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Южно-Российский онкологический журнал

Журнал входит в рекомендованный ВАК РФ перечень рецензируемых научных журналов и изданий для опубликования основных научных результатов диссертаций на соискание учёной степени кандидата и доктора наук.

«Южно-Российский онкологический журнал»: профессиональное медицинское издание. В нем публикуются новости медицинского и фармацевтического сообществ, научно-практические статьи для целевой аудитории – врачей-онкологов. Редакция журнала ставит своей задачей популяризацию научно-исследовательских работ и достижений онкологов Южного федерального округа, анализ процесса глубокой реорганизации здравоохранения в России. Редакция приглашает в качестве авторов всех, кто ищет и находит интересные решения многогранных задач, стоящих перед современной медициной, и хочет поделиться своими мыслями и наблюдениями с коллегами.

Цель: способствовать развитию онкологической медицины Юга России и внедрению её достижений в практику.

Задачи: освещать современные достижения онкологической службы Юга России; содействовать обмену опытом и передовыми знаниями между специалистами; информировать читателей об итогах крупных медицинских форумов.

В журнале размещаются публикации различных рубрик: обзоры литературы, мета-анализы, клинические исследования, наблюдения клинических случаев, обсуждения, анонсы и описания новых методов лечения.

Журнал принимает к публикации: оригинальные статьи, организации здравоохранения, лучевой диагностики, обмен опытом, обзоры, клинические наблюдения.

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Purpose: to promote the development of cancer medicine in the South of Russia and the introduction of its achievements into practice.
Tasks: to highlight the current achievements of the oncology service in the South of Russia; to promote the exchange of experience and advanced knowledge between specialists; to inform readers about the results of major medical forums.
The journal contains publications of various categories: literature reviews, meta-analyses, clinical studies, observations of clinical cases, discussions, announcements and descriptions of new treatment methods.
The journal accepts for publication: original articles, health organizations, radiation diagnostics, exchange of experience, reviews, clinical case reviews.

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ORIGINAL ARTICLE

OWN EXPERIENCE OF SURGICAL TREATMENT FOR ADVANCED CANCER OF THE TONGUE AND THE MOUTH FLOOR

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ABSTRACT

Purpose of the study. Improvement of surgical treatment outcomes in patients with advanced cancer of the tongue and the mouth floor providing radical surgery with preservation of the organ functions.
Materials and methods. Two patients with advanced cancer of the tongue and the mouth floor with metastases to lymph nodes in the neck (St.4 (IVA, pT4a N2b M0), clinical group 2, were operated on according to our special technique.
The surgery was performed under endotracheal anesthesia. After cervical lymph node dissection, the tongue and the mouth floor tissues were resected intraorally. The incisions were made through their entire thickness along healthy tissues. Smears were taken from the dissected tissues for intraoperative pathology consultation control for the presence of cancer cells. The tissues of the mouth floor affected by the tumor were completely removed without going beyond the hyoglossus muscle, since the lingual and hypoglossal nerves go along its outer surface. This allowed radical tumor removal with preservation of the tongue functions.
Results. Patients operated on according to our special technique have been observed for more than 9 months without continued tumor growth and recurrences tumor with preservation of the tongue and the mouth floor functions.
Conclusion. In such patients, ablastics principles are combined with the preservation of the tongue functions. This can be achieved because after removal of the tongue tumor, resection of the mouth floor is performed without going beyond the hyoglossus muscle not affected by the tumor, since the lingual and hypoglossal nerves go along its outer surface. Complying with ablastics, it preserves the tongue functions: chewing, swallowing, articulate speech, taste perception.

Keywords:
advanced cancer, oral organs, mandibulotomy, orostoma, lingual and hypoglossal nerves, hyoglossus muscle

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ОРИГИНАЛЬНАЯ СТАТЬЯ

СОБСТВЕННЫЙ ОПЫТ ХИРУРГИЧЕСКОГО ЛЕЧЕНИЯ РАСПРОСТРАНЕННОГО РАКА ЯЗЫКА И ДНА ПОЛОСТИ РТА

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РЕЗЮМЕ

Цель исследования. Улучшить результаты хирургического лечения больных с распространенным раком языка и дна полости рта обеспечевающего радикальное проведение операции с сохранением функций оперируемых органов.
Материалы и методы. Двое больных с распространенным раком языка и дна полости рта и метастазами в лимфатические узлы шеи St.4 (IVA, pT4a N2b M0) клиническая группа 2, были прооперированы по разработанной нами методике. Операцию проводили под эндотрахеальным наркозом. Сначала осуществлялась шейная лимфодиссекция, далее интраоральным способом резецировался язык с тканями дна полости рта. Разрезы проводились через всю толщу по здоровым тканям. С рассеченной раневой поверхности языка изымались ткани для срочного гистологического исследования – контроля на наличие раковых клеток. Пораженные опухолью ткани дна полости рта полностью удалялись, не выходя за пределы подъязычно-язычной мышцы, по наружной поверхности которой проходят язычный и подъязычный нервы. Это позволяет радикально убрать опухоль с сохранением функций языка.
Результаты. Больные, прооперированные по разработанной методике, находятся под наблюдением без продолженного роста и рецидива опухоли более 9 месяцев, с сохранением функций языка и дна полости рта.
Заключение. У данной категории больных принципы абластики сочетались с сохранением функций языка. Это достигалось тем, что после удаления опухоли языка, резекция дна полости рта проводилась в пределах здоровых тканей сохраняя подъязычно-язычную мышцу, на внешней стороне которой проходят язычный и подъязычный нервы. Тем самым, при соблюдении абластики, сохраняются функции языка: жевание, глотание, членораздельная речь, вкусовое восприятие.

Ключевые слова:
распространенный рак, органы полости рта, мандибулотомия, оростома, язычный и подъязычный нервы, подъязычно-язычная мышца

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INTRODUCTION

Malignant tumors of the oral cavity in the Russian Federation (RF) occupy a leading place among tumors of the head and neck. The dynamics of the morbidity of the population is constantly growing. The absolute number of patients (men and women) in 2010 was 5251, and in 2020–6089. The dynamics of morbidity indicators in these terms amounted to 5.18 and 6.18 with an increase of 28.58 %. At the same time, the rough indicator of the incidence of oral cancer in the Russian Federation per 100,000 population in 2010 was 5.18, and in 2020–6.18. The average annual growth rate of this pathology was 2.47 with an increase of 28.58 %. The gross mortality rate in patients with cancer of the lip, oral cavity and pharynx in 2010 was 6.36, and in 2020–6.50, with an average annual growth rate for this period of 0.46 and an increase of 4.77 [1].

Treatment of advanced cancer of the tongue and the floor of the oral cavity remains an urgent problem, in which an integrated approach is used: surgery, chemotherapy and radiation.

