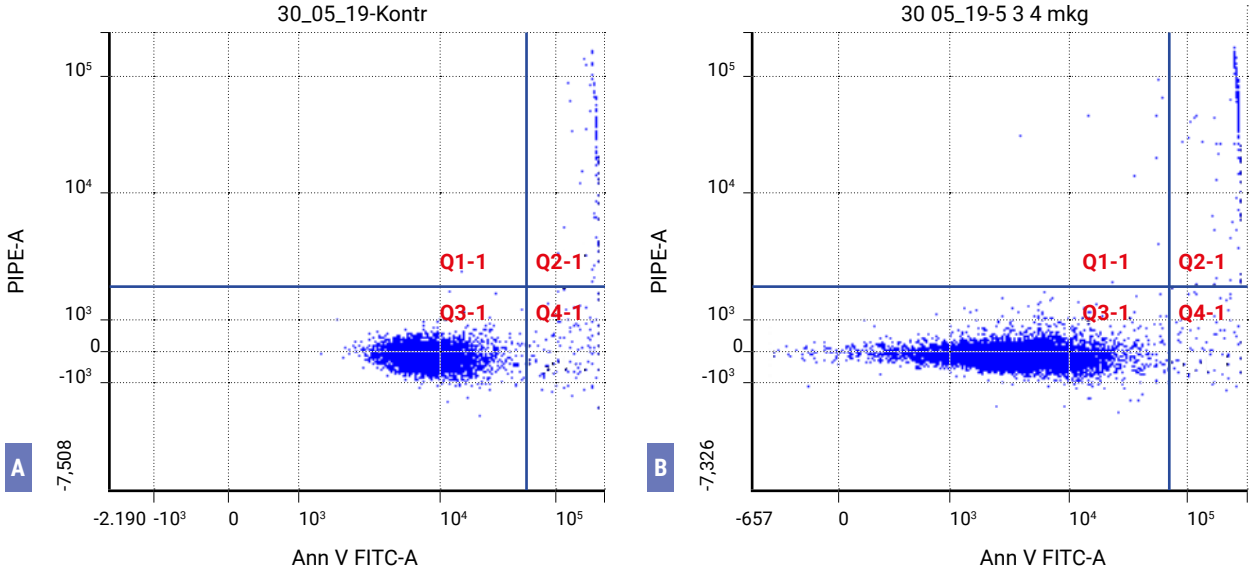


Fig. 8. The effect of 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde on necrosis/apoptosis of the HeLa cell line: A – control specimen; B – experimental specimen, (Q3-1 – living cells, Q4-1 – early apoptosis, Q2-1 – late apoptosis/necrosis, Q1-1 – dead cells)

Table 2. The number of HeLa cells in a state of apoptosis after exposure to isolated secondary metabolites (72 hours exposure)

Compound	Concentration, μg /ml	Alive cells Q3-1	Early-stage apoptosis Q4-1	Late-stage apoptosis / necrosis Q2-1	Dead cells Q1-1
Control		87.0 %	7.2 %	5.8 %	0 %
No. 2	4	78.3 %	13.3 %	8.3 %	0 %
No. 3	4	91.1 %	2.5 %	6.3 %	0 %
No. 5.3	4	94.2 %	2.6 %	3.2 %	0 %

effect of the *P. hibridus* (L.) secon dary metabolites that we obtained, but also used the digital drip PCR method to register molecular genetic changes in loci responsible for suppressing tumor growth and apoptosis in HeLa tumor cells.

The data obtained in the study show mixed results. The most pronounced change in the expression level of the *CASP8* and *CASP3* loci was revealed when exposed to 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde. Cytosolic caspases are cysteine-asparagine proteases, which are the main family of proteins involved in the transmission of cell death signals. Caspases are divided into three groups: initiatory, inflammatory and effector. They are directly involved in the initiation of apoptosis. As is known, the *CASP8* protein, which is an initia-

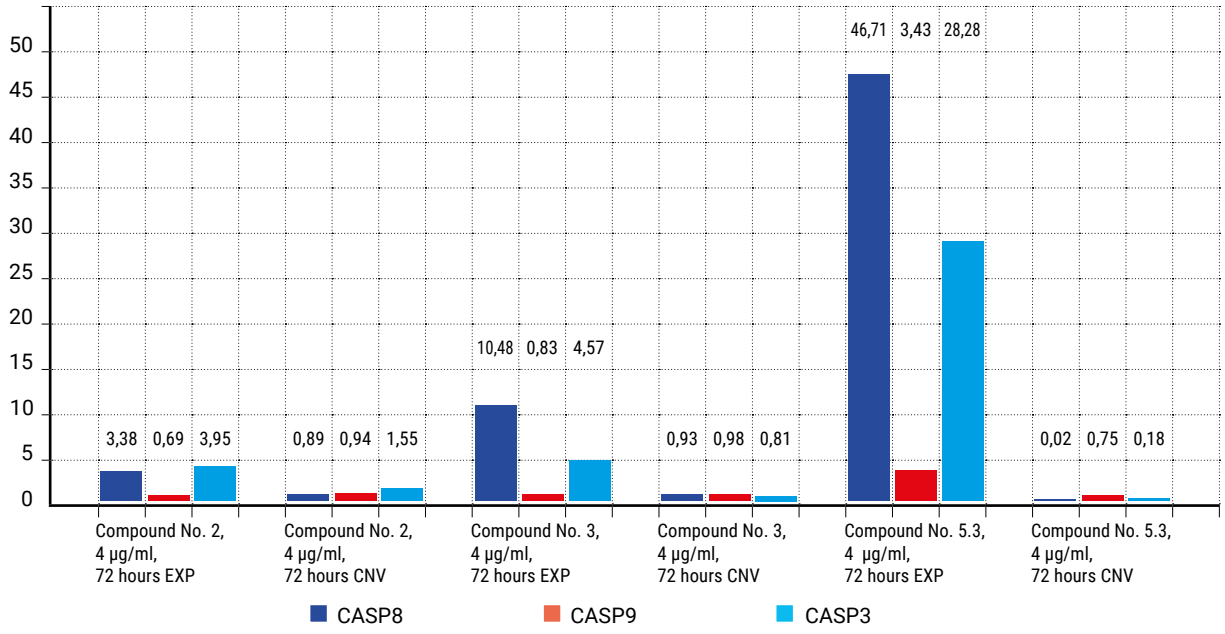


Fig. 9. Changes in the level of replication and expression of the *CASP8*, *CASP9*, and *CASP3* loci under the action of 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one (No. 2), 5-(hydroxymethyl)furan-2-carbaldehyde (No. 3) and 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde (No. 5.3)

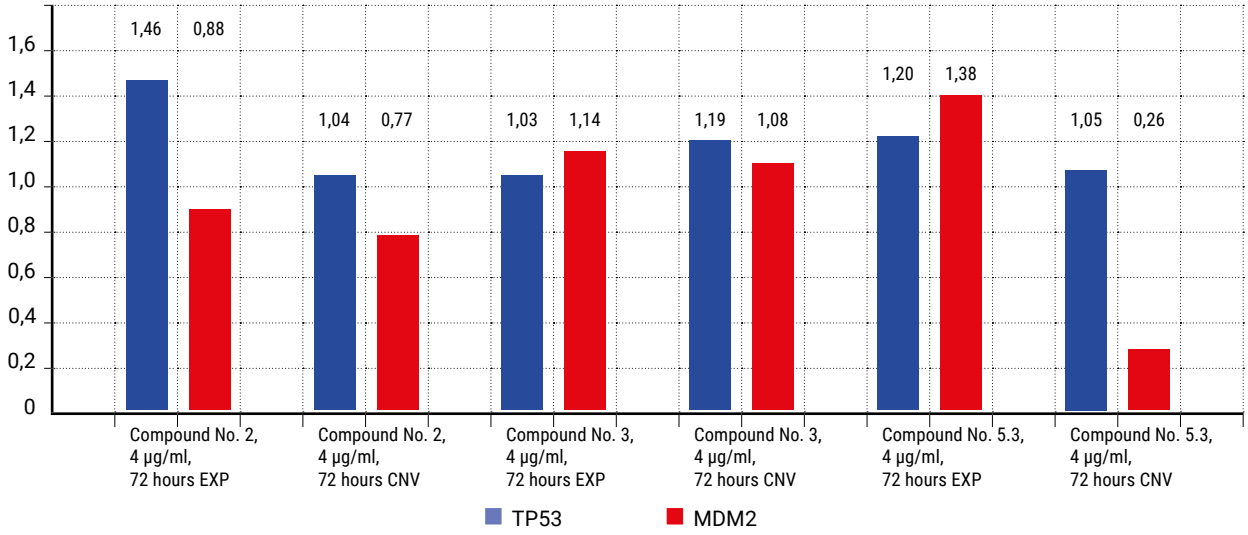


Fig. 10. Changes in the level of replication and expression of *TP53*, *MDM2* loci when exposed to 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one (No. 2), 5-(hydroxymethyl) furan-2-carbaldehyde (No. 3) and 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde (No. 5.3)

tor, is associated with tumor necrosis factor (TNF) located on the cell surface, as well as the FAS ligand (FasL), and induces apoptosis (CD95). Activation of the *CASP8* protein via the external apoptosis pathway triggers BID-mediated activation of BAX and BAK proteins on the outer membrane of mitochondria, which leads to the release of cytochrome C and subsequent activation of CASP9, which, in turn, activates CASP3 and CASP7, thereby performing the process of apoptosis along the mitochondrial pathway [13]. It should be noted that when exposed to 5-(hydroxymethyl)furan-2-carbaldehyde showed a change in the expression level of *CASP8* and *CASP3* of a similar profile.

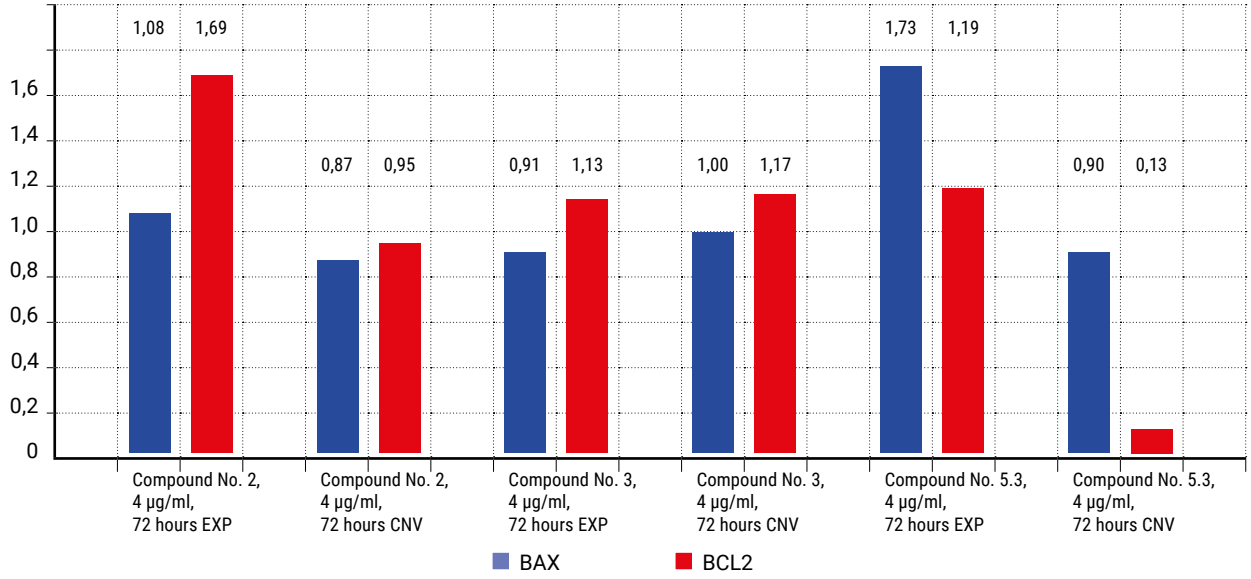


Fig. 11. Changes in the level of replication and expression of *BAX*, *BCL2* loci when exposed to 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one (No. 2), 5-(hydroxymethyl) furan-2-carbaldehyde (No. 3) and 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde (No. 5.3)

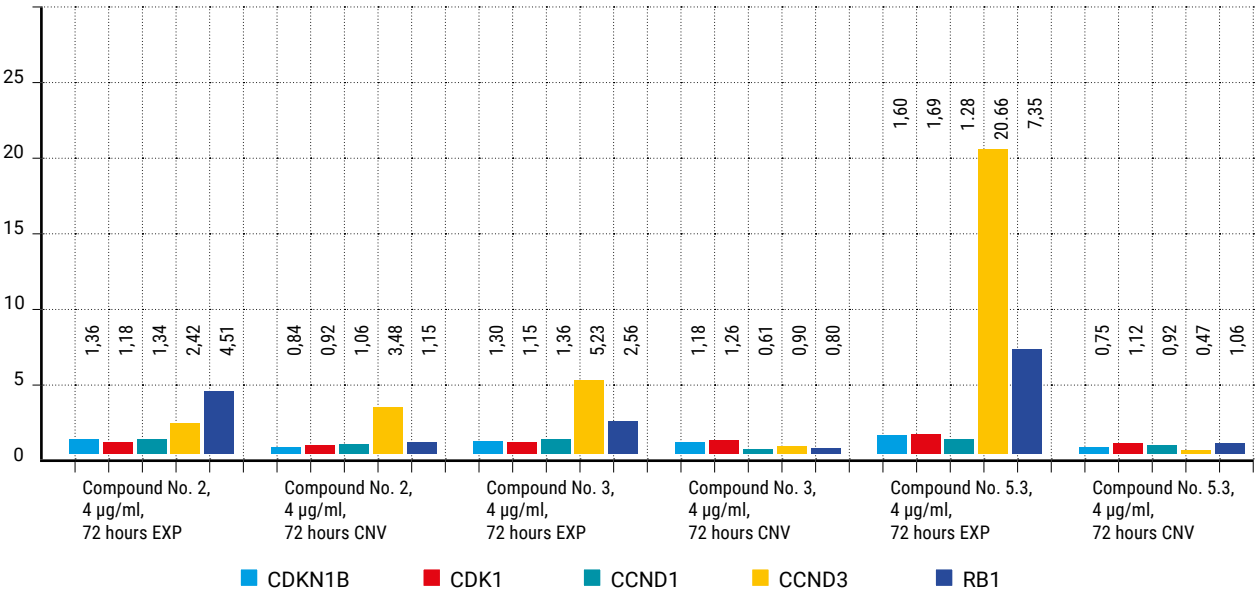


Fig. 12. Changes in the level of replication and expression of *CDKN1B*, *CDK1*, *CCND1*, *CCND3*, *RB1* loci under the influence of 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one (No. 2), 5-(hydroxymethyl)furan-2-carbaldehyde (No. 3) and 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde (No. 5.3)

At the same time, 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde increased the expression level of *TP53* and significantly reduced the expression of *MDM2*, which may indicate a specific targeting of the action of this compound. It should be borne in mind that *TP53* has tumor suppressive activity, which largely explains its ability to induce cell death, including apoptosis, through transcription-dependent and transcription-independent mechanisms [14]. In addition, the nuclear protein p53 transcriptionally activates the expression of many pro-apoptotic genes of the *BCL-2* family, such as *NOXA*, *PUMA*, *BID*, *BAD*, *BIK*, *BAX*, etc., whereas it inactivates the expression of anti-apoptotic *BCL-2*, *BCL–Xl* and *MCL1*, leading to mitochondrial apoptosis [15]. The relationship between changes in the expression level of *TP53* and *BAX* loci was also reflected in the results obtained. As in the case of the *TP53*/*MDM2* locus bundle, exposure to the terpenoid 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde affected the expression level of *BAX*/*BCL2* loci.

The changes in the expression level of CCND3 and RB1 loci under the influence of 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one were also revealed. This compound is the only one used in our study, that led to a decrease in the expression level of the CCND3 locus relative to RB1 (the expression level of RB1 was almost 2 times higher than the expression level of CCND3). As is known, d-type cyclins (d1, d2 and d3) are cell cycle regulators that activate cyclin-dependent kinases cdk4 and cdk6, which are often overexpressed in malignant neoplasms. The CCND3 gene product interacts with the Rb tumor suppressor protein and participates in its phosphorylation. CDK4 activity is associated with this CCND3, which is necessary for the transition of the cell cycle to the G2 phase. Inhibition of CCND3 and cyclin-d-cdk4/6 kinase in tumor cells with a high content of retinoblastoma rb1 protein causes cell cycle arrest. However, reducing only the level of rb1 in tumor cells does not lead to a stop in proliferation [16]. The data on the change in the expression of CCND3 and RB1 are consistent with the data of objective control from photographs obtained using an inverted microscope and data from flow cytofluorometry.

CONCLUSION

The study made it possible to establish the multidirectional effect of secondary metabolites of *P. hibridus* (L.) on the death and apoptosis of HeLa cells. The data obtained by digital drip PCR revealed a maximum increase in the expression of genes responsible for regulating apoptosis (*CASP3*, *CASP8*, *TP53*, *BAX*) under the action of 2,2,8-trimethyldecahydroazulene-5,6-dicarbaldehyde, as well as a change in the expression of *CCND3* and *RB1* genes under the influence of 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one. At the same time, according to cytometry and flow cytofluorometry, a more pronounced proapoptogenic (cytotoxic) effect was detected in 2,4-dihydroxy-2,5-dimethylfuran-3(2H)-one. It should be noted that in our work, the expression index reacted most actively to the studied substances, which, in some cases, was dissonant with both gene replication and the level of mortality and apoptosis of tumor cells. Perhaps chemical modifications of the compounds used by us will have a more pronounced effect both at the molecular genetic level and at the cellular level.

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Enin Ya. S. – concept and design of the study, experiment coduction, writing the manuscript;
Burov O. N. – isolation and verification of compounds from plant material;
Bondarenko E. S. – cytofluorimetric analysis;
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ORIGINAL ARTICLE

Changes in the concentration of freely circulating mutant DNA and wild-type DNA of the *H3F3A* (K27M) gene in the blood and cerebrospinal fluid of children with diffuse midline gliomas during a course of radiation therapy

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ABSTRACT

Purpose of the study. To study the possibility of detecting freely circulating DNA of the *H3F3A* (K27M) gene in blood plasma and cerebrospinal fluid in the lumbar spine in children with diffuse midline gliomas (DMG) during a course of radiation therapy (RT).
Materials and methods. Molecular genetic studies were carried out by digital PCR. 96 samples of lumbar cerebrospinal fluid and 288 samples of peripheral blood plasma from 96 pediatric patients were analyzed. The concentration of circulating tumor (ctDNA) mutant DNA and wild-type DNA of the *H3F3A* (K27M) gene was determined in the studied material against the background of a course of RT. Lumbar cerebrospinal fluid sampling was performed once at the beginning of therapy, blood sampling was performed three times: The 1st test before the start of RT, the 2nd against the background of a total dose 10–15 Gy, and the 3rd after the completion of the RT course. Patients are divided into the following groups: patients with stabilization of brain tumor growth during early magnetic resonance (MR) control 3 months after completion of the course of RT; patients with disease progression during the same follow-up period who underwent radiation or chemoradiotherapy.
Results. When the disease stabilized after a RT course during treatment, the concentration level of both the mutant variant of ctDNA and wild-type ctDNA significantly decreased in the third blood fraction. The absence of changes or an increase in the concentration of mutant ctDNA and wild-type ctDNA of the *H3F3A* (K27M) gene by the end of the course of radiation therapy was typical for patients with disease progression in the form of the appearance of metastatic foci in the central nervous system or continued tumor growth. At the same time, the concentration of wild-type DNA of the *H3F3A* (K27M) gene in the group of patients with progression was higher both in the lumbar cerebrospinal fluid and in the first fraction of blood plasma.
Connclusion. Determination of the concentration and dynamics of circulating tumor DNA of the mutant and wild type of the *H3F3A* (K27M) gene in blood plasma and lumbar cerebrospinal fluid in children with diffuse median gliomas of the brain during radiation therapy is promising from the point of view of predicting the effectiveness of therapy.

Keywords: glioma, diffuse median glioma, digital drip PCR, *H3F3A*, K27M, circulating tumor DNA (ctDNA)

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Compliance with ethical standards: this research has been carried out in compliance with the ethical principles set forth by the World Medical Association Declaration of Helsinki, 1964, ed. 2013. The study was discussed and approved at a meeting by the Scientific Council of the Russian Scientific Center of Roentgenoradiology (Scientific Protocol No. 3/2022, 12/12/2022, Protocol No. 7). Informed consent was received from all the participants of the study

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3.1.6. Онкология, лучевая терапия
ОРИГИНАЛЬНАЯ СТАТЬЯ

Изменения концентрации свободно циркулирующей мутантной ДНК и ДНК дикого типа гена *H3F3A* (K27M) в крови и люмбальном ликворе у детей с диффузными срединными глиомами на фоне курса лучевой терапии

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РЕЗЮМЕ

Цель исследования. Изучить выявляемость свободно циркулирующей ДНК гена *H3F3A* (K27M) в плазме крови и люмбальном ликворе у детей с диффузными срединными глиомами (ДСГ) на фоне курса лучевой терапии (ЛТ).
Материалы и методы. Молекулярно-генетические исследования проводились методом цифровой полимеразной цепной реакции (ПЦР). Проанализировано 96 образцов люмбального ликвора и 288 образцов плазмы периферической крови 96 пациентов детского возраста. В исследуемом материале определялась концентрация циркулирующей опухолевой (цоДНК) мутантной ДНК и ДНК дикого типа гена *H3F3A* (K27M) на фоне проводимого курса ЛТ. Забор люмбального ликвора проводился однократно в начале ЛТ, забор крови – трижды: 1-я проба – до начала ЛТ, 2-я проба – на фоне суммарной очаговой дозы (СОД) 10–15 Грей (Гр), и 3-я – после завершения курса ЛТ. Пациенты, которые получили лучевую или химиолучевую терапию, были разделены на следующие группы: 1-я группа включала в себя пациентов со стабилизацией роста опухоли головного мозга в сроки раннего магнитно-резонансного (МР) контроля, 2-я группа - пациентов с прогрессированием заболевания.
Результаты. При стабилизации заболевания после проведенного курса ЛТ на фоне лечения уровень концентрации как мутантного варианта цоДНК, так и цоДНК дикого типа достоверно снижался в анализе крови при третьем заборе. Отсутствие изменений или увеличение уровня концентрации мутантной цоДНК и цоДНК дикого типа гена *H3F3A* (K27M) к концу курса ЛТ было характерно для пациентов продолженным ростом опухоли с прогрессированием заболевания в виде появления метастатических очагов в центральной нервной системе. При этом концентрация ДНК дикого типа гена H3F3A (K27M) в группе пациентов с прогрессированием была более высокой как в люмбальном ликворе, так и в анализе крови при первом заборе.
Заключение. Определение концентрации и динамики циркулирующей опухолевой ДНК мутантного и дикого типа гена *H3F3A* (K27M) в плазме крови и люмбальном ликворе у детей с диффузными срединными глиомами головного мозга в процессе ЛТ является перспективным с точки зрения прогноза эффективности проводимой терапии.

