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- High-quality published content that includes the latest and trustworthy scientific papers, research or work on oncology issues.

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- Informing readers about the results of major medical forums;
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РЕЦЕНЗИРУЕМЫЙ НАУЧНО-ПРАКТИЧЕСКИЙ

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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.

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- Содействие обмену опытом и передаче передовых знаний между специалистами;
- Информирование читателей о результаты крупных медицинских форумов;
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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. 2

ORIGINAL ARTICLES

СОДЕРЖАНИЕ

ОРИГИНАЛЬНЫЕ СТАТЬИ

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ORIGINAL ARTICLE

Experience of stereotactic radiation therapy and radiosurgical treatment of metastatic vertebral tumors

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ABSTRACT

Purpose of the study. Evaluation of the effectiveness of extracranial stereotactic radiation therapy in various fractionation regimens in the treatment of patients with metastatic vertebral lesions.

Patients and methods. The study included 12 patients with metastatic spinal lesions who underwent extracranial stereotactic radiation therapy (SBRT) on a Novalis Tx linear accelerator, Varian, in radiosurgery mode (SRS; in 1 fraction) and hypofractionation mode (SFD 5Gy, TFD 25Gy, 5 fractions) in the period from 01/01/2020 to 03/31/2022. The assessment of local control was carried out using positron emission tomography – computed tomography (PET-CT) from 18FDG. The intensity of the pain syndrome before and after radiation was assessed using a visual analog pain scale (VAS).

Results. 19 vertebrae with metastatic lesions were irradiated in 12 patients. The SBRT technique in hypofractionation mode was used in 6 (50 %) patients, in radiosurgery (SRS) mode was used in 4 (34 %) patients, in 2 (17 %) patients a combination of irradiation techniques was used on various affected segments of the spinal column. The general tumor volume (GTV) averaged 30.56 = 7.8 km^2. When using the radiosurgical irradiation regimen, SFD ranged from 16 to 18 Gy. When using the hypofractionation technique, the total focal dose (TFD) was 25 Gy, a single focal dose (SFD) was 5 Gy.

Conclusion. Stereotactic radiation therapy and radiosurgery of metastatic vertebral tumors without compression of neural structures provides local tumor control in 92 % of patients within 6 months and in 83 % of patients within 1 year, regression of pain after irradiation – in 67 % of patients.

Keywords: stereotactic radiotherapy, radiosurgery, spinal metastases

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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 Committee on Biomedical Ethics at the National Medical Research Center of Oncology, the Russian Federation Ministry of Health (extract from the protocol of the meeting No. 118 dated 06/02/2022). Informed consent was received from all the participants of the study

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Conflict of interest: Kit O. I. has been the member of the editorial board of the South Russian Journal of Cancer since 2019, however he has no relation to the decision made upon publishing this article. The article has passed the review procedure accepted in the journal. The authors did not declare any other conflicts of interest

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3.1.6. Онкология, лучевая терапия
ОРИГИНАЛЬНАЯ СТАТЬЯ

Опыт стереотаксической лучевой терапии и радиохирургического лечения метастатических опухолей позвонков

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РЕЗЮМЕ

Цель исследования. Оценка эффективности экстракраниальной стереотаксической лучевой терапии в различных режимах фракционирования при лечении пациентов с метастатическим поражением позвонков

Пациенты и методы. В исследование включено 12 больных с метастатическим поражением позвоночника, которым была проведена экстракраниальная стереотаксическая лучевая терапия (SBRT) на линейном ускорителе Novalis Tx, Varian, в режиме радиохирургии (SRS; за 1 фракцию) и режиме гипофракционирования (разовая очаговая доза (РОД) 5Гр, суммарная очаговая доза (СОД) 25Гр, 5 фракций) в период с 01.01.2020 по 31.03.2022 гг. Оценка локального контроля осуществлялась с использованием позитронно-эмиссионной томографии – компьютерной томографии (ПЭТ-КТ) с 18ФДГ. Интенсивность болевого синдрома до и после облучения оценивали по визуально аналоговой шкале боли (ВАШ).

Результаты. У 12 пациентов проведено облучение 19 метастатических пораженных позвонков. Методика SBRT в режиме гипофракционирования была применена у 6 (50 %) больных, в режиме радиохирургии (SRS) использована у 4 (34 %) пациентов, у 2 (17 %) больных на различных пораженных сегментах позвоночного столба применялась комбинация методик облучения. Общий объем опухоли (GTV) в среднем составлял 30,56 ± 7,8 см². При применении радиохирургического режима облучения РОД составляла от 16 до 18Гр. При применении методики гипофракционирования СОД составила 25 Гр, РОД – 5 Гр.

Заключение. Экстракраниальная стереотаксическая лучевая терапия метастатических опухолей позвонков без компрессии невральных структур обеспечивает локальный контроль опухоли у 92 % больных в течение 6 месяцев и у 83 % пациентов в течение 1 года, регресс болевого синдрома после облучения – у 67 % больных.

Ключевые слова: стереотаксическая лучевая терапия, радиохирургия, метастазы в позвоночник

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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 № 118 от 02.06.2022 г.). Информированное согласие получено от всех участников исследования.

Финансирование: финансирование данной работы не проводилось

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INTRODUCTION

An analysis of the literature shows that 30–50 % of cancer patients have metastatic spinal column lesions, including 70–80 % of patients with breast or prostate cancer and 40 % of patients with advanced lung cancer [1]. In one third of patients, the lesion of the vertebrae is symptomatic. Clinical manifestations are most often represented by pain syndrome, varying in intensity. For a long time, conventional radiation therapy (CRT) has been used in the treatment of this group of patients and in the absence of indications for surgical treatment, which has a satisfactory (up to 80 % of cases) analgesic effect, however, local relapses occur in 60–80 % of patients, and the analgesic effect often develops only 2–3 weeks after treatment, especially with radioresistant tumors [2]. Currently, conformal methods of radiation therapy in the treatment of bone metastases are replacing conventional radiation therapy, despite their disadvantages in the form of the need for longer patient preparation, additional diagnostic studies, and high cost [3]. The main difference between conformal radiation therapy and conventional radiation therapy is the creation of an irradiation field of a given shape with minimal impact on surrounding tissues. The possibility of concentrating the radiation dose without increasing it during conformal radiation therapy in the tumor area is, among other things, a way to overcome its radioresistance. Stereotactic body radiation therapy (SBRT) and radiosurgery (SRS) have taken leading positions among conformal methods in the treatment of spinal tumors. In stereotactic radiation therapy, tumor destruction occurs in several large fractions (5–12 Gy each), in stereotactic radiosurgery – by summing up a radical dose (15–21 Gy) in one session.

The purpose of the study was to evaluate the effectiveness of extracranial stereotactic radiation therapy in various fractionation regimes in the treatment of patients with metastatic vertebral lesions.

PATIENTS AND METHODS

The study included 12 patients with metastatic spinal lesions who underwent extracranial stereotactic radiation therapy (SBRT) on a Novalis Tx linear accelerator, Varian, in radiosurgery mode (SRS; for

Table 1. Characteristics of metastatic vertebral tumors in patients

Indicator	Indicator value (*n* = 19)
The location of a metastatic tumor in the spine according to the Tomita classification	
1 type 5 type 7 type	4 (21 %) 1 (5 %) 14 (74 %)
Localization of the tumor in the vertebra	
body body + peduncle of the arch total defeat	13 (68 %) 3 (16 %) 3 (16 %)
The degree of tumor spread according to the Weinstein-Boriani classification	
B + C A + B + C	18 (95 %) 1 (5 %)
Level of lesion	
Cervical Thoracic Lumbar Sacral	2 (10.5 %) 7 (37 %) 8 (42 %) 2 (10 %)

1 fraction) and hypofractionation mode (SFD 5Gy, TFD 25Gy, 5 fractions) in the period from 01/01/2020 to 03/31/2022.

The average age of the patients was 55.47 ± 2.89 years, the ratio of men and women was 2:10. Grade 1b epidural compression was detected in only 1 patient. The stability of the spinal column on the SINS scale averaged 5.0 ± 0.59 points. All patients were neurologically intact (Frankel type E) and functionally intact (70–80 points according to Karnofsky). Pain syndrome before the course of radiation occurred in all patients, the average score according to VAS was 5.4 ± 0.67. According to the histological type of the primary tumor, the distribution was as follows: breast

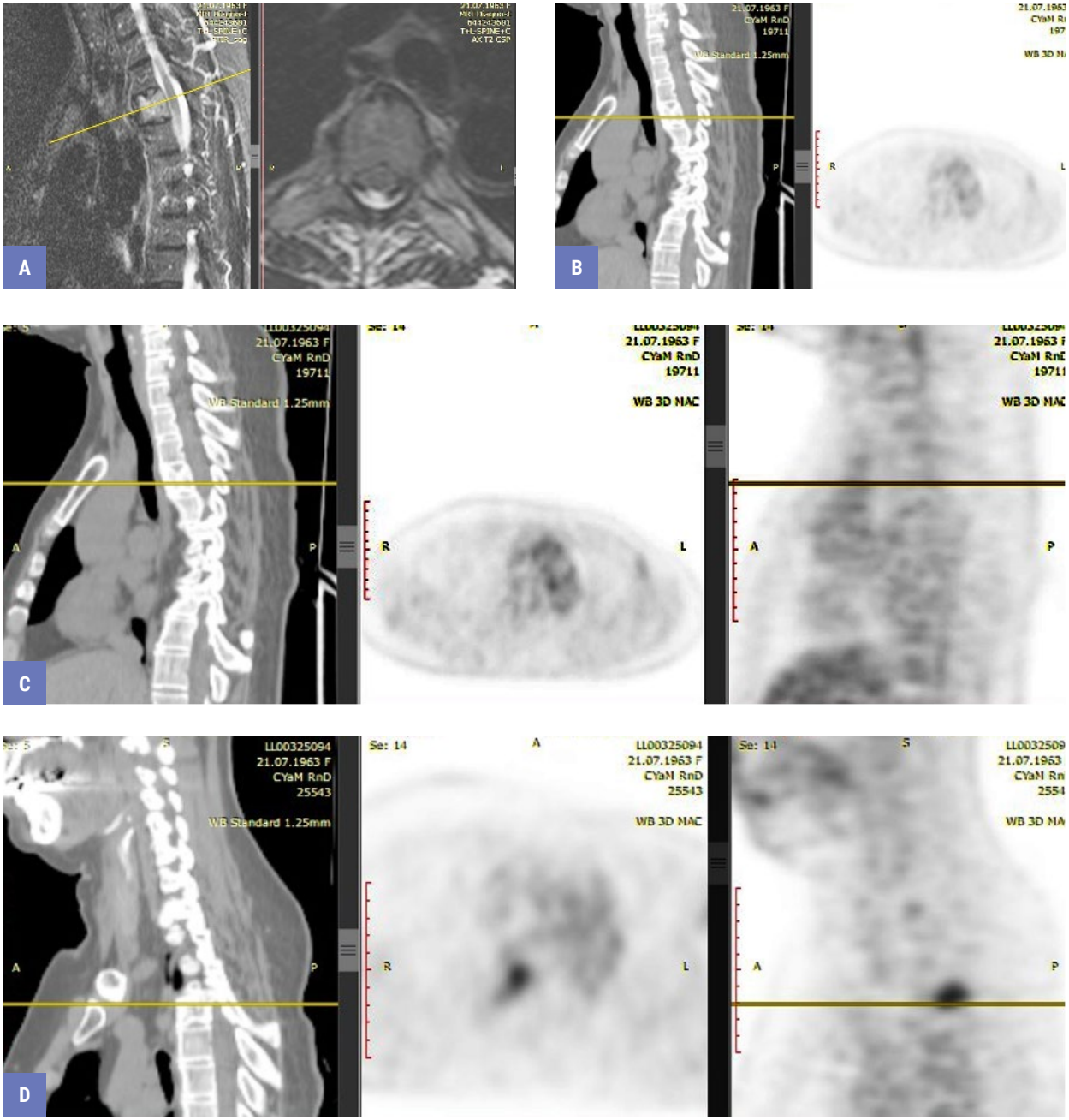

Fig. 1. Assessment of local control after radiation therapy in patient L-o. A, B – MRI and CT data of the thoracic spine before treatment (signs of metastatic lesion of the Th3 vertebra); C – data of the control PET-CT during the observation period 3 months after irradiation (there are no signs of pathological activity in the irradiation zone); D – data of the control PET-CT during the observation period after 12 months after irradiation (signs of recurrence of formation in the area of the vertebral arch peduncle)

cancer – 10 (84 %) patients, skin melanoma – 1 (8 %) patient, without an established primary focus – 1 (8 %) patient. The characteristics of metastatic vertebral tumors in patients with neuroimaging are represented in Table 1.

The general tumor volume (GTV) averaged 30.56 ± 7.8 cm^2. The average radiation dose for single-fraction courses was 26 [13; 16] Gy. When using the hypofractionation technique, the average total focal dose (TFD) was 25 [25; 26] Gy, the average single focal dose (SFD) was 5 [5; 8] Gy.

A single metastatic lesion in the spine at the beginning of treatment was observed in 2 (17 %) patients, in the remaining patients metastatic lesion of the vertebrae was of a multiple nature. In addition to the spine, 6 (50 %) had metastasis to other flat bones of the skeleton, and 4 (34 %) had visceral metastases.

To assess the neurological status and condition of patients, the Frankel and Karnofsky scales were used, the intensity of pain syndrome was assessed using a visually analog pain scale (VAS), and the SINS scale was used to assess instability in the affected spinal-motor segment. All patients were examined on the day of admission, at discharge and every 3 months after completion of the course of radiation therapy. All patients underwent computed tomography (CT) and magnetic resonance imaging (MRI) of the spinal column before treatment, postoperative assessment of local control was carried out using positron emission tomography – computed tomography (PET-CT) with 18F-fluorodeoxyglucose (18-FDG) (Fig. 1).

The irradiation was carried out on the Novalis Tx linear accelerator, Varian. Topometric tomography was previously performed on a Siemens Somatom computed tomograph, and preliminary topometry was processed at the Singo Via virtual simulation station. A full-body vacuum mattress with an ArmShuttle board was used for immobilization and

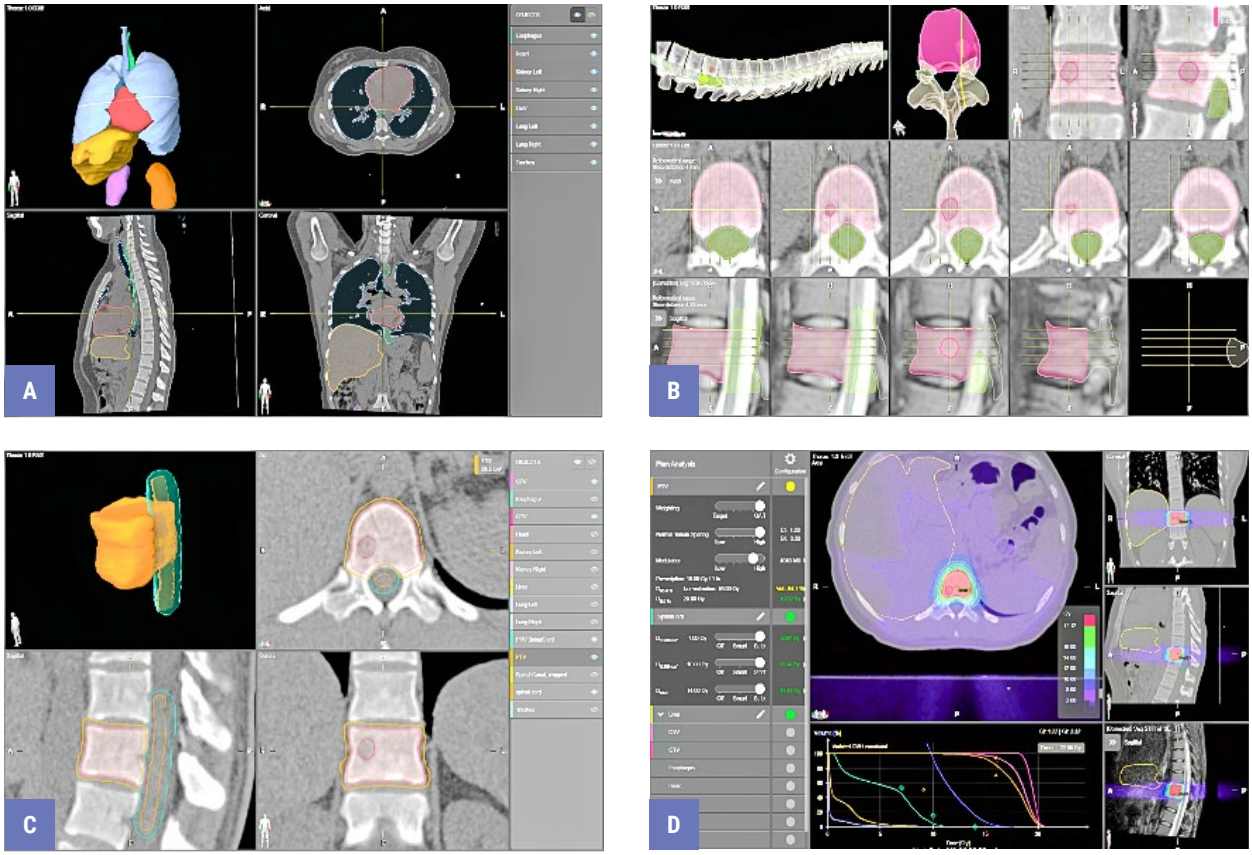

Fig. 2. Radiation planning. A – anatomical segmentation and contouring of critical organs and structures; B – delineation of the GTV volume from images of various modalities and formation of the CTV volume; C – contouring of the spinal cord and the formation of PTV volume; D – dosimetry planning with control of target coverage and load on critical organs and structures, followed by analysis of the calculated irradiation plan

reproducibility of the patient's laying. Using the Elements Brainlab software, segmentation, contouring and formation of a 3D treatment plan for a linear accelerator were performed. The laying and control of the patient's position were performed using orthogonal X-rays using the ExacTrac X-Ray Monitoring BrainLab positioning system. Verification of the calculated stereotactic radiotherapy treatment plan was carried out on a StereoPHAN phantom with a matrix of SRS Mapcheck detectors. Before the radiosurgical treatment session, the absolute dose calibration of the accelerator and the calibration of the positioning system were checked. The dose was delivered using a dynamic volume modulated technique (VMAT).

The clinical volume of the tumor (CTV) was determined in accordance with the International Spine Radiosurgery Consortium Consensus Guidelines [4]. The planned tumor volume (PTV) was calculated by adding a 2 mm edge to the CTV boundaries, minus the PRV (planning risk volume) for the spinal cord (+ 3 mm to the edge of the spinal cord in all directions) and taking into account the location of the risk organs (oropharynx, esophagus, etc.) (Fig. 2).

For each group of indicators, the type of data distribution was determined (histogram construction according to the Kolmogorov – Smirnov test). If the application of the criterion showed a normal distribution of data, the average, the error of the average (M ± m) was used for the description. When the distribution differs from the normal law, the values of the median, 1st and 3rd quartiles (Me [Q1; Q3]) were used for the description. The threshold level of significance for testing statistical hypotheses was assumed to be 0.05.

STUDY RESULTS

In 12 patients, 19 metastatic vertebral tumors were irradiated.

The majority of patients (80 %, *n* = 10) underwent 1 course of radiation therapy, 20 % of patients received 2 courses of radiation. At the same time, irradiation of one vertebra was performed in 8 (67 %) patients, 4 (33 %) received irradiation of two or more segments of the spinal column. The SBRT technique was used in 6 (50 %) patients, radiosurgery (SRS) was used in 4 (34 %) patients. Both SBRT and SRS irradiation techniques were used in 2 (17 %) patients on various affected segments of the spinal column.

The radiation therapy performed was part of the complex treatment in 10 (83 %) patients, combined – in 2 (17 %).