Having more than 50 years of experience in performing operations in this category of patients, we have to listen to the complaints of patients who have undergone extended operations, who have been cured of cancer and are now living. Complaints are mainly about an unsatisfactory quality of life: difficult or probe feeding, illegibility or lack of speech, etc.

The loss of the functions of the oral organs makes patients socially inferior and therefore the issues of their rehabilitation are of paramount importance [2]. There are a lot of publications in the literature on this problem using various methods. This is speech rehabilitation based on increasing the mobility of the stump of the tongue and correcting sound reproduction [3], as well as developed methods of prosthetics of resected organs using various auto-tissues: fascia, musculoskeletal, bone-cartilage, etc. [4].

A detailed acquaintance with the literature on this problem, to a certain extent, served as a basis for us to study the methods already used, as well as to develop new, in our opinion, more effective surgical interventions.

With limited cancer of the tongue and the floor of the oral cavity (T1 and T2 art.), both surgical and radiation methods are used, whereas with common (T3, T4), complex, where operations dominate, is used [5]. Surgical interventions in this category of patients are constantly being improved [6]. At the same time, in some cases it is recommended to remove the contents of the submandibular and, if indicated, the chin triangles in a single block with the primary tumor [7]. At the same time, radical operations, with all their radicalism, as a rule, damaging the afferent and efferent nervous system, disrupt the motor and sensory functions of the tongue and the bottom of the oral cavity.

The innervation of the tongue and the bottom of the oral cavity is complex. It is caused by the presence of a variety of its functions and is carried out by both afferent and efferent pathways through VII,

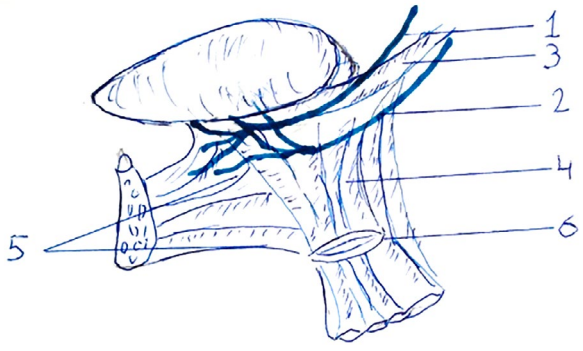

Fig. 1. Topography of sensitive and motor innervation of the tongue.
Note: 1 – *n.lingualis* (lingual nerve), 2 – *n.hypoglossus* (hyoid nerve), 3 – *m.styloglossus* (shield-lingual muscle), 4 – *m.hyoglossus* (hyoid-lingual muscle), 5 – *m.genioglossus* (chin-lingual muscle), 6 – *os.hyoideum* (hyoid bone).

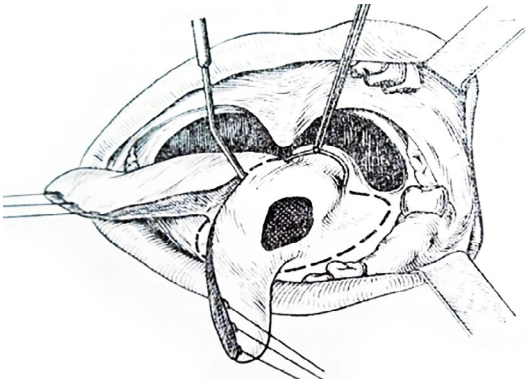

Fig. 2. The generally accepted method of resection of the tongue, the middle third of the half of which is affected by the cancer.

IX, X and XII pairs of cranial nerves. The greatest load falls on the lingual (*n.lingualis*), sublingual (*n.hypoglossus*), lingual (*n.glossopharyngeus*) and upper–laryngeal (*n.laryngeus superior* – branch of the vagus nerve) nerves [8]. *N.lingualis* is one of two branches of the sensitive part of the III branch (*ramus mandibularis*) of the trigeminal nerve (*n.trigeminus* – V pair). It mainly provides afferent communication, whereas efferent communication is carried out by the sublingual (XII pair) nerve (*n.hypoglossus*).

Radical removal of a common (III–IV st.) tumor of the tongue and /or the floor of the oral cavity may be complicated by damage to the nerves of the tongue, the most important of which are the lingual and sublingual (Fig. 1). Their main function is to ensure the sensitivity and motility of the language. For clarity, we present our own sketches on the issue under consideration.

With a widespread cancer of the tongue affecting one of its halves with damage to the hyoid nerve, there is a unilateral violation of its functions – immobilization of the affected tumor of this half of the tongue.

As can be seen from figure 1, the main nerves of the tongue, sensory and motor, pass on the lateral side of the duplicate of the shield-lingual and sublingual muscles, focusing on which, it is possible to avoid injury to these nerves.

If, before the operation, a patient with a widespread cancer process had a mobile tongue and retained sensitivity, then this indicates that the tumor has not spread to the nerves listed above. Therefore, when performing operations in this category of patients, in order to preserve the functions of the tongue, observing ablasty, it is necessary to exclude, as far as possible, their damage.

Operations performed on the tongue and organs of the oral cavity belong to the category of complex, when the radicalism of their performance should be combined with the possibility of preserving the functions of the resected organs: chewing, swallowing and speech.

There is a generally accepted method of surgery for cancer of the tongue affecting part of its back. It provides for the removal of 2 or more anatomical areas within 2–3 cm of healthy tissues (Fig. 2) [7].

As can be seen from the picture presented for these operations, during their resection of healthy tissues, it is quite possible to injure the most important part of it – the non-tumor-affected posterior third of the back of the tongue, the lower part of which is anatomically connected with the bottom of the oral cavity and the nerves passing there. In addition, during hemostasis, carried out by stitching the remaining healthy tissues, the probability of nerve damage also increases.

In cases where the tumor is large and passes to the opposite half of the tongue, when it is removed, using the above methods, providing radicalism, nerve injury on both sides is not excluded.

It is quite clear that the surgeon, first of all, requires radical ablastic surgery. However, it is always necessary to think about its consequences – the usefulness of the patient's subsequent life. Before the operation, the functions of the tongue and the bottom of the oral cavity are checked: chewing, swallowing, speech. Preserved functions after radical surgery speak about the integrity of the nerves.

The aim of the study is to improve the results of surgical treatment of patients with advanced cancer of the tongue and the floor of the oral cavity through radical surgery with possible preservation of the sensory and motor nerves that provide their functions.

PATIENTS AND METHODS

The proposed method of surgery in patients with advanced cancer of the tongue and the bottom of the oral cavity, providing radicalism, allows in the process of its implementation to preserve the motor and sensory nerves not affected by the tumor.