Ключевые слова: глиома, диффузные срединная глиома, цифровая капельная ПЦР, ген *H3F3A*, мутация K27M, циркулирующая опухолевая ДНК (цоДНК)

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INTRODUCTION

Over the past 10 years, there has been a fundamental paradigm shift in the field of diffuse midline gliomas (DMG) diagnosis, where among the most significant discoveries is the K27M mutation in the *H3F3A* or *HIST1H3B* genes, which encode histone variants H3, H3.3 and H3.1. The H3K27M mutation gives odds in gliomagenesis a head start due to persistent clonogenicity and aberrant differentiation and determines the associated changes in histone and DNA methylation [1]. The preservation of proliferative clonogenic states increases the likelihood of acquiring additional mutations in nascent neomorphic cells. In addition, aberrant differentiation can change the organization of tissues and create a microenvironment that promotes the development of tumors. Both of them are potential consequences of the H3K27M mutation, and may contribute to the occurrence of DMG [1]. To date, the detection of the K27M mutation in the *H3F3A* gene is recommended in a number of foreign countries to assess the prognosis of the disease and the choice of treatment tactics [2–4]. The detection of a mutation in DMG is associated with an extremely aggressive clinical course and an unfavorable prognosis, [5–8] regardless of histological examination data, therefore, when the K27M mutation is detected in the *H3F3A* gene, the tumor is classified as grade 4 malignancy [4, 6, 7].

When comparing adult and pediatric patients with central nervous system (CNS) tumors, it was shown that in adults, the K27M mutation occurs with the highest frequency in high-grade gliomas (HGGs) of the thalamus and spinal cord, and in children – with diffuse median gliomas of the brain, while the frequency of the K27M mutation in the *H3F3A* gene can reach 94 % [6, 9, 10]. An important difference is that in children suffering from DMG, the presence of the K27M mutation is an extremely unfavorable prognostic factor, and for supratentorial gliomas in adults, this gene change is not clinically significant. There were no significant differences in survival and clinical course of the disease for adult patients with and without the K27M mutation, H3K27M may be present both in histologically verified HGGs and in low-grade gliomas (LGGs) [9].

In children with HGG with the K27M mutation, they have a more aggressive clinical course in comparison with HGG of a different genetic nature. In this regard, the identification of mutant forms has prognostic value and most studies are focused specifically on the study of the mutant DNA of the *H3F3A* gene in DMGs, especially among children [2]. At the same time, there is practically no information on the prognostic role of determining changes in the concentration of wild-type DNA during treatment and the ratio of concentrations of mutant DNA and wild-type *H3F3A* gene [11] among diffuse midline gliomas, although for tumors of other localizations, an increase in the concentration of wild-type DNA is a poor prognostic factor [12, 13].

In DMGs, due to the peculiarities of the anatomical location of tumors, it is difficult to obtain histological material using surgical intervention. Unfortunately, the use of targeted stereotactic biopsy does not always allow to obtain an adequate amount of material for histological and molecular analysis [14, 15]. When a diffuse tumor is biopsied, several samples are taken from different points, which sometimes does not allow to identify intra-tumor heterogeneity for an accurate diagnosis [16]. The use of a liquid biopsy method aimed at identifying biological markers by analyzing circulating tumor DNA (ctDNA) in blood plasma and lumbar liquor samples makes it possible to determine the molecular profile of a tumor without using traumatic invasive techniques. Modern approaches to monitoring the course of the disease that meet international standards use radiographic imaging – magnetic resonance imaging (MRI) to determine how the tumor reacts to treatment. It is worth noting that performing an MRI examination is an expensive procedure, and often, in the case of pediatric patients, requires the use of an anesthetic aid, which is difficult to access in the regions. In the case of DMG H3K27M, diffuse tumor growth and radiation-induced edema complicate the interpretation of images under dynamic observation. Studies have shown that the levels of tumor biomarkers in biological media, such as blood or cerebrospinal fluid, correlate with the course of the disease. Thus, consistent quantification of these biomarkers can help identify disease progression in advance. The diagnostic potential of using liquid biopsy in children with DMG has not been fully disclosed, although research in this direction is actively underway [7, 17]. In addition, the use of liquid biopsy in pediatric neuro-oncology lags behind similar methods in adults, however, these studies show that the technology has significant potential [7, 17–19].

The most accessible material for the liquid biopsy method is blood plasma, and for many solid tumors, the determination of ctDNA in plasma is an important diagnostic method [6, 7]. However, in DMG, the blood-brain barrier (BBB) significantly restricts the flow of ctDNA into the blood [20], therefore, an alternative source of ctDNA is the lumbar cerebrospinal fluid [7, 21]. In CNS tumors in children, molecular examination of the lumbar cerebrospinal fluid can also be a significant alternative to morphological verification at high risks or inability to obtain biopsy material [19].

To date, the most sensitive method for evaluating ctDNA, which allows obtaining adequate results with a small amount of material, is the digital drip PCR method [7]. In recent years, the number of studies conducted by this method has increased many times, including in CNS tumors [7, 11].

An integrated approach in liquid biopsy studies, especially in DMG, should include a combination of the choice of research material and molecular analysis methods [6, 7]. In recent years, the gold standard has become the study of ctDNA in both plasma and cerebrospinal fluid, which allows us to obtain the most accurate molecular data necessary for the diagnosis and prognosis of the course of the disease.

In this work, we examined the ctDNA of the *H3F3A* (K27M) gene both in blood plasma and in lumbar cerebrospinal fluid in children with diffuse midline gliomas during radiation therapy. Special attention was paid not only to the detectability of mutant ctDNA, but also to the ratio of the amount of mutant ctDNA to wild-type ctDNA – variant allele fraction (VAF), the change in H3.3K27M VAF over time ("delta VAF"), as well as its correlation with various clinical parameters [22]. In our opinion, the study of patients without the H3.3K27M mutation is important, but poorly studied. To date, we have not found information in the available literature on changes in the concentration of wild-type DNA of the *H3F3A* gene against the background of radiation therapy in children, which confirms the relevance of our work and the need for further development of molecular diagnostics and personalized therapy of DMG.

MATERIALS AND METHODS

The study included 96 children with diffuse midline gliomas of the brain who underwent radiation and chemoradiotherapy at the Department of Russian Scientific Center of Roentgenoradiology in the period from 2022 to 2024. The study cohort consisted of 53 (55 %) boys and 43 (45 %) girls aged 18 months to 18 years, the average age at the time of diagnosis was 8 years. Clinical indicators included gender, age, and the nature of disease progression – the appearance of metastatic dropouts in the central nervous system or continued tumor growth, but they had no significant differences in the study groups and associations with DNA concentrations in blood plasma and lumbar liquor ($p > 0.05$). When conducting an instrumental examination of patients based on the results of MRI of the brain natively and with contrast enhancement before the start of therapy, it was found that in all patients the tumors had diffuse growth and median location. Histological examination of tumors in 18 cases showed that HGG prevailed mainly, 6 of them had a K27M mutation in the *H3F3A* gene. The assessment of groups with continued growth and stabilization of the disease was carried out on the basis of MRI data of the brain without and with contrast enhancement (CE), performed within 3–4 months after completion of the course of RT.

The scheme of radiation therapy

Radiation therapy was performed using Varian Clinac 2100 linear accelerators, True Beam, and the Varian Eclipse dosimetric calculation system. During therapy, the traditional version of fractionation of the dose of 1.8–2 Gy was used, with a total focal dose of up to 54 Gy. In the presence of pronounced perifocal edema and symptoms of developing intracranial hypertension, treatment began in the mode of multifractionation in single doses of 1.0–1.1 Gy 2 times a day with an interval between fractions of 4–6 hours with a gradual transition to the usual fractionation mode as the condition stabilizes, but with a correction of the total dose over the period of multifractionation in the direction of its increase equivalent to 54 Gy. In patients with a histologically confirmed diagnosis of HGG, a course of RT was performed with parallel radio modification with temozolomide, 75 mg/m^2, daily against the background of the entire course of RT.

Obtaining research material

During the study, we received samples of peripheral blood plasma and lumbar cerebrospinal fluid from 97 patients. Samples of lumbar cerebrospinal

fluid were taken once against the background of radiation therapy. Blood plasma was taken at three stages: before the start of therapy, during radiation therapy and after completion of the course of radiation therapy.

Isolation of circulating DNA from lumbar cerebrospinal fluid

To isolate ctDNA from the lumbar liquor, we used Sileks kits, which are based on the use of SileksMagNA-Direct particles (particles for selective binding of nucleic acids). The extraction procedure was carried out according to the protocol provided by the manufacturer. The collection of cerebrospinal fluid and the beginning of the procedure for isolation of circulating tumor DNA did not exceed 30 minutes. The lumbar liquor was centrifuged at 1,500 revolutions per minute for 5 minutes, and a superabsorbent fraction with a volume of 0.7 to 2 ml was used to isolate ctDNA. In our work, mutant ctDNA of the *H3F3A* gene was isolated from 96 cerebrospinal fluid samples in 33, and wild-type ctDNA of the *H3F3A* gene was isolated in all 96 cerebrospinal fluid samples (Fig. 1).

Isolation of circulating DNA from blood plasma

Plasma preparation. Plasma was separated immediately after receiving a blood sample. Sileks kits based on SileksMagNA-Direct particles were used to isolate circulating DNA from blood plasma. The isolation procedure was carried out according to the manufacturer's protocol. Of the 288 peripheral blood plasma samples obtained, mutant ctDNA of the *H3F3A* gene was isolated in 29, wild-type ctDNA of the *H3F3A* gene was isolated in all studied samples.

Determination of the K27M mutation in the *H3F3A* gene by digital droplet PCR (ddPCR)

Highly sensitive screening of the *H3F3A* (K27M) mutation using Digital Droplet PCR (ddPCR) technology using the *H3F3A* (K28M) Screening Kit (Bio-Rad, USA) and the QX100 Droplet Digital PCR System (Bio-Rad, USA) was used.

For ddPCR formulation, BioRad reagents were used according to the research protocol. The DNA probes used to detect the amplification products of the studied and normalizing genes were labeled FAM and HEX. The PCR mixture was placed in a droplet generator, where a water-oil emulsion was created from 20 µl of the sample in which the amount of DNA under study was to be determined, and up to 20,000 drops of 1 nl were formed in each tube. In this case, the genetic material is randomly distributed into droplets: both target DNA and background DNA fall into them. The process of distributing the target DNA by droplets is purely random and obeys the law of distribution of small Poisson numbers. Before dividing the sample into drops, it is not nec-

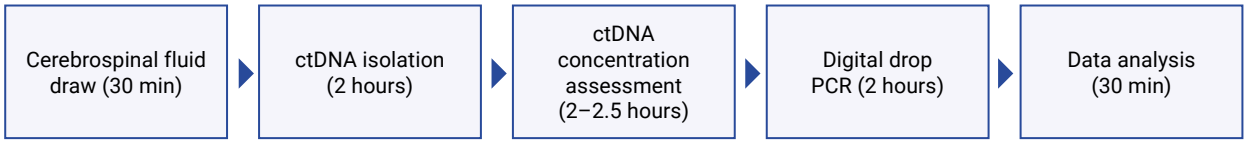

Fig. 1. Isolation of circulating tumor DNA by drip PCR from lumbar cerebrospinal fluid

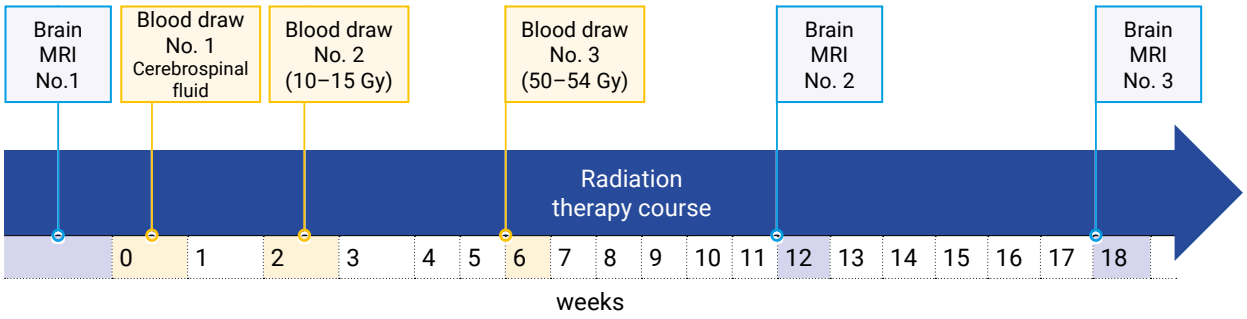

Fig. 2. Study design

essary to dilute it to a concentration so that each drop contains either 0 or 1 copy of the target DNA: when analyzing the results, situations are taken into account when there is more than one copy of the target in one drop. According to the Poisson distribution, either one matrix chain gets into the drop, or none gets into it. Samples were transferred from the droplet generator to the applicator. The amplification was carried out in "real time" mode. After amplification, the tablet was placed in a "BIO-RAD QX 100TM DROPLET READER" device, where the signal from the fluorescent labels was read. In a drop with a matrix, amplification is many times more efficient than with other types of PCR, which is due to the presence of all components of the PCR mixture in the nanoscale. During amplification, enzymatic cleavage of TaqMan probes occurs, as a result of which the fluorescence efficiency of the droplet increases many times. The product accumulated during the amplification is detected in each drop separately, at a rate of 1500 drops/s. Based on the ratio of the total number of microdrops and the number of microdrops in which the fluorescence level exceeds the background, the reader calculates the absolute amount of DNA in one microliter of the sample. The results were recorded in the "Quanta Self 16" program.

The database of clinical cases was formed using electronic databases of Microsoft Excel tables. Statistical processing was carried out using the SPSS software for Windows, version 26.0 (SPSS, Chicago, Illinois, USA) and Statistica, version 13.

The normality of the sample distribution was checked using the Kolmogorov-Smirnov criterion. The reliability of the differences was determined using the Mann-Whitney criterion. The exact one-sided Fisher criterion was used to evaluate qualitative features. The results of comparing quantitative data were considered statistically significant at $p < 0.05$.

STUDY RESULTS AND DISCUSSION

1. Mutation analysis

In recent years, scientists have focused on H3.3K27M mutant DMG [19], since the *H3F3A* (K27M) mutation is more common than G34V/R mutations in children with highly malignant diffuse astrocytomas [23–25]. This mutation is considered as a potential diagnostic marker for the identification of these tumors, similar to the use of IDH1/2 mutations for the diagnosis of diffuse gliomas in adults.

All H3K27M mutations described in DMG in most cases have the same epigenomic consequences for the PRC2 complex (PRC2 – Polycomb repressive complex 2 – conservative protein complex) as a whole [26, 27], despite the different functions and genomic distribution of many variants. It is important to note that the life expectancy of patients largely depends on the type of histone where the K27M mutation is present. Back in 2014, Wu et al. It was found that patients with a mutation in histone H3.1 respond better to radiation therapy, have a less aggressive course and are less likely to have metastases [28]. Therefore, the assessment of the type of histone mutation can be used as a predictive stratification factor in future prospective studies [4].

In this study, variants of allelic fractions (VAF) H3.3K27M were evaluated – the ratio of the concentration of freely circulating mutant DNA to the wild-type DNA of the *H3F3A* (K27M) gene in samples of lumbar liquor, as well as in blood plasma. At the same time, blood plasma samples were taken before the start of radiation therapy (sample 1), during radiation therapy (sample 2) and after the end of radiation therapy (sample 3) (Fig. 2). A total of 8 parameters were evaluated. Thus, we determined the concentration and assessed the dynamics of changes, against the background of radiation therapy, not only mutant DNA, but also wild-type DNA of the *H3F3A* (K27M) gene. It is necessary to understand the molecular features of the development of DMG from various angles in order to make it possible to improve and create new therapeutic strategies.

2. Correlation status of the *H3F3A* (K27M) gene and clinical and pathological characteristics

Previous studies have shown that K27M-mutant DSGs are associated with significantly shorter-term survival [28]. Moreover, in a multivariate analysis that also took into account the effect of treatment, the type of histone H3 mutation was a more accurate predictor of survival duration than the assessment of the clinical and radiological risk of DMG [11, 30].

When analyzing the data we obtained, we established the presence of a significant correlation between progression and mutant ctDNA of the *H3F3A* (K27M) gene obtained from the lumbar liquor fraction (Table 1).

In a recent preclinical study, Grasso et al., investigating the efficacy of panobinostat in DMG, established its effectiveness against cells containing both mutant DNA and wild-type DNA for the *H3F3A* (K27M) gene *in vitro*, although cells with the H3K27M mutation developed resistance to panobinostat within a few weeks after exposure to low doses of the drug. It is worth noting that panobinostat treatment significantly prolongs the survival of mice with tumors without mutation in the *H3F3A* gene [31]. These results led to the initiation of NCT02717455 (clinaltrials.gov), a clinical trial of panobinostat (Phase I–LBH589) conducted by the Pediatric Brain Tumor Consortium (PBTC) for the treatment of children with recurrent or progressive HGG.

In this regard, we paid special attention to the change in the concentration of wild-type DNA for the *H3F3A* (K27M) gene, suggesting that this phenomenon may become one of the effective prognostic markers of tumor progression and the effectiveness of therapy. The analysis of the mutual correlations of the concentrations of wild and mutant DNA of this gene in different fractions of blood plasma showed a number of interesting dependencies, for example, the concentration of mutant DNA K27M in the lumbar liquor had highly reliable correlations with the concentration of the same mutant DNA in the first and second fractions of blood plasma (Table 2).

There was also a high correlation of the concentration of mutant DNA of the *H3F3A* (K27M) gene in the second fraction of blood with cerebrospinal fluid, with the first fraction and the third, as well as with the concentration level of the wild-type gene in the third blood sample (Table 3).

Evaluation of the results of the study of the mutation status in blood plasma and lumbar cerebrospinal fluid of patients using ddPCR showed high

Table 1. Correlation between mutant DNA of the *H3F3A* gene (K27M) and disease progression

Value	Correlations are significant when $p < 0.05$
	Progression
K27Mmut in cerebrospinal fluid	0.271440

Table 2. Correlation between the mutant DNA of the *H3F3A* gene (K27M) in cerebrospinal fluid and the first two blood samples on the background of RT

Value	Correlations are significant when $p < 0.05$
	mut(K27M cerebrospinal fluid)
mut(K27M draw 1)	0.555019
mut(K27M draw 2)	0.384082

Table 3. Correlation between the mutant DNA of the *H3F3A* (K27M) gene in the second blood sample and cerebrospinal fluid draws, the first and third blood samples against the background of RT mutant variant of the gene, as well as the wild-type gene in the third sample

Value	Correlations are significant when $p < 0.05$
	mut(K27M draw 2)
mut(K27M cerebrospinal fluid)	0.384082
mut(K27M draw 1)	0.211165
mut(K27M draw 3)	0.360417
wt(K27M draw 3)	0.390472

informativeness of both blood plasma and lumbar cerebrospinal fluid, which is confirmed in studies where ctDNA are found in the blood and lumbar cerebrospinal fluid in the blood and lumbar cerebrospinal fluid, which are more sensitive and can reflect various types of mutations in glioma cells [11, 32, 33]. At the same time, it was previously stated that the presence of BBB means that the cerebrospinal fluid can provide a more detailed characteristic of the tumor than blood plasma, and contains certain biomarkers that are unlikely to be detected in plasma [34]. However, in our study, we found highly reliable correlations of the studied DNA in blood plasma and lumbar cerebrospinal fluid. At the same time, the concentration of mutant DNA of the *H3F3A* (K27M) gene in the lumbar cerebrospinal fluid also had highly significant correlations with the concentration of wild-type DNA of the *H3F3A* (K27M) gene in the first fraction of blood plasma. Thus, the assessment of VAF in blood plasma has a high prognostic significance in assessing the effectiveness of therapy.

3. Analysis of the dynamics of changes in the concentration of freely circulating DNA of the *H3F3A* (K27M) gene: wild and mutant type in the group with and without progression on the background of radiation therapy

Radiation therapy, as a standard strategy for the treatment of DMG, improves the quality of life of patients, after which 70–80 % of patients experience temporary relief of symptoms, as well as increased survival [24, 35, 36]. However, within 4–9 months, the disease progresses again. Ionizing radiation (AI) used in RT can inhibit tumor growth by inducing DNA damage directly or through reactive oxygen species (ROS) [37]. So far, there are no predictors for assessing the early effect of RT during treatment for children with DMG, only MR control brain monitoring after 1.5–3 months will allow to exclude or confirm the progression of the disease. Accordingly, it is impossible to personalize anti-relapse therapy in time.

