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ORIGINAL ARTICLE

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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.)

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 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

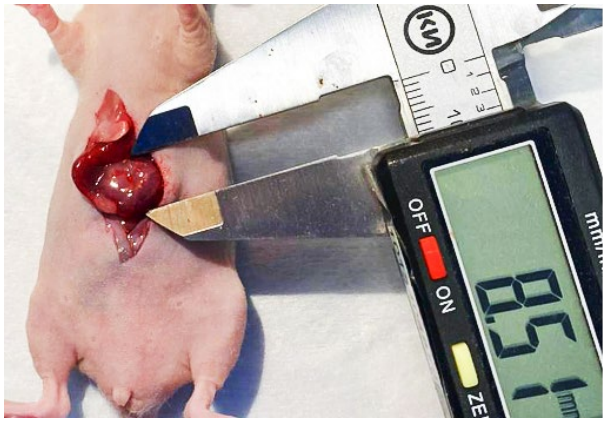

Fig. 1. Measurement of a tumor in the liver of a mouse

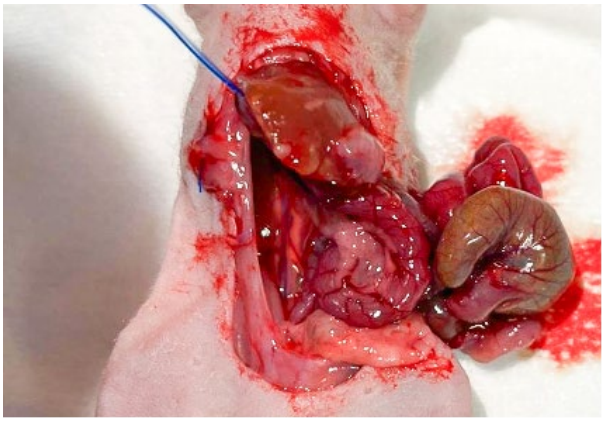

Fig. 2. The process of performing liver blood flow reduction by occlusion of the portal triad to induce hypoxic conditions

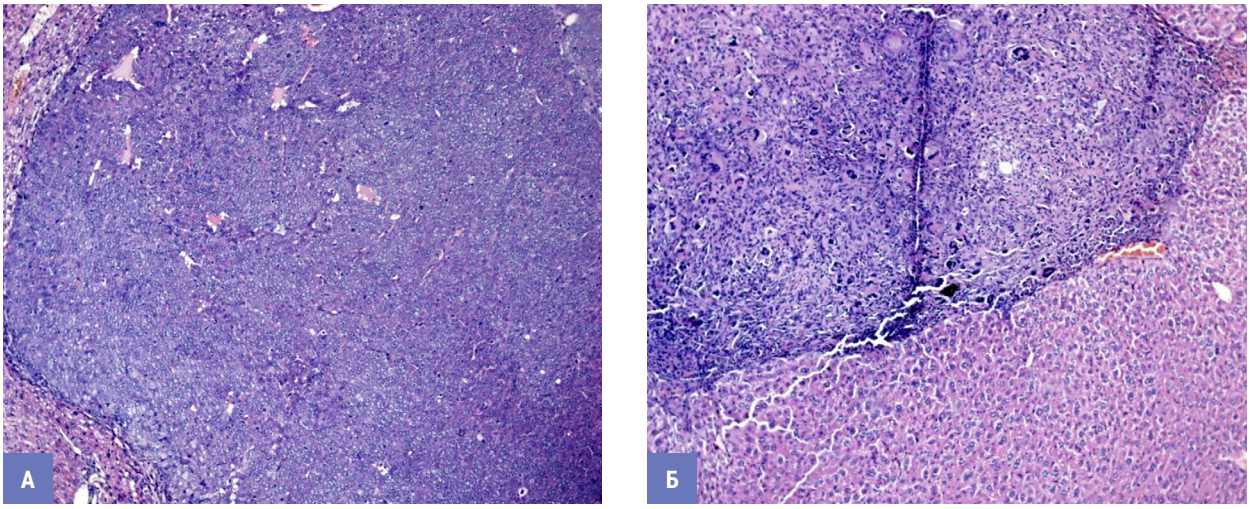

Fig. 3. Histological specimen: morphological picture of hepatocellular carcinoma. A – without hypoxia; B – after hypoxia. Magnification × 100

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-

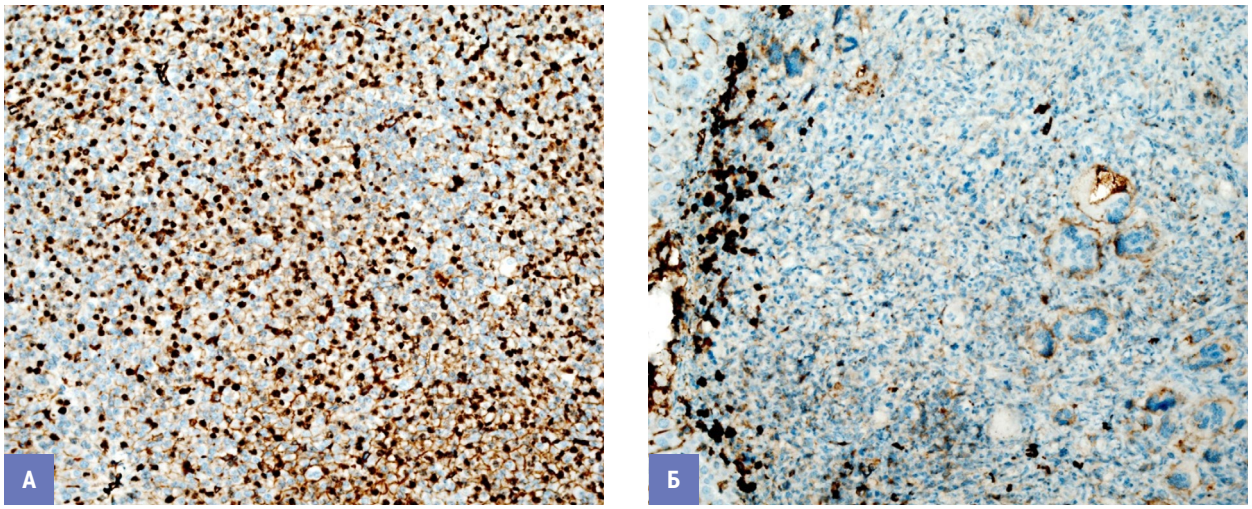

Fig. 4. IHC reaction of the tumor to Ki-67 antibodies (clone SP6). A – without reduction of liver blood flow; B – after reduction of liver blood flow by occlusion of the portal triad. Magnification × 200

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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