The average duration of follow-up was 12.18 ± 2.23 months. Radiological local control (complete, partial response and stabilization of the disease according to the RECIST criteria) was achieved in 11 (92 %) patients within 6 months, in 10 (83 %) – within 1 year. Progression of the underlying disease during the follow-up period was noted in 6 (50 %) patients. The average survival rate before progression was 9.11 ± 2.69 months. A decrease in back pain after irradiation was noted by 8 (67 %) patients, there were no cases of an increase in pain syndrome.

DISCUSSION

Stereotactic radiation therapy and radiosurgery show high efficiency in the treatment of metastatic tumors of the vertebrae. One of the primary goals of irradiation of tumors that do not compress the spinal cord is the treatment of pain syndrome. Vargo J. A. et al. [5] It is proposed to apply certain modes of irradiation, depending on the purpose of the treatment. If the main task is to relieve pain, then preference is given to radiation for 1 fraction (16–18 Gy). In order to achieve long-term local control, preference is given to fractionated SBRT modes (8–9 Gy × 3 fr., or 6–7 G × 5 fr.).

Randomized studies of the analgesic effect of radiation therapy performed in patients using single-fraction SBRT (16–18 or 24 Gy) and mono-multi-fraction CRT (8 Gy for 1 fraction or 30 Gy for 10 fractions) showed no significant difference between the groups of patients 3 months after treatment, and a significantly better effect of SBRT after 6 months [6, 7]. The limitation of these studies was the lesion of no more than 2 adjacent vertebrae and the presence of a distance of at least 3 mm between the edge of the tumor and the spinal cord (no more than 1b degree ESCC), otherwise the groups could not be randomized. Sahgal A. and co-author. [8] In the course of a randomized multicenter study, the advantages of SBRT (two-fraction 24 Gy) over CRT (20 Gy in 5 fractions) in effectiveness against pain syndrome in metastatic spinal in-

jury were also noted, even during the first 3 months after treatment. The results of randomized studies comparing the effectiveness of CRT and SBRT techniques in relation to local tumor control are currently not available in the literature. Meta-analysis conducted by Singh R. et al. [9], which included 3237 patients, showed the presence of local tumor control in 92.9 % of patients after single-factional SBRT (RS) versus 81 % after CRT or 82.1 % after multi-factional CRT.

Local radiation does not prevent the progression of the underlying disease, so it should be used in combination with chemotherapy treatment. During the follow-up period, progression was noted in 50 % of patients, while local control was achieved by the end of the first year after completion of the radiation course in 83 %.

CONCLUSION

Stereotactic radiation therapy and radiosurgery of metastatic vertebral tumors without compression of neural structures provides local tumor control in 92 % of patients for 6 months. and in 83 % of patients within 1 year, regression of pain syndrome after irradiation – in 67 % of patients.

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Zakondyrin D. E. – contributed to the research design development, analysis of the obtained data, writing the text of the manuscript;
Rostorguev E. E. – review of publications on the topic of the article, a set of clinical material, interpretation of the results;
Sakun P. G. – performed data collection, analysis and interpretation, technical editing;
Voshedskii V. I. – took part in research design development, analysis of the data obtained;
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ORIGINAL ARTICLE

Units of fibrinolytic system in mice with urokinase gene knockout in presence of growing B16/F10 melanoma

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ABSTRACT

Purpose of the study. Was to reveal the effect of urokinase gene knockout in male and female mice with transplanted B16/F10 melanoma on the functions of the fibrinolytic system units.

Materials and methods. Male and female mice were used: main group with genetically modified mice C57BL/6-Plautm1.1Bug – ThisPlauGFDhu/GFDhu (uPA-/-); control group with C57Bl/6 (uPA+/+) mice. B16/F10 melanoma was transplanted by the standard methods to the animals, and levels of plasminogen (PG), plasmin (PAP), urokinase receptor uPAR, content (AG) and activity (act) of uPA, t-PA and PAI-I were measured with ELISA (Cussabio, China) in 10 % tumor homogenates and peritumoral area after 3 weeks of tumor growth.

Results. The activity and levels of urokinase in intact uPA-/- animals were significantly (by 100–860 times) inhibited, compared to uPA+/+, but uPAR levels were unchanged in females and were 1.9 times lower in males. PAP levels in uPA-/- mice were 2.1–4.2 times higher than in uPA+/+ animals. The growth of B16/F10 melanoma in uPA-/- mice was slower and metastasizing was suppressed, but their survival was not improved. The dynamics of changes in components of the fibrinolytic system in presence of melanoma growth differed in uPA-/- mice, compared to uPA+/+ animals: PAP levels in tumor samples decreased by over 2 times, uPA levels and activity were not increased, PAI was practically unchanged, but activity of t-PA elevated by 3.8–8.2 times, as well as in uPA+/+ mice.

Conclusion. Despite the suppression of the growth and metastasis of the primary tumor nodes in uPA-/- mice, their average survival was not improved, which indicates that the mechanisms of tumor are complex and there are alternative biological pathways supporting melanoma to survive in conditions of the urokinase gene knockout.

Keywords: urokinase gene knockout, mice, melanoma B16/F10, fibrinolytic system

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3.1.6. Онкология, лучевая терапия
ОРИГИНАЛЬНАЯ СТАТЬЯ

Звенья фибринолитической системы у мышей с нокаутом по гену урокиназы на фоне роста меланомы B16/F10

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РЕЗЮМЕ

Цель исследования. Изучение влияния нокаута по гену урокиназы у мышей обоего пола с перевитой меланомой B16/F10 на функционирование звеньев фибринолитической системы.

Материалы и методы. Были использованы мыши обоего пола: основная группа генмодифицированная линия C57BL/6-Plautm1.1Bug – ThisPlauGFDhu/GFDhu (uPA-/-); группа контроля – линия C57Bl/6 (uPA+/+). Животным по стандартной методике перевивали меланому B16/F10 и через 3 недели роста в 10 % гомогенатах опухоли и ее перифокальной зоне ИФА методом определяли уровень: плазминогена (ПГ), плазмина (PAP), рецептора урокиназы uPAR, содержание (АГ) и активность (акт) uPA, t-PA и PAI-I (Cussabio, Китай).

Результаты. У интактных животных uPA-/- в коже оказалась существенно подавлена, по сравнению с uPA+/+ активность и содержание урокиназы (в 100–860 раз), однако у самок не изменился уровень uPAR, тогда как у самцов снизился в 1,9 раза. Уровень плазмина у uPA-/- мышей был выше в 2,1–4,2 раза, по сравнению с uPA+/+ животными. Рост меланомы B16/F10 у uPA-/- мышей был замедлен, тормозилось метастазирование, однако не увеличивалась продолжительность жизни. Динамика изменений компонентов фибринолитической системы при росте меланомы у uPA-/- мышей отличалась от uPA+/+: в образцах опухоли снижался уровень PAP более чем в 2 раза, не повышался уровень и активность uPA, практически не реагировала PAI, однако, как и у uPA+/+ возрастала активность t-PA в 3,8–8,2 раза.

Заключение. Несмотря на подавление роста первичного узла опухоли и процессов метастазирования у мышей uPA-/-, средняя продолжительность жизни не увеличивалась, что свидетельствует о сложных механизмах опухолевой болезни и наличии альтернативных биологических путей, позволяющих меланоме прогрессировать в условиях нокаута гена урокиназы.

Ключевые слова: нокаут по гену урокиназы, мыши, меланома B16/F10, фибринолитическая система

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Соблюдение этических стандартов: работа с животными проводилась в соответствии с правилами «Европейской конвенции о защите животных, используемых в экспериментах» (Директива 86/609/EEC) и Хельсинкской декларации, а также в соответствии с «Международными рекомендациями по проведению медико-биологических исследований с использованием животных» и приказом Минздрава России от 19.06.2003 г. № 267 «Об утверждении правил лабораторной практики». Комиссией по биоэтике ФГБУ «Национальный медицинский исследовательский центр онкологии» Министерства здравоохранения Российской Федерации от 24.12.2019 г., был одобрен протокол исследования (протокол этического комитета № 15/75) по работе с мышами линии Balb/c Nude

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

The fibrinolytic system is considered one of the leading mechanisms of carcinogenesis, due to the destruction of cell membranes, proliferation, migration and invasion of cells [1].

Urokinase-type (uPA) and tissue-type (t-PA) plasminogen activators are serine proteases that convert plasminogen into plasmin after binding to the uPA receptor (uPAR) [2]. uPA is found on the surface of tumor cells, and its overexpression at the final stage of transformation of malignant cells contributes to the processes of metastasis [3]. The activation of the fibrinolytic system and the formation of plasmin stimulates metalloproteinases, vascular growth factors, this in turn consequently destroys the physical barrier to the migration of tumor cells and stimulates tumor growth [4].

Several researchers believe that understanding the molecular mechanisms of the biological action of the plasmin/plasminogen system and inhibition of angiogenesis by blocking serine proteases may allow improving therapeutic strategies for regulating the growth of malignant tumors and disorders associated with neovascularization [5, 6].

It has been previously shown that changes in the links of the fibrinolytic system of the skin occur in the growth dynamics of B16/F10 transfused melanoma in C57Bl/6 mice with wild type genes, characterized by increased activity of all components of the plasminogen activation system, subsequently leading to an increased content of plasmin in it. The comorbid disease, i. e. chronic neurogenic pain, has a modifying effect on the studied indicators [7–9].

Experimental models of tumors make it possible to find out the causes, study the pathogenesis of the tumor process, develop methods for its prevention and treatment, while the use of various animal lines, including those with genetically determined characteristics, is justified [10]. Models of genetically engineered mice have been successfully used for decades in modeling the tumor process [11]. There are certain types of transgenic mice used in studies of the malignant process in which oncogenes can be constitutively or conditionally expressed. In such animal models, tumor suppressor genes can be suppressed using traditional methods such as retroviral infection, microinjection of DNA constructs and the so-called "gene-directed" transgenic approach. To date, transgenic models have become traditional and are successfully used in carcinogenesis studies [12].

For us, the mice with uPA gene knockout were of the greatest interest, obtained using a molecular genetic method during which changes are made to the nucleotide sequence of the uPA gene, as a result of which urokinase is not bound by the urokinase-type plasminogen activator receptor (uPAR). These mutant animals can be used in the study of inflammation, oncogenesis, and fibrinolysis mechanisms in tumors and surrounding tissues.

The aim of the study was to study the effect of knockout by the urokinase gene in mice of both sexes with B16/F10 transplanted melanoma on the functioning of the fibrinolytic system links.

MATERIALS AND METHODS

The study used genetically modified female and male mice of the C57BL/6-Plautm1.1Bug – ThisPlauGFDhu/GFDhu (uPA-/-) line with an initial weight of females – 24–26 g, 31–33g for males. The rodents were btained from the nursery of laboratory animals "Pushchino" Branch of the Institute of Bioorganic Chemistry named after Academicians M. M. Shemyakin and Yu. A. Ovchinnikov (Pushchino, Moscow region). Animals with urokinase knockout gene (uPA-/-) can be used in studies of chronic tissue inflammation, mechanisms of fibrinolysis, oncogenesis and vascular growth in the tumor and surrounding tissue. Mice of both sexes of the C57BL/6 (uPA+/+) line with an initial weight of 21–23 g obtained from the Andreevka Scientific Center for Biomedical Technologies (FMBA) (Moscow Region) were used as controls. The animals were kept under natural lighting conditions with free access to water and food. The study was conducted in accordance with the "International Recommendations for conducting biomedical research using animals" and the Order of the Ministry of Health of the Russian Federation No. 267 dated 06/19/2003 "On approval of the rules of laboratory practice".

The study was performed on 64 male and 64 female mice. The animals were divided into groups of 10 individuals each: intact females and males of the C57BL/6 line (uPA+/+); intact females and males of the C57BL/6 line -Plautm1.1Bug – ThisPlauGFDhu/GFDhu (uPA-/-); control group females and males of the C57BL/6 line (uPA+/+) 3 weeks after trans-

plantation of melanoma B16/F10; the main group of females and males of the C57BL/6-Plautm1.1Bug – ThisPlauGFDhu/GFDhu (uPA-/-) line 3 weeks after transplantation of melanoma B16/F10. The study period – 3 weeks after the transplantation of melanoma B16/F10 was chosen because it was the stage of mass death for male mice and the beginning of death of females, in addition, after 3 weeks, maximum differences in average tumor volumes in animals of uPA-/- and uPA+/+ lines were noted. Groups of uPA-/- (12 individuals of each sex) and uPA+/+ (25 individuals of each sex) animals were separately identified for the study of average life expectancy.

This work used a cell line of mouse melanoma B16/F10 metastasizing to the lungs, obtained from the N. N. Blokhin Russian Research Center of the Russian Academy of Medical Sciences (Moscow). Melanoma B16/F10 was transferred by subcutaneous injection of 0.5 ml of tumor tissue suspension in saline solution (1:10) into the right hind leg of a mouse according to the standard procedure [13]. With standard grafting, the tumor appears in 100 % of cases, grows quite quickly and on the 12th-16th day of growth metastasizes mainly hematogenously to the lungs (60–90 %), less often to the liver and spleen [14]. For the experiment, the second passage of melanoma B16/F10 transplantation in C57BL mice was used/6.

Tumor growth was assessed by daily measuring its diameters in three mutually perpendicular areas, followed by calculating the volume of the tumor as the product of its three measurements.

Intact animals, as well as mice of the control and main groups, were decapitated 3 weeks after the transplantation of melanoma, and the following were isolated in the cold: tumor, perifocal zone, skin. The samples were mechanically homogenized, 10 % homogenates were obtained from the tissues, prepared on a 0.1M potassium phosphate buffer pH 7.4 containing 0.1 % Twin-20 and 1 % BSA In tissue homogenates, the level of plasminogen (PG), plasmin (PAP), urokinase receptor uPAR, content (AG) and activity were determined using enzyme immunoassay methods (act) uPA, t-PA and PAI-I (Cussabio, China).

Statistical processing of the obtained results was carried out using the Statistica 10.0 program. For all quantitative data, the group arithmetic mean (M) and standard error (m) were calculated. All the results obtained were checked for compliance with the law of normal distribution (Shapiro-Wilk criterion (for small samples)). When the sample corresponded to the normal distribution, parametric statistics were used (Student's criterion), and when there was a discrepancy, nonparametric statistics were used (Wilcoxon-Mann-Whitney criteria). The differences were considered statistically significant at $p < 0.05$.

STUDY RESULTS

It was found that the process of carcinogenesis in genetically modified female mice (uPA-/-) compared with the control (uPA+/+) had features consisting in a reduction in the preclinical period of melanoma development and a decrease in the average volume of tumor nodes at all stages of observation (from 1 to 4 weeks). Single lung metastases were diagnosed in the females of the experimental group, whereas metastatic lung and liver damage was observed in the control group. In males, tumors were characterized by a fairly active, "spasmodic" growth, and their average volume at 4 weeks after transplantation did not differ from those in mice with a normal genome. In uPA-/-males, no visible metastases to internal organs were detected at all stages of the growth of B16/F10 transfused melanoma, but hemorrhages to the lungs were detected. The average life expectancy in uPA-/- and uPA+/+ mice had no significant differences and was 34.67±0.67 versus 30.25 ± 1.67 in females, and 23.33 ± 3.18 versus 22.1 ± 0.82 in males, respectively [15].

Based on the previously obtained results of differences in the growth of malignant tumors in animals with a knockout of the urokinase gene and a wild type of gene, it was interesting to find out what differences in the content and activity of the main links of the fibrinolytic system of the skin are characteristic of animals with a knockout of the urokinase gene (Tables 1, 2).

Compared with animals of the control group, only traces of uPA were recorded in the skin of intact uPA-/- mice of both sexes: a decrease in uPA levels and activity was noted by 100–860 times (Tables 1, 2). In intact uPA-/- females, the high content of PPB attracts attention, exceeding 4.2 times the same indicator in female uPA+/+ mice with a 1.3-fold (p 0.05) reduced PG content.

In intact uPA-/-males, concentrations of both PAP and PG were increased 2.1-fold and 1.8-fold in the skin, compared with those in the skin of intact uPA+/+ mice (Table 2).

In conditions of uPA deficiency with a high content of PAP, an increase in the level of the second activator of PG – tPA was expected, However, its content and activity were reduced only in uPA-/- males by 4.3 times and 1.7 times, respectively. In uPA-/- females, a decrease was revealed, relative to the data in uPA+/+ mice, only tPA activity by 2.5 times, despite an increase in its content by 1.7 times.

The amount of the uPAR receptor in uPA-/- female mice was at the same level as in uPA+/+ mice, whereas in males it was reduced by 1.9 times. Significant differences with the norm were also observed for PAI-1: in females, uPA-/- PAI-I activity and its content were 15.0 and 3.0 times lower than normal, respectively, in males by 4.9 times and 9.8 times, respectively.

Since the average tumor size in female uPA-/- mice was smaller after 3 weeks of the experiment than in uPA+/+ females [15], a comparative analysis of the links of the fibrinolytic system in samples of melanoma of animals with knockout and mice with wild genome type was further performed.

Table 1. The content and activity of fibrinolytic system components in the skin, tumor and perifocal zone of female uPA -/- mice with melanoma B16/F10 3 weeks after transplantation *(M ± m)*

Indicators	Intact mice skin (normal)	Skin	Tumor volume cm³	Perifocal zone
uPA -/- female mice (*n* = 10)				
uPA-act (u/g t)	0.010 ± 0.001[3]	0.009 ± 0.0009[2,3]	0.025 ± 0.002[3]	0.01 ± 0.001[3]
uPA-AG (ng/g t)	0.220 ± 0.02[3]	0.24 ± 0.018[2,3]	0.14 ± 0.011[1,3]	0.73 ± 0.06[1,2,3]
uPAR (pg/g t)	58.20 ± 4.3	56.2 ± 4.7	66.2 ± 5.3[3]	59.5 ± 5.3[3]
PAP (ng/g t)	45.0 ± 3.4[3]	18.75 ± 1.6[1]	19.7 ± 1.4[1,3]	24.4 ± 2.2[1,3]
PG (ng/g t)	7.70 ± 0.6[3]	10 ± 0.97[1,3]	8 ± 0.77[3]	20 ± 1.7[1,2,3]
tPA-act (u/g t)	0.240 ± 0.02[3]	0.16 ± 0.014[1,2,3]	1.95 ± 0.16[1]	0.155 ± 0.014[1,2,3]
tPA-AG (ng/g t)	0.670 ± 0.05[3]	0.34 ± 0.028[1,2,3]	0.69 ± 0.05[3]	1.55 ± 0.13[1,2,3]
PAI-I-act (u/g t)	1.60 ± 0.1[3]	1.85 ± 0.15[2,3]	3.05 ± 0.29[1,3]	5.25 ± 4.4[1,2,3]
PAI-I-AG (ng/g t)	3.30 ± 0.3[3]	2.2 ± 0.17[1.3]	2.7 ± 0.24[3]	2.8 ± 0.22[3]
uPA +/+ female mice (*n* = 10)				
uPA-act (u/g t)	1.6 ± 0.12	2.5 ± 0.2[1]	2.8 ± 0.19[1]	2.6 ± 0.17[1]
uPA-AG (ng/g t)	31.7 ± 2.1	187.5 ± 13[1,2]	335.5 ± 23[1]	186.5 ± 12.5[1,2]
uPAR (pg/g t)	56.06 ± 4.5	65.36 ± 5.6[2]	141.8 ± 11.7[1]	112.0 ± 9.6[1]
PAP (ng/g t)	10.7 ± 0.7	19.9 ± 1.1[1.2]	36.4 ± 1.5[1]	18.9 ± 1.2[1,3]
PG (ng/g t)	10.25 ± 0.9	13.5 ± 1.1[1]	16.7 ± 1.4[1.]	11.03 ± 0.9
tPA-act (u/g t)	0.6 ± 0.04	0.7 ± 0.03[2]	2.2 ± 0.19[1]	0.7 ± 0.06[3]
tPA-AG (ng/g t)	0.4 ± 0.02	2.0 ± 0.15[1,2]	12.3 ± 0.9[1]	2.5 ± 0.13[1,3]
PAI-I-act (u/g t)	24.0 ± 0.16	24.0 ± 1.4[2]	71.1 ± 4.2[1]	81.0 ± 5.8[1]
PAI-I-AG (ng/g t)	9.9 ± 0.4	24.5 ± 1.8[1,2]	79.5 ± 6.3[1]	71.6 ± 5.2[1]

Note: [1] – the differences are statistically significant relative to the norm in animals; [2] – compared with a tumor; [3] – compared with similar samples in uPA+/+ animals ($p < 0.05$)

In the tumor samples of uPA-/- females, compared with the tumor samples of uPA+/+ females, the indicators of the determined factors were significantly lower: the activity and content of uPA by 112 times and by 2396 times, the level of uPAR by 2.1 times, PP and PG by 1.8 times and 2.1 times, the content of tPA by 17.8 times, the activity and content of PAI-1 by 23.3 times and 29 times, respectively. Only the activity of tPA did not have significant differences in tumor samples depending on the urokinase gene.