During the ongoing study, the concentration level and VAF of mutant and wild ctDNA of the *H3F3A* (K27M) gene were studied for groups with stabiliza-

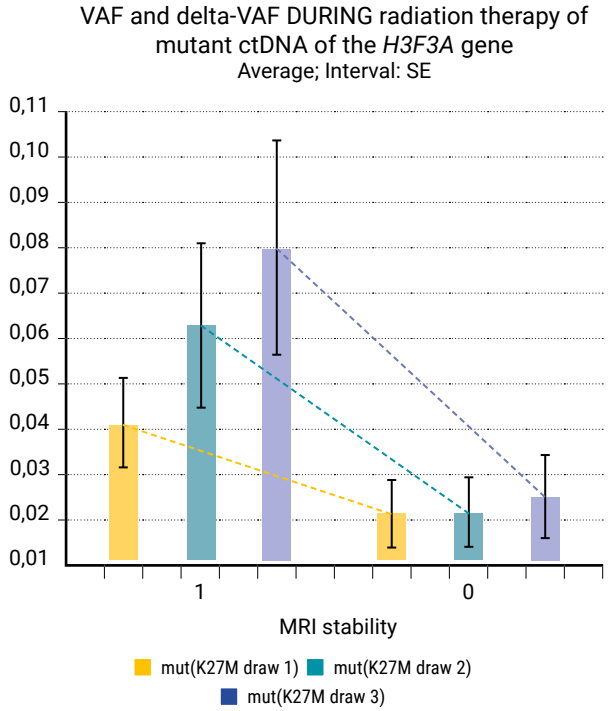

Fig. 3. VAF and delta-VAF against the background of radiation therapy of mutant ctDNA of the *H3F3A* (K27M) gene in blood plasma in a group of patients with continued growth (group 1) and stabilization (group 0), depending on the data of an MR brain study 3 months after completion of treatment

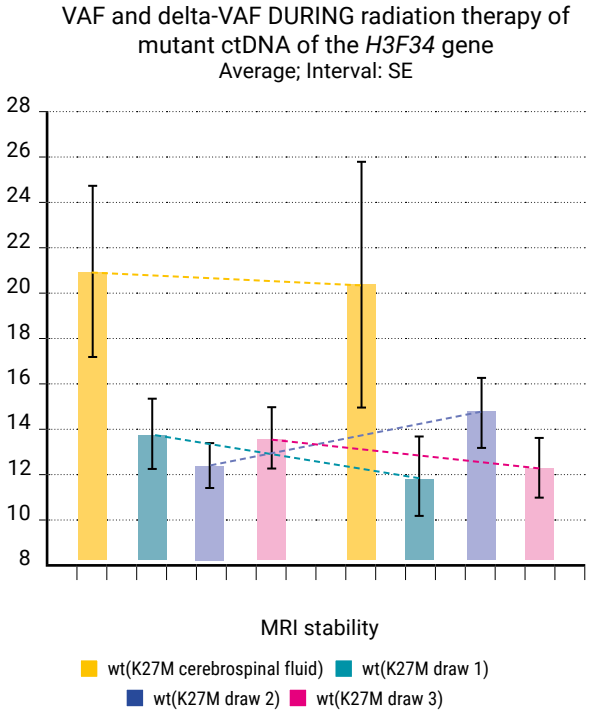

Fig. 4. VAF and delta VAF against the background of radiation therapy of wild-type ctDNA of the *H3F3A* (K27M) gene in cerebrospinal fluid and blood plasma in a group of patients with continued growth (group 1) and stabilization (group 0), depending on the data of an MRI study of the brain 3 months after completion of treatment

tion and progression against the background of RT. In the group of patients with stabilization, the concentration of mutant DNA of the *H3F3A* (K27M) gene in the blood was lower in three blood plasma samples compared with the concentration of mutant ctDNA in the group with early progression. VAF did not tend to significantly increase against the background of RT with stabilization of the disease. Whereas in children with early progression, the concentration of VAF in three plasma samples was 2–3 times higher compared to the group with a favorable prognosis, and the delta of VAF increased 2 times with each subsequent measurement against the background of RT (Fig. 3).

When analyzing the VAF and delta VAF wild-type ctDNA of the *H3F3A* (K27M) gene in cerebrospinal fluid and blood plasma, the following patterns were revealed in the group of patients with progression (group 1) and without progression (group 0), depending on the data of the MR brain examination 3 months after completion. The concentration level of wild-type ctDNA of the *H3F3A* (K27M) gene in the cerebrospinal fluid at the beginning of the course of RT was identical in both groups of patients. At the same time, in the group of patients with early progression (Fig. 4), the wild-type VAF ctDNA had the following pattern: a decrease in the RT process in the second plasma sample and an increase in the third sample against the background of completion of the RT course. Whereas in the group of patients with stabilization of the disease, the VAF of the wild-type ctDNA of the *H3F3A* (K27M) gene differed, namely: an increase in concentration in the second plasma sample and a significant decrease in it at the end of the course of RT. The delta of VAF in blood plasma, first of all, indicates the presence of a significant dependence between these indicators and the course of the disease.

The data obtained prove the diagnostic value of the wild-type ctDNA of the *H3F3A* (K27M) gene, allowing us to significantly expand the possibilities of molecular diagnostics and monitoring the effectiveness of treatment of DMG. In addition, based on the analysis of delta VAF during treatment, it is possible to predict early tumor recurrence after radiation therapy and timely initiation of personalized therapy.

CONCLUSION

1. Studies of VAF in blood plasma and lumbar cerebrospinal fluid of children with tumors diffuse midline gliomas before the start of radiation therapy are comparable and have equal diagnostic value.

2. High plasma concentrations of wild-type DNA of the *H3F3A* (K27M) gene correlate with early progression, which also affects survival rates.

3. Dynamic control of the DNA concentration of both mutant and wild-type *H3F3A* (K27M) gene in blood plasma and lumbar cerebrospinal fluid in children with diffuse midline gliomas during radiation therapy can be used to predict the effectiveness of RT. In addition, based on the analysis of the dynamics of the concentration levels of the wild-type DNA of the *H3F3A* (K27M) gene during treatment, it is possible to predict early tumor recurrence after radiation therapy.

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Bozhenko V. K. – development of the research design, analysis of the data obtained, writing the text of the manuscript, interpretation of the results;
Kudinova E. A. – development of the research design, analysis of the data obtained, writing the text of the manuscript, interpretation of the results;
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Kaminskiy V. V. – review of publications on the topic of the article, a set of clinical material, interpretation of the results;
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ORIGINAL ARTICLE

Urine transcriptomic profile in terms of malignant ovarian tumors

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ABSTRACT

Purpose of the study. Bioinformatic search for transcriptomic markers (based on metabolomic data) and their validation in the urine of serous ovarian adenocarcinoma patients.
Materials and methods. The study included 70 patients with serous ovarian adenocarcinoma and 30 conditionally healthy individuals. The search for metabolite regulator genes and gene regulator microRNAs was performed using the Random forest machine learning method. Ribonucleic acid (RNA) was isolated using the RNeasy Plus Universal Kits. The level of microRNA transcripts in urine was determined by real-time PCR. Differences were assessed using the Mann-Whitney test with Bonferroni correction.
Results. Using the Random forest method, metabolite-regulator gene (47 genes) and metabolite-regulator microRNA (613 unique microRNA) relationships were established. The identified microRNAs were validated by real-time PCR. Changes in the levels of microRNA transcripts were detected: miR-382-5p, miR-593-3p, miR-29a-5p, miR-2110, miR-30c-5p, miR-181a-5p, let-7b-5p, miR-27a-3p, miR-370-3p, miR-6529-5p, miR-653-5p, miR-4742-5p, miR-2467-3p, miR-1909-5p, miR-6743-5p, miR-875-3p, miR-19a-3p, miR-208a-5p, miR-330-5p, miR-1207-5p, miR-4668-3p, miR-3193, miR-23a-3p, miR-12132, miR-765, miR-181b-5p, miR-4529-3p, miR-33b-5p, miR-17-5p, miR-6866-3p, miR-4753-5p, miR-103a-3p, miR-423-5p, miR-491-5p, miR-196b-5p, miR-6843-3p, miR-423-5p and miR-3184-5p in the urine of patients compared to conditionally healthy individuals.
Conclusion. Thus, urine transcriptome profiling allowed both to identify potential disease markers and to better understand the molecular mechanisms of changes underlying ovarian cancer development.

Keywords: microRNAs, polymerase chain reaction, machine learning, bioinformatics, ovarian serous adenocarcinoma, urine, biomarkers

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ОРИГИНАЛЬНАЯ СТАТЬЯ

Транскриптомный профиль мочи при злокачественных новообразованиях яичника

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РЕЗЮМЕ

Цель исследования. Биоинформатический поиск транскриптомных маркеров (на основании метаболомных данных) и их валидация в моче больных серозной аденокарциномой яичников.
Материалы и методы. В исследование было включено 70 пациенток с диагнозом серозная аденокарцинома яичников и 30 условно здоровых индивидуумов. Поиск генов-регуляторов метаболитов и микроРНК регуляторов генов осуществляли с использованием метода машинного обучения Random forest. Выделение рибонуклеиновой кислоты (РНК) производили с помощью набора RNeasy Plus Universal Kits. Уровень транскриптов микроРНК в моче определяли методом полимеразной цепной реакции (ПЦР) в режиме реального времени. Оценку различий проводили с использованием критерия Манна-Уитни с поправкой Бонферрони.
Результаты. С использованием метода Random forest были установлены взаимосвязи метаболит-ген регулятор (47 генов) и метаболит-микроРНК регулятор (613 уникальных микроРНК). Выявленные микроРНК были валидированы методом ПЦР в режиме реального времени. Обнаружено изменения уровня транскриптов микроРНК miR-382-5p, miR-593-3p, miR-29a-5p, miR-2110, miR-30c-5p, miR-181a-5p, let-7b-5p, miR-27a-3p, miR-370-3p, miR-6529-5p, miR-653-5p, miR-4742-5p, miR-2467-3p, miR-1909-5p, miR-6743-5p, miR-875-3p, miR-19a-3p, miR-208a-5p, miR-330-5p, miR-1207-5p, miR-4668-3p, miR-3193, miR-23a-3p, miR-12132, miR-765, miR-181b-5p, miR-4529-3p, miR-33b-5p, miR-17-5p, miR-6866-3p, miR-4753-5p, miR-103a-3p, miR-423-5p, miR-491-5p, miR-196b-5p, miR-6843-3p, miR-423-5p и miR-3184-5p в моче пациенток относительно условно-здоровых индивидуумов.
Заключение. Таким образом, транскриптомное профилирование мочи позволило как выявить потенциальные маркеры заболевания, так и лучше понять молекулярные механизмы изменений, лежащих в основе развития рака яичников.

Ключевые слова: микроРНК, полимеразная цепная реакция, машинное обучение, биоинформатика, серозная аденокарцинома яичника, моча, биомаркеры

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Соблюдение этических стандартов: в работе соблюдались этические принципы, предъявляемые Хельсинкской декларацией Всемирной медицинской ассоциации (World Medical Association Declaration of Helsinki, 1964, ред. 2013). Исследование одобрено Комитетом по биомедицинской этике при ФГБУ «Национальный медицинский исследовательский центр онкологии» Министерства здравоохранения Российской Федерации (выписка из протокола заседания № 15 от 14.06.2022 г.). Информированное согласие получено от всех участников исследования

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Конфликт интересов: все авторы заявляют об отсутствии явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи

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INTRODUCTION

Ovarian cancer (OC) currently takes one of the leading positions in terms of morbidity and mortality in the world and the Russian Federation among gynecological malignancies [1, 2]. OC includes many subtypes of tumors, each of which has distinctive biological and clinical characteristics. According to the WHO classification, serous carcinoma, endometrioid carcinoma, mucinous carcinoma, light cell carcinoma, malignant Brenner tumor, serous-mucinous carcinoma, undifferentiated carcinoma and mixed epithelial carcinoma are distinguished [3, 4].

In most patients, rheumatoid arthritis is sporadic, usually detected late, and the overall 5-year survival rate is only 30–40 %. Early detection of OC is the most important factor in improving patient survival [6]. New methodological approaches, including modern molecular biology approaches, are needed for early detection and improved diagnosis of this disease. The application of genomics and metabolomics has opened a new chapter of research, which will allow the development of new tools for early diagnosis and monitoring of the course of oncological diseases. Advances in metabolomic approaches using liquid or gas chromatography combined with high-resolution mass spectrometry (MS) have opened new prospects for simultaneous detection and identification of biomarkers in biological samples [7].

Our earlier study [8] of the urine metabolomic profile by ultrahigh-performance liquid chromatography with mass spectrometric detection showed that patients with serous ovarian carcinoma have an imbalance in the content of certain fatty acids and their derivatives, acylcarnitines, phospholipids, amino acids and their derivatives, as well as some derivatives of nitrogenous bases. At the same time, 26 metabolites with abnormal concentrations in urine may have some potential as non-invasive biomarkers of breast cancer in women belonging to high-risk groups.

Thus, it was shown that 14 metabolites (kynurenine, phenylalanyl valine, lysophosphatidylcholine (18:3), lysophosphatidylcholine (18:2), alanyl leucine, lysophosphatidylcholine (20:4), L-phenylalanine, phosphatidylinositol (34:1), 5-methoxytryptophan, 2-hydroxymyristic acid, 3-oxocholic acid, lysophosphatidylcholine (14:0), indolacrylic acid, lysophosphatidylserine (20:4)) have significantly higher concentrations compared to conditionally healthy individuals. The content of 12 compounds, on the contrary, was reduced (L-beta-aspartyl-L-phenylalanine, myristic acid, decanoyl carnitine, aspartyl-glycine, malonylcarnitine, 3-hydroxybutyrylcarnitine, 3-methylxanthine, 2,6-dimethylheptanoyl carnitine, 3-oxododecanoic acid, N-acetylproline, L-octanoylcarnitine, capriloylglycine) [8].

The determination of a number of the above compounds with high accuracy in urine samples is a procedure that requires expensive equipment and is feasible only in a small number of medical institutions. In this regard, it is extremely important to switch to more accessible predictive markers, for example, transcriptomic data. In this regard, the level of microRNA transcripts in urine is of particular interest [9].

microRNAs are short non-coding RNAs that regulate gene expression by catalyzing the destruction of mRNA, or by inhibiting the translation of mRNA into protein. Mature microRNA is a single-stranded RNA of the order of 22 nucleotides in size, obtained from a primary transcript. microRNAs are transcriptional regulators and modulate gene expression by interacting with complementary nucleotide sequences of target mRNAs [10]. microRNAs make a significant contribution to the initiation and development of various molecular events, including the initiation of oncogenesis, progression, and metastasis of tumors, which makes microRNAs potential biomarkers for assessing the progression and prognosis of cancer [11]. The study of the microRNA-mRNA regulatory network is of great importance both for elucidating the molecular mechanisms underlying carcinogenesis and for creating a panel of new biomarkers.

The purpose of the study was the bioinformatic search for transcriptomic markers (based on metabolomic data) and their validation in the urine of patients with serous ovarian adenocarcinoma.

MATERIALS AND METHODS

The prospective study included 70 patients diagnosed with ovarian cancer (serous adenocarcinoma of low (n = 30) and high grade malignancy (n = 30), T1a – 4, T1b – 3, T1c – 5, T2a – 3, T2b – 5, T3a – 14, T3b – 6, T3c – 30) and 30 conditionally healthy volunteers (without any known pathologies) who make up the control group.

Urine samples were used as objects of research. Before the study, the patients gave informed consent to the scientific use of biological samples. Urine was collected before the start of treatment.

Evaluation of microRNA expression

500 µl of urine sample was mixed with 900 µl of QIAzol reagent (QIAGEN). Further isolation of total RNA was carried out using the RNeasy Plus Universal Kits kit according to the manufacturer's protocol. To identify mature microRNAs and small U6 RNAs, the method proposed by Balcells I. and co-authors was used [12]. The isolated total RNA was used in a reverse transcription reaction, which was performed simultaneously with polyadenylation of RNA using specific OC primers. Next, the obtained complementary DNA was detected using real-time polymerase chain reaction (PCR) (PCR-RV).

The design of specific oligonucleotide primers was carried out using the Balcells I. algorithm [12]. Several sets of oligonucleotides were selected for each micro-RNA, from which those characterized by the highest efficiency of reverse transcription and PCR were selected. The effectiveness of reverse transcription was evaluated by the values of threshold cycles (Ct) obtained by analyzing synthetic analogues of microRNA and mRNA (Biosan CJSC, Russia) taken at a known concentration. The efficiency of amplification (E) for each system was evaluated by constructing a calibration curve, using the dilution analysis of the corresponding RNAs isolated from clinical samples according to the protocol described above (the average value of E was 2.0). The stability of expression for the selection of reference genes was evaluated using the geNorm algorithm [13]. The initial list of proposed normalizers for microRNAs included: miR-191 (expression of this microRNA was the most stable in 13 compared tissues [14]); miR-23a (as a normalizer suitable for the analysis of cervical samples [15] and U6 (traditionally used as a separate standard for normalization of microRNA expression data). Using the geNorm algorithm, U6 was selected to normalize microRNA expression data.

A reverse transcription reaction was performed separately for each microRNA in one repeat. For reverse transcription, a reaction mixture containing 1x poly(A) buffer (BioLabs), 10 U/µl Reverse Transcriptase MMLV (Synthol), 0.1 mM dNTPs (Synthol), 0.1 mM ATP (BioLabs), 1 µM OC primer, 0.5 U/µl Poly(A) polymerase (BioLabs) and 1 mcg of total RNA. The reaction was carried out for 15 minutes. at 16 oC, 15 min. at 42 °C, the reverse transcriptase was then inactivated for 2 min. at 95 °C.

The change in the relative expression of microRNA was evaluated by PCR-RV. Amplification was performed in 20 µl of a PCR mixture containing 1x PCR buffer, 0.25 mM dNTPs, 2 mM MgCl2, 1 unit act. Taq-DNA polymerase, 500 nM of direct and reverse primers. OC-qPCR formulation of each sample was performed in three repeats. The resulting mixtures were incubated in a CFX 96 amplifier (Bio-Rad Laboratories, USA) according to the following program: 2 minutes 94 °C, 50 cycles: denaturation at 95 °C for 10 seconds, annealing and elongation – 63 °C for 30 seconds. The results corresponding to Ct > 40 were found to be negative.

The relative expression (RE) was calculated using the formula $RE = 2^{-\Delta\Delta Ct}$. The normalization of the results was carried out according to the reference locus and the expression level of the corresponding target microRNAs in the samples of the control group, sequentially according to the scheme given below:

1. Normalization by reference locus:
$\Delta C(t) = C(t_{target} - C(t)_{reference}$, where $C(t)_{reference}$ is C(t) of the reference locus.

2. Calculation of $E^{-\Delta C(t)}$ for each microRNA for each patient of the control group and the main group.

3. Calculation of the median $E^{-\Delta C(t)}$ for each locus for the control group and the main group.

4. Normalization for the control group and the result as a multiplicity of changes: $RE = E^{-\Delta C(t)median}$ of the main group $/E^{-\Delta C(t)median}$ of the control group (which is identical to $RE = E^{-\Delta\Delta C(t)}$ [16].

Statistical and bioinformatic data processing

The differences were assessed using the Mann-Whitney criterion for a threshold level of statistical significance of $p < 0.05$, and the Bonferroni correction was used to account for multiple comparisons. The data analysis was carried out in the Python programming language using the SciPy library [17].

The search for metabolite regulatory genes and microRNA regulators of genes was carried out using the Random forest machine learning method, which combines the Breiman bagging method and the random subset method. The result of the "ran-

dom forest" model is the predicted probability that the target gene or microRNA is the true regulator of a particular metabolite [18].

LASSO (Least Absolute Shrinkage and Selection Operator), a penalized logistic regression in the R programming language in the Rstudio shell, was used to select minimal sets of microRNAs The importance of variables was determined by counting the number of bootstrap models with a non-zero coefficient of the variable [19].