Taking into account the anatomical features of the oral cavity organs, which limit the view of the surgical field and complicate the operation, especially with advanced (IV st.) cancer, it is recommended to perform a mandibulotomy beforehand [6]. If the patient refuses to carry it out or at the third stage of the disease, the operation is performed intraoral.

The operation is performed under endotracheal anesthesia through a pre-imposed tracheostomy. The tongue affected by the tumor is brought out as much as possible. In case of unilateral location of the tumor or when it spreads to the other half of the tongue, dissection of healthy tissues is performed by retreating 2.0 cm from the edge of the tumor in accordance with its shape and size, preserving the unaffected tissues as much as possible (Fig. 3).

From the middle and edges of the healthy tongue tissue left after resection, 3 tissue fragments are taken for urgent histological examination for the presence of cancer cells. In their absence, the operation continues, and when malignant cells are detected, the resection zone expands by another 1.0 cm with repeated histological examination. Let us repeat that it is easier to fulfill these requirements with a pre-performed mandibulotomy.

When the tumor spreads to the tissues of the bottom of the oral cavity with preserved functions of the tongue, which indicates the integrity of the tumor with sensitive and motor nerves, the operation is carried out radically with the preservation of these nerves. To do this, focusing on the duplicate of the shield-lingual and sublingual muscles, on the lateral surface of which these nerves pass, under visual and manual control carried out both in the oral cavity and externally on the neck, the tumor tissue is removed medially above the above-named muscle duplicate.

With a tumor process spreading to one half of the tongue and the bottom of the oral cavity with damage to the sublingual and lingual nerves, which makes it immobile, the operation is carried out radically to healthy tissues, including the above-mentioned muscle duplicate. With a tumor affecting both halves of the tongue, the immobile half is radically removed, while the other, mobile, is operated according to the presented method. Its radical implementation later, depending on the nature of the operation, returns to the patient certain functions of the language sufficient for natural nutrition and speech.

Our relatively small experience (two patients with advanced cancer of the tongue and the bottom of the oral cavity with unilateral immobility of the tongue, who were informed about the scope of the operation and agreed to it) revealed the expediency of using these operations, since, observing radicalism, the functions of the language were preserved. In the postoperative period, these patients underwent radiation and chemotherapy. The collection of clinical material continues.

Clinical observation

Patient K. born in 1999 was admitted to the Department of Head and neck Tumors of the National Medical Research Centre for Oncology, Rostov-on-Don, with the diagnosis: cancer of the tongue with spread to the bottom of the oral cavity and metastases to the lymph nodes of the neck (squamous cell carcinoma St.4 (IVA, pT4a N2b M0)). Concomitant disease: leukemia, condition after treatment, stabilization of the process. He considers himself ill for about 6 months, when a tumor appeared on the left half of the tongue. I did not go to the doctor, I was treated independently with mouthwash. Pathology in the mouth was regarded as stomatitis on the background of leukemia. When examined by a dentist, a tumor of the tongue was revealed. Sent to an oncologist.

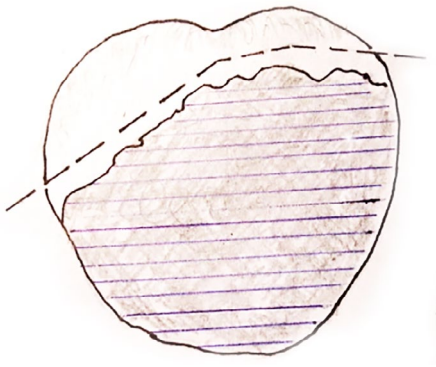

Fig. 3. Resection of the tongue with advanced cancer affecting both its halves. Dissection lines are carried out within healthy tissues, in accordance with the shape of the tumor, which allows you to preserve healthy tissues of the tongue as much as possible.

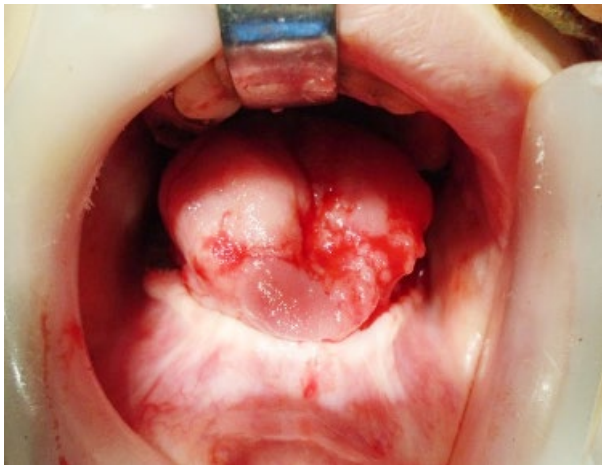

Fig. 4. Patient K. 1999. Cancer of the tongue with lesions of the anterior, middle and partially posterior third of the left half, as well as the anterior third of the right half.

Local status: symmetrical face. In the submandibular region on the left, enlarged, slightly mobile lymph nodes 2.5 × 3.0 cm and on the neck, along the internal jugular vein 2.0 × 3.0 cm. Opening the mouth is painful, but in full. Teeth on the upper and lower jaws are missing after leukemia. The left half of the tongue is motionless. Its anterior, middle and partially posterior third are affected by a tumor spreading to the anterior third of its right half (Fig. 4).

After the examination, the patient was operated on. A tracheotomy with intubation and subsequent radical surgery under general endotracheal-intubation anesthesia was performed under local anesthesia. Cervical lymphadenectomy (I–V levels) with ligation of the external carotid artery was performed. Taking into account the patient's refusal of mandibulotomy, the operation was continued by intraoral method. The middle third of the right, unaffected by the tumor, half of the tongue is stitched and brought out. Under visual and manual control, resection of the left half of the tongue and partially the right half was performed with the preservation of its middle and posterior third of the dorsum and lateral edge, retreating from the tumor by 2.0 cm. The parts of the tongue affected by the tumor were removed in a single block. No malignant cells were found in the tissues taken for urgent histological examination from the left part of the tongue. Squamous cell carcinoma was detected in the removed tissues of the tongue and the bottom of the oral cavity.

Under visual-manual control of the bottom of the oral cavity and external-manual control of the neck, tumor-affected tissues were identified, which were removed without going beyond the hyoid-lingual muscle not affected by the tumor. In a similar way, focusing on the right duplicate of the shield-lingual and sublingual muscles, the tumor of the bottom of the oral cavity was removed without going beyond the sublingual muscle. A smear was also taken from the abandoned tissues for urgent cytological examination for the presence of cancer cells, which did not reveal their presence. The defect of the tissues of the tongue and the bottom of the oral cavity is sutured in layers with the formation of the stump of the tongue, which is up to half of its former volume. Healing took place by primary tension. The postoperative period proceeded without complications. On the 5th day, the movement of the tongue stump was partially restored (Fig. 5). Nutrition was carried out with the help of a naso-esophageal probe, which was removed on the 10th day, after which, with partial restoration of the function of the tongue, the tracheostomy was decanulated (Fig. 6). By the end of the second week, after removal of stitches, she was discharged home.