That said, 3 weeks after the transplantation of melanoma B16/F10 in uPA+/+ females in tumor samples, compared with the corresponding intact skin, an increase in all studied parameters of the fibrinolytic system was noted, whereas in uPA-/- females in melanoma samples such stimulation was not detected, except for an increase in activity, but not the content tPA.

There were also differences in the studied parameters in the perifocal zone and the skin unaffected by tumor growth. Thus, in the perifocal zone of uPA -/- females after 3 weeks of melanoma growth, uPA activity and level were lower than in the perifocal zone of uPA+/+ mice by 260 and 255 times, respectively, and the concentration of uPAR was also 1.9 times lower. The level of tPA, as well as its activity, were

Table 2. Content and activity of fibrinolytic system components in the skin, tumor and perifocal zone in male uPA -/- mice with melanoma B16/F10 3 weeks after transplantation *(M ± m)*

Indicators	Intact mice skin (normal)	Skin	Tumor volume cm^3	Perifocal zone
uPA -/- male mice				
uPA-act (u/g t)	0.010 ± 0.001[3]	0.009 ± 0.0007[2,3]	0.013 ± 0.0011[1,3]	0.015 ± 0.001[1,3]
uPA-AG (ng/g t)	0.250 ± 0.02[3]	0.25 ± 0.018[2,3]	0.10 ± 0.009[1,3]	0.39 ± 0.03[1,3]
uPAR (pg/g t)	56.90 ± 4.3[3]	67.4 ± 5.9	90.8 ± 7.6[1]	67.55 ± 5.5[2]
PAP (ng/g t)	30.0 ± 2.5[3]	18.13 ± 1.4[1,3]	14.4 ± 0.9[1,3]	18.13 ± 1.7[1]
PG (ng/g t)	12.50 ± 0.9[3]	9.1 ± 0.77[1,3]	10 ± 0.07[3]	12.2 ± 0.8
tPA-act (u/g t)	0.320 ± 0.02[3]	0.17 ± 0.015[1,2,3]	1.22 ± 0.78[1.3]	0.17 ± 0.014[1,2,3]
tPA-AG (ng/g t)	0.70 ± 0.05[3]	0.66 ± 0.06[2,3]	0.46 ± 0.04[1.3]	0.88 ± 0.071[2,3]
PAI-I-act (u/g t)	2.60 ± 0.2[3]	1.77 ± 0.13[1,2,3]	2.87 ± 0.21[3]	2.37 ± 0.18[3]
PAI-I-AG (ng/g t)	4.10 ± 0.3[3]	2.43 ± 0.21[1,2,3]	3.9 ± 0.33[3]	4.8 ± 0.43[3]
uPA +/+ male mice				
uPA-act (u/g t)	1.561±0.10	1.65 ± 0.143	2.7 ± 0.21	1.9 ± 0.17
uPA-AG (ng/g t)	215.3 ± 16.8	181.6 ± 17.1[3]	300.4 ± 24	210.3 ± 19
uPAR (pg/g t)	110.3 ± 6.5	65.13 ± 5.7[1]	73.48 ± 5.3[1]	85.96 ± 7.2[1]
PAP (ng/g t)	14.52 ± 0.9	30.7 ± 2.9	48.8 ± 4.1	19.8 ± 1.7
PG (ng/g t)	6.851 ± 0.5	15 ± 1.2	21.6 ± 1.9	13 ± 1.1
tPA-act (u/g t)	0.551 ± 0.04	0.86 ± 0.07	2.4 ± 0.19	0.8 ± 0.06
tPA-AG (ng/g t)	2.981 ± 0.2	4.8 ± 0.38[1]	11.6 ± 0.9	5.5 ± 0.42
PAI-I-act (u/g t)	12.61 ± 1.02	52.5 ± 4.7	41.3 ± 3.8	59.4 ± 5.6
PAI-I-AG (ng/g t)	40.0 ± 3.5	28 ± 2.5	39 ± 3.4	54.8 ± 4.5

Note: [1] – the differences are statistically significant relative to the norm in animals; [2] – compared with a tumor; [3] – compared with similar samples in uPA+/+ animals ($p < 0.05$)

1.6 times and 4.5 times lower than in animals without knockout, respectively. The activity of PAI-I and its content were reduced by 15.4 times and 25.6 times. Despite this, the level of PAP and PG in the perifocal zone of uPA-/- females turned out to be 1.3 times and 1.8 times higher than in uPA+/+ females, respectively.

That said, in the perifocal zone in female uPA+/+ mice, after 3 weeks of melanoma growth, almost all links of the fibrinolytic system (with the exception of PG and tPA activity) exceeded the indicators in the skin of intact animals, whereas in uPA-/- mice in the perifocal zone, compared with the skin of intact animals, only an increase in the content was detected PG, uPA and tPA, without increasing their activity, as well as increased activity of PAI-I.

In the skin of uPA-/- females unaffected by tumor growth, almost all indicators of the fibrinolytic system were reduced, except for the absence of differences in PAP and uPAR, compared with skin samples in uPA+/+ females. Thus, in skin samples with tumor growth in females, uPA-/- activity and uPA content were lower by 277.8 times and 781.3 times; tPA activity and content by 4.4 times and 5.9 times; PAI-I activity and content by 13 times and 11.4 times, respectively. After 3 weeks of tumor growth, the dynamics of changes in the studied parameters in unaffected skin in uPA+/+ females, compared with intact mice, generally corresponded to the orientation in the tumor and perifocal zone – activation of the fibrinolytic system was observed, whereas in uPA-/- females, on the contrary, either no changes or a decrease in the level of RAR, activity and the content of tPA and the content of PAI-I, compared with intact mice of the same line.

At the stage 3 weeks after transplantation, the volumes of primary tumors in uPA-/- males were smaller than in animals with wild type genes [15].

In the tumor samples of uPA-/- males, compared with similar samples in uPA+/+ males, the level of plasmin was reduced by 3.4 times and plasminogen by 2.2 times (Table 2). The activity and content of uPA in males with urokinase gene knockout were 208 times and 3004 times lower than in wild-type animals, respectively, and the content and activity of tPA were 25.2 times and 2 times lower, respectively. In addition, a decrease by 14.4 times and 10 times in the activity and concentration of PAI-I was revealed as well.

Only the uPAR content did not differ depending on the state of the urokinase gene. So if in males uPA+/+ in tumor samples, compared with intact skin (normal), almost all the studied parameters of the fibrinolytic system have increased, with the exception of the receptor level, in melanoma in males uPA-/- on the other hand only an increase in tPA and uPAR activity was noted.

In the perifocal zone of uPA-/- males, compared with the perifocal zone of uPA+/+ males, the activity and level of uPA were 127 times and 538 times lower, and tPA was 25 times and 11.4 times, and PAI-I was 25 times and 8.8 times, respectively. At the same time, the level of uPAR, plasmin and PG in the perifocal zone did not carry any significant differences depending on the state of the urokinase gene. It turned out that with tumor growth in uPA+/+ males in the perifocal zone, the content of PAP, PG, as well as the activity and content of tPA and PAI-I increased compared with the skin of the corresponding intact animals, whereas in males uPA-/- either did not change compared with intact skin, or decreased.

In the samples of unaffected skin in uPA-/- males with melanoma B16/F10, compared with the indicators in unaffected skin in uPA+/+ males, the concentrations of PAP and PG were on average 1.7 times lower, the activity and content of uPA 183 times and 726 times, the activity and content of tPA 5.1 times and by 7.3 times, the activity and concentration of PAI-I by 29.7 times and 11.5 times. Only the uPAR level had no significant differences. At the same time, it should be noted that in uPA+/+ males and in mice with urokinase gene knockout, only the absence of changes in the activity and content of iRA, as well as a decrease in PAI-I levels, turned out to be the same in unaffected skin with melanoma growth, as well as a decrease in the level of PAI-I, compared with the indicators of healthy skin of the corresponding intact animals. The rest of the studied parameters changed in different directions – in uPA-/- males either decreased (PAP, PG, tPA activity) or did not change (PAI-I activity, tPA content, uPAR), whereas activation was detected in uPA+/+ (with the exception of uPAR).

DISCUSSION

Currently, it is known that urokinase (uPA) is secreted in many malignant cells, including pancreatic, breast, and colorectal cancers, and its expression often correlates with the prognosis of the

disease [4, 16]. The biological role of this protease is to bind to the uPAR receptor to stimulate the proteolytic cascade and convert inactive proteases such as plasmin and matrix metallopeptidase 9 (MMP-9) into active forms, thereby endowing tumor cells with the ability to destroy the components of the extracellular matrix, activate the growth and metastasis of tumor cells [17–19]. Therefore, the role of uPA in migration, invasion and metastasis of tumor cells is undeniable [18].

Previously, we received confirmation of the effect of urokinase gene knockout on the tumor process, namely, significant suppression of tumor volume growth and metastasis in animals of both sexes [15]. We found that in intact uPA-deficient mice of the C57BL/6-PlautmI.IBug-ThisPlau6FDhu/GFDhu line, almost the entire cascade of PG regulators was suppressed in the skin (with the exception of the urokinase receptor uPAR and tPA content only in females). We expected to detect an increase in the activity of a number of enzymes, but in intact uPA-/- mice, an increased content of plasmin alone was recorded. With uPA deficiency in C57BL/6-PlautmI.IBug-ThisPlau6FDhu/GFDhu mice, plasmin activity could have found other targets in our experiment. We believe that an increase in the content of PAP in knockout mice is a kind of compensation, contributing to a sharp decrease in urokinase, cleavage of its receptor.

Despite the significant suppression of the fibrinolytic system in mice, uPA-/- transfused melanoma grew, and although it had significantly smaller volumes (especially in females) and rarely metastasized (males had no visible metastases), the life expectancy of animals of the two lines did not have significant differences. In addition, the level of plasmin in the skin of intact uPA-/- mice exceeded the values in animals of the C57BL/6 line. These points prove the presence of alternative biological pathways that melanoma "uses" for its survival in conditions of knockout of the urokinase gene.

One of the alternative pathways in urokinase gene knockout conditions may be uPAR, known as CD-87, which is highly expressed in various tumor cells, and various signals regulated by uPAR play an important role in neoplasm proliferation and metastasis, tumor-related glycolysis, as well as tumor microenvironment and angiogenesis [20]. There is evidence that it is uPAR that regulates the migration of melanoma cells by assembling them in complex regulatory units with transmembrane receptors [21].

Our study showed that on the background of significant suppression of urokinase, the level of the uPAR receptor in intact skin in females did not change, and in males. Although an almost twofold decrease in its concentration was detected. However, its content in the tumor and surrounding tissues on the background of the growth of melanoma B16/F10 has no significant differences from those in animals with wild the type of genome. It is known that uPAR competes with uPA for participation in many non-proteolytic biological processes, such as migration, adhesion, cell proliferation and angiogenesis [22]. Thus, uPA-/- uPA functions could be performed by uPAR in mice. In our study, the uPA-/- urokinase receptor level in the studied samples did not change in relation to the parameters in intact animals, whereas in males the tumor samples increased, which was accompanied by large volumes of melanoma in males, compared with females.

Series of studies confirm that a decrease in uPAR expression on the cell surface mitigates the development of characteristic cancer signs caused by PIK-3CA and KRas mutations in colorectal cancer [23], and by interacting with uPA and IGF1R, uPAR is able to enhance the malignant potential of triple negative breast cancer [24]. Clinical observations are confirmed by experimental studies in which knockout of the uPAR gene in mice leads to G2/M arrest, thereby suppressing cell proliferation [25]. There are studies on the possibility of using uPA inhibitors to slow tumor growth and metastasis [26].

It is believed that overexpression of uPAR in human melanoma cells controls the invasive and glycolytic phenotype. uPAR-mediated pathways have already been established, including the integrin-dependent association of uPAR with at least four IL-TKR systems: *EGFR, IGFR, PDGFR* and *MET* [27]. The results of our studies showed a significant increase in the level of uPAR in tumor samples and its perifocal zone in uPA+/+ mice, whereas in uPA-/- females such a pattern was not observed and only in uPA-/- males the concentration of the urokinase receptor increased in tumor samples. The complexity of various molecular pathways allows malignant cells to continue to proliferate and migrate even in conditions of urokinase deficiency, using uPA-independent pathways of proteolytic

activation of angiogenesis factors. This was confirmed by our previous studies of angiogenesis factors in animals with urokinase gene knockout, demonstrating an increased content of VEGF-A and especially VEGF-C in unaffected skin in female uPA-/- mice [28].

At the same time, it should be considered that knockout by the urokinase gene is a kind of artificially induced genetic comorbid disease, as a result of which the fibrinolytic system is suppressed not only in the skin, but also in other organs and systems. The involvement of the fibrinolytic system in various physiological processes, wound healing, as well as in the preservation of brain neurons after various ischemic injuries indicates a possible insufficiency of these processes in uPA-/- animals.

CONCLUSIONS

The fact that in uPA-/- mice, despite the extremely small volumes of the primary tumor and rare metastasis, tumor disease caused the death of animals at the same time as in uPA+/+ animals, indicates a significant effect of tumor disease on all regulatory systems of the body, regardless of the size of the neoplasm. Our study confirmed the claim that the use of drugs that inhibit the urokinase pathway may be promising in the treatment of the disease by slowing the growth of neoplasm volume and its metastasis, but is not a panacea, since the effect of a malignant tumor on the body is much more complex, therefore further studies of the pathogenesis of malignant growth are required.

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ORIGINAL ARTICLE

Features of anorectal function after radiation therapy in patients with rectal cancer

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ABSTRACT

Purpose of the study. To study the function of the sphincter in patients with rectal cancer after chemoradiotherapy using the method of high-resolution anorectal manometry.
Patients and methods. The study included 30 patients with cancer of the middle and lower ampullary rectum, who underwent combined treatment at the National Medical Research Center of Oncology. The patients underwent a course of neoadjuvant gamma radiation therapy using capecitabine. High-resolution anorectal manometry was performed before the start of treatment and 2 months after completion of chemoradiotherapy to study the functional parameters of the sphincter apparatus. The severity of anorectal dysfunction was assessed using the Wexner anal incontinence scale.
Results. According to high-resolution anorectal manometry, the average pressure of the anal canal at rest decreased by 1.4 times ($p < 0.05$), and the average absolute compression pressure with voluntary contraction decreased by 1.2 times ($p = 0.0012$) after neoadjuvant chemoradiotherapy. A comparative assessment of the maximum absolute compression pressure at this stage of treatment did not allow us to trace a significant difference between its value before the start of radiation therapy and 2 months after its completion ($p > 0.05$). An increase in threshold sensitivity volumes was noted in 23 patients ($p = 0.16$). The use of the Wexner scale didn't show a statistically significant change in the median scores according to the results of patient surveys following the completion of treatment (5.2 vs. 5.5 points, $p > 0.05$).
Conclusions. Radiation therapy has an effect on anorectal function, which may contribute to the occurrence of low anterior resection syndrome after surgical treatment. For this reason, it is now necessary to carefully consider the risks of developing anorectal dysfunction. Equally important is the use of methods for the prevention of low anterior resection syndrome for patients who have received combined treatment for rectal cancer.

Keywords: low anterior resection syndrome, high-resolution anorectal manometry, neoadjuvant chemoradiotherapy

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Compliance with ethical standards: the work followed the ethical principles set forth in the World Medical Association Declaration of Helsinki, 1964, ed. 2013. The study is approved by the Committee on Biomedical Ethics at the National Medical Research Center for Oncology (extract from the protocol of the meeting No. 28 dated 09/09/2022). Informed consent was received from all participants of the study

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ОРИГИНАЛЬНАЯ СТАТЬЯ

Особенности аноректальной функции после лучевой терапии у больных раком прямой кишки

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РЕЗЮМЕ

Цель исследования. Изучение функции сфинктерного аппарата у больных раком прямой кишки после химиолучевой терапии с помощью метода аноректальной манометрии высокого разрешения.

Пациенты и методы. В исследовании принимали участие 30 больных раком среднеампулярного и нижнеампулярного отделов прямой кишки, проходивших комбинированное лечение в ФГБУ «Национальный медицинский исследовательский центр онкологии» Министерства здравоохранения Российской Федерации. Пациентам выполнялся курс неоадьювантной дистационной гамма-терапии с применением капецитабина. Для изучения функциональных параметров сфинктерного аппарата выполняли аноректальную манометрию высокого разрешения до начала лечения и через 2 мес. после завершения химиолучевой терапии. Степень выраженности аноректальной дисфункции оценивали с использованием шкалы анальной инконтиненции Wexner.

Результаты. После проведения неоадьювантной химиолучевой терапии по данным аноректальной манометрии высокого разрешения показатель среднего давления анального канала в состоянии покоя снижался в 1,4 раза ($p < 0,05$), а среднее абсолютное давление сжатия при волевом сокращении уменьшалось в 1,2 раза ($p = 0,0012$). Сравнительная оценка максимального абсолютного давления сжатия на данном этапе лечения не позволила проследить достоверного отличия между его значением до начала лучевой терапии и через 2 мес. после ее завершения ($p > 0,05$). У 23 пациентов было отмечено увеличение пороговых объемов чувствительности ($p = 0,16$). Применение шкалы Wexner не показало статистически значимого изменения медианы баллов по результатам опросов пациентов после завершения лечения (5,2 против 5,5 баллов, $p > 0,05$).

Заключение. Лучевая терапия оказывает влияние на аноректальную функцию, что может способствовать возникновению синдрома низкой передней резекции после хирургического лечения. По этой причине в настоящее время необходимо учитывать риски развития аноректальной дисфункции. Не менее важным является использование методов профилактики синдрома низкой передней резекции для больных, получивших комбинированное лечение по поводу рака прямой кишки.

Ключевые слова: синдром низкой передней резекции, аноректальная манометрия высокого разрешения, неоадьювантная химиолучевая терапия

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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 № 28 от 09.09.2022 г.). Информированное согласие получено от всех участников исследования

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INTRODUCTION

In 2020, 1,931,590 new cases of colorectal cancer and 935,173 deaths were detected worldwide, while in Russia these morbidity and mortality rates were 77,213 and 42,079 cases, respectively [1]. Statistical data indicate that the problem of diagnosis and treatment of rectal cancer continues to be relevant.

The standard of treatment for patients with locally advanced cancer of the middle and lower ampullary parts of the rectum is neoadjuvant chemoradiotherapy followed by surgical treatment with total mesorectumectomy. An important aspect in the treatment of rectal cancer remains an organ-preserving strategy using nerve-sparing techniques [2]. Maintaining the integrity and functional activity of the sphincter avoids the need for the formation of a lifelong colostomy and improves the quality of the patients' lives [3].

The use of radiation therapy at the first stage of combined treatment can reduce the risk of local recurrence by reducing the size of the tumor process and improve the long-term survival of patients [4, 5]. Modern modified radiation therapy not only reduces the size of the primary tumor, but also reduces the area of radiation for surrounding tissues [6]. Nevertheless, neoadjuvant chemoradiotherapy may negatively affect the work of the sphincter of the rectum [7]. Due to the increase in the number of sphincter-preserving surgical interventions and non-adjuvant radiation therapy in recent years, more and more attention has been paid to functional results [8, 9]. Systematic reviews consider radiation therapy as one of the significant risk factors for intestinal dysfunction [10].