Table 1. Metabolites and genes regulating metabolic pathways

Metabolites*	Regulator genes	Enzymes
Kinurenin	*KYNU, KMO, KYAT3, IDO*	kynureninase, kynurenine-3-monooxygenase, kynurenine aminotransferase 3, indolamine-pyrrole-2,3-dioxygenase
Phenylalanine-Valine	*PAH*	Phenylalanine hydroxylase
Myristic acid	*PPARA, PPARGC1A, CYP4Z1, IYD, FASN, PLA2G5, LGALS13*	fatty acid synthase, beta-ketoacyl synthase domain, calcium-dependent phospholipase A2, soluble lectin 13 binding galactoside
Lysophosphatidylcholine (18:3), (18:2), (20:4), (14:0)	*PLA2G2A, PLB1, LPCAT1*	phospholipase A2, lysophospholipase, LPC-acyltransferase
Decanoyl carnitine	*ACADM, ACADS, CROT*	Acyl-CoA dehydrogenase, Carnitine octanoyltransferase (Carnitine O-Octanoyltransferase)
Malonyl carnitine	*CPT1, CPT1A, ACADM*	palmitoyl-CoA transferase, malonyl-CoA decarboxylase
Alanine-Leucine	*GAL, PGA3*	galanin, pepsinogen A
3-hydroxybutyrylcarnitine	*ACADM, CRAT*	3-hydroxyacyl CoA dehydrogenase, carnitine-O-acetyltransferase
3-methylxanthine	*PDE4D*	cAMP-specific 3',5'-cyclic phosphodiesterase 4D
L-Phenylalanine	*PAH, DDC*	phenylalanine hydroxylase, DOPA decarboxylase
Phosphatidylinositol (34:1)	*PIK3CA, PIK3CB, PIK-3C2A, PLCB1, PIGL*	phosphatidylinositol-3-kinase, 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta-1, N-acetylglucosaminylphosphatidylinositol-de-N-acetylase
2,6 dimethylheptanoyl carnitine	*ACADM, CRAT*	3-hydroxyacyl CoA dehydrogenase, carnitine-O-acetyltransferase
5-methoxytryptophan	*TPH1*	tryptophanhydroxylase
3-oxododecanoic acid	*FASN, OXSM*	3-oxoacyl synthase, fatty acid synthase
2- hydroxymyristic acid	*NMT1*	n-myristoyl transferase 1
3-oxocholic acid	*FABP6*	gastropine
Indolylacrylic acid	*KYAT1*	kynurenine aminotransferase 1 (kynurenine aminotransferase 1)
N-acetylproline	*APEH*	N-acylpeptide hydrolase
L-octanoylcarnitine	*CROT, COT, CPT2, CPT1*	Carnitine-O-octanoyltransferase, carnitine-O-palmitoyltransferase 2(Carnitine O-octanoyltransferase, Carnitine O-palmitoyltransferase 2)
Capriloyl glycine	*ACADM, ODC1, GLYATL1*	3-hydroxyacyl CoA dehydrogenase, ornithine decarboxylase 1, Glycine N-acyltransferase
Lysophosphatidylserine	*GPR34, PLA1A*	lysophosphatidylserine new receptor 1, phospholipase a1

Note: * – the list of metabolites based on the data of the article [8]

Table 2. Metabolites, regulatory genes of metabolic pathways and microRNAs interacting with them

	Metabolites	Regulator genes	microRNA
1	Kinurenin	*KYNU, KMO, KYAT3, IDO1*	***KMO***:miR-30b-3p, miR-153-5p, miR-149-3p, miR-363-5p, miR-624-3p, miR-937-5p, miR-1233-3p, miR-1238-3p, miR-1972, miR-3200-5p, miR-4319, miR-3689a-5p, miR-3689b-5p, miR-4478, miR-3689e, miR-4695-5p, miR-4724-5p, miR-664b-3p, miR-5684, miR-6758-5p, miR-6780a-5p, miR-6799-5p, miR-6856-5p, miR-6867-5p, miR-6883-5p, miR-6894-5p, miR-6894-5p, miR-7106-5p, miR-7106-5p, miR-1273h-5p, miR-12122. ***KYNU***:miR-30a-3p,miR-200c-3p, miR-382-5p, miR-382-5p, miR-2117, miR-3654, miR-4652-3p, miR-4743-3p, miR-6739-3p, miR-6879-3p, miR-6885-3p, miR-10397-5p, miR-4638-5p, miR-30a-3p, miR-200c-3p, miR-382-5p, miR-382-5p, miR-2117, miR-3654, miR-4652-3p, miR-4743-3p, miR-6739-3p, miR-6879-3p, miR-6885-3p, miR-10397-5p. ***KYAT3***: miR-5692c, miR-5692b, miR-5692c, miR-5692b, miR-5692c, miR-5692b. ***IDO1***: miR-593-3p, miR-891a-3p, miR-5683, miR-6728-3p
2	Phenylalanine-Valine	*PAH*	miR-23a-3p, miR-4502, miR-12132
3	Myristic acid	*PPARA, PPARGC1A, CYP4Z1, IYD, FASN, PLA2G5, LGALS13*	***IYD:*** miR-760, miR-29a-5p, miR-208a-5p, miR-30b-3p, miR-184, miR-195-3p, miR-320a-3p, miR-373-5p, miR-483-3p, miR-551b-5p, miR-643, miR-646, miR-1224-5p, miR-320b,miR-922, miR-1202, miR-1205, miR-1287-3p, miR-513c-3p, miR-1321, miR-3144-3p, miR-3152-5p, miR-3185, miR-3191-5p, miR-3199, miR-514b-5p, miR-4279, miR-3663-5p, miR-3681-5p, miR-3689a-3p, miR-3689b-3p, miR-4429, miR-4452, miR-3689c, miR-4531, miR-4533, miR-3972, miR-3976, miR-451b, miR-4731-5p, miR-4796-3p, miR-4799-3p, miR-5003-3p, miR-5195-3p, miR-5588-5p, miR-6509-3p, miR-6737-5p, miR-6737-3p, miR-6752-3p, miR-6764-3p, miR-6779-5p, miR-6780a-5p, miR-6824-3p, miR-6829-5p, miR-6830-5p, miR-6849-5p, miR-6849-3p, miR-6882-5p, miR-6894-3p, miR-7106-5p, miR-7844-5p, miR-8052, miR-8069, miR-8078, miR-146a-5p, miR-607, miR-3614-5p, miR-4482-3p, miR-197-3p, miR-744-3p, miR-3187-3p, miR-3652, miR-4420, miR-4430, miR-4633-5p, miR-4642, miR-4781-3p, miR-5698, miR-6499-3p, miR-6787-3p, miR-6843-3p, miR-6848-3p, miR-588, miR-4423-5p, miR-6501-5p. ***CYP4Z1:*** miR-2110, ***FASN:*** miR-30c-5p, ***LGALS13:*** miR-4650-3p. ***PLA2G5:*** miR-765, miR-3682-3p, miR-4533,miR-2467-3p,miR-4786-3p,miR-1253,miR-3191-5p,miR-6847-5p,miR-11181-3p,miR-3916. ***PPARA:***miR-181a-5p, miR-181b-5p, miR-20b-5p, miR-181d-5p, miR-22-3p, miR-140-5p, miR-372-3p, miR-330-5p, miR-331-3p, miR-345-3p, miR-520d-3p, miR-551b-5p, miR-619-5p, miR-622, miR-2113, miR-665, miR-939-3p, miR-1976, miR-3116, miR-3183, miR-4251, miR-3690, miR-550b-2-5p, miR-4436a, miR-4443, miR-4515, miR-4717-5p, miR-4723-5p, miR-4745-5p, miR-4749-5p, miR-4755-3p, miR-5591-5p, miR-6126, miR-6131, miR-6134, miR-6505-3p, miR-6734-3p, miR-6744-3p, miR-6753-3p, miR-6766-5p, miR-6791-5p, miR-6805-3p, miR-6817-5p, miR-6852-5p, miR-6873-3p, miR-6880-5p, miR-7151-3p, miR-8071, let-7b-5p, let-7e-5p, miR-224-3p, miR-302a-3p, miR-326, miR-335-3p, miR-429, miR-511-5p, miR-8085, miR-10394-5p, miR-10524-5p, miR-9851-5p, miR-7107-3p, miR-7110-3p, miR-7155-3p, miR-7158-3p, miR-7976, miR-1233-5p, miR-4651, miR-6757-5p, miR-6778-5p, miR-27a-5p, miR-34a-5p, miR-130b-5p, miR-196b-5p, miR-607, miR-1249-5p, miR-3689d, miR-5006-5p, miR-6756-5p, miR-6788-5p, miR-6797-5p, miR-6851-5p. ***PARGC1A:*** let-7a-5p, let-7b-5p, let-7c-5p, let-7e-5p, miR-23b-3p, miR-138-5p, miR-409-5p, miR-487a-3p, miR-193b-3p, miR-4458, miR-6884-5p, miR-23a-3p, miR-193a-3p, miR-485-3p, miR-3666, miR-3681-3p, miR-211-5p, miR-485-5p, miR-342-5p, miR-452-5p, miR-511-5p, miR-508-5p, miR-573, miR-659-3p, miR-764, miR-1825, miR-2116-3p, miR-2682-3p, miR-3929, miR-4436a, miR-4649-5p, miR-4664-5p, miR-4713-5p, miR-4728-3p, miR-122b-3p, miR-4768-5p, miR-4769-3p, miR-5003-5p, miR-5006-3p, miR-5011-5p, miR-5591-3p, miR-5685, miR-6124, miR-6740-3p, miR-6818-3p, miR-6833-3p, miR-6845-3p, miR-6892-3p, miR-7110-5p, miR-7703, miR-7850-5p, miR-8075, miR-8485, miR-148a-5p, miR-214-3p, miR-222-3p, miR-9898, miR-6083
4	Lysophosphatidylcholine	*PLA2G2A, PLB1, LPCAT1*	***LPCAT1***: miR-27a-3p, miR-370-3p, miR-4739, miR-4768-3p, miR-4783-3p. ***PLB1***: miR-3162-5p, miR-4529-3p, miR-4740-5p. ***PLA2G2A***: miR-765, miR-3652, miR-6134, miR-6745, miR-6756-5p, miR-6769a-5p, miR-6785-5p, miR-6769b-5p, miR-7847-3p

Table 2. (Continuation) Metabolites, regulatory genes of metabolic pathways and microRNAs interacting with them

	Metabolites	Regulator genes	microRNA
5	Decanoyl carnitine	*ACADM, ACADS, CROT*	***CROT***: miR-33a-5p, miR-373-3p, miR-33b-5p, miR-17-5p, miR-500a-5p, miR-501-5p, miR-1250-3p, miR-4659b-3p, miR-219b-5p, miR-4795-3p, miR-6807-3p, miR-6867-5p, miR-522-3p, miR-4325, miR-5004-3p, miR-6833-5p, miR-221-3p. ***ACADM***: miR-4437, miR-5580-3p, miR-6529-5p, miR-3184-5p, miR-4704-3p. ***ACADS***: miR-484
6	Malonyl carnitine	*CPT1A, ACADM*	***ACADM***: miR-4437, miR-5580-3p, miR-6529-5p, miR-3184-5p, miR-4704-3p. ***CPT1A***: miR-653-5p, miR-328-3p, miR-6866-3p, miR-1296-3p, miR-1322, miR-6883-5p, miR-7-2-3p, miR-335-3p, miR-520a-3p, miR-4310, miR-4287, miR-6718-5p, miR-6785-5p, miR-6869-3p, miR-7856-5p, miR-93-5p, miR-4293, miR-4322, miR-4707-3p, miR-24-3p, miR-6849-3p
7	Alanine-Leucine	*GAL, PGA3*	***GAL***: miR-922, miR-4742-5p, miR-4753-5p, miR-4436b-3p, miR-5004-5p, miR-5089-3p, miR-15b-5p, miR-138-1-3p, miR-302d-5p, miR-6810-5p, miR-3976. ***PGA3***: miR-2467-3p, miR-1909-5p, miR-6743-5p, miR-1913, miR-2115-5p, miR-4646-5p, miR-5006-5p, miR-6857-5p, miR-11399, miR-5008-5p, miR-4649-3p,miR-423-5p, miR-3679-5p, miR-423-3p, miR-1296-3p, miR-3126-5p, miR-6759-3p, miR-3180, miR-6763-5p, miR-769-3p, miR-3139, miR-5571-5p, miR-6768-5p, miR-761, miR-3151-5p, miR-18a-5p, miR-4672, miR-6873-3p, miR-6875-3p, miR-3156-5p, miR-6771-5p, miR-6879-5p, miR-3945
8	3-hydroxybutyrylcarnitine	*ACADM, CRAT*	***ACADM***: miR-4437, miR-5580-3p, miR-6529-5p, miR-3184-5p, miR-4704-3p. ***CRAT***: miR-936, miR-1207-5p, miR-6764-5p, miR-7150, miR-10392-3p
9	3-methylxanthine	*PDE4D*	***PDE4D***: miR-18a-5p, miR-31-5p, miR-148a-3p, miR-301a-3p, miR-148b-3p, miR-875-5p, miR-6766-3p, miR-26a-5p, miR-103a-3p, miR-107, miR-139-5p, miR-362-3p, miR-339-5p, miR-18b-5p, miR-448, miR-487a-3p, miR-4429, miR-203a-3p, miR-211-3p, miR-124-3p, miR-149-5p, miR-99b-3p, miR-372-3p, miR-373-5p, miR-520a-3p, miR-520d-3p, miR-625-5p, miR-641, miR-1301-3p, miR-449c-5p, miR-1266-5p, miR-1321, miR-1912-3p, miR-2114-5p, miR-3125, miR-3187-5p, miR-4261, miR-4280, miR-3646, miR-3689a-5p, miR-3689b-5p, miR-3922-5p, miR-4446-3p, miR-3689d, miR-3689e, miR-4492, miR-4502, miR-4511, miR-3977, miR-4646-5p, miR-4675, miR-4698, miR-4741, miR-4756-5p, miR-4768-3p, miR-5193, miR-1295b-5p, miR-5589-3p, miR-6500-3p, miR-548az-5p, miR-6504-5p, miR-6511a-5p, miR-6512-5p, miR-6809-5p, miR-6809-3p, miR-6829-5p, miR-6839-5p, miR-6859-5p, miR-5787, miR-6077, miR-6796-3p, miR-6860, miR-7114-5p, miR-7151-3p, miR-8080, miR-8081, miR-8086, miR-195-5p, miR-3136-5p, miR-6080, miR-6888-5p, miR-340-5p, miR-4439, miR-3148, miR-6857-3p, miR-497-5p
10	L-Phenylalanine	*PAH, DDC*	***PAH***: miR-23a-3p, miR-4502, miR-12132. ***DDC***: miR-875-3p, miR-3166, miR-4502, miR-3158-3p

STUDY RESULTS

Bioinformatic analysis of the relationship of the urine metabolomic profile with gene and microRNA expression. Using the Random forest machine learning method implemented in the R programming language, the analysis of the metabolomic data from the article [8] was carried out, as well as the Human Metabolome Database (HMDB, https://hmdb.ca/metabolites).

At the first stage, the metabolite-enzyme and enzyme-gene regulator relationships were established. The results are presented in table 1.

The results of the metabolite-gene-microRNA relationship are presented in Table 2 and Fig. 1–2. It can be seen that the content of metabolites detected in urine is regulated by a complex network of rna and microRNA interactions. For two metabolites, myristic acid and phosphatidylinositol, 237 and 143 micro-RNAs were detected, respectively, regulating the content of these substances in biological fluids.

Thus, bioinformatic analysis has determined a list of 613 unique microRNAs involved in the regulation of the concentration of 21 metabolites. Of the 613 microRNAs, only the microRNAs with the maximum

Table 2. (Continuation) Metabolites, regulatory genes of metabolic pathways and microRNAs interacting with them

	Metabolites	Regulator genes	microRNA
11	Phosphatidylinositol (34:1)	*PIK3CA, PIK3CB, PIK3C2A, PLCB1, PIGL*	***PIGL***: miR-4651, miR-5087, miR-6499-3p, miR-6739-3p, miR-6764-5p, miR-212-5p, miR-659-3p, miR-3189-3p, miR-3934-3p, miR-378g, miR-4519, miR-6819-5p. ***PIK3C2A***: miR-503-5p, miR-301b-3p, miR-6838-5p, miR-23a-5p, miR-29a-5p, miR-23b-5p, miR-510-5p, miR-1264, miR-2113, miR-1286, miR-3619-3p, miR-4423-3p, miR-4436a, miR-4484, miR-1343-3p, miR-6074, miR-6760-3p, miR-6867-5p, miR-212-5p, miR-150-5p, miR-378a-5p, miR-518a-5p, miR-1224-3p, miR-764, miR-6821-3p. ***PIK3CA***: let-7i-5p, let-7e-5p, miR-19a-3p, miR-19b-3p, let-7g-5p, miR-152-3p, miR-202-5p, miR-4429, miR-198, miR-548e-5p, miR-548o-3p, miR-2114-5p, miR-4430, miR-4493, miR-4659b-3p, miR-122b-5p, miR-4803, miR-5006-3p, miR-6797-3p, miR-1972, miR-2116-5p, miR-3157-5p, miR-3191-5p, miR-514b-5p, miR-4303, miR-4277, miR-3606-5p, miR-3614-3p, miR-3679-3p, miR-676-5p, miR-378g, miR-4446-5p, miR-4477b, miR-4486, miR-4652-3p, miR-6819-5p, miR-6857-5p, miR-6868-3p, miR-6893-3p, miR-7162-3p, miR-10526-3p, miR-12126, miR-139-5p, miR-422a. ***PIK3CB***: miR-23b-3p, miR-362-5p, miR-3666, miR-3064-5p, miR-4465, miR-199a-3p, miR-199b-3p, miR-212-5p, miR-150-5p, miR-6504-5p, miR-204-3p, miR-671-5p, miR-1263, miR-3646, miR-4430, miR-4682, miR-5093, miR-6165, miR-6715a-3p, miR-7850-5p, miR-9500, miR-130b-5p, miR-3619-5p, miR-32-3p, miR-623, miR-542-5p, miR-548j-5p, miR-544b, miR-3614-5p, miR-3652, miR-548aw, miR-5703, miR-8077, miR-2117. ***PLCB1***: miR-103a-3p, miR-107, miR-423-5p, miR-3129-5p, miR-139-5p, miR-124-3p, miR-138-1-3p, miR-302c-5p, miR-876-5p, miR-1244, miR-1322, miR-548s, miR-4267, miR-3692-3p, miR-4433a-3p, miR-4436a, miR-3978, miR-4647, miR-4659a-3p, miR-4670-3p, miR-5194, miR-548az-3p, miR-6783-3p, miR-6860, miR-7151-5p, miR-8056, miR-8063, miR-502-3p
12	2,6 dimethylheptanoyl carnitine	*ACADM, CRAT*	***ACADM***: miR-4437, miR-5580-3p, miR-6529-5p, miR-3184-5p, miR-4704-3p. ***CRAT***: miR-936, miR-1207-5p, miR-6764-5p, miR-7150, miR-10392-3p
13	5-methoxytryptophan	*TPH1*	***TPH1***: miR-320a-3p, miR-450a-2-3p, miR-320b, miR-2110, miR-4435, miR-5693, miR-5702, miR-6830-5p, miR-12118
14	3-oxododecane acid	*FASN*	miR-30c-5p
15	2-hydroxymyristic acid	*NMT1*	***NMT1***: miR-181a-5p, miR-214-3p, miR-491-5p, miR-432-5p, miR-922, miR-1202, miR-1205, miR-1972, miR-2110, miR-2682-3p, miR-3160-5p, miR-3176, miR-4303, miR-4291, miR-4447, miR-3972, miR-4667-5p, miR-4690-3p, miR-4700-5p, miR-23b-3p, miR-615-3p
16	3-oxocholic acid	*FABP6*	***FABP6***: miR-208a-5p, miR-330-5p, miR-196b-5p, miR-3180-3p, miR-3181, miR-4278, miR-3689f, miR-4754, miR-4786-3p, miR-5190, miR-5195-3p, miR-6745, miR-6751-5p, miR-6769a-5p, miR-6771-5p, miR-6792-5p, miR-6821-5p, miR-7156-3p, miR-10226, miR-10392-5p
17	Indolylacrylic acid	*KYAT1*	***KYAT1***: miR-423-5p, miR-6842-5p, miR-597-3p, miR-4710, miR-6741-5p, miR-6796-5p, miR-4447, miR-193b-3p
18	N-acetylproline	*APEH*	miR-1289
19	L-octanoylcarnitine	*CROT, CPT2*	***CROT***: miR-33a-5p, miR-373-3p, miR-33b-5p, miR-17-5p, miR-500a-5p, miR-501-5p, miR-1250-3p, miR-4659b-3p, miR-219b-5p, miR-4795-3p, miR-6807-3p, miR-6867-5p, miR-522-3p, miR-4325, miR-5004-3p, miR-6833-5p, miR-221-3p. ***CPT2***: miR-433-3p, miR-6843-3p, miR-6848-3p, miR-208a-5p, miR-6742-3p, miR-34a-5p
20	Capriloyl glycine	*ACADM, ODC1, GLYATL1*	***ACADM***: miR-4437, miR-5580-3p, miR-6529-5p, miR-3184-5p, miR-4704-3p. ***ODC1***: miR-423-5p, miR-3184-5p, miR-7973, miR-193b-3p. ***GLYATL1***: miR-1207-5p, miR-4668-3p, miR-4742-3p, miR-4999-5p, miR-664b-3p, miR-6846-3p, miR-6893-3p
21	Lysophosphatidylserine (20:4)	*GPR34, PLA1A*	***PLA1A***: miR-3153, miR-7110-3p, miR-6754-5p, miR-6887-3p. ***GPR34***: miR-3193, miR-2909, miR-4738-5p, miR-486-3p, miR-6808-5p

interaction strength with the mRNA of the genes regulating the content of metabolites were selected. The final list contained 91 microRNAs, presented in Table 3.

Features of the content of microRNA transcripts in the urine of patients with serous ovarian adenocarcinoma

The generated list of 91 microRNAs regulating the activity of 37 genes was used for validation by real-time PCR on urine samples of patients and conditionally healthy volunteers.

A statistically significant ($p < 0.005$) change in the transcript level of 47 microRNAs relative to conditionally healthy volunteers was found in the urine of patients with serous ovarian adenocarcinoma (Fig. 3).