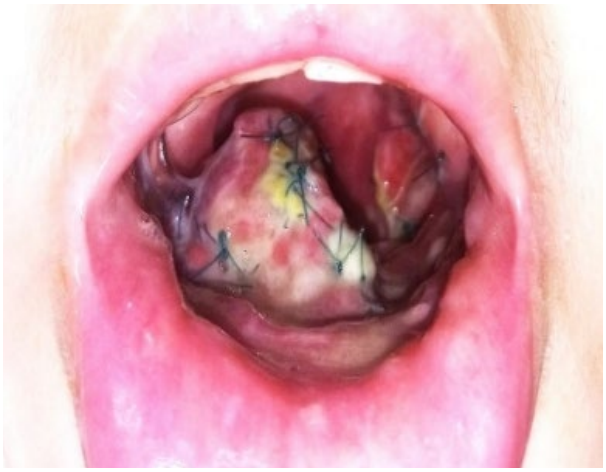

Fig. 5. The same patient. 5 days after the operation. The movements of the tongue stump are limited. Nutrition through the nasoesophageal probe. Breathing through the tracheostomy and natural pathways (mouth and nose).

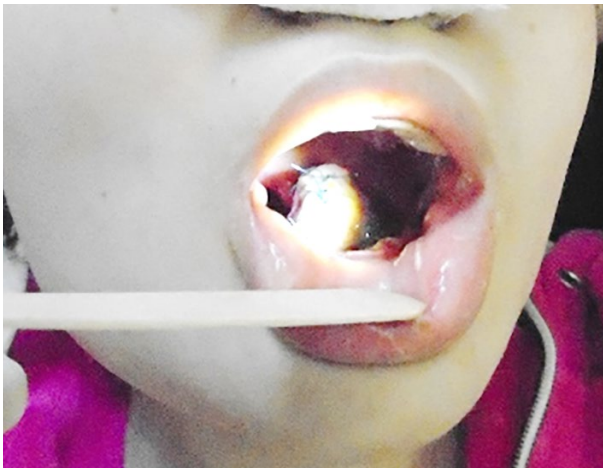

Fig. 6. The same patient. 10 days after the operation. Breathing through the tracheostomy and natural pathways (mouth and nose). The lability of the tongue stump is sufficient for feeding liquid food. She was discharged in a satisfactory condition for postoperative chemoradiotherapy at her place of residence. After 1 month after discharge, natural nutrition and respiration were restored. He is under observation after surgery without continued growth and relapse for 9 months.

RESEARCH RESULTS AND DISCUSSION

The problem of treating patients with advanced cancer of the tongue and the floor of the oral cavity remains difficult to this day. This is due to the structural features of these organs and, first of all, their innervation. The most important for the life of a patient with this disease are the lingual (*n.lingualis*) and sublingual (*n.hypoglossus*) nerves responsible for sensory and motor functions. Radical removal of a common (III–IV st.) tumor of the tongue and/or the floor of the oral cavity may be complicated by damage to these nerves. At the same time, the functions of the language are violated. Speaking about the topography of the tongue and the bottom of the oral cavity with muscles and nerves, it should be noted that the maxillohyoid muscle (*m.mylohyoideus*) forms the diaphragm of the mouth, which divides the bottom of the oral cavity into two floors – upper and lower. If the patient's sensitivity and mobility of the tongue were preserved before the operation, then this indicates the integrity of the nerves. This means that in this category of patients, the tumor process has not spread to the bottom of the oral cavity or partially captured the tissues of only its upper floor, without spreading to the nerves. In these cases, the surgeon removes only the tumor, without delving into the projection of the location of the nerves. Practice has shown that the early detection of cancer depends much on the patient himself, which is especially pronounced in an oncological situation. Usually, patients turn to doctors at the first signs of the disease, while another category of patients suffers troubles associated with it, and only with its progression they turn to a doctor with an already widespread or even neglected disease. This is especially pronounced in patients with oral cancer, when patients see their pathology and, realizing the danger, suffer. And only when there is pronounced discomfort with eating and speech and the process has already spread, go to the doctor. It should be noted that during the initial examination of patients with III–IV st., cases with bilateral damage to the nerves of the tongue are practically not detected. At the same time, as a rule, in patients with an already widespread process, with one half of the tongue immobile, the other remains intact. When examining such a patient from the IV st., regional metastases and a tumor affecting one of the halves of the tongue with partial spread to the other are usually detected. Clinical examination almost always confirms this situation and does not exclude the possibility of the need for radical surgery with bilateral nerve resection in this category of patients. In these cases, the patient must be warned about possible consequences before the operation. Difficulties arise when the cancer process spreads to both halves of the tongue with the immobility of one of its halves. The proposed method of surgery is intended primarily for this category of patients, but it can also be used in cases with a less common process. For the orientation of the surgeon during the operation, after dissection of the tumor-affected tongue, it is necessary to orient yourself with the topography of the location of the nerves. To do this, during the operation, after dissecting the tissues of the tongue and obtaining a pathologic and histological conclusion about the absence of cancer cells in its preserved tissues, all attention should be paid to orientation in the topography of the muscles and nerves of the floor of the oral cavity. Manually and visually, both intraoral approach and external, on the neck, above the hyoid bone, determine the location of the combination of the schiloglossus (*m.stiloglossus*) and hyoglossus (*m.hyoglossus*) muscles. At the same time, manually, in the projection of these muscles, the topography of the sublingual-lingual muscle is determined, along the lateral surface of which nerves pass. Subsequently, the removal of the affected tissues of the bottom of the oral cavity is carried out to the medial edge of the hyoid-lingual muscle not affected by the tumor process. This allows for a radical operation without injuring the nerves, which preserves the function of the tongue.

CONCLUSION

In patients with advanced cancer of the tongue and the floor of the oral cavity, with the defeat of one of its halves, the principles of ablasty should, if possible, be combined with the preservation of their functions. This is achieved by the fact that after radical removal of the tumor of the tongue, resection of the bottom of the oral cavity is carried out ablastically, without going beyond the hyoid-lingual muscle not affected by the tumor, on the outside of which the lingual and hyoid nerves pass. This, while performing an ablastic operation, preserves the functions of the tongue: chewing, swallowing, articulate speech, taste perception and salivation.