According to a number of authors, the effect of radiation therapy is associated with the development of fibrous changes in structures and tissues exposed to radiation [6, 11]. By reducing the elasticity of the rectum by thickening its wall, radiation therapy leads to a deterioration in long-term functional results [12]. When the primary tumor is located close to the anal canal, the sphincter apparatus is often also in the field of high radiation doses, which can affect the tone of the sphincters, reducing the contractility of the locking apparatus [10].

Changes in the functioning of the sphincter apparatus may include an increase in the frequency of urges and a deterioration in the control of gas discharge and defecation with the possible development of incontinence [13]. Similar clinical manifestations may occur with varying frequency in patients with rectal cancer after low anterior resection [14]. Therefore, it is relevant to study anorectal function at different stages of treatment in order to develop individual methods for preventing the development and correction of these symptoms.

The anorectal dysfunction symptom assessment system includes various questionnaires, the most widely used of which are the low anterior resection syndrome and Wexner scales. In the analysis of the randomized clinical trial of FOWARC, neoadjuvant radiation therapy was associated with a worse low anterior resection syndrome score and quality of life [15]. However, the question of the effect of neoadjuvant therapy on functional outcomes in patients after combined treatment of rectal cancer currently remains controversial.

An objective assessment of the function of the sphincter apparatus of the rectum can be obtained by performing high-resolution anorectal manometry. This method of investigation represents the pressure distribution in the anal canal, both at rest and when performing physiological tests. The advantage of high-resolution anorectal manometry is the use of a higher physiological resolution created by the increased density of sensitive sensors and their location around the circumference [16]. High-resolution anorectal manometry displays changes in anorectal activity at rest and with various functional tests in the form of a colored contour graph [17].

The purpose of the study: to study the indicators of the functional state of the sphincter apparatus in patients with rectal cancer after chemoradiotherapy using the method of high-resolution anorectal manometry.

PATIENTS AND METHODS

The analysis of changes in the anorectal function of the sphincter was performed in patients undergoing observation and treatment in the period from 2022 to 2023 at the National Medical Research Center for Oncology, Ministry of Health of the Russian Federation. The study included 30 patients with a confirmed diagnosis of cancer of the middle and lower ampullary rectum. At the time of treatment, the average age of patients was 63.2 years

(patients ranged in age from 40 to 76 years). At the same time, 60 % of men (18 patients) and 40 % of women (12 patients) were men. According to the results of histological analysis, adenocarcinoma with a predominance of a moderately differentiated tumor form was observed in patients (56.7 %). In 16 patients (53.3 %), the primary tumor site was located at a distance of < 5 cm from the anorectal junction. The median distance from the lower edge of the tumor to the anodermal junction was 6.5 cm (3–10 cm).

Patients underwent conformal remote radiotherapy with a single focal dose of 2 Gy 5 times a week to a total focal dose of 50–54 Gy per primary tumor focus and 44 Gy on the path of regional metastasis. Radiation therapy was accompanied by modification with capecitabine at a dosage of 1,650 mg/m^2 per day orally in two doses on the days of the sessions.

To study the functional parameters of the rectal sphincter, high-resolution anorectal manometry was performed using water perfusion technology with an 8-channel catheter of the WMP Solar GI device (MMS, Holland). The study was conducted before the start of treatment and 2 months after the end of chemoradiotherapy. Anorectal manometry was performed according to a standard procedure in the position of a patient with bent knee and hip joints. The level of average anal pressure in the anal canal at rest and the levels of average and maximum compression pressure were assessed. To study the sensitivity and reservoir function of the rectum, the first rectal sensation, the volume at the first urge to defecate and the maximum tolerable volume when filling the balloon with air were recorded.

The severity of dysfunction of the sphincter apparatus of the rectum was assessed according to clinical gradation using the Wexner anal incontinence scale. The results of the scale are presented in the form of points from 0 to 20, while intestinal incontinence is established when 12 points or more are scored.

According to the Shapiro-Wilk criterion, the parameters considered in the study had a distribution different from normal. Statistical data processing was carried out using the Statistica 13.0 package. Quantitative data in our study were represented by the median (Me) and quartile values Q1 and Q3 in the Me (Q1 – Q3) format. The nonparametric Mann-Whitney criterion was used to compare the variables of two samples (before and after chemoradiotherapy).

STUDY RESULTS

Since the onset of rectal cancer in patients, the clinical manifestations of tumor lesions have ranged from episodes of intestinal discomfort and irregular stools to involuntary defecation. Of the total study group, 16 cases (53.3 %) had loose stools more often than 5 times a day, 12 patients (40 %) had false urges to defecate, 7 patients (23.3 %) had manifestations of anal incontinence in the form of cases of uncontrolled gas discharge and 4 of them (13.3 %) had incontinence intestinal contents.

Functional changes in the internal anal sphincter reflect the parameters of resting anal pressure. When comparing the obtained indicators, the level of average anal pressure at rest in patients has decreased by 1.4 times after completion of the course of chemoradiotherapy ($p < 0.05$) (Fig. 1).

A similar trend was observed when estimating the average absolute compression pressure with voluntary contraction. 2 months after the completion of radiation therapy, its index in patients decreased by 1.2 times ($p = 0.0012$). A comparative assessment of the maximum absolute compression pressure at this stage of treatment did not allow us to trace a significant difference between its value before the onset of radiation therapy and 2 months after its completion ($p > 0.05$). The values of the obtained parameters of high-resolution anorectal manometry are presented in Table 1.

Attention was drawn to a decrease in the endurance of volitional contraction and an increase in muscle fatigue during functional tests. Upon completion of chemoradiotherapy, there was a decrease in the median duration of sphincter contraction from an average of 22 seconds from the initial state to 18 seconds. Also, an increase in threshold sensitivity volumes was noted in 23 patients, but no statistical difference was found when comparing these indicators ($p = 0.16$).

The study of anorectal function on the Wexner scale did not show a statistically significant change in the median scores according to the results of patient surveys after completion of the neoadjuvant stage of treatment (5.2 points and 5.5 points before treatment and after radiation therapy, respectively, $p > 0.05$). Before the start of treatment, a minimum score of 2 points on the Wexner scale was observed in 11 patients (36.7 %), while in 7 of them (23.3 %) after

radiation therapy, the minimum threshold increased to 4 points. At the same time, the number of patients with a maximum score of 13 points on the Wexner scale did not change after radiation therapy (13.3 %).

DISCUSSION

Chemoradiotherapy at the first stage of treatment in patients with cancer of the middle and lower ampullary rectum increases the possibility of performing organ-preserving treatment and improves oncological treatment results by reducing the frequency of local tumor recurrence by less than 6 % [13]. However, along with this advantage, the use of radiation therapy followed by anterior rectal resection and total mesorectomectomy is associated with higher rates of intestinal dysfunction [18]. The development of anorectal dysfunction of varying severity after combined treatment of rectal cancer was associated with a deterioration in the quality of life in 19–52 % of patients [19].

There are many works in the modern literature describing a more significant effect of combined treatment on the sphincter apparatus of the rectum compared with surgical intervention [13, 20]. Surgical trauma can cause neurogenic damage to the locking apparatus due to mobilization, especially with low rectal resections [21]. Intraoperative trauma in the form of anal dilation can affect both the external and internal anal sphincters with a transient zone and the so-called "hemorrhoidal cushion" [22]. However, there are much fewer studies providing data on the direct effect of neoadjuvant radiation therapy on the sphincter apparatus [9, 10]. In this study, the effect of radiation therapy on the function of the sphincter apparatus of the rectum was evaluated in accordance with manometric parameters and clinical manifestations.

Anorectal function is a complex physiological mechanism, an important role in the implementation of which belongs to the sphincter apparatus of the rectum. The activity of the smooth muscles of

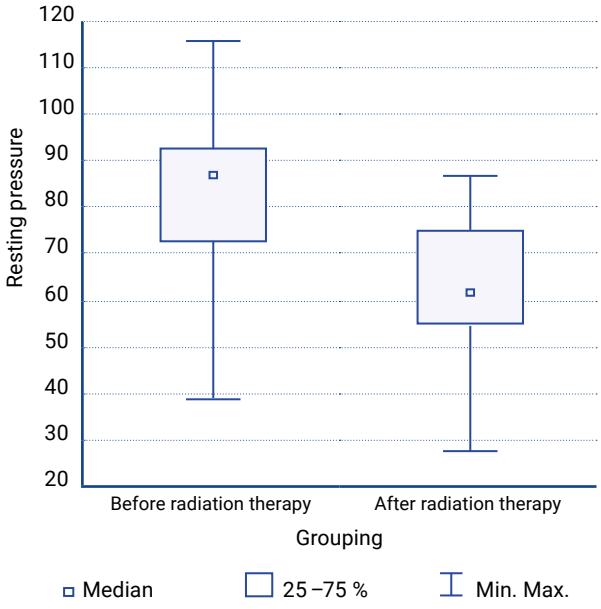

Fig. 1. Comparative assessment of the resting pressure index in the anal canal

Table 1. Parameters of anorectal manometry of the subjects studied during radiation therapy

Parameter	Before the start of treatment Me (Q1; Q3)	After radiation therapy Me (Q1; Q3)	*p*
Average anal pressure at rest (mmHg)	87 (73; 92)	61 (55; 74)	$p < 0.05$
Average absolute compression pressure (mmHg)	154 (128; 173)	124 (102; 139)	$p = 0.0012$
Maximum anal compression pressure (mmHg)	196 (161; 221)	176 (149; 139)	$p > 0.05$
Endurance Test time (sec)	22 (17; 25)	18 (11; 23)	$p > 0.05$
Threshold sensitivity volume (ml)	35 (28; 49)	46 (41; 54)	$p = 0.16$
Threshold sensitivity volume (ml)	35 (28; 49)	46 (41; 54)	$p = 0.16$

the internal anal sphincter maintains pressure in the anal canal at rest. While the striated musculature of the external anal sphincter and pelvic floor is involved in the implementation of arbitrary contraction, especially over a long period [23]. The coordinated functioning of the anal sphincters and the ampoule of the rectum provides the possibility of adequate implementation of the locking function [24].

The results of the analysis demonstrate a decrease in resting pressure after a neoadjuvant course of radiation therapy, which is confirmed by information from other studies found in modern literature [25, 26]. At the same time, the above data did not reveal a significant change in the work of the external anal sphincter compared with the work of the internal sphincter. Also, several publications showed no changes in the work of the external anal sphincter after radiation therapy [26–28]. However, in our study, a decrease in the average values of anal compression pressure was observed, which can be considered as a possible prerequisite for a decrease in the strength and endurance of arbitrary contraction.

Randomized studies demonstrate a decrease in resting pressure in the postoperative period after neoadjuvant radiation therapy due to deterioration of the internal sphincter [29, 30]. Irradiation is associated with damage to the sacral plexus and with fibrous changes in the muscle fibers of the sphincters [31]. The greater susceptibility to radiation exposure of the internal sphincter compared with the external one may be due to such features as a smaller number of muscle fibers and innervation by a thin network of nerve fibers of the pelvic plexus [28].

Changes in the locking apparatus of the rectum were also noted during morphological examination. Histological analysis revealed damage to the myenteric plexus of the internal anal sphincter, and there was also a tendency to increased collagen deposition in this structure [32].

The pathogenetic aspects of the effect of radiation therapy on the blocking function are studied in many studies. In the work of Rahbari N. N. et al., (2013) it was found that radiation therapy can not only cause difficulties in performing total mesorectomies, but also reduce the ability of irradiated tissues to repair, thereby leading to an increase in a number of complications in patients after low anterior rectal resection [33]. The literature has also described the relationship between radiation therapy and the development of colorectal cancer failure (Kit O. I. et al., 2018) [34].

On the other hand, the pathogenetic factor of the negative effect of radiation therapy on the function of anal sphincters is vascular fibrosis, pelvic and musculoskeletal plexus [11, 32, 35]. Some researchers describe a malfunction of the function of the internal anal sphincter, which is not even included in the radiation field, which can also affect the capacity and pliability of the rectum [36]. The above facts contribute to the development of anorectal dysfunction and the occurrence of anal incontinence in some of the treated patients.

Irradiation of the rectum causes weakening of the anal sphincter, as well as impaired processing of anorectal sensory stimuli [36]. According to a study conducted by van der Sande M. E. et al. (2019), the relationship between the dose of radiation therapy and the severity of anorectal dysfunction in patients with rectal cancer was monitored [37].

Clinical manifestations of the negative effects of radiation therapy on the function of the rectal occlusion apparatus may be characterized by a specific pattern. Most authors report a higher frequency of loose stools and urge to defecate after radiation therapy, signs of anal incontinence in the form of incontinence of gases and intestinal contents and laundry contamination are less common [13, 26, 28]. However, according to the data obtained in our study, there was no significant difference in the clinical picture before and after radiation therapy. The most frequent complaints, as well as before the start of treatment, were frequent loose stools and false urge to defecate. Only a small number of patients had cases of uncontrolled gas discharge and incontinence of intestinal contents.

De Nardi and co-authors studied 39 patients with rectal cancer before and after radiation therapy. The results of anorectal manometry showed a significant decrease only in resting pressure after the treatment. When assessing incontinence on the Wexner scale before neoadjuvant therapy, 5 patients already had mild incontinence with an average score of 3, and after neoadjuvant therapy, 11 reported incontinences with an average score of 3.8 [38].

When assessing the quality of the function of the sphincter apparatus according to the Wexner scale, no significant difference in the median scores was

noted in our work according to the results of the patient survey. This fact suggests that radiation therapy does not always cause deterioration of clinical symptoms on the part of the evacuation function.

Thus, in our study, changes in the manometric parameters of the internal and external anal sphincters, a decrease in the endurance of contractions of the rectal locking apparatus were not accompanied by significant clinical manifestations. However, the changes we have identified may become a prerequisite for the development of anal incontinence after completion of treatment.

CONCLUSION

Radiation therapy may affect the function of the rectal occlusion apparatus, especially the internal anal sphincter. These changes may contribute to the formation of low anterior rectal resection syndrome after surgery. For this reason, it is now necessary to consider the risks of developing anorectal dysfunction. Equally important is the use of methods for the prevention of low anterior resection syndrome for patients who have received combined treatment for rectal cancer.

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ORIGINAL ARTICLE

Hypoxia effect on proliferative activity of cells in orthotopic xenograft of hepatocellular carcinoma of the liver in the experiment

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ABSTRACT

Purpose of the study. The purpose of this research was to investigate the effect of *in vivo* hypoxic conditions on the proliferative potential of HepG2 liver cancer cells.

Materials and methods. Human liver cancer cells of the HepG2 line have been cultured. The HepG2 cell suspension was injected subcutaneously into mice in an amount of 5×10^6 to obtain a xenograft. Tumor nodes that had reached the required size were divided into fragments and transplanted into the orthotopic site. Balb/c nude mice with implanted HepG2 liver cancer xenograft were used in this experiment. The mice with tumor implanted in the liver were divided into two groups, intact and hypoxic. Mice from the second group underwent liver blood flow reduction by occlusion of the portal triad for 20 minutes. Tumor nodes were extracted for histological and immunohistochemical staining for proliferation marker Ki-67 on the 4th day after the procedures. The proportion of positively stained cells was calculated, and the results were statistically analyzed using the Statistica 10.0 software.

Results. Orthotopic models of liver cancer in Balb/c Nude mice were obtained. Histological and immunohistochemical studies were carried out. Histological analysis showed that hepatocellular carcinoma is characterized by an average degree of differentiation. In the tissues of these xenografts, by using immunohistochemical analysis for the proliferation marker Ki-67, it was possible to identify statistically significant differences between the two groups, i. e. intact and the one with reduction of blood flow. The proportion of immunopositive cells was 65 [65–70] % and 19 [15–25] %, respectively.

Conclusion. A tendency to decreased proliferative activity of tumor cells after hepatic blood flow reduction, i. e. hypoxia exposure, was demonstrated. Our data indicate that the proliferative activity of tumor cells is directly related to the microenvironment, and to the hypoxic environment in particular. Further study of the effect of hypoxia on the processes of growth and development of malignant tumors may contribute to a deeper understanding of the biological features of tumors and their treatment.

Keywords: hypoxia, liver, HepG2, proliferation, Ki-67, *in vivo*

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Compliance with ethical standards: when performing this study, all manipulations with laboratory animals were carried out in compliance with the Rules and Regulations for Carrying Out Animal Research Work. The study was approved by the Ethics Committee of the National Medical Research Center for Oncology (Protocol No. 4/108 dated 02/10/2021)

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3.1.6. Онкология, лучевая терапия
ОРИГИНАЛЬНАЯ СТАТЬЯ

Влияние гипоксии на пролиферативную активность клеток ортотопического ксенографта гепатоцеллюлярной карциномы печени в эксперименте

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РЕЗЮМЕ

Цель исследования. Оценить пролиферативную активность клеток рака печени HepG2 при моделировании гипоксических условий *in vivo*.

Материалы и методы. Культивировали клетки рака печени человека линии HepG2. Для получения ксенографта клеточную суспензию HepG2 вводили мышам подкожно в количестве 5×10^6. Достигшие необходимого размера опухолевые узлы делили на фрагменты и трансплантировали в ортотопический сайт. В работе использовали мышей линии Balb/c Nude, которым имплантировали ксенографт рака печени HepG2. Мышей с прижившейся опухолью в печени делили на две группы – интактная и с гипоксией. Мышам из второй группы выполняли редукцию кровотока печени путем окклюзии портальной триады в течение 20 мин. На 4-е сутки после проведенных манипуляций опухолевые узлы извлекали для выполнения гистологического и иммуногистохимического окрашивания на маркер пролиферации Ki-67. Вычисляли долю позитивно окрашенных клеток и проводили статистический анализ результатов с помощью пакета программ Statistica 10.0.

Результаты. Были получены ортотопические модели рака печени у мышей линии Balb/c Nude. Проведены гистологическое и иммуногистохимическое исследования. Гистологический анализ показал, что гепатоцеллюлярная карцинома характеризуется средней степенью дифференцировки. В тканях данных ксенографтов с помощью иммуногистохимического анализа на маркер пролиферации Ki-67 удалось выявить статистически значимые различия между двумя группами – интактной и с редукцией кровотока. Доля иммунопозитивных клеток составила 65 [65–70] % и 19 [15–25] % соответственно.

Заключение. Продемонстрирована тенденция к снижению пролиферативной активности опухолевых клеток после редукции кровотока печени, то есть воздействия гипоксии. Полученные нами данные свидетельствуют о том, что пролиферативная активность клеток опухоли напрямую связана с микроокружением, в частности, с гипоксической средой. Дальнейшее изучение воздействия гипоксии на процессы роста и развития злокачественных образований может способствовать более глубокому пониманию биологических характеристик опухолей и их лечения.

Ключевые слова: гипоксия, печень, HepG2, пролиферация, Ki-67, *in vivo*

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Соблюдение этических стандартов: при выполнении данного исследования все манипуляции с лабораторными животными проводились в соответствии с «Правилами проведения работ с использованием экспериментальных животных». Исследование одобрено этическим комитетом ФГБУ «Национальный медицинский исследовательский центр онкологии» Министерства здравоохранения Российской Федерации (протокол № 4/108 от 10.02.2021 г.)

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INTRODUCTION

The hypoxic environment, characterized by low oxygen content, plays a crucial role in the processes of cell survival and reprogramming. This fact is confirmed by numerous studies on the evolution and development of organisms [1, 2]. Particularly, it has been established that the normal development of mammals occurs under conditions of hypoxia (moderate to severe), which regulates many aspects of ontogenesis and morphogenesis. In addition, it is known that the oxygen gradient is an important regulator of cellular processes in both physiological and many pathological conditions, including malignant diseases [3].