A significant increase ($p < 0.05$) in the level of miR-382-5p by 1.9 times, miR-593-3p by 3.4 times, miR-29a-5p by 2.6 times, miR-2110 by 2.5 times,

Table 3. The final list of microRNAs involved in the regulation of the concentration of metabolites

	Metabolites	microRNA
1	Kinurenin	***KMO***: miR-30b-3p, miR-153-5p, miR-149-3p, miR-363-5p. ***KYNU***: miR-30a-3p, miR-200c-3p, miR-382-5p, miR-382-5p. ***KYAT3***: miR-5692c, miR-5692b, miR-5692c. ***IDO1***: miR-593-3p, miR-891a-3p.
2	Phenylalanine-Valine	***PAH:*** miR-23a-3p, miR-4502, miR-12132
3	Myristic acid	***IYD***: miR-760, miR-29a-5p. ***CYP4Z1***: miR-2110, ***FASN***: miR-30c-5p, ***LGALS13***: miR-4650-3p. ***PLA2G5***: miR-765, miR-3682-3p. ***PPARA***: miR-181a-5p, miR-181b-5p, miR-20b-5p. ***PPARGC1A***: let-7a-5p, let-7b-5p, let-7c-5p
4	Lysophosphatidylcholine	***LPCAT1***: miR-27a-3p, miR-370-3p, miR-4768-3p. ***PLB1***: miR-3162-5p, miR-4529-3p. ***PLA2G2A***: miR-765, miR-3652
5	Decanoyl carnitine	***CROT***: miR-33a-5p, miR-373-3p, miR-33b-5p, miR-17-5p. ***ACADM***: miR-4437, miR-5580-3p, miR-6529-5p. ***ACADS***: miR-484
6	Malonyl carnitine	***ACADM***: miR-4437, miR-5580-3p, miR-6529-5p. ***CPT1A***: miR-653-5p, miR-328-3p, miR-6866-3p
7	Alanine-Leucine	***GAL***: miR-922, miR-4742-5p, miR-4753-5p. ***PGA3***: miR-2467-3p, miR-1909-5p, miR-6743-5p
8	3-hydroxybutyrylcarnitine	***ACADM***: miR-4437, miR-5580-3p, miR-6529-5p
9	3-methylxanthine	***PDE4D***: miR-18a-5p, miR-31-5p, miR-148a-3p
10	L-Phenylalanine	***PAH***: miR-23a-3p. ***DDC***: miR-875-3p, miR-3166
11	Phosphatidylinositol (34:1)	***PIGL***: miR-4651, miR-5087, miR-6499-3p. ***PIK3C2A***: miR-503-5p, miR-301b-3p. ***PIK3CA***: let-7i-5p, let-7e-5p, miR-19a-3p. ***PLCB1***: miR-103a-3p, miR-107, miR-423-5p
12	2,6 dimethylheptanoyl carnitine	***ACADM***: miR-4437, miR-5580-3p, miR-6529-5pю ***CRAT***: miR-936, miR-1207-5p
13	5-methoxytryptophan	***TPH1***: miR-320a-3p, miR-450a-2-3p, miR-320b
14	3-oxododecanoic acid	***FASN:*** miR-30c-5p
15	2-hydroxymyrystine acid	***NMT1***: miR-181a-5p, miR-214-3p, miR-491-5p
16	3-oxocholic acid	***FABP6***: miR-208a-5p, miR-330-5p, miR-196b-5p
17	Indolylacrylic acid	***KYAT1***: miR-423-5p, miR-6842-5p, miR-597-3p
18	N-acetylproline	***APEH:*** miR-1289
19	L-octanoylcarnitine	***CROT***: miR-33a-5p, miR-373-3p, miR-33b-5p, miR-17-5p. ***CPT2***: miR-433-3p, miR-6843-3p
20	Capriloyl glycine	***ACADM***: miR-4437, miR-5580-3p, miR-6529-5p. ***ODC1***: miR-423-5p, miR-3184-5p. ***GLYATL1***: miR-1207-5p, miR-4668-3p
21	Lysophosphatidylserine (20:4)	***PLA1A***: miR-3153, miR-7110-3p. ***GPR34***: miR-3193, miR-2909

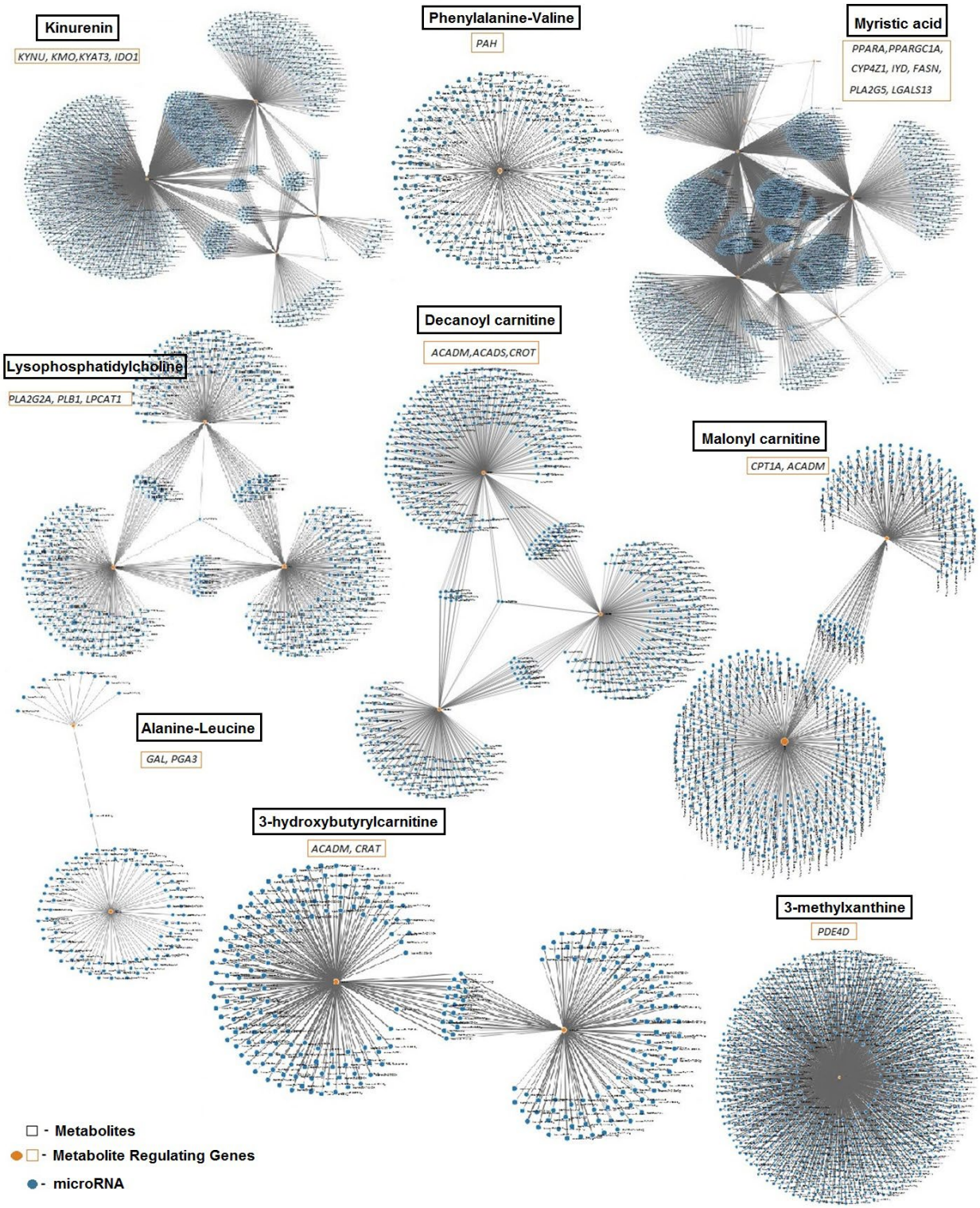

Fig. 1. Metabolites, regulatory genes of metabolic pathways and microRNAs interacting with them

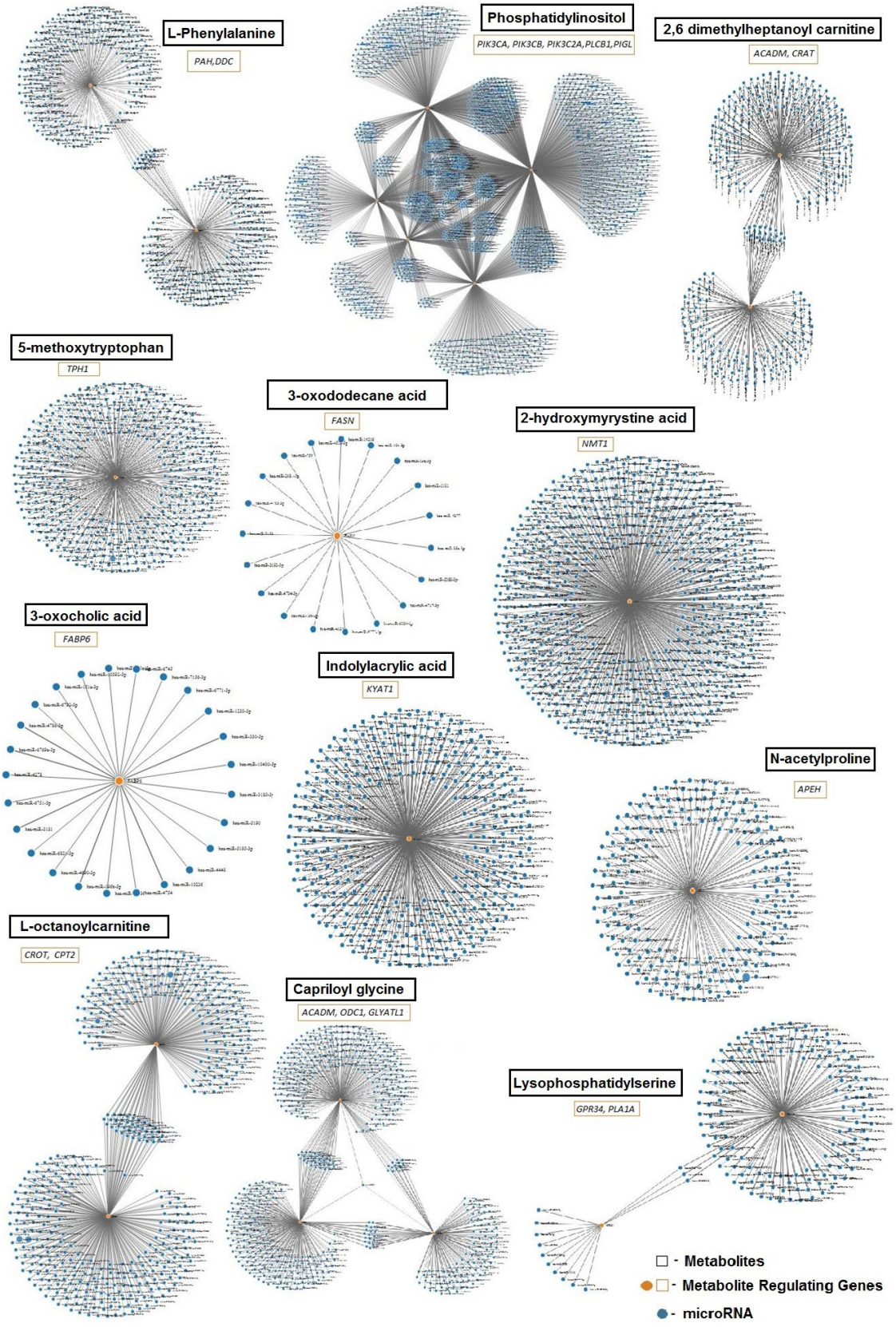

Fig. 2. Metabolites, regulatory genes of metabolic pathways and microRNAs interacting with them

miR-30c-5p by 2.9 times, miR-181a-5p by 2.6 times was found, let-7b-5p 2.6 times, miR-27A-3p 1.9 times, miR-370-3p 2.6 times, miR-6529-5p 2.5 times, miR-653-5p 2.2 times, miR-4742-5p 2.4 times, miR-2467-3p 2.6 times, miR-1909-5p 3.5 times, miR-6743-5p 4.9 times, miR-875-3p 2.3 times, miR-19a-3p 4.9 times, miR-208a-5p 2.6 times, miR-330-5p 3.2 times, miR-1207-5p by 3.5 times, miR-4668-3p by 4.2 times, miR-3193 is 2.6 times higher than their level in the urine of conditionally healthy individuals.

There was also a significant decrease ($p < 0.05$) in the level of miR-23a-3p by 20.0 times, miR-12132 by 4.0 times, miR-765 by 1.8 times, miR-181b-5p by 4.0 times, miR-4529-3p by 1.8 times, miR-33b-5p by 3.1 times, miR-17-5p by 4.6 times, miR-6866-3p by 1.7 times, miR-4753-5p by 14.3 times, miR-103a-3p by 19.6 times, miR-423-5p by 3.0 times, miR-491-5p by 1.7 times, miR-196b-5p by 5.0 times, miR-6843-3p 2.3 times, miR-423-5p 4.6 times and miR-3184-5p 2.6 times relative to their urine levels in conditionally healthy individuals.

Thus, the microRNA profile miR-382-5p, miR-593-3p, miR-29a-5p, miR-2110, miR-30c-5p, miR-181a-5p, let-7b-5p, miR-27a-3p, miR-2110, miR-30c-5p, miR-181a-5p, let-7b-5p, miR-27a-3p, miR-370-3p, miR-6529-5p, miR-653-5p, miR-4742-5p, miR-2467-3p, miR-1909-5p, miR-6743-5p, miR-875-3p, miR-19a-3p, miR-208a-5p, miR-330-5p, miR-1207-5p, miR-4668-3p, miR-3193, miR-23a-3p, miR-12132, miR-765, miR-181b-5p, miR-4529-3p, miR-33b-5p, miR-17-5p, miR-6866-3p, miR-4753-5p, miR-103a-3p, miR-423-5p, miR-491-5p, miR-196b-5p, miR-6843-3p, miR-423-5p and miR-3184-5p are differential for patients and conditionally healthy individuals.

DISCUSSION

In our study, using machine learning methods, links were established between metabolites that changed the concentrations of relatively healthy donors and genes encoding proteins involved in the synthesis and degradation of these metabolites, as well as links between metabolite regulatory genes and microRNA regulators of these genes.

Bioinformatics analysis has identified a list of 613 unique micro-RNAs involved in the regulation of the concentration of 21 metabolites. Of the 613 microRNAs, only the microRNAs with the maximum interaction strength with the mRNA of the genes regulating the content of metabolites were selected. The final list contained 91 micro-RNAs, of which 47 changed the level of their transcripts in urine (validated by PCR).

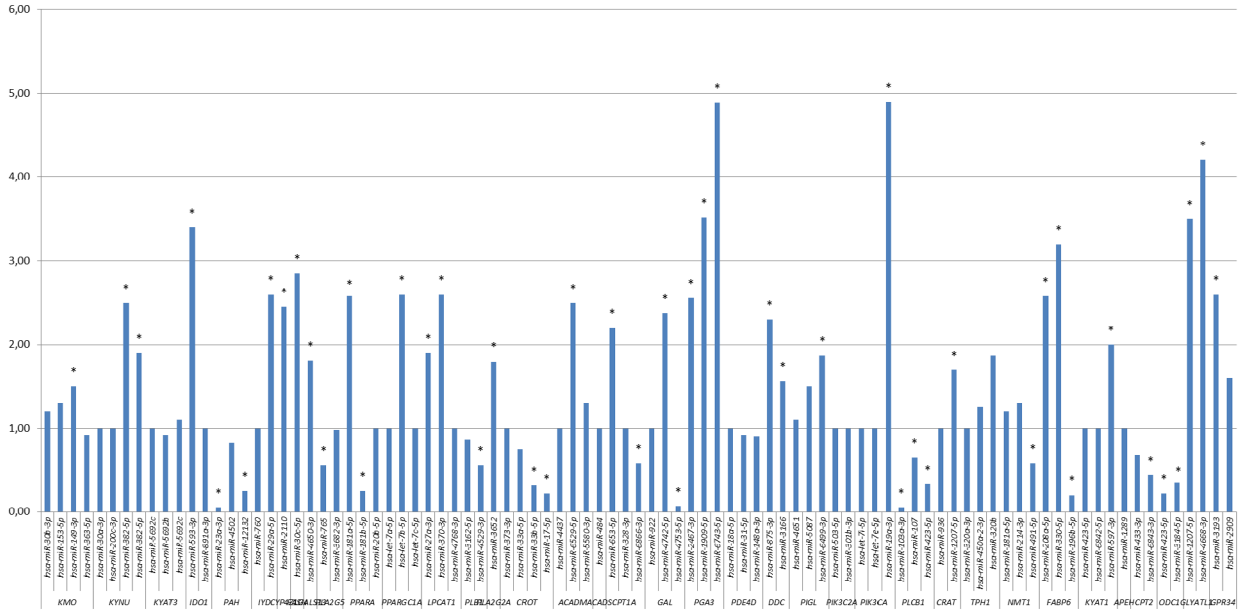

Fig. 3. Changes in the level of 91 micro-RNA transcripts in the urine of patients with serous ovarian adenocarcinoma
Note: * – statistically significant ($p < 0.05$) change in the transcript level relative to the control group

The level of transcripts miR-382-5p, miR-593-3p, miR-29a-5p, miR-2110, miR-30c-5p, miR-181a-5p, let-7b-5p, miR-27a-3p, miR-370-3p changed most significantly in patients with OC miR-6529-5p, miR-653-5p, miR-4742-5p, miR-2467-3p, miR-1909-5p, miR-6743-5p, miR-875-3p, miR-19a-3p, miR-208a-5p, miR-330-5p, miR-1207-5p, miR-4668-3p, miR-3193, miR-23a-3p, miR-12132, miR-765, miR-181b-5p, miR-4529-3p, miR-33b-5p, miR-17-5p, miR-6866-3p, miR-4753-5p, miR-103a-3p, miR-423-5p, miR-491-5p, miR-196b-5p, miR-6843-3p, miR-423-5p and miR-3184-5p relative to their urine levels in conditionally healthy individuals.

According to a number of authors, changes in the expression level of some of these microRNAs are associated with serous ovarian cancer: hsa-miR-382-5p, hsa-miR-27a-3p, hsa-miR-1207-5p, hsa-miR-423-5p [20], hsa-miR-593-3p [21], hsa-miR-29a-5p [22, 23] and hsa-miR-30c-5p [24, 25] and hsa-miR-30a-5p [26].

Nevertheless, the micro-RNA panel we identified (miR-382-5p, miR-593-3p, miR-29a-5p, miR-2110, miR-30c-5p, miR-181a-5p, let-7b-5p, miR-27a-3p, miR-370-3p, miR-6529-5p, miR-653-5p, miR-4742-5p, miR-2467-3p, miR-1909-5p, miR-6743-5p, miR-875-3p, miR-19A-3p, miR-208a-5p, miR-330-5p, miR-1207-5p, miR-4668-3p, miR-3193, miR-23A-3p, miR-12132, miR-765, miR-181b-5p, miR-4529-3p, miR-33b-5p, miR-17-5p, miR-6866-3p, miR-4753-5p, miR-103a-3p, miR-423-5p, miR-491-5p, miR-196b-5p, miR-6843-3p, miR-423-5p and miR-3184-5p) is unique and in this combination in literary sources not represented.

Obviously, transcriptomic imbalance begins in tissues and leads to a metabolic imbalance, which eventually affects the composition of body fluids, including urine.

Modern clinical oncogynecology has a serious need for effective biomarkers, changes in the levels of which can serve as evidence of the occurrence of a malignant process. Non-invasive and inexpensive PCR analysis of micro-RNA in urine makes it a particularly attractive screening tool. The application of this approach may allow for frequent testing of women belonging to high-risk groups and ensure long-term patient monitoring.

CONCLUSION

Bioinformatics analysis revealed a list of 613 unique microRNAs involved in the regulation of 21 metabolites. At the same time, the level of transcripts of 38 microRNAs (miR-382-5p, miR-593-3p, miR-29a-5p, miR-2110, miR-30c-5p, miR-181a-5p, let-7b-5p, miR-27a-3p, miR-370-3p, miR-6529-5p, miR-653-5p, miR-4742-5p, miR-2467-3p, miR-1909-5p, miR-6743-5p, miR-875-3p, miR-19a-3p, miR-208a-5p, miR-330-5p, miR-1207-5p, miR-4668-3p, miR-3193, miR-23a-3p, miR-12132, miR-765, miR-181b-5p, miR-4529-3p, miR-33b-5p, miR-17-5p, miR-6866-3p, miR-4753-5p, miR-103a-3p, miR-423-5p, miR-491-5p, miR-196b-5p, miR-6843-3p, miR-423-5p and miR-3184-5p) urine has diagnostic potential in ovarian cancer and is the basis for further research.. Thus, transcriptomic profiling of urine made it possible both to identify potential markers of the disease and to better understand the molecular mechanisms of changes underlying the development of OC.

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Filippov F. E. – conducting the experiment, writing the manuscript;
Porkhanova N. V. – statistical data processing;
Maksimov A. Yu. – editing the manuscript.

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ORIGINAL ARTICLE

Metabolomic profile of malignant ovarian tumors

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ABSTRACT

Purpose of the study. Investigate the metabolomic profile in tissues of patients with serous ovarian adenocarcinoma.
Materials and methods. The study included 100 patients with serous ovarian adenocarcinoma. Chromatographic separation of metabolites was performed on a Vanquish Flex UHPLC System chromatograph, which was coupled with an Orbitrap Exploris 480 mass spectrometer. Differences were assessed using the Mann-Whitney test with Bonferroni correction.
Results. In ovarian tumor tissue, 20 compounds had abnormal concentrations compared to normal tissue: increased levels of kynurenine, phenylalanylvaline, lysophosphatidylcholine (18:3), lysophosphatidylcholine (18:2), alanylleucine, L-phenylalanine, phosphatidylinositol (34:1), 5-methoxytryptophan, lysophosphatidylcholine (14:0), indoleacrylic acid and decreased levels of myristic acid, decanoylcarnitine, aspartylglycine, malonylcarnitine, 3-methylxanthine, 3-oxododecanoic acid, 2-hydroxymyristic acid, N-acetylproline, L-octanoylcarnitine and capryloylglycine.
Conclusion. A significant metabolic imbalance was found in ovarian tumor tissue, expressed in abnormal concentrations of fatty acids and their derivatives, acylcarnitines, amino acids and their derivatives, phospholipids and nitrogenous base derivatives. The concentrations of these 20 metabolites in tissues can serve as diagnostic markers of ovarian cancer. Thus, metabolomic tissue profiling allowed both to identify potential markers of the disease and to better understand the molecular mechanisms of changes underlying the development of this disease.

Keywords: metabolites, ultra-high performance liquid chromatography and mass spectrometry, ovarian serous adenocarcinoma, biomarkers

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Compliance with ethical standards: the research study is carried out in compliance with the ethical principles set forth by World Medical Association Declaration of Helsinki, 1964, ed. 2013. The study was approved by the Committee on Biomedical Ethics at the National Medical Research Center for Oncology (extract from the minutes of the meeting No. 17 dated 06/28/2022). Informed consent was received from all participants of the study

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ОРИГИНАЛЬНАЯ СТАТЬЯ

Метаболомный профиль злокачественных опухолей яичника

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РЕЗЮМЕ

Цель исследования. Изучение метаболомного профиля в тканях у больных серозной аденокарциномой яичников.
Материалы и методы. В исследование было включено 100 пациенток с диагнозом серозная аденокарцинома яичников. Хроматографическое разделение метаболитов проводили на хроматографе Vanquish Flex UHPLC System, который был сопряжен с масс-спектрометром Orbitrap Exploris 480. Оценку различий проводили с использованием критерия Манна Уитни с поправкой Бонферрони.
Результаты. В опухолевой ткани яичника 20 соединений имели аномальную концентрацию по сравнению с нормальной тканью: обнаружено увеличение содержания кинуренина, фенилаланил-валина, лизофосфатидилхолина (18:3), лизофосфатидилхолина (18:2), аланил-лейцина, L-фенилаланина, фосфатидилинозитола (34:1), 5-метокситриптофана, лизофосфатидилхолина (14:0), индолакриловой кислоты и снижение содержания миристиновой кислоты, деканоилкарнитина, аспартил-глицина, малонилкарнитина, 3-метилксантина, 3-оксододекановой кислоты, 2-гидроксимиристиновой кислоты, N-ацетилпролина, L-октаноилкарнитина и каприлоилглицина.
Заключение. В опухолевой ткани яичника обнаружен значительный метаболомный дисбаланс, выраженный в аномальных концентрациях жирных кислот и их производных, ацилкарнитинов, аминокислот и их производных, фосфолипидов и производных азотистых оснований. Концентрации этих 20 метаболитов в тканях могут служить диагностическими маркерами рака яичников. Таким образом, метаболомное профилирование тканей позволило как выявить потенциальные маркеры заболевания, так и лучше понять молекулярные механизмы изменений, лежащих в основе развития данного заболевания.