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Donskaya A. K. – technical editing, reference preparation, illustration preparation.
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ORIGINAL ARTICLE

PRACTICAL EXPERIENCE OF A LUNG CANCER PRIMARY CELL CULTURE COLLECTION CREATION AT THE NATIONAL MEDICAL RESEARCH CENTRE FOR ONCOLOGY

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ABSTRACT

Purpose of the study. Testing of new chemotherapeutic agents in translational and biology medicine needs studies on immortalized cell lines. However, such models do not always have the biological properties of a tumor *in situ*, in contrast to primary cell cultures. Primary cultures of lung cancer cells have biological, morphological and molecular characteristics close or identical to tumor cells *in vivo*. Obtaining collections of primary lung cancer cell lines is an important task in creating various models for preclinical studies.

Materials and methods. The materials are represented by postoperative tumor samples obtained from 25 patients with newly diagnosed lung cancer without prior treatment. The following methods were used to obtain primary cultures: enzymatic dissociation in Hanks' solution with the addition of 300 units/ml collagenase I (Thermo Fisher Scientific, USA), enzymatic dissociation using the Brain Tumor Dissociation Kit (Miltenyi Biotec, Germany) and 150 units/ml. ml of collagenase I, as well as the method of explants. The following methods were used to remove fibroblasts: the use of the FibrOut™ system (CHI Scientific, USA), magnetic separation of fibroblasts using Anti-Fibroblast MicroBeads (Miltenyi Biotec, Germany), and cold trypsinization.

Results. We have obtained 15 primary lung cancer cell cultures that have passed the zero order passage. In this work, the method of enzymatic dissociation turned out to be the most effective. Incubation of lung tumor samples with collagenase for 1 hour preserves the viability and adhesiveness of the cells. The explant method did not show its effectiveness for long-term cultivation, there was no migration of tumor cells to plastic. Magnetic separation, as a method of removing stromal components of fibroblasts, showed the greatest efficiency, while maintaining the viability of tumor cells.

Conclusion. The obtained primary cell cultures of lung cancer can be used for many tasks of experimental oncology: studies of the biological characteristics of lung cancer, development of preclinical models for the studies on new chemotherapeutic drugs.

Keywords:
primary cell culture, lung cancer, mechanical dissociation, enzymatic dissociation, collagenase, explant method, fibroblast removal

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ОРИГИНАЛЬНАЯ СТАТЬЯ

ОПЫТ СОЗДАНИЯ КОЛЛЕКЦИИ КЛЕТОЧНЫХ КУЛЬТУР НЕМЕЛКОКЛЕТОЧНОГО РАКА ЛЕГКОГО В ФГБУ «НМИЦ ОНКОЛОГИИ» МИНЗДРАВА РОССИИ

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РЕЗЮМЕ

Цель исследования. Тестирование новых химиотерапевтических агентов в трансляционной и биомедицине нуждается в исследованиях на иммортализованных клеточных линиях. Однако такие модели не всегда обладают биологическими свойствами опухоли *in situ*, в отличие от первичных культур клеток. Первичные культуры клеток рака легкого обладают близкими или идентичными опухолевым клеткам *in vivo* биологическими, гистологическими и молекулярными характеристиками. Получение коллекций первичных клеточных линий рака легкого является важной задачей в создании различных моделей для доклинических исследований.

Материалы и методы. В работе использовали послеоперационные образцы опухоли, полученные от 25 пациентов с впервые выявленным раком лёгкого без предварительного лечения. Для получения первичных культур использовали следующие методы: ферментативной диссоциации в растворе Хэнкса с добавлением 300 ед./мл коллагеназы I (Thermo Fisher Scientific, США), ферментативной диссоциации с использованием набора Brain Tumor Dissotiation Kit (Miltenyi Biotec, Германия) и 150 ед./мл коллагеназы I, а также метод эксплантатов. Для удаления фибробластов использовали следующие методы: применение системы FibrOut™ (CHI Scientific, США), магнитная сепарация фибробластов с использованием Anti-Fibroblast MicroBeads (Miltenyi Biotec, Германия) и метод холодной трипсинизации.

Результаты. Получено 15 первичных клеточных культур рака легкого, прошедших нулевой пассаж. В данной работе наиболее эффективным оказался метод ферментативной диссоциации. Инкубирование образцов опухоли легкого с коллагеназой в течение 1 ч. сохраняет жизнеспособность и адгезивную способность клеток. Метод эксплантатов не показал своей эффективности в целях долгосрочного культивирования, отсутствовала миграция клеток опухоли на пластик. Магнитная сепарация, как метод удаления стромальных компонентов фибробластов, показала наибольшую эффективность, сохраняя жизнеспособность клеток опухоли.

Заключение. Полученные первичные клеточные культуры рака легкого могут быть использованы для многих задач экспериментальной онкологии: исследования биологических особенностей рака легкого, разработки доклинических моделей исследования новых химиотерапевтических препаратов.

Ключевые слова:
первичная клеточная культура, рак легких, механическая диссоциация, ферментативная диссоциация, коллагеназа, метод эксплантатов, удаление фибробластов

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INTRODUCTION

Lung cancer is one of the most common oncological diseases with a high mortality rate in the Russian Federation as well as in the world. In Russia, the incidence of lung cancer is among the top three, while in men it ranks first and accounts for 16.5 % of the total number of all oncological pathologies [1]. Being a heterogeneous disease, lung cancer includes several subtypes that are important for the clinical and pathological course of the disease. Histologically, lung tumors are divided into two main histotypes: small cell carcinoma (SCC) and non-small cell lung cancer (NSCLC). Small cell lung cancer accounts for 15–20 % of primary lung tumors and is the most aggressive form of malignant neoplasms of this localization. Non-small cell lung cancer accounts for about 80 % of the total number of lung tumors and is divided into four histological subtypes: lung adenocarcinoma, squamous cell carcinoma, large cell lung cancer and bronchial carcinoid tumor [2]. Molecular genetic subtypes of NSCLC are determined by the presence or absence of mutations in the EGFR, KRAS, translocations in the ALK, ROS1 genes, which allows based on these data to make decisions about the appointment of targeted therapy, and PD-L1 protein expression to make the decision about immunotherapy [3]. Despite all the successes in the treatment of lung cancer achieved through the use of radiation, chemotherapy, and immunotherapy, the prognosis for patients remains disappointing: SCC is characterized by early metastasis and the overall five-year survival rate is about 5 %, NSCLC has better prognosis, however, given the fact that the disease is more often diagnosed at stage III–IV, the five-year survival rate remains low and is 15–19 % [4]. The search for new drugs includes screening libraries of antitumor compounds on primary cultures and permanent cancer cell lines [5]. Primary culture consists of tumor cells placed in the culture medium after mechanical or enzymatic disaggregation or as a result of migration from explants. The primary cell culture that has passed the first passage, i.e. passed the first replanting after the cells isolated from the tumor sample have attached to the culture plastic, is called a cell line. A permanent cell line is a transformed *in vitro* cell line that has overcome the so–called Hayflick limit – the limit number of divisions determined by the length of telomeres [6]. Permanent lung cancer cell lines were first obtained in the 80s of the twentieth century, 25 years after the creation of the first cervical cancer cell line (HeLa). After the development of serum-free media, for example, ACL4 and HITES media, the introduction of lung cancer cell lines into culture reached its peak. Currently, more than 200 permanent cell lines of this nosology are known [7; 8].