Sudden and short-term effects of hypoxia (from several minutes up to 72 hours), resulting from fluctuations in tumor perfusion, are accompanied by functional and structural defects in the vascular network of the tumor. Such exposure can lead to the formation of high levels of reactive oxygen species (ROS), which can damage cells [4]. Hypoxia can also cause the growth of cancer cells to stop, slow down proliferation and, subsequently, their death. It has been shown that hypoxia-induced factors directly affect the proliferative activity of tumor cells [5]. The most widely used marker of proliferation in both normal and tumor cells is the Ki-67 protein. It participates in the cell cycle, being involved in ribosome biogenesis, heterochromatin organization and mitotic chromosome separation [6]. The Ki-67 index makes it possible to assess the degree of malignancy of the tumor and predict the course of the disease in combination with other factors. A direct correlation has been established between the number of tumor cells expressing Ki-67 and the stage of malignant diseases [7, 8]. The proliferative potential and survival of cancer cells can be modulated by creating hypoxic conditions, which is actively used in such therapeutic procedures as transarterial embolization and transarterial chemoembolization [9]. However, it is also known that hypoxia is crucial for the survival of cells resistant to low-oxygen environments, characterized by resistance to therapeutic effects and increased invasive ability [10]. On this matter, a comprehensive study of the tumor's response to hypoxia, as well as an understanding of its positive and negative effects, will expand the understanding of the mechanisms of interaction of cancer cells and the features of their microenvironment.

The effect of the level of oxygenation is studied using various approaches, including *in vitro*, it is also possible to use methods of isolated primary tumors, but they do not accurately reflect the real parameters of the tumor microenvironment [11]. An analysis of the literature data shows that the most reliable and trustworthy data can be obtained using *in vivo* methods that allow more accurately, compared with other research approaches, to model the effect of hypoxia on the activity of malignant neoplasms and their proliferative potential, which may be important for planning further translational studies [12, 13].

The purpose of the study was to evaluate the proliferative activity of HepG2 liver cancer cells in modeling hypoxic conditions *in vivo*.

MATERIALS AND METHODS

Laboratory animals and their maintenance

For this experiment, mice with Balb/c Nude immunodeficiency (*n* = 14) 10–12 weeks old and weighing 25–27 g have been used and obtained from the vivarium National Medical Research Center for Oncology, the Russian Federation Ministry of Health. The mice were in an IVC system (individually ventilated cages), food and water were provided without restrictions. All work with experimental animals was carried out in accordance with the ethical principle of the European Convention for the Protection of Vertebrates Used for Experiments or Other Scientific Purposes (ETSN 123, Strasbourg, March 18, 1986). This experiment was approved by the decision of the local bioethical committee of the National Medical Research Center for Oncology.

Culture of human liver cancer cells

Human liver cancer cells of the HepG2 line were cultured in accordance with a standard procedure using a culture medium for DMEM cells with the addition of veal serum (Gibco, Thermo Fisher Scientific) at a concentration of 10 %, as well as 1 % penicillin and streptomycin. Cultivation was carried out in a CO_2 incubator (Thermo Fisher Scientific, 8000W) at a humid atmosphere of 37 °C, 5 % CO_2.

Creating an orthotopic model of liver cancer

Initially, before conducting the experiment, we created a liver cancer xenograph by subcutaneously

injecting a 5×10^6 cell suspension of HepG2 into Balb/c Nude mice (n = 2). When the obtained subcutaneous xenographs reached a diameter of 1–1.5 cm, the mice were euthanized, the tumor nodes were extracted and divided into fragments about 1 × 1 × 1 mm in size for further transplantation into the liver. Access to the liver was carried out by performing laparotomy on pre-anesthetized recipient animals. An incision was made in the left lobe of the liver, after which the previously obtained tumor fragments were placed into the parenchyma of the left lobe of the liver using anatomical tweezers. The wound was sewn up with a wound stitch after the manipulations.

Creating hypoxic conditions by reducing liver blood flow

A control laparotomy was performed to measure the volume of tumor nodes 2 weeks after the tumor fragments were implanted into the liver of mice. To determine the size of the tumor node, the following formula was used: $V = LW^2/2$, L for the length of the tumor, W for the width of the tumor. Then the animals were divided into 2 groups (n = 6 for each), the distribution criterion was the size of the tumor node, while the values of the average volume of tumor nodes in the groups differed with a minimum interval. The first group is intact, the second group is with a reduction in liver blood flow. To provide access to the liver and its blood vessels, the mice of the second group underwent laparotomy. Then, to occlude the vessels of the portal triad of the liver, a needle with suture material was inserted under them and blood flow was reduced for 20 minutes using the tension of the suture material. After that, the tension of the suture material was removed to restore blood supply to the liver and the surgical wound was sutured in layers. The mice of the first group underwent a control laparotomy without blood flow reduction.

Euthanasia

On the 4th day after the surgical manipulations, the animals were euthanized to extract tumor nodes. Euthanasia was performed by dislocation of the cervical vertebrae.

Histological and immunohistochemical (IHC) studies

The resulting tumor material was fixed in 10 % formalin for 24 hours, then enclosed in paraffin, sections were made using a rotary microtome, which were subsequently dewaxed according to a standard protocol. Hematoxylin and eosin staining was performed for histological examination. IHC staining was performed automatically in the BenchMark ULTRA Ventana immunohistostainer according to the protocols of manufacturers attached to the antibodies used. Antibodies Ki-67 (clone SP6), CellMarque were used in a 1:200 dilution. To analyze the expression of Ki-67 by tumor cells, the proportion of cells with colored nuclei (percentage of the total number of tumor cells) in at least 10 random fields of view was calculated.

Statistical analysis

The results obtained during the experiment were analyzed using the Statistica 10.0 software package. The data are presented in the form of the median, 25th and 75th percentiles. A comparative analysis of the differences in Ki-67 nuclear staining between the groups was carried out with the Mann-Whitney statistical criterion.

STUDY RESULTS

During the experiment, orthotopic models of liver cancer in Balb/c Nude mice were obtained by implanting a fragment of the xenograft of the HepG2 cell line directly into the left lobe of the liver [14]. A control laparotomy made it possible to demonstrate that all animals developed tumor nodes in the left lobe of the liver. The measurement results showed that 2 weeks after implantation of the HepG2 xenograft fragment into the liver, the size of intrahepatic tumor nodes was 130.27 [42.88–345.3] mm^3. (Fig. 1).

After performing the control laparotomy procedure, the animals were divided into 2 groups. In order to induce hypoxic conditions, group 2 animals underwent reduction of liver blood flow (Fig. 2). For this, the right lobe of the liver was shifted closer to the diaphragm, which facilitated free access to the portal triad. Clamping the vessels of the portal triad with suture material made it possible to achieve a reduction in the blood flow of the liver and the tumor node located in it, which was visually confirmed by a change in the color of the liver, as a result of insufficient blood supply, the organ became paler. After the restoration of blood supply, the liver turned maroon again.

The results of histological examination showed a focus of hepatocellular carcinoma in the liver tissues, characterized by an average degree of differentiation, represented by solid-trabecular structures, in the thickness of which the vessels are located. Necrosis foci are locally present. The cellular composition is represented by large epithelial cells resembling hepatocytes. Large polymorphic nuclei with granular chromatin and well-distinguishable nucleoli are visible inside the cells. Mitosis figures are also found, including atypical forms (Fig. 3).

In the immunohistochemical study of the expression of the Ki-67 proliferation marker in the tissues of liver cancer xenographs, the number of immunopositive cells was 65 [65–70] % (Fig. 4A), in the group with blood flow reduction, the number of stained nuclei was statistically significantly less, which amounted to 19 [15–25] % ($p < 0.001$) (Fig. 4B).

DISCUSSION

It is known that hypoxia is an important factor that can contribute to the formation of cellular plasticity and tumor heterogeneity, affecting the phenotype and cell functions. However, despite the impressive array of data presented in the scientific literature, it is possible to observe a lack of correlation between different methods of studying the effect of oxygen

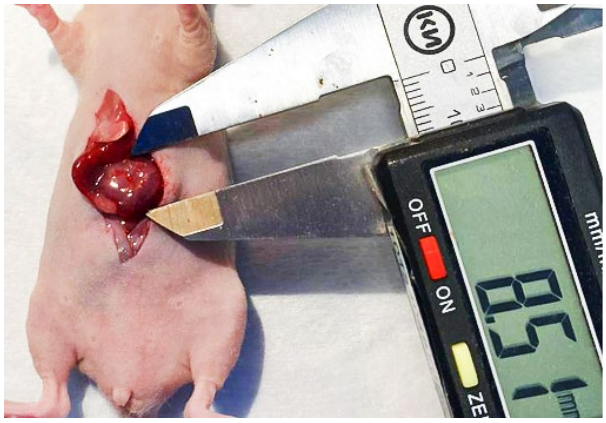

Fig. 1. Measurement of a tumor in the liver of a mouse

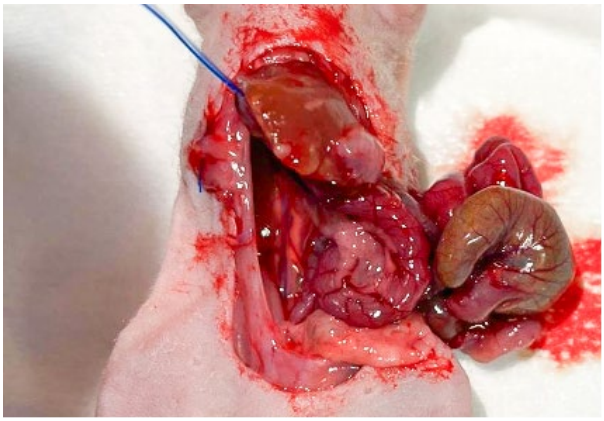

Fig. 2. The process of performing liver blood flow reduction by occlusion of the portal triad to induce hypoxic conditions

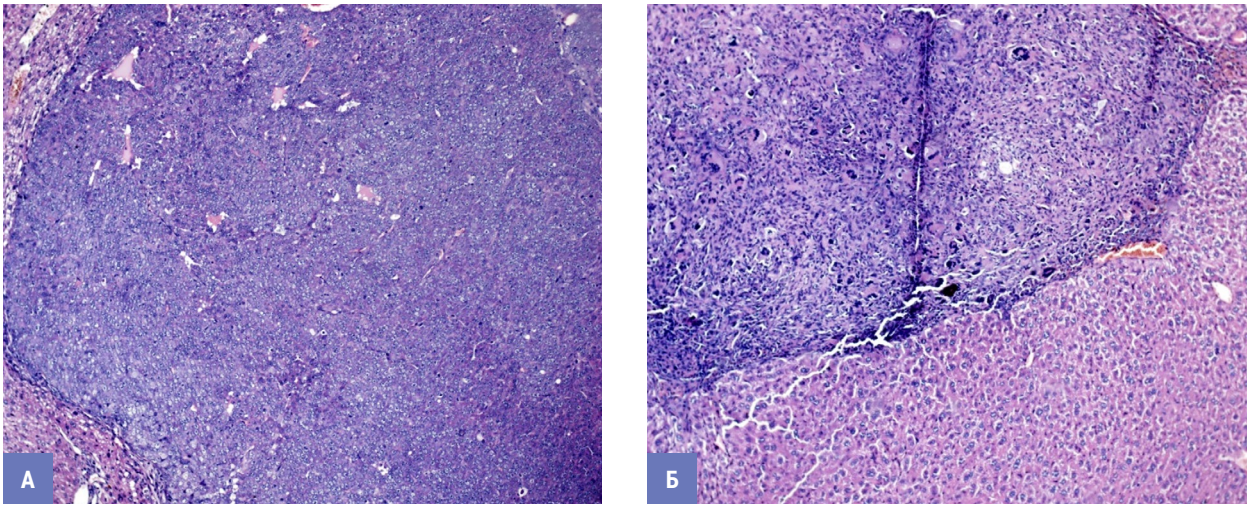

Fig. 3. Histological specimen: morphological picture of hepatocellular carcinoma. A – without hypoxia; B – after hypoxia. Magnification × 100

levels, since they all provide information about different diseases, non-uniform time and topological points of sampling of tumor material, or, for example, blood oxygenation. From this point of view, the use of animal models allows, as far as possible, to bring uniformity to the experimental conditions and obtain reproducible results by performing serial experiments. Considering the listed advantages of the *in vivo* approach, we performed an experiment to study the effect of low oxygenation on liver cancer cells. The results of the IHC study showed that in tumor samples of animals with reduced blood flow, a lower value of the Ki-67 proliferation marker was observed. An analysis of the literature data showed that in the works of other authors there is a direct connection between hypoxia and the proliferative potential of tumor cells. For example, a study of endometrial tumors showed that the expression level of Ki-67 is inversely correlated with the expression level of hypoxia-induced factor (HIF-1a), which indicates low cell proliferative activity in conditions of oxygen deficiency. In addition, such a correlation may contribute to reducing the effect of anti-cancer drugs such as metformin [15]. Also, in the work on visualization of hypoxia of cancer cells in animals and cancer patients, it was found that tumor cells in effusions and micrometastases were in a state of high hypoxia and low proliferation, regardless of the type of tumor. In this work, samples of human and animal tumor cells were examined by IHC for HIF-1a, glucose transporter (GLUT-1) and proliferation marker (Ki-67). In addition, it has been convincingly demonstrated that ascites is an environment with a very low level of oxygenation since the cells floating in it do not have adequate blood supply and can survive only through glycolysis pathway. It is important to note that the authors mention that tumor hypoxia is a driving factor in resistance to radiation therapy and chemotherapy [16].

In this study, a low level of Ki-67 expression was noted in tumor samples of animals with blood flow reduction, however, zoning in the location of positively colored cells was observed in tumor tissues. Cells expressing Ki-67 were concentrated along the tumor zones directly in contact with intact liver tissue. It is known that the so-called "invasion front" of a tumor is formed by cells located on its surface, and they form patterns of invasion and tumor spread. Given this fact, it can be assumed that cells that have retained their proliferative potential, despite the effects of hypoxic conditions resulting from blood flow reduction, and located along the edge of the tumor node, may have an increased invasive potential. In addition, the observed zonality of Ki-67 expression may probably be related to proximity or distance from blood vessels with reduced blood flow.

CONCLUSION

This study shows that liver tumors of mice subjected to the liver blood flow reduction procedure were characterized by lower Ki-67 values. The ob-

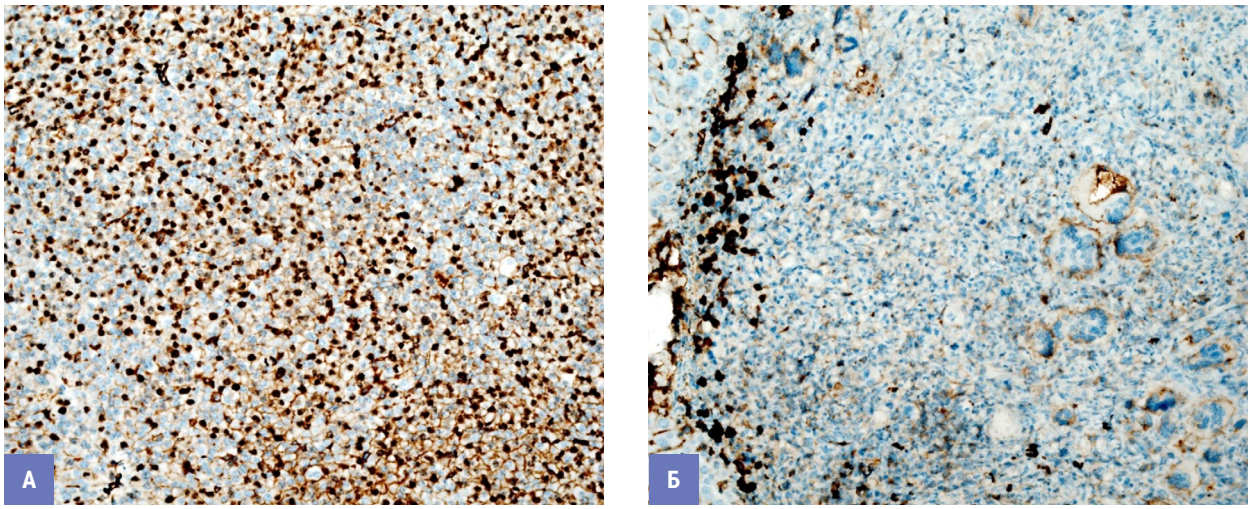

Fig. 4. IHC reaction of the tumor to Ki-67 antibodies (clone SP6). A – without reduction of liver blood flow; B – after reduction of liver blood flow by occlusion of the portal triad. Magnification × 200

tained data indicate that the proliferative activity of tumor cells is directly related to the microenvironment, particularly to the hypoxic environment. Further study of the effects of hypoxia on the growth and development of malignant tumors may contribute to a deeper understanding of the biological characteristics of tumors and the approaches to their treatment.

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Contribution of the authors:

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Trifanov V. S. – editing the text;
Shulga A. A. – text writing, statistical analysis;
Goncharova A. S. – search for literature data, writing a text;
Gurova S. V. – conducting an experiment;
Ulyanova E. P. – histological analysis;
Maksimov A. Yu. – text editing.

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ORIGINAL ARTICLE

Dynamics of saliva cytokine levels during intraoperative photodynamic therapy in patients with locally advanced oral cancer

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ABSTRACT

Purpose of the study. Assessment of the level of certain cytokines in the saliva of patients with primary locally advanced cancer of the oral mucosa in addition to surgical treatment with intraoperative PDT (IPDT).
Patients and methods. Patients with primary locally advanced cancer of the oral mucosa T3-4aN0-2M0 were divided into 2 groups: the main group (30 patients) underwent radical tumor removal supplemented with IPDT and the control group (30 patients) without addition. IPDT was performed using Latus-T (farah) and a chlorin E6 photosensitizer. Cytokine levels were determined in unstimulated whole saliva the day before, on the 3rd and on the 7th day after the operation by the ELISA multiplex analysis method.
Results. A similar dynamic of the cytokine profile of patients of both groups was shown: on the 3rd day after surgery, the levels of G-CSF, IL-6, MIP-1β increased, and GM-CSF and IFN-γ decreased compared with baseline values. On the 7th day, the dynamics of G-CSF, GM-CSF, IL-6 persisted, while IL-8, IL-10, IL-12 changed to the opposite.
Intergroup differences were revealed in the level of IL-1β – on day 3, an increase in the main group and a decrease in the control group. The level of IL-7 on day 7 decreased sharply in the control group and increased statistically significantly in patients receiving IPDT. The main group showed a 4.8-fold increase in IL-8 on day 3 and its 3.6-fold drop on day 7 with the opposite dynamics in the control group. The TNF-α level increased only in the main group on day 7, and in the control group it decreased by 3 and recovered on day 7. On day 3, the MCP-1 level increased in the main group and decreased in the control group. The level of IL-17 in the main group increased on the 3rd day with a further decrease below the baseline, and in the control group it decreased on the 3rd day, followed by a recovery on the 7th. An increase in IL-5 and IL-13 levels on day 3 was noted only in the control group, however, the level of IL-5 in both study periods in the main group was lower than in the control group.
Conclusion. IPDT in patients with primary locally advanced oral cancer causes changes in the cytokine composition of saliva during the first week after surgery, some of which can be associated with an elongation of the relapse-free period in such patients.