Ключевые слова: метаболиты, ультравысокоэффективная жидкостная хроматография и масс-спектрометрия, серозная аденокарцинома яичника, биомаркеры

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Соблюдение этических стандартов: в работе соблюдались этические принципы, предъявляемые Хельсинкской декларацией Всемирной медицинской ассоциации (World Medical Association Declaration of Helsinki, 1964, ред. 2013). Исследование одобрено Комитетом по биомедицинской этике при ФГБУ «Национальный медицинский исследовательский центр онкологии» Министерства здравоохранения Российской Федерации (выписка из протокола заседания № 17 от 28.06.2022 г.). Информированное согласие получено от всех участников исследования

Финансирование: финансирование данной работы не проводилось. Работа выполнена с использованием научного оборудования ЦКП ФГБУ «Национальный медицинский исследовательский центр онкологии» Министерства здравоохранения Российской Федерации: https://ckp-rf.ru/catalog/ckp/3554742/

Конфликт интересов: все авторы заявляют об отсутствии явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи

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INTRODUCTION

In the last decade, among oncogynecological diseases, ovarian cancer has occupied leading positions in terms of morbidity and mortality in the world and Russia [1, 2]. Malignant ovarian tumors are divided into many histological subtypes, each of which has distinctive biological and clinical characteristics. There are serous carcinoma, endometrioid carcinoma, mucinous carcinoma, light cell carcinoma, malignant Brenner tumor, serous-mucinous carcinoma, undifferentiated carcinoma and mixed epithelial carcinoma. Serous adenocarcinoma is the most common subtype [3, 4].

The overall five-year survival rate of ovarian cancer patients does not exceed 40 %, which is due to late diagnosis. To date, the sensitivity and specificity of the main diagnostic methods of this disease are insufficient to detect it at an early stage [5, 6]. New approaches are needed to improve diagnosis. Metabolomics methods based on high-resolution liquid chromatography and mass spectrometry (MS) open up new prospects for the detection and identification of biomarkers in the femtomolar and attomolar ranges.

So in the work of Y. Ahmed-Salim and co-authors analyzed the results of 32 publications in the field of metabolic research in ovarian cancer. Most studies have reported a violation of the regulation of phospholipids and amino acids: histidine, citrulline, alanine and methionine. At the same time, combinations of more than one metabolite as a panel in various studies achieved higher sensitivity and specificity for diagnosis than a single metabolite; for example, combinations of various phospholipids [7].

In [8], the role of histidine and citrulline in the development of ovarian cancer was confirmed, and new lipid compounds (lysophosphatidylcholine C16:1, phosphatidylcholine C32:2, C34:4 and C36:6) potentially involved in cancer metabolism were discovered.

However, such studies in ovarian cancer are not numerous compared to genomic and transcriptomic ones, and most of them were performed on equipment with lower resolution and a principle of operation different from Orbitrap technology [9], and biological fluids of patients, such as urine [10] or blood [11].

The purpose of the study was to study the metabolic profile of tissues of patients with serous ovarian adenocarcinoma in order to identify potential diagnostic markers of the disease.

MATERIALS AND METHODS

The study included 100 patients diagnosed with serous ovarian adenocarcinoma (T3a-c). Samples of normal and tumor tissue obtained at the stage of surgical treatment were used as objects of research. The average age of the patients was 54.2 years.

Analysis of metabolites by the HPLC-MS method

Surgical biopsies of tumor and normal ovarian tissue were used for analysis, which were stored in liquid nitrogen until the moment of metabolic and molecular genetic studies. The samples were homogenized at a temperature no higher than 4 °C. The homogenate was mixed with 600 µl of acetonitrile LC-MS (Merck, Germany)/methanol LC-MS (Merck, Germany) in a ratio of 3/1, was stirred for 15 minutes using a vortex and incubated for 15 hours at –20 °C. Proteins were precipitated by centrifugation at 16000 g 0 °C for 30 minutes. The supernatant was transferred to clean Eppendorf tubes. The solvent was evaporated at 45 °C for 4 hours on a SpeedVac vacuum evaporator (Eppendorf). The resulting dry precipitate was dissolved in 300 µl of 95 % acetonitrile LC-MS solution (Merck, Germany) with the addition of 0.1 % formic acid (Merck, Germany). To better dissolve the sediment, the samples were treated with ultrasound in an Elmsonic P 120 H ultrasonic bath (ELMA, Germany). Further, the samples were centrifuged for 30 min at 16000 g and the resulting supernatant was used for chromatomass spectrometric analysis.

Chromatographic separation of metabolites was performed on a Vanquish Flex UHPLC System Thermo Fisher Scientific chromatograph. The chromatograph was paired with the Orbitrap Exploris 480 mass spectrometer, which has an electrospray ionization source. A sample of metabolites in a volume of 2 µl was divided on a Hypersil GOLD™ C18 column (1.9 µm, 150 × 2.1 mm), eluents: A – 0.1 % formic acid LC-MS (Merck, Germany), B – acetonitrile LC-MS (Merck, Germany) containing 0.1 % formic

acid (Merck, Germany). The following elution gradient was used: 1 min – 5 % of eluent B, 15 min – linear gradient of eluent B from 5 to 95 %, 2 min – 95 % of eluent B, 0.5 min – change of eluent composition to 5 % of eluent B, 3 min – 5 % of eluent B. The flow of eluents is 200 µl/min.

Mass spectrometric analysis was performed on an Orbitrap Exploris 480 (Thermo Fisher Scientific) mass spectrometer with an electrospray ionization source. The mass spectrometer was configured for priority ion detection in the m/z range from 67 to 1000 Da with a resolution of 60,000. The spectra were taken in the detection mode of positively charged ions. The time to remove one spectrum is 20 minutes. Additional MS settings were as follows: ion sputtering voltage = –3.5 kV; capillary temperature = 320 °C; sample heater temperature = 300 °C; protective gas = 35; auxiliary gas = 10 and radio frequency S-lens – 50.

For mass spectrometric peaks to be identified, compliance with specific metabolites from the Human Metabolome Database was established (http://www.hmdb.ca) and Metlin (Scripps Center for Mass Spectrometry, USA; http://metlin.scripps.edu). For this purpose, an accurately measured mass of the chemical compound was used. Bioinformatic analysis was performed using Compound Discoverer Software (Thermo Fisher Scientific, USA) and analysis of biochemical pathways using the KEGG PATHWAY Database.

Statistical data processing

The differences were assessed using the Mann-Whitney criterion for a threshold level of statistical significance of $p < 0.05$, and the Bonferroni correction was used to account for multiple comparisons. The data analysis was carried out in the Python programming language using the SciPy library [12].

STUDY RESULTS

During the conducted metabolomic profiling, 100 samples of serous ovarian adenocarcinoma and 100 samples of conditionally normal ovarian tissues were analyzed. 750 metabolites were identified. For metabolites whose intensities in the mass spectra differed statistically significantly relative to normal tissue, P-value and FoldChange were determined (Table 1).

According to the data obtained, the metabolome of the tumor tissue of patients with serous ovarian carcinoma differed significantly from samples of normal ovarian tissue of the same patients. In the tumor tissue of the patients, 10 metabolites (kynurenine, phenylalanyl valine, lysophosphatidylcholine (18:3), lysophosphatidylcholine (18:2), alanyl leucine, L-phenylalanine, phosphatidylinositol (34:1), 5-methoxytryptophan, lysophosphatidylcholine (14:0), indolacrylic acid) had significantly higher concentrations In comparison with conditionally normal tissue, the concentration of 10 compounds (myristic acid, decanoyl carnitine, aspartyl-glycine, malonylcarnitine, 3-methylxanthine, 3-oxododecanoic acid, 2-hydroxymyristinic acid, N-acetylproline, L-octanoylcarnitine, caprylylglycine), on the contrary, was reduced.

Thus, it was found that the concentrations of myristic acid, 2-hydroxymyristic acid and 3-oxododecanoic acid in tumor tissue were statistically significantly ($p < 0.01$) reduced by 2.6 times, 4.8 times and 1.4 times, respectively, compared with normal tissue. The levels of decanoyl carnitine, malonylcarnitine and L-octanoyl carnitine in tumor tissue were statistically significantly ($p < 0.0001$) lower by 5.3 times, 1.5 times and 6.7 times, respectively, than in normal tissue. Statistically significantly ($p < 0.00000005$), the concentration of a number of phospholipids in tumor tissue in patients with ovarian cancer was increased relative to normal ovarian tissue: lysophosphatidylcholine (18:3) by 2.1 times, lysophosphatidylcholine (18:2) by 3.4 times, phosphatidylinositol (34:1) by 4.1 times and lysophosphatidylcholine (14:0) by 1.9 times. Statistically significant ($p < 0.01$) changes in the concentration of some amino acids and their derivatives were also found: an increase in the concentration of kynurenine by 6.1 times, phenylalanyl valine by 2.2 times, alanyl leucine by 1.6 times, L-phenylalanine by 1.8 times, 5-methoxytryptophan by 1.6 times and indolacrylic acid by 1.5 times relative to normal tissue, as well as a decrease in the concentration of N-acetylproline by 1.7 times, caprylylglycine by 1.5 times and aspartyl glycine by 5.0 times, respectively, relative to normal ovarian tissue. A change in the content of nitrogenous base derivatives in ovarian tumor tissue was also detected, i.e. a 2.3-fold decrease in the concentration of 3-methylxanthine ($p < 0.0001$).

DISCUSSION

The HPLC-MS method identified 750 metabolites of various classes, while the concentration of 10 metabolites in the tumor tissue was significantly increased compared to conditionally normal tissue, and the concentration of 10 compounds was lowered on the contrary.

Fatty acids and their derivatives

In the tumor tissue, the concentrations of most fatty acid derivatives – myristic acid, 2-hydroxymyristic acid and 3-oxododecanoic acid were reduced compared to conditionally normal tissue. Tumor cells are characterized by a profound restructuring of the metabolism of lipids and fatty acids. In some types of tumors, the utilization of fatty acids increases, while in others it is suppressed [13, 14].

Myristic acid ($CH_3(CH_2)_{12}COOH$, FoldChange 0.38, p = 0.0000241) is a saturated fatty acid with an aliphatic long chain, present in almost all living organisms [15]. Abnormal levels of myristic acid can increase the risk of tumors [16]. It is involved in the implementation of several antitumor mechanisms,

Table 1. The difference between the metabolomic profile of tumor tissue and normal in patients with serous ovarian adenocarcinoma

Metabolites	m/z	FoldChange, tumor/normal tissue	*p*-value
1. Fatty acids and their derivatives			
Myristic acid	231.2	0.38	0.00002410
2-hydroxymyristic acid	267.2	0.21	0.00001000
3-oxododecanoic acid	237.1	0.74	0.01000060
2. Acylcarnitines			
Decanoyl carnitine	316.2	0.19	0.000000003
Malonyl carnitine	230.1	0.65	0.000100002
L-octanoylcarnitine	288.2	0.15	0.000004011
3. Phospholipids			
Lysophosphatidylcholine (18:3)	518.3	2.05	0.000000002
Lysophosphatidylcholine (18:2)	521.3	3.40	0.000000051
Lysophosphatidylcholine (14:0)	468.3	1.89	0.000000003
Фосфатидилинозитол (34:1)	430.8	4.11	0.000000001
4. Aminoacids and their derivatives			
Alanine-Leucine	185.1	1.55	0.000900000
Phenylalanine-Valine	265.2	2.15	0.000100000
L-Phenylalanine	166.1	1.84	0.000000002
Kinurenin	209.1	6.07	0.000001000
Aspartyl-glycine	208.1	0.20	0.000012400
5-methoxytryptophan	217.1	1.61	0.000004200
Indolylacrylic acid	171.1	1.49	0.010189400
N-acetylproline	140.1	0.59	0.000085630
Capriloyl glycine	202.1	0.65	0.010212890
5. Derivatives of nitrogenous bases			
3-methylxanthine	167.1	0.44	0.000100000

such as the production of myristoleic acid, which causes apoptosis, and in the synthesis of ceramides de novo. According to a number of authors, the content of myristic acid in biological fluids and tissues is inversely associated with the risk of colorectal cancer. However, the mechanisms underlying this relationship have not been fully studied [17-20].

2-hydroxymyristic acid ($C_{14}H_{28}O_3$, FoldChange 0.21, p = 0.00001) is a fatty acid containing an aliphatic chain carrying a hydroxyl substituent at position 2, is a derivative of myristic acid. The physiological function of hydroxy fatty acids remains largely unknown. They have been shown to play a specific role in signaling to cells [21]. 2-Hydroxymyristinic acid is metabolically activated in cells to form 2-hydroxymyristoyl-CoA, a potent inhibitor of myristoyl-CoA [22]. Currently, the main mechanisms by which 2-hydroxylation of fatty acids is associated with metabolic adaptation and tumor growth remain unclear [23].

3-oxododecanoic acid ($C_{12}H_{22}O_3$, FC = 0.74, p = 0.01000060) is a fatty acid that is a 3-oxo derivative of decanoic acid. In the human body, 3-oxododecanoic acid participates in a number of enzymatic reactions [24]. Keto-fatty acids are often reported as artifacts of fatty acid oxidation, but relatively rarely as natural fatty acids. 3-Keto-fatty acids, found as secondary components of animal tissues, are usually intermediates of β-oxidation.

For the beta-oxidation of fatty acids by mitochondria, the presence of carnitine, an important cofactor of metabolic processes, is an indispensable condition. There are more than 1,000 types of acylcarnitines in the human body, the general function of which is to transport acyl groups of organic acids and fatty acids from the cytoplasm to the mitochondria so that they can be broken down during beta oxidation to produce energy [25]. This is one of the most efficient ways of energy production in cells, therefore, tissues with high energy consumption mainly depend on the utilization of fatty acids [26].

Cancer is a pathological condition characterized by high energy consumption. Glucose and glutamine as energy substrates are considered a distinctive feature of tumor cells, and the metabolic switch that allows their use in almost anaerobic conditions is known as the Warburg effect [27]. The canonical interpretation of the Warburg effect implies that cells bypass the mitochondrial respiratory chain to synthesize ATP even with sufficient oxygen supply [28]. However, it is obvious that the Warburg effect needs to be considered in a more general metabolic context, which also includes the utilization of fatty acids in accordance with the effectiveness of these substrates in terms of ATP output. Metabolic flexibility is a phenomenon observed in different types of cancer and within the same type of cancer at different stages of progression. Carnitine-induced fatty acid oxidation plays a critical role in the production of NADH, FADN2, NADPH, and ATP, which can contribute to the development of tumors [29].

Acylcarnitines

Metabolic reprogramming of tumor cells regulates the content of acylcarnitines with different chain lengths in order to create a balance between production, energy consumption and synthesis of metabolic intermediates to meet the requirements of rapid proliferation [30]. Acylcarnitines have cytotoxicity and immunomodulatory properties that can be used by the tumor for growth and survival in situ [31]. Thus, a change in the level of malonylcarnitine is associated with the risk of developing breast cancer.

Malonylcarnitine is a metabolite that accumulates with a specific violation of fatty acid oxidation caused by a violation of the intake of long-chain acylcarnitine esters into the mitochondria and insufficiency of the mitochondrial respiratory chain with a deficiency of complex 11 and malonyl-CoA decarboxylase [32].

L-octanoylcarnitine is a physiologically active form of octanoylcarnitine [33], which is found in deficiency of medium chain acyl-CoA dehydrogenase (MCAD). L-octanoylcarnitine is involved in lipid peroxidation (HMDB: HMDB0000791), fatty acid metabolism (HMDB: HMDB0000791), mitochondrial beta oxidation of short-chain saturated fatty acids (HMDB: HMDB0000791) and lipid transport (HMDB: HMDB0000791). Changes in its concentrations have been recorded in blood and faeces in colorectal cancer, Crohn's disease and ulcerative colitis [34].

Decanoyl carnitine is classified as an acylcarnitine with a medium chain length. A change in the concentration of decanoyl carnitine was found in renal cell carcinoma and breast cancer [35].

The study of fluctuations in the content of acylcarnitines can contribute to a better understanding of the mechanisms of oncological diseases and the development of methods for their diagnosis and treatment.

Phospholipids

In this study, an increase in the concentration of lysophosphatidylcholines and phosphatidylinositol was observed in ovarian tumor tissue. Lysophospholipids are secreted by various types of cells, including tumor cells. These chemical compounds play an important role in the development, activation, and regulation of the immune system [36]. Changes in the composition and content of phospholipids and lysophospholipids have previously been shown in prostate cancer and are considered as potential biomarkers [37]. Lysophospholipids function as signaling molecules through their specific membrane receptors. In addition, some of the lysophospholipids have tumor-promoting activity and are therefore called "oncolipids" [38]. Recent studies have shown that phospholipids are candidates for PH biomarkers. Several comprehensive prospective studies of lipids have been conducted, such as lysophosphatidylcholines, phosphatidylcholines, ceramides and sphingomyelins, the concentrations of which differ in patients with rheumatoid arthritis compared with healthy ones [39, 10].

Lysophosphatidylcholines, also called lysolecithins, are a class of chemical compounds formed from phosphatidylcholines by the enzyme phospholipase A2. Lysophosphatidylcholines are the most common phospholipids in the blood and key lipids in various pathophysiological conditions such as inflammation, endothelial activation and atherogenesis [40]. Among other properties, they act as a signaling molecule released by apoptotic cells to attract phagocytes, which then phagocytize apoptotic cells [41].

Phosphatidylinositols are minor phospholipids of the inner membrane layer of eukaryotic cells, important components of intracellular signaling pathways. Phosphatidylinositol is a substrate for a variety of signaling kinase molecules that can attach a phosphate group to inositol. The main biological functions of phosphatidylinositols are a membrane stabilizer (HMDB: HMDB0009799) and a molecular messenger (signaling molecule (HMDB: HMDB0009799)). Phosphatidylinositols are involved in such important signaling pathways and processes as fatty acid metabolism (HMDB: HMDB0009799), lipid peroxidation (HMDB: HMDB0009799), apoptosis, cell adhesion [42], cell migration and proliferation [43]. Their content increases in the blood (HMDB: HMDB0009799) in a number of oncological diseases, including breast cancer, colorectal cancer and stomach cancer [44].

Amino acids and their derivatives

An abundant supply of nutrients, such as amino acids, is necessary for the increased metabolic needs of tumor cells that maintain high proliferative activity [45].

The alteration of tryptophan metabolism in cancer via the kynurenine pathway has attracted widespread attention as a mechanism by which tumors can elude immune control [45].

Kynurenine (β-(o-aminobenzene)-α-aminopropionic acid) is an intermediate product of the enzymatic breakdown of tryptophan and the biosynthesis of nicotinic acid in the human body. During enzymatic oxidation, kynurenine is converted to 3-hydroxykynurenine. The pathway of L-tryptophan biotransformation with the formation of "kynurenine" metabolites plays an important role in the mechanisms of immunoregulation and "negative" control of immune inflammation [46].

In addition to the main pathways of tryptophan catabolism, there are secondary ones, one of them leads to indolylacrylic acid ($C_{11}H_9NO_2$, indolacrylate), the biological role of which in animals is still unclear [47]. Stimulating the production of indolacrylic acid can promote anti-inflammatory reactions and have therapeutic value [47]. In our study, the level of indolacrylic acid is elevated in ovarian tumor tissue. The production of indolacrylic acid may contribute to the development of anti-inflammatory reactions [47]. It has been shown to selectively affect breast cancer cells, but does not affect untransformed primary fibroblasts. In our study, an increase in indolacrylic acid was accompanied by an increase in the content of kynurenine.

5-methoxytryptophan ($C_{12}H_{14}N_2O_3$), which is an endothelial factor with anti-inflammatory properties, is synthesized from L-tryptophan by 2 enzymes: tryptophan hydroxylase-1 and hydroxyindole-O-methyltransferase [48]. It controls the migration and acti-

vation of macrophages by inhibiting NF-kB [49], and also regulates epithelial-mesenchymal transition and metastasis [50].

Changes in the metabolism of another aromatic amino acid, *phenylalanine* and its derivatives, are also associated with inflammation and immune activation. Neurauter G. et al showed that the concentration of phenylalanine in serum in patients with ovarian carcinoma correlates with the concentration of markers of immune activation and the development of oxidative stress [51].

We also found a decrease in the content of aspartyl glycine dipeptide in tumor tissue. This compound is probably a product of incomplete breakdown of proteins and peptides. It is known that some dipeptides have physiological or cellular signaling effects, although most of them are simply short-lived intermediates on the way to specific amino acid degradation pathways. Some dipeptides are also considered as biomarkers of diseases [52].

Concentrations of *N-acetyl-L-proline* ($C_7H_{11}NO_3$) and caprylylglycine also decrease. N-acetylproline is a biologically available N-terminal form of the proteinogenic alpha amino acid L-proline. N-terminal acetylation of proteins is a widespread and highly conserved process in eukaryotes, which is involved in the protection and stability of proteins [53]. A number of studies have shown the association of N-acetyl-L-proline with colorectal cancer [54] and metastatic melanoma [55]. Caprylylglycine is a lipid amino acid consisting of caprylic acid and glycine. Acylglycines are usually minor metabolites of fatty acids [55].

Nitrogenous base derivatives and steroids

In our study, a decrease in the concentration of 3-methylxanthine was found in ovarian tumor tissue. 3-methylxanthine ($C_6H_6N_4O_2$) is a methyl derivative of purine with a ketone group (3,7-dihydropurine-2,6-dione). Some evidence suggests that methylxanthines have antitumor effect [56]: they inhibit *PI3K/Akt/mTOR* and stimulate *PTEN*, promoting apoptosis and autophagy [57].

CONCLUSION

A significant change in metabolism was found in the ovarian tumor tissue, presented in abnormal concentrations of fatty acids and their derivatives, acylcarnitines, amino acids and their derivatives, phospholipids and derivatives of nitrogenous bases. Concentrations of these metabolites in tissues can serve as diagnostic markers of ovarian cancer. Thus, the metabolic profiling of tissues allowed both to identify potential markers of the disease and to better understand the molecular mechanisms of changes underlying the development of this disease.