The use of permanent cell lines makes it possible to evaluate the direct cytotoxic and cytostatic effects, the organ-specific toxicity of the tested chemical compounds and preparations, to determine their mechanism of action, as well as target proteins and genes [9]. However, permanent cell culture *in vitro* after prolonged subcultivation cannot demonstrate the heterogeneity of the original tumor *in vivo*, which occurs due to the presence in the cellular composition of the primary tumor of several subclones genotypically different from each other, which is characteristic, in particular, for non-small cell lung cancer. The heterogeneity of lung cancer is characterized by differences in the rate of cell growth, their karyotype, the presence of cell surface receptors, enzyme production, gene expression [10], sensitivity to various cytostatics [11]. It is known that differences in the biological characteristics of tumor cells underlie the metastatic progression of the primary tumor, its acquisition of resistance to targeted and chemotherapy, the occurrence of relapses. Neglecting tumor heterogeneity at the early stages of preclinical studies is one of the reasons for the failure of clinical trials of new antitumor drugs, which leads to large economic costs and slowing progress in this area [3; 12].

Keeping this in mind, a more adequate source for the creation of cellular models of tumor growth are primary cell cultures, in which the heterogeneity of the tumor is reproduced at the level of its histopathological, molecular and genetic features. The creation of collections of primary lung cancer cultures is a common practice in various scientific institutions around the world. Such collections serve to reproduce the population features of the disease [13], to study the response of NSCLC to chemotherapy [14] and to study various aspects of oncogenesis [15; 16].

There are many methods for obtaining primary cell cultures, which researchers are constantly optimizing depending on the characteristics of the tumor material and the goals facing the study. A key step in the process of creating a cell culture

is to obtain, during enzymatic or mechanical tissue disaggregation, a pool of viable tumor cells, with a composition close to the heterogeneous cellular composition of the original tumor. For tumors with a dense structure, which include lung cancer, the method of enzymatic dissociation is preferred, while the composition and viability of the resulting cells directly depend on the reagents used and fermentation conditions [17]. One of the most popular components of mixtures for enzymatic dissociation of dense tumor tissue is collagenase I, which has a high specificity with respect to collagen fibers, the main protein of the extracellular matrix, leaving intact the proteins of cell membranes, which is important for preserving the viability and biological characteristics of isolated cells [18]. Collagenase I is often used in the production of primary lung cancer cell lines, both in pure form [19] and in a mixture with other enzymes (trypsin) [13]. As an alternative to tissue dissociation, the explant method is used, which is based on the migration of intact cells to culture plastic from small fragments of the tumor. The advantage of this approach is the high survival rate of cells with the most complete preservation of the heterogeneity of their composition, however, the effectiveness of obtaining a cell line is limited by the mobility of tumor cells [6; 14]. Obtaining primary cultures is a rather complex process due to the small number of initial tumor cells, as well as the partial loss of cell viability after tumor resection and the use of methods disaggregation of the material [17].

Regardless of the method of obtaining the primary culture of lung cancer, as well as other malignant neoplasms with a pronounced fibrous component, contamination by stroma cells is a big problem, of which the most numerous are fibroblasts, which *in vitro* quickly switch to division and thereby are able to suppress tumor cells. There are a number of ways to combat fibroblasts in culture, which can be divided into three groups: 1) the use of cytostatics specific for fibroblasts; 2) using the properties of differential adhesion of primary culture cells and 3) cell sorting. Each of these methods has its own advantages and disadvantages [20].

The purpose of the study was to evaluate the effectiveness of various methods of isolating tumor cells when creating a collection of cell cultures of non-small cell lung cancer.

MATERIALS AND METHODS

In the period from March to September 2021, tumor material from 25 patients with non–small cell lung cancer (lung adenocarcinoma) was selected for research, of which 20 samples were obtained from the primary focus and 5 samples from brain metastasis.

The patients were treated in the Department of thoracic surgery and in the Department of Neuro-Oncology at the National Medical Research Centre for Oncology in 2021. The histological diagnosis was confirmed in the pathology and anatomical department of National Medical Research Centre for Oncology. The patients were aware of their participation in the scientific study and signed an informed consent to the collection of biological material. The study was approved by the local ethical committee National Medical Research Centre for Oncology Protocol No. 6/1 of February 10, 2020.

Samples from the operating room were transferred in Hanks solution (HBSS, Gibco, USA) with the addition of 1 % penicillin-streptomycin (Biolot, Russia) at a temperature of +4–8 °C to the Laboratory of Cellular Technologies National Medical Research Centre for Oncology in an interval of no more than 20 minutes after removal of the tumor drug. To work out the technique of obtaining viable cells, we selected several variants of enzymatic dissociation protocols, as well as the explant method. In 15 cases, we used the procedure of enzymatic dissociation using pure collagenase I in the case of the primary focus (n = 10) and using a combination of collagenase I and enzymes for dissociation of brain tissue in the case of samples obtained from metastases (n = 5). In 10 cases, the explant method was used to obtain a cell culture from the primary focus. A schematic representation of the protocols and methods used in the work is shown in Figure 1. In all cases, a standard culture medium was used for growing primary cell lines, which is a DMEM medium (Gibco, USA) with the addition of 10 % FBS (HyClone, USA), 1 % insulin-transferrin-sodium selenite (Biolot, Russia), 10 ng/ml FGF-2 (Miltenyi Biotec, Germany), 10 ng/ml EGF (Miltenyi Biotec, Germany), 1 % NEAA (Gibco, USA).

Visual examination of cell morphology and photofixation were performed using an inverted Axio Vert microscope. A1 (Carl Zeiss Microscopy, Germany).