Keywords: intraoperative photodynamic therapy, cytokines, saliva, primary locally advanced oral cancer

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Compliance with ethical standards: the work followed the ethical principles set forth by the World Medical Association Declaration of Helsinki, 1964, ed. 2013. The study was approved by the Ethics Committee of the National Medical Research Center of Oncology (extract from the protocol of the meeting No. 15 dated 10/12/2021). Informed consent was received from all participants of the study

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3.1.6. Онкология, лучевая терапия
ОРИГИНАЛЬНАЯ СТАТЬЯ

Динамика уровней цитокинов слюны при проведении интраоперационной фотодинамической терапии у больных местно-распространенным раком полости рта

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РЕЗЮМЕ

Цель исследования. Оценка уровня некоторых цитокинов в слюне больных первичным местно-распространенным раком слизистой оболочки полости рта при дополнении хирургического лечения интраоперационной ФДТ (ИФДТ).
Пациенты и методы. Пациенты первичным местно-распространенным раком слизистой оболочки полости рта T3-4aN0-2M0 были распределены в 2 группы: основная группа (30 больных) – проведено радикальное удаление опухоли, дополненное ИФДТ и контрольная группа (30 пациентов) – без дополнения. ИФДТ проводили с помощью «Латус-Т» (фара) и фотосенсибизатором хлорин Е6. Уровни цитокинов определяли в нестимулированной цельной слюне за сутки до, на 3-и и на 7-е сутки после проведения операции методом ИФА мультиплекс-анализа.
Результаты. Показана сходная динамика цитокинового профиля больных обеих групп: на 3-и сутки после операции уровни G-CSF, IL-6, MIP-1β повышались, а GM-CSF и IFN-γ снижались по сравнению с исходными показателями. На 7-е сутки характер динамики G-CSF, GM-CSF, IL-6 сохранялся, а IL-8, IL-10, IL-12 менялся на противоположный. Межгрупповые различия выявлены по уровню IL-1β – на 3-и сутки повышение в основной и снижение в контрольной группе. Уровень IL-7 на 7-е сутки резко снижался в контрольной группе и статистически значимо повышался у больных, получавших ИФДТ. В основной группе показано 4,8-кратное повышение IL-8 на 3-и сутки и его 3,6-кратное падение на 7-е с противоположной динамикой в контрольной. Уровень TNF-α возрастал только в основной группе на 7-е сутки, а в контрольной отмечено его снижение на 3-и и восстановление на 7-е сутки. На 3-и сутки уровень MCP-1 возрастал в основной и снижался в контрольной группе. Уровень IL-17 в основной группе нарастал на 3-и сутки с дальнейшим снижением ниже исходного, а в контрольной группе снижался на 3-и сутки с последующим восстановлением на 7-е. Нарастание уровней IL-5 и IL-13 на 3-и сутки отмечены только в контрольной группе, однако, уровень IL-5 в оба срока исследования в основной группе был ниже, чем в контрольной.
Заключение. ИФДТ у больных первичным местно-распространенным раком полости рта вызывает изменения цитокинового состава слюны в течение первой недели после операции, часть из которых можно связать с удлинением безрецидивного периода у таких пациентов.

Ключевые слова: интраоперационная фотодинамическая терапия, цитокины, слюна, первичный местно-распространенный рак полости рта

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INTRODUCTION

Currently, it is recognized that under the influence of inflammation, neoplastic and stromal cells interact and control the evolution of the tumor, producing cytokines that modulate the antitumor immune response [1–3]. The similarity of the cytokine pattern for tumors of different localization and histogenesis has been demonstrated [4], however, there is no consensus on whether cytokines play a decisive pro-oncogenic or anti-oncogenic role [5]. Both can be assumed based on the known biological properties of cytokines as stimulants, on the one hand, proliferation and neo angiogenesis, and on the other hand, the immune response.

In recent decades, the participation of various pro- or anti-inflammatory cytokines of the tumor microenvironment of squamous cell carcinoma of the oral cavity in the oncogenesis of a tumor of this localization has been shown [6–8]. Most studies describe elevated levels of IL-1β, 6, 8, 10, TNF-α in patients with oral cancer compared with healthy ones at both the local and systemic levels [9, 10]. Moreover, a higher content of IL-1β and IL-6 was demonstrated in tumors of the oral mucosa than in areas of unchanged mucosa [11]. Literature data show that hyperproduction of cytokines by tumor cells and its microenvironment in patients with oral cancer is one of the causes of tumor spread.

Thus, when developing approaches to the treatment of oral cancer, it is important to analyze the local and systemic cytokine status of such patients, as well as the induction of an antitumor immune response. The latter can be achieved with photodynamic therapy (PDT), one of the mechanisms of antitumor effectiveness of which is immunogenic cell death [12]. PDT induces it through the release of damage-associated molecular patterns (DAMPs) [13]. PDT-induced acute inflammation and immunogenic cell death are considered the initial step in the implementation of its immunostimulating effect [14], which manifests itself in a tumor-specific immune response [15]. The role and place of immune mechanisms in the realization of the antitumor effect of PDT are summarized in a recent review [14].

The purpose of the study was to assess the level of certain cytokines in the saliva of patients with primary locally advanced cancer of the oral mucosa in addition to surgical treatment with intraoperative PDT (IPDT).

PATIENTS AND METHODS

The study group included 60 patients with primary locally advanced cancer of the oral mucosa who were being treated by the Department of Head and Neck Tumors of the Federal State Budgetary Institution "National Medical Research Center of Oncology" of the Ministry of Health of the Russian Federation. The average age of the patients was 63.1 ± 14.3 years. The distribution of patients by gender was as follows: male – 48 people, female – 12 people. In all patients, the diagnosis was morphologically verified and corresponded to stages III–IV (T3-4aN0-2M0) of cancer. According to the morphological structure, the tumors were represented by squamous cell carcinoma, of which moderate–differentiated – 79 %, highly differentiated – 16 % and low–differentiated – 5 %.

The patients were randomized into 2 groups: the main group (30 patients) – patients who underwent radical tumor removal supplemented with IPDT and the control group (30 patients) – without the supplement. The patients in the groups were comparable in age, gender and location of the primary focus. All patients underwent surgical intervention in the volume of radical removal of the tumor of the tongue and oral mucosa with cervical lymphadenectomy in a volume adequate to the prevalence of the tumor process, on the affected side or bilateral (with damage to the anterior parts of the oral cavity).

The study was approved by the Ethics Committee of the National Medical Research Center of Oncology Protocol No. 15 dated 10/12/2021, all patients signed a voluntary informed consent.

Intraoperative PDT was performed in accordance with the developed and patented method (RF Patent No. 2797433) [15]. At the first stage, patients with locally advanced cancer of the mucous membrane of the oral cavity and tongue underwent surgical treatment, and then, after hemostasis of the postoperative wound of the oral cavity, PDT was performed after covering the healthy surrounding tissues around the surgical field with sterile eight-layer gauze wipes. Latus-T (headlight) was used for the session, with parameters: wavelength 662 nm,

power density 45 MW, light energy 200–300 J/cm^2. The lamp was placed in such a way that the area of laser light emission covered both the bed of the removed tumor and the edges of the surgical wound. The duration of exposure was calculated depending on the size of the bed of the removed tumor of the oral cavity according to the formula: T = T0 × nw/kp, where T0 is the tabular value of the irradiation time, nw is a coefficient showing how many times the energy density WS (J/ cm^2) to be collected by the surface differs from the tabular WS/0 = 100 J/cm^2: nw = WS/100, kp is a coefficient showing how many times the laser power differs from the tabular P0 = 100 MW: kp = P/100. After completion of the photodynamic therapy stage, plastic surgery of the postoperative defect was performed. Chloride E6 was used as a photosensitizer, which was administered intravenously in a single dose of 2 mg per 1 kg of patient weight 3–3.5 hours before the expected end time of tumor removal. The immediate results of complex treatment supplemented with IPDT were evaluated in patients with locally advanced oral mucosal cancer.

Cytokine levels (G-CSF, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, MCP-1, MIP-1β, TNF-α) were determined in saliva by ELISA multiplex analysis (Bio-Plex Pro Human Cytokine Assays 17-Plex Panel, Bio-Rad, USA). The collection of unstimulated whole saliva for the study was carried out from 8 to 9 a. m. the day before the operation, on the 3rd and on the 7th day after it. The results were expressed in pg/ml.

Statistical analysis of the study results was carried out using the Statistica 12.0 program (StatSoft Inc., USA), MedCalc (version 9.3.5.0). Since the distribution was not normal, the Mann-Whitney criterion was used to compare intergroup indicators, and the Wilcoxon criterion was used to compare indicators in dynamics; the differences were considered statistically significant at $p < 0.05$. The data are presented as a median with upper and lower quartiles (Me [LQ, UQ]).

STUDY RESULTS AND DISCUSSION

The results of the study are shown in Fig. 1A-1G, 2A-2G.

On the 3rd day after surgery in patients of both groups, the levels of G-CSF, IL-6, MIP-1β increased (Fig. 1A), and GM-CSF and IFN-γ decreased (Fig. 1B) compared with baseline values; the content of the remaining cytokines was multidirectional in the compared groups of patients.Some of the indicators changed statistically significantly in only one of the groups, which is apparently related to the effect of PDT (Fig. 1B, 1G).

On the 7th day, the dynamics of G-CSF, GM-CSF, IL-6 persisted, IL-8, IL-10, IL-12 changed to the opposite, the levels of other cytokines changed in the main and control groups in different ways (Fig. 2A-D).

Differences were revealed between the main and control groups in terms of colony-stimulating factors: an increase in G-CSF and a decrease in GM-CSF on both the 3rd and 7th days after surgery compared with baseline values, and in the main group, changes in G-CSF are less pronounced than in the control group, and in both The duration of the study was at lower values (Fig. 1A, 2A). So, in the control group, the level of G-CSF increased 22.8 times on the 3rd day after surgery, and 13 times in the main group, and on the 7th day it was 22.2 and 4.8 times higher than the initial one, respectively, i. e. the decrease on the 7th day after the initial increase by 3 occurred only after the effect of PDT.

IL-1β levels on day 3 increased in the main group from 410.1 [321.9; 522.6] to 550.8 [528.7; 590.6] pg/ml and decreased in the control group to 102.1 [22.1; 159] pg/ml, and on the 7th the reverse pattern was observed (Fig. 1B, 2B).

There was a 3.3-fold increase in IL-6 in the saliva of patients of both groups on day 3 compared with the baseline, which continued on day 7 with higher rates in the main group (1856.2 [1753.1;1975] versus 1356.9 [1261.3; 1450.7] pg/ml, $p < 0.05$), (Fig. 1A, 2A).

The dynamics of IL-7 consisted in an increase in the level of this cytokine in the saliva of patients of both groups on the 3rd day after surgery, statistically significant only in the control group (by 53 %) and multidirectional on the 7th day – a sharp drop (to 0) in the control group and a continued increase that reached statistical significance (15.3 [13.6; 17.2] versus 10.2 [6.8; 13.5] before treatment; $p < 0.05$) in patients receiving IPDT.

In the main group, there was a pronounced (4.8-fold) transient increase in IL-8 content on day 3 and its 3.6-fold drop on day 7; in the control group, on the contrary, it decreased on day 3 and increased on day 7, exceeding the indicator of the main group

in the last period (Me 2883.1 [2621.8;3338.2] and 2006.4 [1934.3;2310.4] pg/ml, respectively, $p < 0.05$), (Fig. 1A, 2B). The TNF-α level increased only in the main group on day 7, and in the control group it decreased by 3 and recovered on day 7 (Fig. 1D, 2D). On the 3rd day, there was a multidirectional dynamics of MCP-1 levels: an increase in the main group and a decrease in the control group (Fig. 1D), and on the 7th in both groups, the indicators become 3.5 and 4.6 times lower, respectively, than the initial one (Fig. 2D). The content of MIP-1β has a similar dynamics in both groups, increasing by 3 and returning to the baseline on the 7th day after surgery (Fig. 1A, 2G).

There was a decrease in IFN-γ and IL-4, expressed in the main group only on the 7th day with indicators at this time lower than the control ones (Fig. 2B). The median level of IFN-γ in the main group on day 7 was 4.7 [4.2; 5.2] pg/ml, and in the control group 6.8 [6.3; 7.4] pg/ml versus 10.6 [8.4; 12.4] pg/ml before treatment; for IL-4, the indicators were 4.1 [3.2; 4.7] and 8,6 [7,2; 8,9] versus 10,9 [8,7; 14,2]

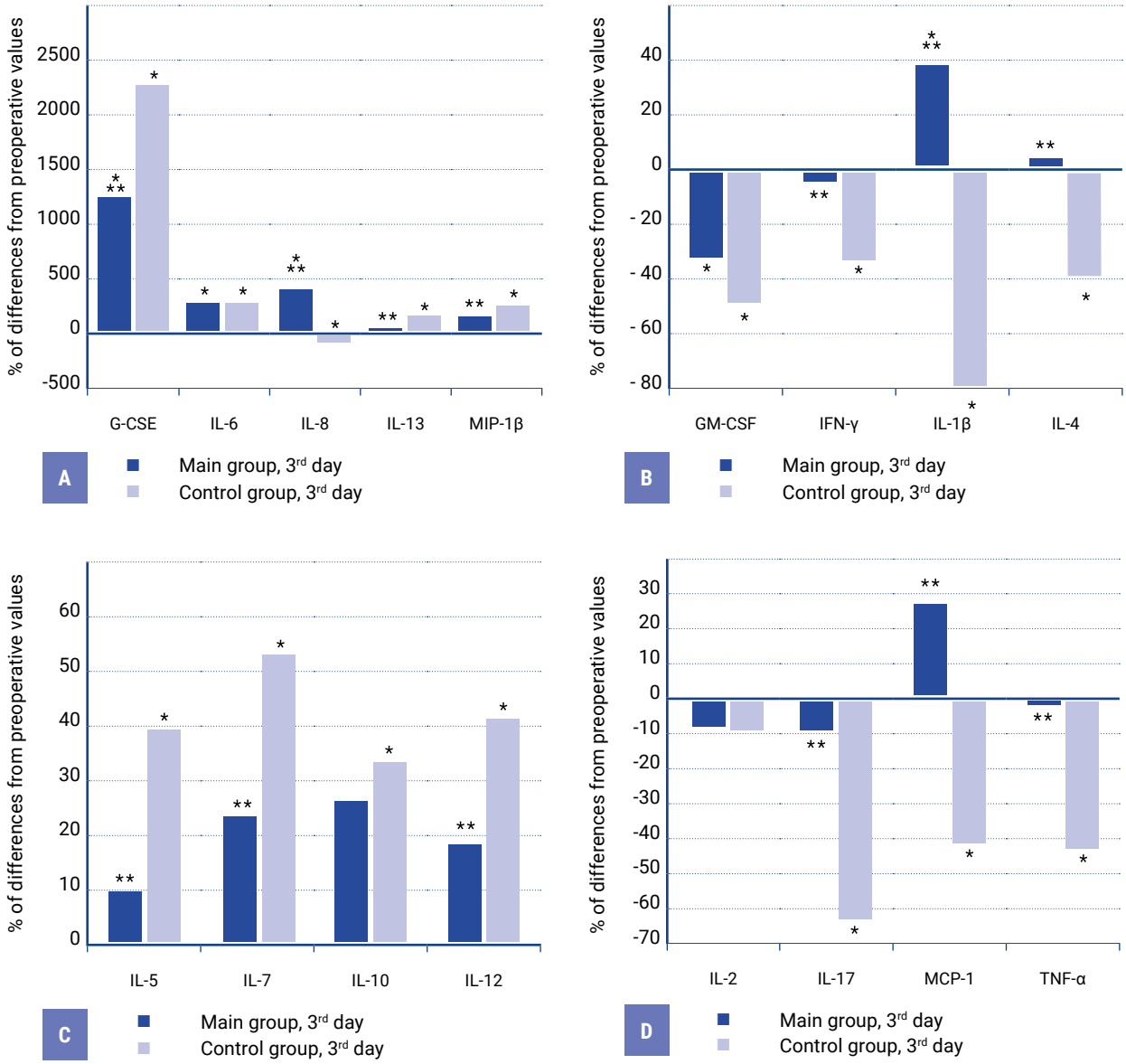

Fig. 1. The concentration of cytokines in the saliva of patients on the 3rd postoperative day.
Note: * – statistically significant differences compared to the level before surgery, $p < 0.05$; ** – statistically significant differences compared to the level of the control group, $p < 0.05$

pg/ml, respectively; for both cytokines, the differences between the indicators of the main and control groups are statistically significant ($p < 0.05$). The dynamics of IL-2 levels in the form of an increase was detected only in the control group on day 7 (Me 29.6 [26.9; 30.7] versus 21.4 [17.4; 25.5] pg/ml before treatment, $p < 0.05$), (Fig. 2B).

A decrease in IL-12 levels occurs in both groups on day 7 (Fig. 2B), and it is less pronounced in the main group than in the control group (Me 11.7 [10.6; 12.8] and 7.9 [7.2; 9.3], respectively, versus 14.7 [13.5; 16.7] pg/ml before treatment; $p < 0.05$) only for the control group), which can be regarded positively, given the role of this cytokine in the polarization of T-helper cells in Th1, providing cellular immunity.

IL-17 levels demonstrate different phases of changes: in the main group, a transient increase on the 3rd day with a further decrease below the initial one, and in the control group, a decrease on the 3rd day followed by a recovery on the 7th (Fig. 1G, 2G). The increase in the levels of cytokines polarizing

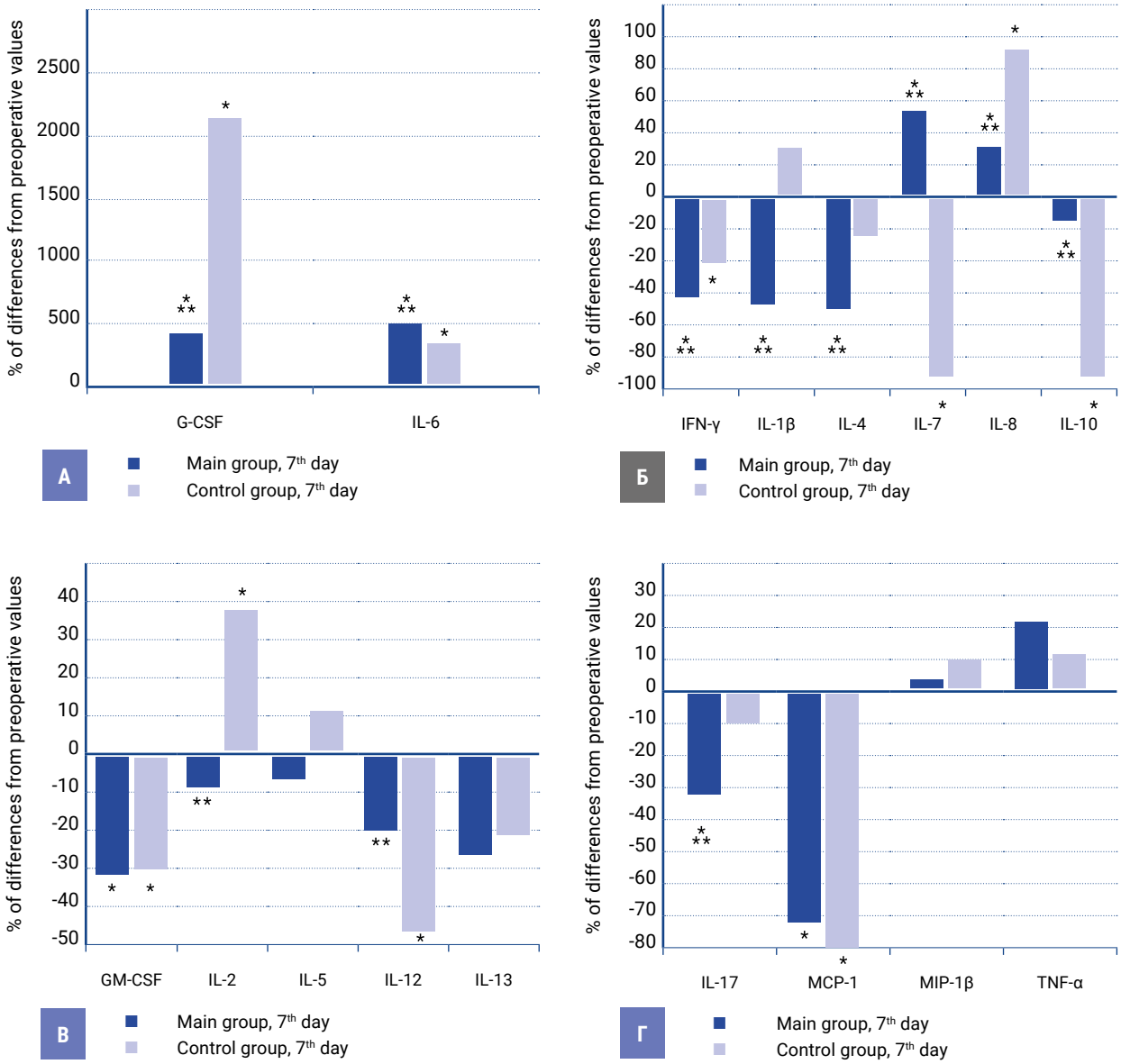

Fig. 2. The concentration of cytokines in the saliva of patients on the 7th postoperative day.
Note: * – statistically significant differences compared to the level before surgery, $p < 0.05$; ** – statistically significant differences compared to the levels in the control group, $p < 0.05$

CD4+ in Th2, IL-5 and IL-13 on day 3, noted only in the control group, is also transient. However, the level of IL-5 in both study periods in the main group was lower than in the control group (Fig. 1B, 2b): on day 3, 136.3 [130.2; 144.4] versus 173.6 [168.7; 176.8] pg/ml, on day 7, 117.4 [106.6; 125.2] versus 138.5 [131.7; 147.2] pg/ml, respectively, at both periods $p < 0.05$), which, taking into account the data described in the literature on its association with the recurrence of some tumors [16], is probably one of the positive aspects of the action of IPDT.