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REVIEW

Modern strategy of metastatic colorectal cancer treatment (literature review)

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ABSTRACT

Metastatic lesions account for about 50–60 % of all cases of colorectal cancer (CRC). Currently, the prognosis for metastatic CRC has significantly improved due to the advent of effective drug therapy and the expansion of surgical treatment options. In this regard, the study of modern directions of treatment of metastatic CRC is of particular interest.

In this study, both literature data and obtained treatment results of patients with metastatic colorectal cancer have been analyzed (PubMed, Scopus, eLibrary databases were used) at the National Medical Research Centre for Oncology.

Currently, many factors should be taken into account when planning therapy for patients with metastatic CRC: the characteristics of the tumor itself (biology and localization of the tumor, tumor burden, RAS, BRAF mutational status), the patient (age, performance status, functional state of organs and systems, comorbidity, patient attitude, expectations and preferences) and the treatment itself (toxicity, flexibility of the treatment program, socio-economic factors, quality of life). With a resectable process, surgical treatment with adjuvant or perioperative chemotherapy, and with potentially resectable liver metastases, with massive prevalence, unfavorable prognosis – to carry out the most active drug therapy taking into account the mutational status of the tumor in order to transfer the process to a resectable one. In case of widespread colorectal cancer, drug therapy lines are consistently carried out, the selection of which is based on the goals of therapy, the type and time of primary therapy, the mutation profile of the tumor, and the toxicity of drugs.

Patients with metastatic liver and/or lung lesions should be considered through the prism of surgical treatment, since it is surgical intervention that can significantly improve the results of treatment of patients. Therefore, patients with potentially resectable metastases should receive the most effective treatment and be operated on as soon as the process becomes resectable. At the same time, modern chemotherapy and targeted therapy are an integral part of the treatment of patients with metastatic colorectal cancer.

Keywords: review, metastatic colorectal cancer, liver resection, chemotherapy, targeted therapy

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ОБЗОР

Современные хирургические стратегии лечения метастатического колоректального рака (обзор литературы)

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РЕЗЮМЕ

Метастатическое поражение составляет около 50–60 % всех случаев колоректального рака (КРР). В настоящее время прогноз в отношении метастатического КРР значительно улучшил-ся в связи с появлением эффективной лекарственной терапии и расширением возможностей хирургического лечения. В связи с этим особый интерес представляет изучение современных направлений лечения метастатического КРР.

В данном исследовании был проведен анализ данных литературы (использовались базы данных PubMed, Scopus, eLIBRARY) и собственных результатов лечения больных метастатическим колоректальным раком в ФГБУ «Национальный медицинский исследовательский центр онкологии» Министерства здравоохранения Российской Федерации.

В настоящее время при планировании терапии пациентов с метастатическим КРР следует учи-тывать многие факторы: характеристики самой опухоли (биология и локализация опухоли, tumour burden, мутационный статус RAS, BRAF), пациента (возраст, performance status, функ-циональное состояние органов и систем, коморбидность, отношение пациента к различным ме-тодам лечения, ожидания и предпочтения) и самого лечения (токсичность, гибкость програм-мы лечения, социоэкономические факторы, качество жизни). При резектабельном процессе хирургическое лечение с адъювантной или периоперационной химиотерапией, а при потенци-ально резектабельных метастазах в печень, при массивной распространенности, неблагоприят-ном прогнозе – проводить максимально активную лекарственную терапию с учетом мутаци-онного статуса опухоли с целью перевода процесса в резектабельный. При распространенном КРР последовательно проводят линии лекарственной терапии, выбор которой основывается на целях терапии, виде и времени первичной терапии, мутационном профиле опухоли, токсично-сти препаратов.

Пациенты с метастатическим поражением печени и/или легких должны рассматриваться через призму оперативного лечения, поскольку именно хирургическое вмешательство способно зна-чимо улучшить результаты лечения пациентов. Поэтому пациенты с потенциально резекта-бельными метастазами должны получать максимально эффективное лечение и оперироваться, как только процесс станет резектабельным. При этом современная химиотерапия и таргетная терапия являются неотъемлемой частью лечения больных метастатическим КРР.

Ключевые слова: обзор, метастатический колоректальный рак, резекция печени, химиотерапия, таргетная терапия

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INTRODUCTION

Colorectal cancer (CRC) is the third most common type of malignant neoplasm worldwide and the second leading cause of cancer death [1, 2]. In Russia, the incidence of CRC was 124 per 100 thousand population in 2022 [3]. Metastatic lesions are observed in 50–60 % of all CRC cases, while metachronous metastases occur in 20–50 %, and synchronous metastases account for 15–30 % of cases, and there is also a relationship between the latter and the worst prognosis [4, 5]. The most common localization of metastases is the liver, followed by the lungs, peritoneum and distant lymph nodes.

At the same time, metachronous metastases occurring after treatment are most often observed (40 %), synchronous metastases account for 25 % of cases and are associated with a worse prognosis. In 30 % of patients, metastatic lesion is limited only to the liver with a primary resectable process in 1/3 of these patients. In 70 % of patients with metastatic CRC, liver damage is primarily unresectable or there are extrahepatic metastases [6, 7]. These data indicate that the majority of CRC patients will sooner or later have distant metastases, and these will mainly be liver metastases.

Nevertheless, the current situation is not so tragic, since the prognosis for metastatic CRC has improved significantly due to the advent of effective drug therapy and the expansion of surgical treatment options [8, 9].

The purpose of the study was to study modern treatment options for metastatic colorectal cancer.

Metastatic CRC drug therapy

Many studies have been devoted to the choice of drug therapy for metastatic CRC. There are modern chemotherapy and immunotherapy regimens (targeted drugs, immune checkpoint inhibitors), as well as methods of local exposure (radiofrequency thermal ablation, chemoembolization).

The long-term results of using FOLFOX and FOLFIRI schemes turned out to be comparable, but the advantages of the FOLFOX scheme in line 1 in terms of the frequency of liver resections were revealed [10]. A retrospective PRIME study conducted in England among 512 patients showed that the addition of EGFR inhibitors to chemotherapy regimens increases the frequency of R0 liver resections by 60 % in patients with unresectable metastases, and these patients can be cured [11]. This conclusion was also confirmed by a meta-analysis conducted by Petrelli F. and coauthors [12]. The FIRE-3 study conducted among 353 patients compared the use of different therapy regimens for metastatic CRC and found that the best response was achieved in the group of patients receiving cetuximab and FOLFIRI after 3.5 months of treatment [13]. The meta-analysis also confirmed that for patients with metastatic CRC with wild type RAS, the best treatment strategy in the 1st line of therapy is chemotherapy + cetuximab [14]. In wild type RAS/BRAF and left–sided localization of the primary tumor, anti-EGFR should be used, in right-sided – bevacizumab [15]. Achieving resectability of liver metastases increases the 5-year survival rate from 9 to 42 % [16].

Surgical strategies for CRC metastatic liver damage

The requirement for liver resection in CRC metastases is currently beyond doubt and has been proven by LiverMetSurvey International Registry data, according to which, out of 23 thousand patients, the 5-year survival rate in the presence of liver resection was 42 %, without resection – 9 %. In the review by Simmonds R. S. et al. [17], based on the analysis of 30 studies, it was found that the 5-year survival rate of CRC patients with liver metastases with liver resection R0/R1 was 30–32 %, with resection R2 – 7 %, without liver resection – 0 %. A meta-analysis conducted by Kanas G. P. et al. [18] showed that the 5-year survival rate of patients with metastatic CRC undergoing liver resection was 38 %. Therefore, surgery should be the goal for the majority of these patients [6].

The criteria for resectability of liver metastases were determined by Adam R. They were divided into technical and oncological [19]. The technical absolute criteria are the impossibility of R0 resection with less than or 30 % of the remaining liver tissue, as well as the presence of unresectable extrahepatic metastases. The technical relative criteria are the possibility of R0 resection only using other procedures, as well as R1 resection. The oncological criteria are unresectable extrahepatic metastases, 5 or more liver metastases, and tumor progression.

Prognostically unfavorable factors after liver resection for CRC metastases are: R1 resection, ex-

trahepatic metastases, more than 1 metastasis, size more than 5 cm, CEA above 200 ng/ml, metastases in the lymph nodes of the primary tumor, non-event interval less than 12 months, bilateral liver damage [16, 19], BRAF mutation [20].

Oncological and surgical criteria are taken into account when choosing treatment tactics for initially resectable liver metastases. In the absence of surgical difficulties and favorable oncological prognostic factors. Surgical treatment without preoperative chemotherapy is recommended (adjuvant therapy is possible). In case of adverse oncological factors, pre- or perioperative chemotherapy is necessary. In case of surgical difficulties, systemic chemotherapy is recommended regardless of oncological factors [21].

Adam R. suggests determining the tactics of treatment of CRC with liver metastases depending on the presence of symptoms of the primary tumor: with an asymptomatic tumor with resectable metastases, simultaneous resection of the liver and primary focus is possible, however, if there are risk factors, then chemotherapy is performed first, then liver resection, and only then resection of the primary focus is performed; with symptomatic tumors with resectable metastases undergo resection of the primary lesion, followed by chemotherapy followed by liver resection. In case of an asymptomatic tumor with unresectable metastases, chemotherapy is recommended, followed by step-by-step resection of the liver and primary focus when the process is transferred to a resectable one. In case of a symptomatic tumor with unresectable metastases, resection of the primary focus is performed, chemotherapy followed by liver resection when the process is transferred to a resectable one [7].

Aigner F. and co-author. They make their own adjustments to the treatment regimen for metastatic CRC: in an asymptomatic resectable process with the presence of risk factors after neoadjuvant chemotherapy, simultaneous surgical interventions should be performed instead of two-stage ones [22].

The views of oncologists are currently attracted by a group of asymptomatic tumors with conditionally resectable liver metastases, which is recommended to undergo the most active neoadjuvant chemotherapy with the maximum frequency of objective response (duplets, triplets) using targeted drugs (depending on the RAS status) with an assessment of the effect every two months [11, 23].

At the same time, patients with conditionally resectable liver metastases undergoing drug therapy should undergo surgery immediately upon reaching resectability, without waiting for a full response. This position is due to several factors. Firstly, 83 % of metastases that disappeared during chemotherapy will cause the progression of the disease, and secondly, there is also a danger of hepatotoxicity [18, 22], to reduce the development of which it is recommended to limit preoperative therapy to 2–3 months [24].

In addition, the frequency of postoperative complications directly depends on the number of therapy courses performed: 13.6 % – without chemotherapy, 19 % – after 5 courses of therapy, 45.4 % – after 6–9 courses of therapy, 61.5 % – after 10 or more courses of therapy [25, 26]. At the same time, early tumor reduction (≥ 20 % or ≤ 30 %) at 6–8 weeks of therapy is an indicator of sensitivity to treatment and is associated with a high frequency of liver resections and an increase in overall patient survival [11, 27].

The volume of surgical intervention on the liver for CRC metastases

The question of an adequate amount of liver surgery for CRC metastases is a matter of debate. Anatomical and atypical resections are possible here. Previously, the advantage was given to extensive liver resections with a large margin from the edge of metastasis due to the better results of extensive interventions. However, the era of highly effective drug therapy has made it possible to equalize the survival of patients with anatomical and non-anatomical liver resections for CRC metastases [28].

In 2019, a meta-analysis of 18 studies was published, including 7,081 patients, comparing parenchymal-preserving and extensive liver resections [29]. It turned out that the overall and relapse-free survival in these groups of patients was comparable, which allowed the authors to conclude that parenchymal-preserving surgery is an adequate method of treating metastatic CRC. Parenchymal-preserving and anatomical liver resections for metastases were compared with the same results in 12 studies involving 2505 patients [30].

Currently, the attitude towards the negative edge of liver resection has also changed. Thus, Kokudo N. [31] found CRC micrometastases in the liver parenchyma in only 2 % of patients within 5 mm or more of the macroscopic border of the tumor. Fur-

ther studies have shown that there is no significant difference in patient survival when the distance from the resection line is less than and more than 1 cm. Even a clearance of 1 mm did not increase the time before the recurrence of liver metastasis, and the 5-year overall survival rate was 33 % [31]. In addition, with modern chemotherapy, even R1 resection has no prognostic value for patient survival [32].

Parenchymal-preserving liver resections have also opened up wide opportunities for the successful application of laparoscopic techniques in CRC metastases [33–35].

Repeated liver resections for metastatic CRC

The point of preserving the liver parenchyma is not only the possibility of chemotherapy, but also repeated liver resections in the event of new metastases. The expediency of repeated liver resections has been proven in studies, in particular, in the work of Schmidt T. [36], in which, when analyzing the data of 578 patients, it was found that without repeated liver resections with recurrence of metastatic lesion, the 5-year overall survival was 36.7 %, with repeated liver resections – 56.6 %, with resections for the third time – 53.2 %.

At the same time, the established prognostic factors for repeated resections are not applicable, only the pT stage of the primary tumor and the metachronism of the lesion are important [37]. A meta-analysis of 7,200 patients from 27 studies showed that repeated operations benefit patients with a long relapse-free period with solitary, small, unilobar lesion and absence of extrahepatic metastases [36]. Therefore, patients for repeated liver resections should be carefully selected.

In cases of impossibility of resection or RTT of metastatic liver damage, it is possible to use other methods of local exposure such as regional intraarterial chemoinfusion, embolization therapy, which allow achieving a median overall survival of 45.6 months compared with 40.5 months with systemic therapy alone [38].

Metastatic lung and ovarian lesions in CRC

Metastatic lung disease is the second favorite localization of distant CRC metastasis. The importance of active surgical tactics in this case was demonstrated in a study by Onaitis M. W. et al. [39], in which thoracic intervention made it possible to achieve 28 % 3-year recurrence-free and 78 % overall survival when analyzing data from 378 patients. The factors of negative prognosis for lung metastases are: a short recurrence-free interval, metastatic lymph node lesion, the presence of more than 1 metastatic lesion in the lungs, high CEA, lung resection R1, large metastases, liver metastases in the anamnesis [39, 40].

Clinical recommendations for the treatment of metastatic liver damage in CRC are also applicable for metastatic lung damage [39]. Combined and sequential resections of the liver and lungs are possible with encouraging results in a selected group of patients with solitary metastatic lesion [41].

Metastatic ovarian lesions often occur in women, while they are more often detected with damage to other organs, a single lesion occurs only in 24 % of women. The prognosis for ovarian metastases is worse than in the liver, and the median survival is only 23 months [42].

The choice of treatment tactics for metastatic CRC

The ESMO-ASIA 2019 consensus on the treatment of metastatic CRC summarized current views and recommended surgical treatment with adjuvant or perioperative chemotherapy in a resectable process, and with potentially resectable liver metastases, with massive prevalence, and an unfavorable prognosis, to carry out the most active drug therapy taking into account the mutational status of the tumor in order to transfer the process to a resectable one [21]. In case of unresectable CRC, drug therapy lines are consistently carried out, the choice of which is based on the goals, type and time of primary therapy, the mutational profile of the tumor, and the toxicity of the drugs. Studies have found a correlation between an increase in the median survival of metastatic CRC and the use of all three main cytotoxic agents (fluorouracil/leucovorin, oxaliplatin, irinotecan) during therapy [43].

At the same time, many factors should be taken into account when planning therapy for patients with metastatic CRC. These are the characteristics of the tumor itself (biology and localization of the tumor, tumour burden, RAS mutations, BRAF), the patient (age, functional state of organs and systems, comorbidity, patient attitude, expectations and preferences) and the treatment itself (toxicity, flexibility of the treatment program, socio-economic factors, quality

of life) [21]. That is, a balance is needed between the risks and benefits of metastatic CRC therapy, and much attention was paid to this issue at ASCO 2020.

CONCLUSION

Therefore, patients with colorectal cancer with metastatic liver and/or lung damage should be considered through the prism of surgical treatment, since it is surgical intervention that can significantly improve the results of treatment of patients. Therefore, patients with potentially resectable metastases should receive the most effective treatment and be operated on as soon as the process becomes resectable. At the same time, modern chemotherapy and targeted therapy, depending on the mutation status and localization of the tumor, are an integral part of the treatment of patients with metastatic CRC.

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REVIEW

Extraneural metastases of a cerebral glioma in a child: case report with literature review

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ABSTRACT

Malignant gliomas make up 25 % of the central nervous system (CNS) tumors in adults and 8–15 % in children. About half of such gliomas have a median localization and are designated by the term "diffuse midline gliomas" (DMG). DMG in children are typically localized in the area of the pons; in 78 % of such cases a heterozygous somatic mutation H3K27M is present. The prognosis of H3K27M-mutant DSG is very unfavorable, with 2-year overall survival rate being less than 10 %. One of the ways of progression of gliomas leading to the death of patients is the spread of the tumor in the form of metastases. Malignant gliomas metastasize mainly into various structures of the CNS (according to autopsies – in about 20 % of patients with glioblastomas), the probability of their metastases to other organs (so-called extraneural metastases), according to some evaluations, is quite rare and doesn't exceed 2 %. In our practice since 1993, which counts 1700 children with malignant gliomas, including 830 patients with DMG, we've observed only one patient with extraneural metastases. The article describes this case of a child who died of the progression of the DMG's extraneural metastases, despite the fact that chemoradiotherapy had achieved its stabilization in the CNS. This patient with the initial lesion of the pons and cerebellum had massive metastasis to the lymph nodes: supraclavicular, mediastinal, retroperitoneal and inguinal ones, as well as to both pleural cavities, which occurred about one year after treatment of the progression, which had manifested in the form of continued growth of the primary tumor and its dissemination in the central nervous system. The article provides literature data on the frequency, clinical manifestations and possible treatment approaches for extraneural metastasis of brain gliomas. Extraneural metastases of those tumors occur most often to the bones, lymphatic system, lungs, abdominal organs, soft tissues. The effective treatment for extraneural metastases of gliomas has not been developed yet, which makes it urgent to solve this problem through multicenter studies.

Keywords: malignant glioma, brain, extraneural matastases, radiotherapy, chemotherapy

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3.1.6. Онкология, лучевая терапия

ОБЗОР

Экстраневральное метастазирование глиомы головного мозга: описание случая у ребенка и обзор литературы

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РЕЗЮМЕ

Глиомы высокой степени злокачественности среди всех опухолей центральной нервной системы (ЦНС) составляют до 25 % у взрослых и 8–15 % у детей. Примерно половина из них в детском возрасте имеет срединную локализацию и обозначается термином «диффузные срединные глиомы» (ДСГ). При срединных глиомах у детей, локализующихся в области моста, в 78 % случаев отмечается гетерозиготная соматическая мутация H3K27M. Прогноз H3K27M-мутантной ДСГ весьма неблагоприятный: 2-летняя общая выживаемость менее 10 %. Один из путей прогрессирования глиом, ведущий к гибели больного – это распространение опухоли в виде метастазов. Злокачественные глиомы метастазируют, главным образом, в различные структуры ЦНС (по данным аутопсий – примерно у 20 % пациентов с глиобластомами); вероятность возникновения их метастазов в других органах (так называемых, экстраневральных метастазов), по некоторым оценкам, весьма мала – не более 2 %. В нашей практике, насчитывающей 1700 детей со злокачественными глиомами, включая 830 пациентов с ДСГ (пролеченных за период с 1993 года), нам встретился лишь один больной с экстраневральными метастазами. В настоящей статье мы описываем этот случай: ребенок умер от прогрессирования экстраневральных метастазов ДСГ, несмотря на то что при химиолучевом лечении удалось достичь ее стабилизации в ЦНС. У этого больного с исходным поражением моста головного мозга и мозжечка имело место массированное метастазирование в лимфатические узлы: надключичные, медиастинальные, забрюшинные, паховые, а также в обе плевральные полости, которое произошло примерно через год после лечения прогрессирования, проявлявшегося в виде продолженного роста первичной опухоли и ее диссеминации в ЦНС. В статье приводятся данные литературы о частоте, клинических проявлениях и возможных подходах к лечению при экстраневральном метастазировании глиом головного мозга. Чаще всего наблюдается экстраневральное метастазирование этих опухолей в кости, лимфатическую систему, легкие, органы брюшной полости, мягкие ткани. Эффективного лечения при возникновении экстраневральных метастазов глиом не разработано, что делает актуальным решение этой проблемы путем многоцентровых исследований.

Ключевые слова: злокачественная глиома, головной мозг, экстраневральные метастазы, лучевая терапия, химиотерапия

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Соблюдение этических стандартов: в работе соблюдались этические принципы, предъявляемые Хельсинкской декларацией Всемирной медицинской ассоциации (World Medical Association Declaration of Helsinki, 1964, ред. 2013). Исследование одобрено Комитетом по биомедицинской этике при ФГБУ «Российский научный центр рентгенорадиологии» Министерства здравоохранения Российской Федерации (выписка из протокола заседания № 2 от 09.12.2022 г.). Информированное согласие получено от всех участников исследования

Финансирование: финансирование данной работы не проводилось

Конфликт интересов: все авторы заявляют об отсутствии явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи

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INTRODUCTION

Malignant gliomas are one of the most complex and aggressive forms of neuro-oncological diseases. Gliomas of a high grade of malignancy among all tumors of the central nervous system (CNS) account for up to 25 % in adults and 8–15 % in children. About half of them in childhood have a median localization and are designated by the term "diffuse midline gliomas" (DMG). In midline gliomas in children localized in the pontin area, a heterozygous somatic mutation H3K27M is noted in 78 % of cases. The prognosis of H3K27M-mutant DMG is very unfavorable, with 2-year overall survival rate being less than 10 % [1–5].

One of the ways of progression of gliomas leading to the death of the patient is the metastatic spread of the tumor. Malignant gliomas metastasize mainly into various structures of the central nervous system (in about 20 % of patients with glioblastomas according to autopsies), the probability of their metastases in other organs (so-called extraneural metastases), according to some estimates, is very small and doesn't exceed 2 % [6, 7]. Extraneural metastasis, although a rare phenomenon in diffuse midline gliomas, is of critical importance in the context of prognosis and further patient management tactics, since it can significantly worsen the quality of life and reduce survival rate. The presented clinical case highlights the need for a multidisciplinary approach to the diagnosis and treatment of this pathology, as well as makes the questions about the molecular genetic mechanisms underlying the extraneural spread of the tumor crucial. A review of the available literature reflects current trends in research in this field. When compiling the review, we used Scopus, MEDLINE, and Web of Science databases.