Protocol No. 1. *Enzymatic dissociation in a solution of Hanks and collagenase I (Thermo Fisher Scientific, USA).* The protocol used corresponds to the usual practice of enzymatic treatment of lung tumor tissue [19]. The tumor fragments were placed in a Petri dish (d = 35 mm) (Eppendorf, Germany) with Hanks solution (with Ca^{2+} and Mg^{2+} ions) (Gibco, USA) and the samples were fragmented with a surgical blade to a size of 1–2 mm^3, after which 300 units/ml of collagenase I were extracted (Thermo Fisher Scientific, USA) and incubated for 1 hour in a thermostat at 37 °C and 5 % CO_2. At the end of cultivation, the sample was resuspended by passing through a plastic tip several times, resulting in a homogeneous cell suspension. Next, 3 ml of standard culture medium was introduced, centrifuged for 5 min at 300g and the supernatant was decanted. 5 ml of DMEM nutrient medium (Gibco, USA) was added to the cell sediment and resuspended, after which the suspension of tumor cells was passed through a sterile nylon filter (d = 70 nm) (Becton Dickinson, USA). Cell viability was calculated and determined in the Goryaev chamber with a 0.4 % solution of trypan blue (Biolot, Russia). The samples, depending on the number of cells, were passed into vials with a culture surface area of 25 cm^2 or 75 cm^2 (Thermo Fisher Scientific NUNCtm EasYFlasktm, Denmark) in a nutrient medium for primary cell lines of the Primary Cancer Culture System (PromoCell®, Germany) and placed in a CO_2 incubator for further cultivation at 37 °C. and 5 % CO_2. On the 3rd day of cultivation, 2 ml of fresh nutrient medium Primary Cancer Culture System was added. Further, the replacement of the nutrient medium was carried out once every three days. After a month of cultivation, the nutrient medium for the primary cell lines was replaced with a standard culture medium.

Protocol No. 2. *Enzymatic dissociation with the combined use of Brain Tumor Dissociation Kit (Miltenyi Biotec, Germany) and 150 units/ml of collagenase I (Thermo Fisher Scientific, USA).* The samples were crushed with scalpels to a size of 1–2 mm^3. For enzymatic dissociation, the Brain Tumor Dissociation Kit (Miltenyi Biotec, Germany) was used according to the manufacturer's instructions. Cultured for 2 hours in a CO_2 incubator (Binder, Germany) at 37 °C and 5 % CO_2 on a mechanical stirrer, programming the device according to the instructions for the kit. Further, 150 units/ml of collagenase I were added to the suspension of enzymes and tumor fragments and incubated for another 18 hours at +4…+8 °C. After the incubation time, the sample was washed, filtered, and cells were counted and passed, as indicated in Protocol 1.

Protocol No. 3. *Explants method.* The tumor material was fragmented with a scalpel up to 2 mm in a 35 mm diameter Petri dish (Eppendorf, Germany) with 5 ml of culture medium for the cultivation of primary cell lines of the Primary Cancer Culture System (PromoCell®, Germany), after which the obtained explants were cultured in an incubator at 37 °C, 5 % CO_2. After a few weeks of cultivation, the non-adhesive explants were transferred from a Petri dish, crushed by pipetting and then washed, filtered, counted and passed cells, as indicated in protocol 1.

Removal of fibroblasts from the primary culture was carried out by several methods widely used in the practice of obtaining cell lines from solid tumors [20].

Method No. 1. *Removal of fibroblasts using the FibrOut™ system (CHI Scientific, USA).* The FibrOut™ system was introduced into vials with primary lung cancer cell cultures at the rate of 1 ml of the system to 500 ml of culture medium. Further, the cells were cultured for 3–5 days until the fibroblasts were completely detached, after which the medium was replaced with a standard culture medium.

Method No. 2. *Magnetic separation of cells using a set of Anti-Fibroblast MicroBeads (Miltenyi Biotec, Germany).* At the first stage, primary cultures were removed using a trypsin-versin solution (1:1) (Biolot, Russia) at a temperature of 37 °C, then fibroblasts from the cell suspension were separated on a magnetic column according to the manufacturer's instructions. The negative fraction depleted by fibroblasts was passaged in a standard culture medium and placed in a CO_2 incubator for further cultivation at 37 °C and 5 % CO_2.

Method No. 3. *Cold trypsinization.* The spent nutrient medium was decanted from culture vials with primary cultures, after which a cooled trypsin-versene solution (1:1) was introduced (Biolot, Russia) (+4…+8 °C) and incubated for 3 minutes at room temperature, observing the process of detaching cells from the bottom of the vial with a microscope. The fibroblasts separated from the bottom of the vial were carefully washed with a DMEM medium containing 5 % FBS and removed from the vial. Then a standard

culture medium was introduced into the vial with the remaining cells and placed in a CO_2 incubator for further cultivation at 37 °C and 5 % CO_2.

RESEARCH RESULTS

In all cases, with enzymatic dissociation of the material, we were able to obtain a suspension with a proportion of viable cells of about 90 %. Moreover, the viability was not reduced even in samples obtained from metastases, despite their prolonged incubation at a reduced temperature (+4 … +8 °C). On the 2nd day after exposure to enzymes, a small number of attached cells were observed, while tumor cells formed clusters, and fibroblasts were observed in the form of single spindle-shaped cells (Fig. 2A). As the cultivation time increased, the number of adhered cells grew, fibroblasts occupied the entire surface