The importance of chronic inflammation and its accompanying cytokine production in tumor growth is known. The pro-oncogenic role of most known cytokines in neoplasia has been repeatedly described, realized through the activation of various transcription factors and signaling pathways, stimulation of proliferation, neoangiogenesis, epithelial-mesenchymal transition, and other processes [4]. However, it seems difficult to distinguish the importance of cytokines for malignant growth and for the immune response, of which they are an integral component, as a result of which their antitumor activity is carried out. Probably, a lot depends on their number, ratio, expression of receptors to them, etc. Unlike chronic, acute inflammation, moreover, is not microbial, but induced by a physical factor such as PDT, apparently exhibits an inhibitory effect on tumor growth both directly by destroying its cells and indirectly through the immune system [15]. In this regard, PDT-induced hyperproduction of cytokines in the lesion, especially after removal of the tumor, not only contributes to its sanitation, accelerating wound cleansing and healing, but also may have positive long-term consequences associated with an increase in the recurrence-free period due to the destruction of residual tumor cells and induction of an immune response to DAPMs. Our results partially confirm this. Oxidative stress caused by PDT stimulates the release of proinflammatory cytokines into the tumor microenvironment, which also affects their content in saliva. At the beginning of PDT-induced inflammation, tumor vessels become permeable to adhesion proteins (ICAM 1, VCAM 1, selectins), thus contributing to massive infiltration of the tumor by immune cells producing a wide range of cytokines, of which the literature emphasizes the importance of TNF-α, IL-6, IL-1β, as well as heat shock proteins, metabolites of arachidonic acid [17]. In this regard, cytokines that have a pro-inflammatory effect, in particular, the ability to increase vascular permeability, and are usually considered pro-oncogenic due to their stimulating effect on neoangiogenesis, have a positive value in PDT for the realization of its effect. The comparative dynamics of saliva cytokines obtained by us in patients of the main and control groups suggests earlier peaks in IL-1β and IL-8 concentrations occurring on the 3rd day after intraoperative PDT and high local levels of TNF-α and IL-6, which persist throughout the follow-up period. On the contrary, anti-inflammatory cytokines, for example, IL-10 and TGF-β, suppress the effects of PDT [18]. It is known about the importance of IL-7 in inflammation that its local content increases due to the production of macrophages, dendritic cells, fibroblasts, and its target are T lymphocytes expressing a receptor for it. IL-7 stimulates mainly Th1 and Th17 lymphocytes mediating cellular immunity [19]. A recent review on the role of IL-7 in tumors indicated that it inhibits the growth of melanoma, enhances the action of IFN-γ, restores the activity of CD8+ T lymphocytes by reducing their expression of PD-1 [20]. Based on this, it can be assumed that the decrease in the level of this cytokine observed by us after a transient increase in the saliva of patients in the control group is prognostically unfavorable for the state of local cellular immunity, and, on the contrary, its stable increase in the saliva of patients receiving IPDT reflects stimulation of the T-cell link.

The role of granulocytes and related cytokines in the body of the tumor carrier is twofold. There are numerous literature data on the pro-oncogenic effect of neutrophils [21], as well as chemokines that attract them to the focus. Neutrophilic "traps" promote the survival of circulating tumor cells in the blood and tumor metastasis [22, 23]. Nevertheless, neutrophils, as participants in the generation of ROS, are given important importance in the action of PDT [24]. However, since G-CSF promotes the release of immature granulocytes with immunosuppressive effects (MDSC) from the bone marrow into the peripheral blood and tissues, we consider a lower level of it in our patients receiving IPDT compared with the control group as a positive moment.

CONCLUSION

Intraoperative use of PDT in patients with locally advanced oral cancer causes changes in the cytokine composition of saliva that develop during the first week after surgery, some of which can be associated with the resulting clinical effect, consisting in prolongation of the relapse-free period in such patients (RF Patent No. 2797433). However, further studies are required to clarify the role of cytokines in the antitumor effectiveness of the applied photodynamic effect in patients with locally advanced oral cancer.

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ORIGINAL ARTICLE

Status and molecular genetic parameters of papillomavirus infection: individual characteristics and associative links with clinical and morphological factors of cervical cancer

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ABSTRACT

Purpose of the study. Study of the characteristics of human papillomavirus (HPV) infection, comparison of HPV status, molecular and genetic parameters of HPV high risk (HR) with the clinical and morphological characteristics of cervical cancer.
Materials and methods. The study included 240 patients with morphologically verified cervical cancer stages I–III, in whom the presence of HPV DNA of 14 genotypes was examined before treatment; upon detection, viral load (VL), the presence and degree of DNA integration into the genome of the host cell were examined.
Results. A number of statistically significant associative relationships have been identified between the molecular and genetic parameters of HPV infection and clinical and morphological indicators of the tumor process, in particular the relationship of HPV-negative CC with age and stage of the disease; HPV infection with several genotypes and HPV genotype – with the histological type of tumor; VL – with age, stage and histological type of tumor. Significant associative connections have been established between the molecular genetic parameters of the virus itself: genotype and level of VL, genotype and integration of HPV DNA into the host genome, as well as a negative linear correlation between VL and the degree of integration.
Conclusion. The obtained data on the relationship between the molecular and genetic parameters of HPV infection and traditional prognostic factors can become the basis for further research on the development of prognostic models for the purpose of personalizing multimodal treatment programs.

Keywords: human papillomavirus (HPV), high carcinogenic risk (HCR), cervical cancer (CC), HPV genotype, multiple infection, viral load, HPV status, virus DNA integration into the cell genome

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Compliance with ethical standards: the work followed the ethical principles set forth in the World Medical Association Declaration of Helsinki, 1964, ed. 2013. The study was approved by the local Ethics committee of the A. F. Tsyb Medical Radiological Research Centre – Branch of the National Medical Research Radiological Centre of the Ministry of Health of the Russian Federation, Obninsk, Russian Federation (extract from the protocol of the meeting No. 103 dated 09/17/2015). All patients signed an informed consent to participate in the study

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3.1.6. Онкология, лучевая терапия
ОРИГИНАЛЬНАЯ СТАТЬЯ

Статус и молекулярно-генетические параметры папилломавирусной инфекции: индивидуальные особенности и ассоциативные связи с клинико-морфологическими факторами рака шейки матки

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РЕЗЮМЕ

Цель исследования. Изучение особенностей папилломавирусной (ВПЧ) инфекции, сопоставление ВПЧ-статуса, молекулярно-генетических параметров ВПЧ высокого канцерогенного риска (ВКР) с клинико-морфологическими характеристиками рака шейки матки (РШМ).

Материалы и методы. В исследование были включены 240 больных с морфологически верифицированным РШМ I–III стадий, у которых до начала лечения исследовали наличие ДНК ВПЧ 14 генотипов, при выявлении – вирусную нагрузку (ВН), наличие и степень интеграции ДНК в геном клетки-хозяина.

Результаты. Выявлен ряд статистически значимых ассоциативных связей между молекулярно-генетическими параметрами ВПЧ-инфекции и клинико-морфологическими показателями опухолевого процесса, в частности связь ВПЧ-негативного РШМ с возрастом и стадией заболевания; ВПЧ-инфицирования несколькими генотипами и генотипа ВПЧ – с гистологическим типом опухоли; ВН – с возрастом, стадией и гистологическим типом опухоли. Установлены значимые ассоциативные связи между молекулярно-генетическими параметрами самого вируса: генотипа и уровня ВН, генотипа и интеграции ДНК ВПЧ в хозяйский геном, а также отрицательная линейная корреляция между ВН и степенью интеграции.

Заключение. Полученные данные о взаимосвязи молекулярно-генетических параметров ВПЧ-инфекции с традиционными прогностическими факторами могут стать основой для дальнейших исследований по разработке прогностических моделей с целью персонализации мультимодальных лечебных программ.

Ключевые слова: вирус папилломы человека (ВПЧ), высокий канцерогенный риск (ВКР), рак шейки матки (РШМ), генотип ВПЧ, множественная инфекция, вирусная нагрузка, ВПЧ – статус, интеграция ДНК вируса в клеточный геном

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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 локальным этическим комитетом МРНЦ им. А. Ф. Цыба – филиал ФГБУ «НМИЦ Радиологии» Минздрава России (выписка из протокола заседания № 103 от 17.09.2015 г.). Все пациенты подписали добровольное информированное согласие на участие в исследовании

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INTRODUCTION

Cervical cancer (CC) ranks first among malignant neoplasms of the female genital organs [1]. Annually, more than 600 thousand new cases are detected in the world and about 342 thousand deaths from this pathology are registered [2]. In the Russian Federation, breast cancer is the leading cause of death from cancer in the female population aged 30–39 years (21.5 %) [3].

Human papillomavirus (HPV) of high carcinogenic risk (HCR) is a proven factor in the development of breast cancer [4]. Among the total number of patients with breast cancer, 88–95 % are HPV-positive, according to various authors [5, 6]. The most common HPV genotypes, according to most literature sources, are types 16 and 18, which are collectively detected in almost 75–85 % of cases of HPV-positive breast cancer [6–9]. In 2020, the World Health Organization introduced a new classification of cervical epithelial tumors based on the presence/absence of HCR HPV, the so-called HPV status [10, 11]. The cited sources indicate that HPV-negative status is an indicator of an unfavorable prognosis of the effectiveness of treatment, but, as noted above, the proportion of such patients is small, which dictates the need to search for prognostic markers in the majority other breast cancer patients with HPV-positive status. It is known that HPV infection is characterized by significant diversity at the molecular genetic level, and, importantly, some of its parameters can affect the sensitivity of tumor cells to antitumor effects (according to the results of studies on cell cultures *in vitro*). In this regard, it could be assumed that studying the features of HPV infection in cervical cancer can provide additional information for stratification of patients in a prognostic aspect, will allow to personalize multimodal treatment programs for breast cancer and, ultimately, improve the effectiveness of treatment.

Data on the relationship between the clinical and morphological characteristics of breast cancer and the molecular genetic parameters of HPV infection are widely presented in the literature. The authors report the presence of an association between HPV status and lymphovascular invasion [12], HPV HCV genotypes and the morphological form of the tumor, the relationship of HPV type 18 with the presence of deep stromal invasion and lymph node damage [13]. Some researchers pay attention to a statistically significant relationship between high viral load (VL) and the risk of metastatic lymph node damage, tumor size [14], others – to the correlation of low VL with the stage of the disease and enlarged lymph nodes [15]. However, the heterogeneity of the samples with the lack of a comprehensive assessment of the relationship of the entire spectrum of molecular genetic parameters of HPV infection with prognostically known clinical and morphological factors often determines the contradictory nature of the data obtained and makes it relevant to further studies in homogeneous groups of patients with breast cancer with the inclusion of the maximum number of criteria studied.

MATERIALS AND METHODS

The study on the topic of HPV infection features, the comparison of HPV status, molecular genetic parameters of HPV HCR with the clinical and morphological characteristics of the tumor process was performed in 240 patients with morphologically verified stage I–III breast cancer (FIGO) who underwent examination and treatment in the department of radiation and combined methods of treatment of gynecological diseases of the A. F. Tsyb Medical Radiological Research Centre – Branch of the National Medical Research Radiological Centre of the Ministry of Health of the Russian Federation, Obninsk, Russian Federation [16]. The study is a retrospective-prospective cohort, conducted in accordance with the protocol approved by the local ethics committee of the A. F. Tsyb Medical Radiological Research Centre – Branch of the National Medical Research Radiological Centre of the Ministry of Health of the Russian Federation, Obninsk, Russian Federation (Protocol No. 103 dated 09/17/2015). The ethical principles set forth by the Helsinki Declaration of the World Medical Association (World Medical Association Declaration of Helsinki, 1964, ed. 2013). Prior to inclusion in the study, the patients signed a voluntary informed consent to participate in the study and determine *in vitro* the parameters of HPV infection in the biomaterial of the cervix. The inclusion criteria were: morphologically verified stage I–III breast cancer, lack of specialized treatment for this disease; non–inclusion criteria – pregnancy, stage IV breast cancer, specialized treatment

for this disease in the anamnesis; exclusion criteria – refusal of patients from further participation in the study. The average age of the patients was 47.2 ± 12.0 years. Locally advanced forms of breast cancer (stages II and III of the disease) prevailed – in total in 186 (77.5 %) patients. According to the morphological structure of the tumor, squamous cell carcinoma of various degrees of differentiation was most often verified in patients – in 216 (90 %). According to the form of growth, endophytic and mixed prevailed, respectively, in 59 (24.6 %) and 136 (56.6 %) patients; according to the variant of the spread of the tumor process, parametric in various variations and metastatic, respectively, in 174 (93.5 %) and 66 (66.7 %) patients.

The presence of HPV DNA of 14 genotypes was studied in all 240 patients before treatment: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68. Biopsies and/or joint scrapings of the epithelium of the cervical canal (endocervix) and the outer wall served as the material for the study cervical surfaces (exocervix) taken before the start of treatment. All stages of the subsequent analysis of the obtained biomaterial samples were performed on domestic test systems produced by the Federal State Budgetary Institution of the Central Research Institute of Epidemiology of Rospotrebnadzor. DNA isolation was carried out by the sorbent method using a set of reagents "DNA-sorb-AM". The presence, differentiated determination of the genotype and quantitative load of HPV was carried out by multiplex PCR with the detection of a fluorescent signal over four channels in real time on the Rotor Gene amplifier (Corbett Research, Australia) using the reagents "HPV Amplification HCR genotype-titer FL". In this test system, the viral genes E1, E6, E7 and the β-globin cellular gene are amplified. Only data for samples with a positive result of β-globin analysis are considered valid. This gene serves as an internal control of the reaction (EQ), and also allows you to estimate the number of cells in a sample (1 cell contains 2 β-globin molecules) and normalize the results of amplification of viral genes for the same number of cells. The results of the study were processed in the Excel software add-in attached to the test system and interpreted in accordance with the following criteria: a) logarithm (lg) of the number of HPV DNA copies per 105 cells less than 3 (VL < 3) – low viral load; b) lg of the number of copies of HPV DNA per 105 cells is equal to or more than 3, but less than 5 (3 ≤ VL < 5) – moderate viral load; c) lg of the number of copies of HPV DNA per 105 cells is more than or equal to 5 (VL ≥ 5) – high viral load. In case of multiple infection, the quantitative load of all established HPV HCV genotypes was determined, the highest indicators corresponded to the leading genotype of the virus.

The presence of HPV DNA integration was assessed by the ratio of the number of genomic equivalents of the E7/E2 virus, taking into account the standard deviation and the coefficient of variation of the data in accordance with the developed algorithm [17]. Its principle is based on the fact that the E7 gene remains intact during the integration of viral DNA into the DNA of the host cell, respectively, its amount in both forms of the virus – episomal and integrated – is the same. In most cases, the E2 gene is destroyed during integration and its amount decreases. The analysis was performed using TagMan technology in real-time multiplex PCR format using a set of reagents that allows differentiated determination of the number of E2 and E7 viral genes and the β-globin cellular gene. In one test tube, sections of the E7 and E2 virus genes and a section of human β-globin DNA, ICS, were amplified. At the same time, standard samples with known concentrations of HPV 16 and 18 DNA and β-globin DNA were amplified in each experiment. All samples, both clinical and standard, were amplified in three repeats. For each of the repeats, the amount of E7 and E2 was calculated using calibration curves and a regression equation obtained on standard samples in accordance with the program for amplification of these genes. The degree of HPV DNA integration was estimated by the formula (1 – E2/E7) × 100 %. The absence of an amplification signal for the E2 gene in the presence of such a signal for the E7 gene corresponds to 100 % integration of viral DNA into the cell genome.

Statistical data processing was performed using the Statistica 10.0 software package (StatSoft, Inc.). For descriptive statistics, average values and standard error (SE) were used. The comparison of groups by qualitative characteristics was carried out using the Fisher criterion, by quantitative characteristics – using the Mann-Whitney U-test. Spearman's nonparametric correlation method with the calculation of the rank correlation coefficient (r) was used to evaluate

the linear relationships between variables. Multivariate analysis was performed using the Agglomerative clustering (AGNES) method with the construction of tree diagrams – dendrograms.

STUDY RESULTS AND DISCUSSION

HPV status and genotype

The presence of HPV HCR was registered in the overwhelming number of patients in the study cohort – in 215 (89.6 %) out of 240. The average age of HPV-infected patients with breast cancer was 46.7 ± 11.8 years, which is much lower than in Europe (54 ± 14 years) [9]. The average age of patients in whom HPV HCR was not detected was 50.6 ± 14.0 years and did not differ from that of HPV-infected patients ($p > 0.05$). However, HPV-negative breast cancer was 3.5 times more common among patients over 55 years of age ($p = 0.004$) (Fig. 1), which is consistent with data from other studies [18, 19]. There was a statistically significant increase in the frequency of HPV-negative forms of the disease at stage III (18.2 %) compared with stages II (3.4 %) and I (7.4 %) (respectively $p = 0.001$ and $p = 0.05$), as mentioned by domestic researchers [18].

Among all the genotypes found in patients with breast cancer, prevailed 16 (62,6 %), 18 (13 %) and 45 (6.1 %) types of HPV HCR, followed by 31 (4,1 %), 33 (2,8 %), 39 and 56 types (2.5 % each) (Fig. 2). The total proportion of other types of HPV HCR (35, 51, 52, 58, 59, 66, 68) It was 6.4 %. A similar share distribution in the countries of the European region, in particular the Russian Federation, is reported in numerous publications, which also indicate the prevalence of HPV genotypes 16 and 18 in 70–75 % of cases [18–20]. In the study group of 215 HPV-positive patients, genotypes or their combinations with the dominant genotype belonging to the phylogenetic group A9 were most often found (16, 31, 33, 35, 52, 58) – in 76.7 % of cases. The share of representatives of the A7 group (18, 39, 45, 59) was more than 3.4 times lower – 22.3 %. The remaining 2 groups A5 (51) and A6 (56, 66) were represented in isolated cases (0.5 %). The peak occurrence of group A9 genotypes occurred at a young age – up to 30 years (78.6 %), and A7 – in the age category up to 45 years (31.3 %), however, without statistically significant differences, which is consistent with the results of multifactorial analysis [21], although some studies demonstrate the presence of a link between the HPV genotype and the age of patients with breast cancer [9].