The study purpose was to describe the case of extraneural metastases of malignant glioma in a child and compare it with the literature data, which will allow us to outline the directions of further research on this problem.

CASE REPORT

Patient N., 3.5 year old boy, began to have clubfoot and developed wide base gait in 2021. He was consulted by an orthopedist. The diagnosis was made: flat-valgus deformity of the feet. During the following month, there was a deterioration in gait in the form of an increase in shakiness, he began to limp on his left leg, began to stumble, and the motor skills of his left hand deteriorated.

From the anamnesis of life: a child from IVF pregnancy, which proceeded without any abnormalities. The delivery was urgent at 38 weeks. He was observed by a neurologist for perinatal central nervous system damage, myotonic syndrome. Prior to the present disease, he suffered from acute respiratory viral infections, right-sided purulent otitis media.

An MRI of the brain was performed on 08/03/2021: in the area of the Varolian bridge (mainly in its left half) with extension to the left pedicle and hemisphere of the cerebellum, an extensive zone of diffuse signal change was visualized, hyperintensive on T2, FLAIR, iso-hypointensive on T1, with linear restriction of diffusion along the periphery, without contrast enhancement, with the presence in the hemisphere cerebellar areas of cystic transformation. The bridge of the brain, the pedicle and the hemisphere of the cerebellum were enlarged in volume, with compression and displacement of the IV ventricle laterally to the right, displacement of the left amygdala of the cerebellum caudal to the level of the foramen magnum, displacement of the medulla oblongata ventrally; along the anterior surface of the bridge was an exophytic component with compression of the prepontine cistern, fouling of the main artery. Conclusion: the picture of a diffuse lesion of the brain stem, the left hemisphere of the cerebellum, which may correspond to a diffusely growing glial tumor (fibrillar astrocytoma? anaplastic astrocytoma?), with signs of mass effect, descending axial wedging, occlusive internal hydrocephalus.

Neurological status on 08/04/2021: one-time vomiting occured, meningeal symptoms were negative, pupils, eye slits were symmetrical, there was no violation of the volume of movement and position of the eyeballs, deviations of the head and tongue, phonation and swallowing disorders; there was horizontal fine nystagmus when looking to the sides; tendon reflexes D < S, left Achilles reflex with clonus; superficial and deep muscle sensitivity was preserved; gait was atactic, with a wide base of support; the finger-nasal test with pronounced intention; in the Romberg pose was unstable, fell.

Ultrasound of the abdominal cavity and kidneys on 08/04/2021: no pathological signs were detected.

Chest X-ray (in the posterior direct projection, lying position) on 08/04/2021: reduction of pneumatiza-

tion with increased pulmonary pattern in the projection of the medio-basal sections of both pulmonary fields was noted. Conclusion: hypostatic changes in the lungs.

He started on decongestant therapy with dexamethasone on 08/04/2021. During the treatment of the treatment, the general cerebral symptoms regressed, and status-coordination disorders persisted. MRI of the brain and spinal cord (native and with contrast enhancement) 08/12/2021: in addition to the MRI data from 08/03/2021, there were no signs of metastasis in the structures of the central nervous system.

The patient's mother refused the stereotactic biopsy or partial removal of the tumor offered by the neurosurgeon. Consultation by a radiologist revealed: considering the anamnesis data, MRI signs, the patient's age, the unresectable nature of the tumor, conformal radiation therapy was indicated. Surgical intervention was performed in the volume of installation of a ventricular-peritoneal shunt (VPS) on 09/06/2021. From 09/08/2021 to 10/18/2021, he received a course of radiation therapy to the area of the initial lesion in hyperfractionation mode (single focal dose of 1 Gy 2 times a day with an interval between fractions of 4 hours, 5 days a week), total dose of 54 Gy. Since November 2021, he has been under dynamic observation. PET/CT scan of the brain on 11/29/2021 revealed: the PET picture, together with the MRI results, corresponded to the features of diffuse glioma of the brain stem and left hemisphere of the cerebellum with low amino acid uptake (without PET signs of anaplasia). At the end of April 2022, after acute respiratory viral infection, bilateral purulent otitis media and sinus thrombosis developed.

30.05.2022 MRI of the central nervous system (native and with contrast enhancement): the picture of multidirectional dynamics was noted: stabilization of tumor growth of the brain stem and the left hemisphere of the cerebellum, negative dynamics in the form of metastatic lesions along the ventricular system and spinal cord.

Surgery was performed on 06/17/2022 with a VPS revision. A stereotactic biopsy of a tumor of the lateral ventricle was performed on 06/24/2022. Histological and immunohistochemical examination on 06/24/2022 showed: tumor cells express GFAR, OLIG2, vimentin, focally S100. There was a reaction with anti-NF in numerous axons, which indicated a diffuse type of tumor growth. Single cells were weakly positive with anti-p53. CD34 expression was only in the vascular endothelium. The proliferative index of Ki-67 reached 60–70 %, focally higher.

From 07/08/2022 to 08/16/2022, radiation therapy was performed in the volume of craniospinal irradiation, single radiation dose of 1.6 Gy, total dose of 35.2 G. From 08/08/2022 to 08/10/2022, total dose was increased to 40.0 Gy for the entire volume of the spinal cord, in parallel from 08/08/2022 to 08/16/2022, a boost was performed on the area of the ventricles of the brain, single radiation dose of 1.8 G, total dose of 46 Gy. Simultaneously with the course of radiation, he received chemotherapy with temozolomide 75 mg/m^2 daily as monotherapy.

A molecular study under the One Foundaion program revealed amplifications: PDGFRA, MDM4, PIK-3C2B, as well as ATM – R3008C. Considering the revealed amplification of PDGFRA, the patient was offered imatinib therapy, which was not carried out for organizational reasons. According to the control MRI data, there was a positive dynamics of tumor foci in the central nervous system, with further stabilization. From September 2022 to June 2023, 8 courses of temozolomide monotherapy were performed. After that, treatment was interrupted due to the development of an acute respiratory viral infection.

Since mid-July 2023, the mother began to notice that the child was limping on his left leg. CT scans of the organs of the thoracic cavity, abdominal cavity and pelvis (native and with contrast enhancement) revealed conglomerates of lymph nodes of the supraclavicular, subclavian region on the right, anterior thoracic wall, paraaortic group at the infrarenal level with spread to the inguinal canal on the left; massive right-sided pleural effusion; left-sided pleural effusion; pyelocalicectasia on the left, decreased function of the left kidneys. Considering the obtained data, the presence of a systemic disease, lymphoma, was suspected at this point. MRI of the central nervous system showed stabilization of the size of tumor foci compared to previous studies.

In August 2023, the child underwent a pleural puncture, as well as a puncture biopsy of the bone marrow with its morphological examination and immunophenotyping: data for systemic blood disease were not received. A tumor population of cells was detected in the pleural fluid, which, according to immunophenotyping data, did not express mark-

ers specific to lymphoproliferative diseases. On 08/21/2023, a biopsy of the inguinal lymph node was performed, its histological examination from 09/01/2023 revealed signs of a malignant tumor with a glial phenotype. Conclusion: extraneural metastasis of the primary tumor of the central nervous system could not be excluded. Protocol of immunohistochemical examination dated 09/01/2023 of inguinal lymph node tissues: neoplastic cells diffusely expressed OLIG2, H2K27me3, CD56, SOX-10, INI1. Subtotal expression of NKX2.2, GFAP, vimenin was detected. Weak focal expression of NSE, FLI1, S100, cyclin D1. Tumor cells were negative in reactions with antibodies to panCK, EMA, TLE1, WT1, SMA, mean-A, BCOR, ERG, synapophysin, myogenin, p53, chromogranin A, TdT, desmin, CD20, CD3, CD99, MSA. Protocol of molecular genetic research dated 09/01/2023: during the FISH study, no amplification of the N-MYC gene, deletion of the SRD (1p36) gene, deletion of the KMT2A (MLL) gene, rearrangements of the FOXR2 gene, translocation of the EWSR1 gene were found.

Palliative irradiation of tumor conglomerates in areas of extraneural metastases was proposed, but the patient did not come for this treatment. He died on 01/11/2024 on the background of the progression of extraneural metastases.

DISCUSSION AND LITERATURE REVIEW

The patient case reported above with an initial lesion of the pons and cerebellum (DMG) had massive metastases to the lymph nodes: supraclavicular, mediastinal, retroperitoneal, inguinal, as well as to both pleural cavities, which occurred about one year after treatment of progression, which had manifested in the form of continued growth of the primary tumor and its dissemination in the central nervous system. It is not surprising that before biopsy and molecular genetic studies, the development of a second tumor, including lymphoma, was not ruled out. It is noteworthy that at the stage of diagnosis of the progression of DMG, a stereotactic biopsy was performed, which, of course, was necessary to clarify the diagnosis, nevertheless, could potentially contribute to the spread of the tumor, as indicated by some publications listed below. At the initial diagnosis in 2021, before the start the special treatment, the patient underwent a complete examination, including ultrasound of the abdominal cavity, kidneys, chest X-ray, according to which no signs of extraneural metastases were detected.

The first extraneural metastasis of glioblastoma was described by Davis L. back in 1928. He called it "spongioblastoma." The primary tumor in the 31-year-old patient was localized in the left hemisphere of the brain. Histologically confirmed metastases developed in the soft tissues of the upper limb and in the scapular region on the right, as well as in the left lung; they appeared approximately 5.5 months after partial removal of the primary tumor [8].

Pietschmann S et al. (2015) analyzed 109 articles and abstracts published in English or German for the period from 1928 to 2013 (85 years), which reported a total of 150 patients with extraneural metastases of malignant gliomas of the brain. It is noteworthy that more than half of the publications they analyzed (describing 95 cases) were made after 1993. The age of patients at the initial diagnosis ranged from 4 to 83 years (median 42 years). There were only four children (under the age of 18) in this combined cohort. The majority of patients had a pathomorphological diagnosis of glioblastoma (137, that is, 91.3 %), the remaining 13 (8.7 %) had gliosarcoma. The time from the initial diagnosis to the detection of extraneural metastases from these publications was accurately determined for 71 patients. Of these, 7 (that is, one in ten) had a primary tumor and metastases diagnosed simultaneously. Taking into account these patients, the period from the diagnosis of glioblastoma or gliosarcoma to the detection of metastases ranged from 0 to 81 months (median 9 months). The localization of metastases was diverse: in 52 cases they developed in the organs of the chest (including in 45 patients – in the lungs), in 31 cases in the organs of the abdominal cavity and retroperitoneal space (including in 23 patients – in the liver). In addition, metastases to bone or bone marrow were described in 53 patients, to lymph nodes in 51, to muscles and other soft tissues in 35, to skin in 11, to thyroid and parathyroid glands in 6, to other organs (including eyes and mammary glands) in 4. A significant proportion patients had several localizations of metastases. In the publications included in this review [9], a sufficient description of treatment after detection of metastases was provided for 60 out of 150 patients (40 %), in the rest, treatment was either not

reported or it did not include antitumor methods. Twenty-nine patients after the detection of metastases were treated with any one antitumor method: 17 of them underwent surgery, 4 had radiation and 8 had chemotherapy. In 31 patients in such a situation, various combinations of these methods were used, most often: chemotherapy + radiotherapy (in 15) and surgery + chemotherapy + radiotherapy (in 10). In this review [9], it was noted that extraneural metastases of malignant glial tumors are more often found in relatively young people, which can serve as one of the arguments for the use of active antitumor treatment in them. However, the authors did not reveal a clear increase in survival with such treatment tactics. Overall survival in extraneural metastases was slightly better than survival in CNS metastases, although this difference did not reach the confidence limit. The authors were unable to formulate any specific recommendations for the treatment of patients with extraneural metastases of malignant glial tumors, since in the combined cohort analyzed by them, treatment was very diverse and selected individually [9].

Of high interest is the work of De Martino L. et al, 2023 [10], which describes the authors' own observation of two children with extraneural metastases of diffuse midline gliomas. In one of them, an 11-year-old boy, diffuse midline bridge glioma was confirmed by stereotactic biopsy. Loss of H3K27me3 and expression of a protein associated with H3K27M mutation were detected in tumor cells. At the first stage of treatment, the patient underwent induction chemotherapy with vinorelbine and nimotuzumab, followed by irradiation of the tumor zone in the mode of conventional fractionation, total dose 54 Gy (according to the VMAT method). 5 months after diagnosis, CT and MRI scans revealed extensive metastasis to the soft meninges of the brain and spinal cord, as well as extraneural metastases: in the sternum, vertebrae and pelvic bones. Bone metastases were confirmed by examining a biopsy of the left iliac bone. He died a month later. The second patient, a girl of the same age, was diagnosed with median glioma in the region of the IV ventricle with the H3K27M mutation. Total resection of the tumor was performed, the diagnosis was confirmed on the basis of histological and molecular genetic examination of its tissue. As in the first patient, after chemotherapy with vinorelbine and nimotuzumab, she underwent local radiation therapy (total dose 54 Gy, VMAT technique). However, 3 months after the end of the course of treatment, she was diagnosed with progression in the area of the original tumor, as well as in the ependyma of the ventricles of the brain and its soft membranes. Craniospinal irradiation (CSI) was performed in the mode of classical fractionation, total dose 36 Gy, followed by 15-month chemotherapy with irinotecan and bevacizumab. After completion of CSR, according to MRI data, a partial response of tumor foci in the brain was detected, however, signs of metastases in the vertebrae appeared. CT scan of the whole body made it possible to detect osteosclerotic foci not only in the spine, but also in the ribs, sternum, pelvic bones, in both shoulder and femur bones. PET/CT with ^{18}F-FDG revealed foci of moderate hyperfixation in the bones, however, repeated biopsies of these foci did not reveal metastases. Subsequently, she developed a lesion of a large number of intra-thoracic and abdominal lymph nodes, as well as pleural effusion, the study of which by drip digital polymerase chain reaction revealed a mutation H3.3A (c.83A>T, p.K28M), although cytological examination of pleural effusion of tumor cells did not detect. The patient died 2 years after the diagnosis of median glioma, that is, 3 months after the detection of pleural effusion. The authors of the description of these two patients, while studying the medical literature, found publications about 12 similar patients: their age ranged from 4 to 36 years, the localization of extraneural relapses was diverse: bones, lymph nodes, lungs, pleural cavity, liver, peritoneum, muscles. One of these patients had abdominal metastasis due to the spread of tumor cells along the ventriculo-peritoneal shunt [11]. It is noteworthy that the descriptions of 12 cases collected in the mentioned review by De Martino L et al. (2023), were published no earlier than 2014, that is, starting from the time when the concept of "diffuse midline glioma" was formed. The authors of this review express concern that, probably, the frequency of ectraneural metastases in patients with diffuse midline gliomas is underestimated, since they are not routinely examined to identify such metastases. The article points to the possibility of increasing the risk of extraneural metastases in connection with surgical interventions on primary tumors. The following expressed in the article may explain why extraneural metastases are rare in malignant gliomas. Probably, outside the

central nervous system, glioma cells are most often destroyed by the immune system, and in the brain these cells are protected, being in a microenvironment favorable them. This hypothesis is confirmed by descriptions of cases of extraneural glioblastoma metastases in patients who had previously undergone organ transplantation [12, 13].

The literature review made in the mentioned article by De Martino L et al. [10], did not include the work of Chinese researchers Ge X et al [5], which describes the development of extraneural metastases of diffuse median glioma of the brain stem region in a 9-year-old boy. His primary tumor was diagnosed based on CNS MRI data followed by stereotactic biopsy. The histological picture corresponded to anaplastic astrocytic glioma (grade IV malignancy according to World Health Organization (WHO)), immunohistochemical examination revealed a protein formed by the H3K27M mutation. Initially, there was no dissemination in the brain and spinal cord. Due to hydrocephalus, ventriculo-peritoneal bypass surgery was performed. The patient underwent local irradiation of the tumor area, total dose 50 Gy for 25 fractions with simultaneous of chemotherapy with temozolomide at a daily dose of 75 mg/mg^2. A month after the end of irradiation, MRI showed a slight decrease in tumor volume. The patient received adjuvant chemotherapy with temozolomide: 5 cycles of 5 days, every 28 days, the daily dose of this drug was 150 mg/mg^2 in the first cycle, 200 mg/m^2 in all subsequent cycles. 2 months after the end of chemotherapy, the patient complained of lowering of the right corner of the mouth, as well as back and lumbar pain. MRI revealed the progression of the primary tumor, dissemination in the spinal cord and along the soft meninges, as well as foci of destruction of the lumbar and sacral vertebrae with pathological contrast enhancement. PET-CT revealed a diffuse increase in metabolism in the cervical, thoracic and lumbosacral spine. The patient soon developed neck pain and urinary retention. Despite an attempt at chemotherapy (one cycle with vincristine and cyclophosphamide), no improvement was achieved, and the patient died 1 month after the detection of tumor metastases.

The authors of all the above publications emphasize that effective treatment programs for patients with extraneural metastases of malignant gliomas of the central nervous system have not yet been developed. This is due to both the relative rarity of such cases and their severity. Therefore, at least isolated reports that indicate the possibility of prolonging the life of such patients are so valuable.

As an example of such an observation, one can cite the description of the case made by Yang G et al [14]. Initially, an MRI scan revealed a tumor lesion of the right temporal and occipital lobes in the form of several nodes in a 58-year-old man. In the tissue of a totally removed tumor, histological examination revealed signs of glioblastoma with areas of oligodendroglioma. After the combined treatment (surgery, local radiation therapy, total dose 60 Gy in conventional fractions and chemotherapy with temozlolomide), no tumor remnants were observed according to MRI. However, six months after the end of treatment, a local relapse was detected, repeated radiation therapy was performed to its area, total dose 30 Gy in five fractions and several cycles of bevacizumab. It was possible to achieve a partial response and an improvement in the quality of life. However, after 2 years, while there was stabilization of tumor foci in the brain, multiple histologically confirmed metastases were found in the right lung, and then in the bones. Since PD-L1 expression was detected in lung metastasis tissue, and a slight increase in lung foci was noted during treatment with bevacizumab and temozolomide, it was decided to add pembrolizumab to the treatment. 5 cycles were performed with this drug. As a result, a partial response was noted in the lung, with a stable state of tumor tissue in the brain. Temozolomide was discontinued due to fatigue syndrome and lack of methylation of the MGMT promoter in tumor tissue; treatment with bevacizumab and pembrolizumab continued. However, after a few months, bone metastases developed, but the patient continued to receive the same treatment due to the lack of any alternatives at the disposal of his doctors. The progression of metastases continued. However, the authors believe that these drugs allowed to slow down this process. As a result, the patient lived quite a long time after the diagnosis of pulmonary metastases: 27 months, while in a series of patients with glioblastoma extraneural metastases published by Noch EK et al [15], the average life expectancy after their detection was 5 months (from 1 to 16 months), it is noteworthy that pembrolizumab was not used in them.

Undabeitia J et al [16] describe a case of extraneural glioblastoma metastases in a 20-year-old

patient. The primary tumor was localized in the right temporal region of the brain; its total removal was performed, followed by chemoradiotherapy. Metastases to both lungs and pleural cavities, as well as to the pancreas and vertebrae, occurred 5 months after surgery. Lung metastases were in the form of infiltrations, and they were confirmed by biopsy. Chemotherapy with irinotecan and bevacizumab was attempted, but the patient died.

In the case report by Kim A. V. et al. a 16-year-old patient is described, whose glioblastoma was initially localized in the left parietal lobe of the brain. 6 months after the operation, supplemented by local radiation and chemotherapy, it metastasized to the V cervical vertebra, which was confirmed by histological and immunohistochemical examination of the tissue of this vertebra [17].

Razmologova O. Yu. and Sokolova T. V. reported a case of glioblastoma metastases in the lungs at autopsy in a 64-year-old patient who died shortly after surgery on glioblastoma of the left parietal lobe of the brain. These metastases were confirmed by immunohistochemical method with determination of glial fibrillary acid protein expression [18]. A similar patient was described by Zhetpisbaev B. and Isakhanova B.: a 53-year-old man underwent partial removal of a tumor from the temporal lobe of the right hemisphere, in the postoperative period there was a deterioration in the condition in the form of depression of consciousness, unstable hemodynamics, the patient died. Histological examination of the removed tumor tissue revealed glioblastoma. While examining the left lung, a tumor focus was accidentally discovered, which, according to histological and immunohistochemical studies, corresponded to glioblastoma metastasis [19].

The information we have provided on the problem of extraneural metastases of brain gliomas is based on two major reviews [9 and 10] and descriptions of individual cases that were not included in them. In the last 20–30 years, there has been an increasing trend in the number of such publications. This can be explained by the improvement of various components of neuro-oncology, especially pathomorphology and diagnostic radiology. In addition, the development of surgical techniques, radiation therapy and drug treatment gives patients a chance to prolong their life span, during which such metastases can manifest. Despite the relative rarity of the occurrence of extraneural metastases in patients with malignant gliomas, there is still reason to believe that evaluations of their frequency are underestimated. Apparently, they remain unrecognized in many patients during tumor progression in the central nervous system, nevertheless, exacerbating the severity of the disease. The validity of this assumption is confirmed by the cases of occult metastases in the lungs [18 and 19].

CONCLUSION

In summary, extraneural metastases of malignant gliomas of the brain are rare, but their probability should be taken into account both when making an initial diagnosis and during subsequent control examinations, especially in patients who have undergone various surgical interventions on tumors or bypass operations. Despite the steadily increasing number of publications on this topic, there is still no accurate information about the frequency of such metastases, about the optimal ways of their early diagnosis, and effective therapeutic tactics have not been developed in case of their occurrence. In addition, the reason for the rarity of extraneural metastases in CNS tumors is not clear; a meticulous study of this issue could shed light on aspects of the pathogenesis of these tumors, and therefore open up new directions for their therapy. To solve these problems, multicenter studies involving the efforts of the leading neuro-oncological clinics are highly needed.

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