In squamous cell carcinoma, the prevalence of genotypes of group A9 (80.0 %) ($p = 0.0002$) with the dominance of HPV 16 (74.3 %) ($p = 0.0002$) was noted; in adenocarcinoma, groups A7 (66.7 %) ($p = 0.0003$) with the predominance of HPV 18 (60.0 %) ($p < 0.0001$). HPV type 16 (86 %) was sig-

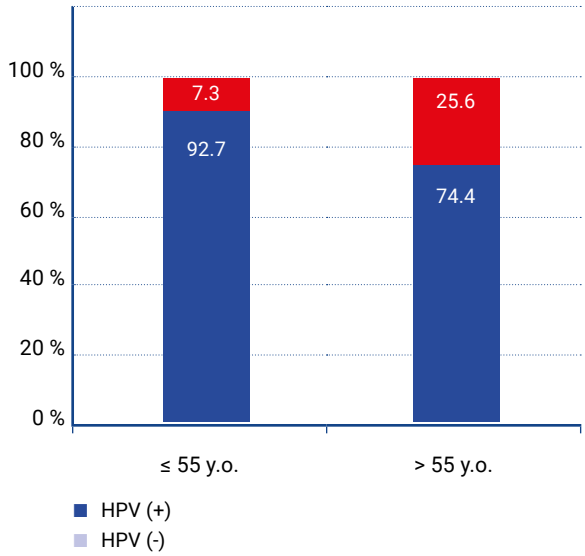

Fig. 1. Features of high risk HPV infection in patients with breast cancer, depending on age

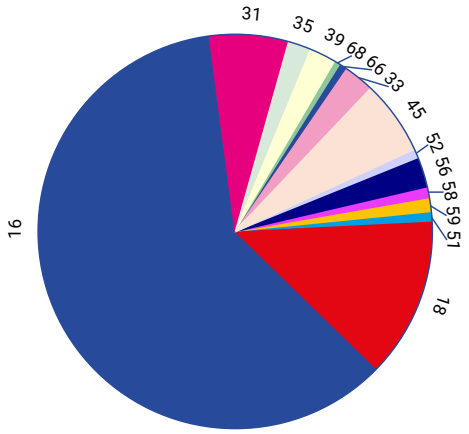

Fig. 2. Prevalence of 14 high risk HPV genotypes in patients with breast cancer, including cases of multiple infection

nificantly more common among HPV 16/18-associated squamous cell carcinomas, and HPV type 18 (64.3 %) in adenocarcinoma (p = 0.0001). A similar associative relationship of phylogenetic groups and, accordingly, genotypes with the histological type of tumor has been revealed in other studies [9, 22]. The distribution of the most common phylogenetic groups (A9 and A7) did not significantly differ depending on the stage of the disease, the form of tumor growth, and in patients with locally advanced breast cancer, including the variant of the spread of the tumor process (presence/absence of infiltration of parametria, metastatic variant) (p > 0.05), which is also confirmed by the results of other research [23].

Infection with several types of HPV HCV (multiple infection) was detected in 25 (11.6 %) of 215 HPV-infected patients (19–2 genotypes, 6–3 genotypes). There were no statistically significant differences in the incidence of single or multiple HPV infection depending on age, stage of the disease, form of growth and variant of tumor spread, which is confirmed in the study by N. Jing et al. (2003) [24]. However, it should be noted that multiple infection occurred only in patients with morphologically verified squamous cell carcinoma (p < 0.0001 when compared with the glandular morphotype of the tumor, p = 0.038 when compared with undifferentiated cancer), this pattern was noticed by other researchers [25].

Viral load

Viral load was determined in 199 HPV-positive patients with stage I–III breast cancer, 175 (87.9 %) of them with a single infection, 24 (12.1 %) cases with multiple infection. In the study group, high VL was most often observed (average level 6.4 ± 1.3) – in 142 (71.4 %) cases. In 50 (25.1 %) patients, VL was moderate (average level 4.4 ± 0.54), and only in 7 (3.5 %) it was low (average level 2.4 ± 0.1). When comparing the data on VL obtained during the processing of various biological materials – cervical scrapings and biopsies of the same patients (n = 47) – a fairly high correlation of these indicators was revealed among themselves (r = 0.72, p < 0.0000001) (Fig. 3).

There was a statistically significant increase in the proportion of cases with low VL with increasing age (r = 0.86, p = 0.04), and no cases of low VL were detected in the age group under 30 years (Fig. 4).

In HPV 16, high virus load was most common, and in HPV 18, moderate and high loads were observed with almost the same frequency (Fig. 5): the average VL level in HPV 16 (6.0 ± 1.7) turned out to be statistically significantly higher than the same indicator in HPV 18 (5.0 ± 1.1) (p < 0.001). This pattern was maintained for the phylogenetic groups to which these genotypes belonged: 6.0 ± 1.6 and 4.9 ± 1, respectively, for the genotypes of the A9 and A7 groups (p < 0.001).

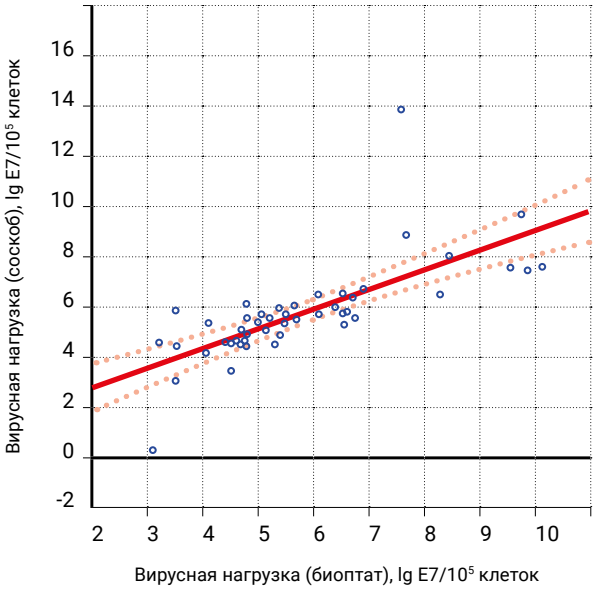

Fig. 3. Analysis of the correlation dependence of the HPV viral load in epithelial samples and corresponding biopsies of the cervix of patients with CC

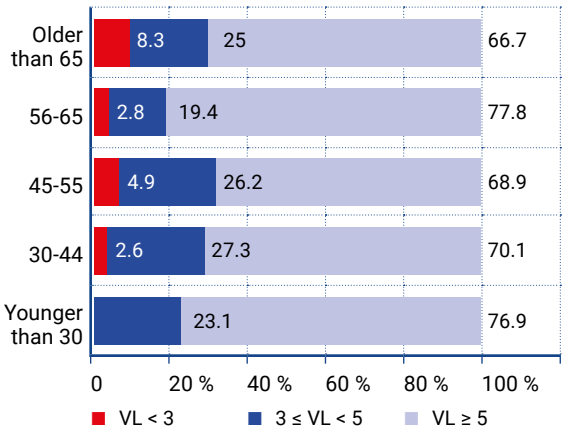

Fig. 4. Viral load (VL) in patients with CC depending on age

In stage III of the disease, the average VL level (6.2 ± 1.6) was significantly higher than in stage I (5.4 ± 1.9) and II (5.4 ± 2.1), respectively, $p = 0.006$ and $p = 0.02$. Our data are consistent with the latest results of domestic studies on relatively low VL in the early stages of the disease [19, 26].

In squamous cell carcinoma, more cases of high VL (73.9 %) ($p = 0.08$) were detected, and in adenocarcinoma – low load (13.3 %) ($p = 0.07$) (Fig. 6). Accordingly, the average level of VL was higher in squamous cell carcinoma (5.8 ± 1.6) compared with adenocarcinoma (5.0 ± 1.6) ($p = 0.10$). The relationship of low load with cervical adenocarcinoma and HPV type 18 is also noted by other authors [27].

According to our data, there were no statistically significant differences in the level of VL in different forms and variants of the spread of the tumor process.

HPV DNA Integration 16/18

The presence of virus DNA integration, both complete and partial, was studied in patients infected with HPV types 16 and 18 (140 and 28 cases, respectively), which are the most aggressive and account for the vast majority of all genotypes detected in breast cancer. Such patients accounted for 78.1 % of all HPV-positive cases in our study. In the studied cohort, the majority of patients revealed the integration of virus DNA (integrated form) – in 101 out of 168 people (60.1 %), which confirms the results of a number of studies on the high incidence of invasive PCV virus in the integrated state [26, 28]. In the remaining 67 (39.9 %) patients, there was a lack of integration of HPV DNA into the cellular genome (episomal form according to the criterion of preserving the E2 gene in an intact state). It should be noted that the failure to integrate HPV DNA into the genome of the host cell in accordance with the algorithm described above cannot be unambiguously interpreted as the presence of only the episomal form of the virus, since such integration can occur with the participation of various other viral genes [29–30]. However, this process is mainly associated with a violation of the integrity of the E2 gene of the virus [31], which is explained by the high availability of this viral gene for various types of genetic rearrangements. Moreover, we have obtained data suggesting a higher biological significance of the E2-mediated pathway of integration of the viral genome into the cellular one, as opposed to integration invoVLing other viral genes [32].

A comparative analysis of the data on the degree of integration of viral DNA in scrapings and biopsies of the cervix obtained from the same patients ($n = 47$) revealed a fairly high correlation of the indicators with each other: the correlation coefficient $R = 0.89$ at a significance level $p < 0.000001$ (Fig. 7).

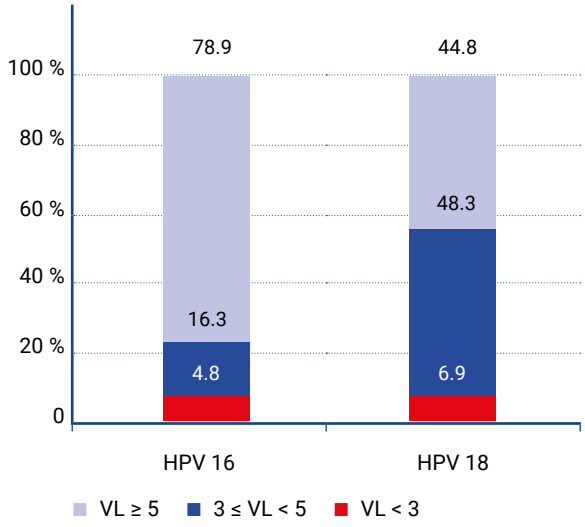

Fig. 5. Viral load (VL) in HPV-positive patients with CC, depending on the genotype of the virus

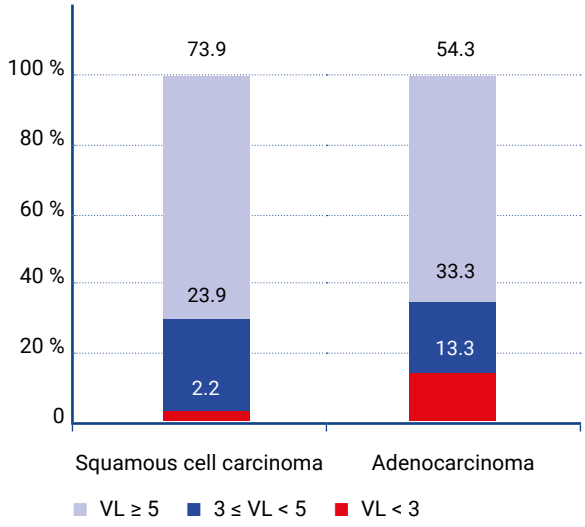

Fig. 6. Viral load (VL) in HPV-positive patients with CC depending on the morphological form of the tumor

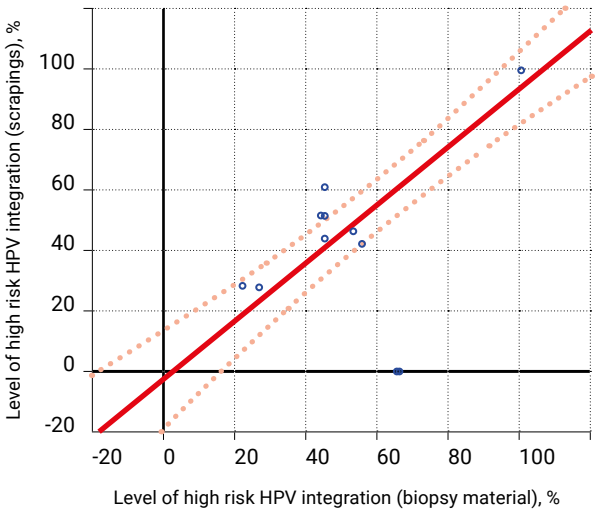

Fig. 7. Analysis of the correlation dependence of the degree of high risk HPV integration in epithelial scrapings and corresponding biopsies of the cervix of patients with cervical cancer. The degree of integration varies from 0% (the episomal form of the virus) to 100 % (full integration of viral DNA into the cellular genome). The intermediate values correspond to the mixed form of high risk HPV– the presence of both episomal and integrated forms; the quantitative indicator – the degree of integration corresponds to the proportion of integrated forms of high risk HPV

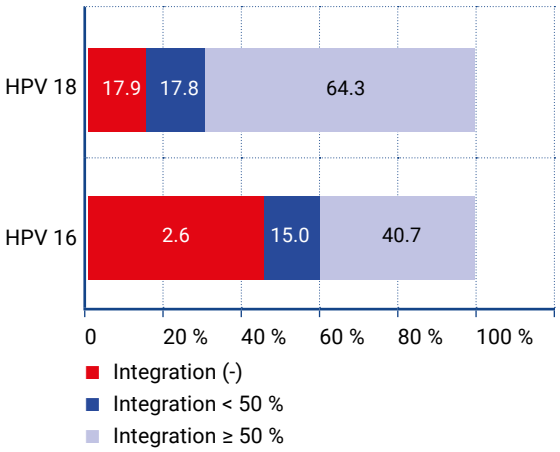

Fig. 8. Physical status and degree of integration of HPV 16 and HPV 18 DNA in patients with CC

Table 1. Distribution of CC patients depending on the qualitative and quantitative parameters of HPV 16/18

Viral form	Episomal abs (%)	Integrated abs (%)	
Viral load		< 50 %	≥ 50 %
VL < 3 (*n* = 5)	1 (20.0)	0	4 (80.0)
3≤ VL < 5 (*n* = 39)	11 (28.2)	5 (12.8)	23 (59.0)
VL ≥ 5 (*n* = 124)	55 (44.4)	21 (16.9)	48 (38.7)

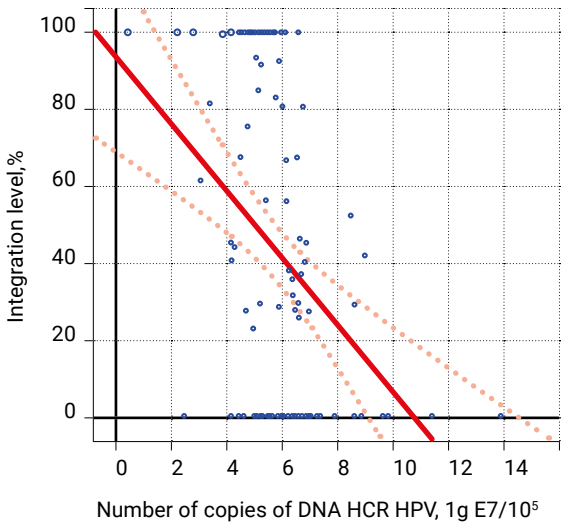

Fig. 9. Correlation analysis of the molecular genetic parameters of HPV infection in CC patients (n = 168): 0 % – lack of integration (episomal form of the virus), 100 % – complete integration of HPV DNA into the genome of the host cell. The intermediate values correspond to the mixed form of HCR HPV – the presence of both episomal and integrated forms; the quantitative indicator – the degree of integration – corresponds to the proportion of integrated forms of HPV 16/18

Taking into account these data, as well as similar results of a comparative analysis of HCV, it is possible to recommend the use of scraping of the cervical epithelium for the molecular genetic study of HPV parameters, since the informative value of the material obtained by this method is not inferior to the informative value when performing a more traumatic procedure – cervical biopsy.

The integrated form of HPV HCR was most common in patients over 65 years of age – in 66.7 % of cases, while in 44.4 % of cases it was in the form of complete (100 %) integration. When infected with HPV 18, compared with HPV 16, integrated forms of the virus prevailed (82.1 % and 55.7 %, respectively, p = 0.01) with a predominance of highly integrated (DNA integration ≥ 50 %) forms (64.3 % and 40.7 %, respectively, p = 0.019), a high percentage of which was full (100 %) integration HPV DNA (50.0 % vs. 20.7 %, p = 0.003) (Fig. 8). The more frequent detection of HPV type 18 in the integrated state compared with HPV type 16 is also reported in foreign studies [33].

The analysis of the presence/absence and degree of integration depending on clinical and morphological characteristics did not reveal statistically significant associative relationships, which is consistent with the literature data [34].

Associative relationship of viral load and HPV DNA status 16/18

The molecular genetic parameters of viral infection were studied in 168 HPV 16/18-positive patients with stage I–III breast cancer. As the HCV increased, there was an increase in the proportion of episomal and a decrease in the proportion of highly integrated forms of the virus (Table 1).

Low viral load only in a single case (20.0 %) accompanied the liposomal form of the virus; all other cases of low viral load (80.0 %) were combined with 100 % integration. Previously, we had established an inverse linear correlation between VL and the degree of integration of HPV DNA into the cellular genome [35]. Subsequently, the sample of patients was significantly increased, and this pattern remained with high significance (r = – 0.41, p < 0.0001) (Fig. 9).

Multivariate analysis

In order to study possible associative relationships between various parameters characterizing the tumor process and HPV infection, a multidimensional exploratory analysis was performed using the clustering method, which allowed us to identify the most interrelated parameters – the morphological form of the tumor and HPV status (Fig. 10), and in HPV-positive breast cancer – the morphological form

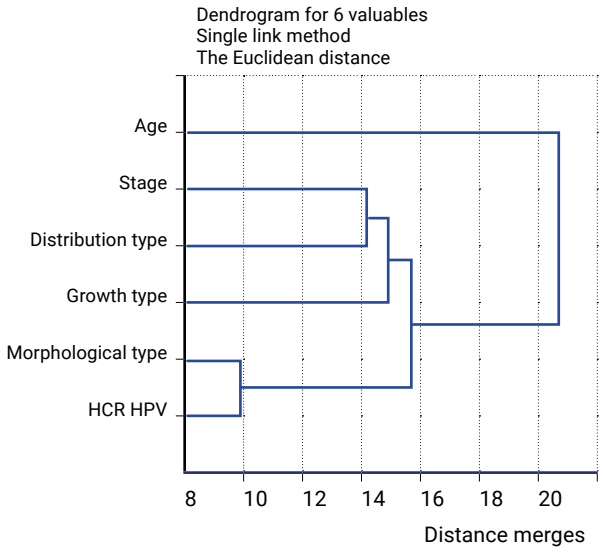

Fig. 10. Dendrogram of CC patients with HPV status (n = 240)

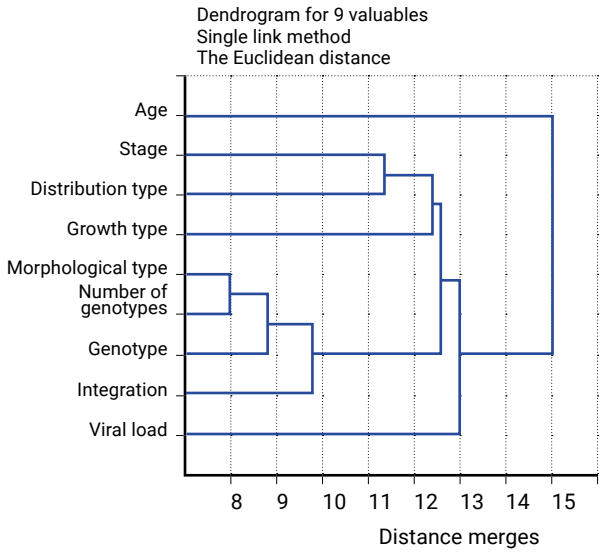

Fig. 11. Dendrogram of HPV-associated CC patients taking into account the entire spectrum of molecular genetic parameters of HCR HPV (n = 174)

of the tumor and the following features of HPV infection: the number of HPV HCV genotypes present, genotypes 16 and 18, the physical status of viral DNA – the presence/absence of integration into the genome of the host cell (Fig. 11).

Thus, multifactorial exploratory analysis made it possible to detect associative relationships that were not obtained by pairwise comparison of various factors, but which could be assumed indirectly when studying the results of a single-factor analysis.

CONCLUSION

The study of possible associative relationships between a wide range of molecular genetic parameters of HPV infection and the clinical and morphological characteristics of a malignant tumor of the cervix revealed the presence of correlations between HPV status, HPV genotype, the number of genotypes present and a known prognostic factor – the morphological form of cervical cancer. At the same time, our work shows the absence of a relationship between such molecular genetic parameters of HPV infection as the genotype and the level of integration of virus DNA into the cellular genome with the main traditional factor in predicting the effectiveness of treatment – the stage of the disease. This fact suggests the possibility of a prognostic value of these parameters independent of the stage and justifies the expediency of conducting further studies to assess the prognostic value of the level of integration of HPV DNA of various genotypes (primarily the most common types 16 and 18) as potential independent biomarkers for predicting the effectiveness of treatment of breast cancer.

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