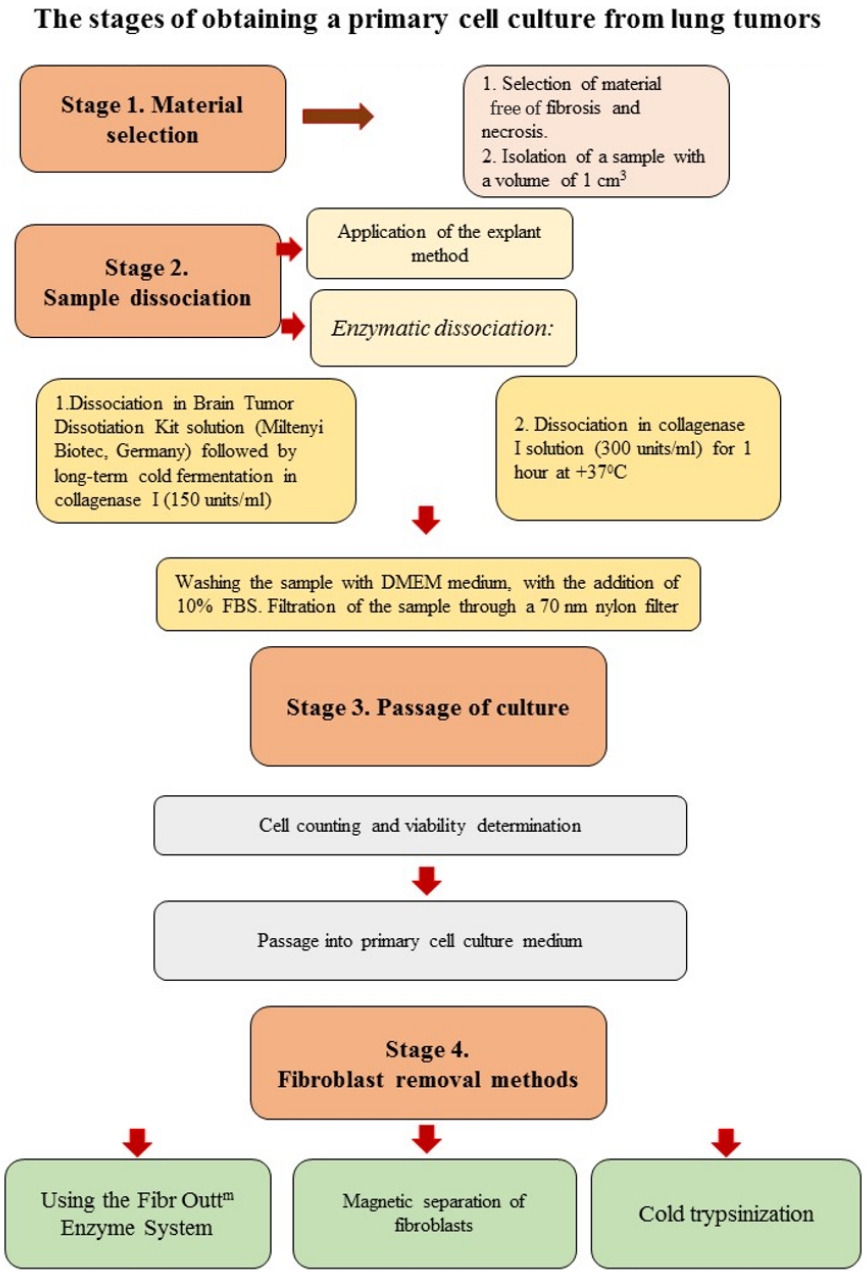

Fig. 1. Diagram that shows the stages of obtaining primary lung cancer cell lines.

of the vial, and tumor cells formed single clusters of rounded shape (Fig. 2B). The first replacement of the nutrient medium was carried out for 5 days. cultivation, then every 3 days. Elimination of erythrocytes and cellular detritus from the nutrient medium occurred during decanting of the waste medium.

Explants were attached to the bottom of the vial for 2 days. In the explant samples, after attachment to the surface of the Petri dish, only a small number of migrated cells were observed, which also showed no signs of proliferation even after several weeks of cultivation. Taking advantage of the fact that the fragments of tumor tissue became loose after a long stay in the nutrient medium, the remnants of explants were subjected to mechanical dissociation. The resulting cell sediment was passed through a 70 nm nylon filter and passed into culture vials with a surface area of 25 cm^2. The proportion of viable cells with this method of obtaining primary lung cancer culture was 70–80 %. After 2 days, the cells attached to the surface of the vial, and after 7 days. Their proliferation was observed after cultivation (Fig. 2C).

Working with samples of lung cancer metastases to the brain, contamination was often noted with cells morphologically similar to glial cells, having a fusiform shape and translucent processes forming a network (Fig. 2D). Unlike fibroblasts, these cells did not proliferate, their pool was depleted after each passage of primary cell lines. With a large number of glial cells, an additional mechanical cleaning of the sample was carried out using a scrapper under control in an inverted microscope in a laminar box.

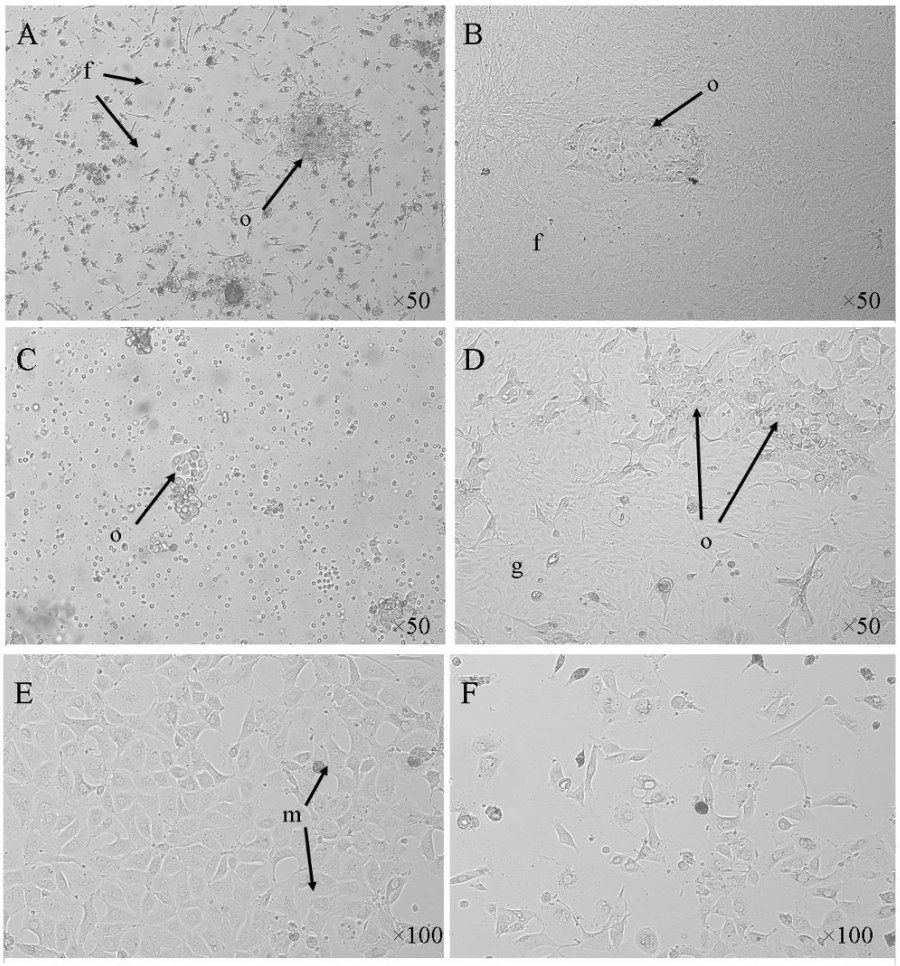

Fig. 2. View of primary lung cancer cell lines. A – cells after enzymatic dissociation on the 2nd day of cultivation. B – cell proliferation after 7 days of cultivation. C – proliferation of cells from the explant after disaggregation. D – primary lung cancer culture obtained from brain metastasis. E is a proliferating primary culture of epithelial morphology that has passed the zero passage. F – degradation of the cell line: vacuolization of tumor cells. Designations: *o* – tumor cells, *f* – fibroblasts, *g* – glial cells, *m* – mitoses.

In all cultures obtained from samples of locally advanced lung cancer, contamination with fibroblasts was observed (Fig. 2B), for the elimination of which several purification methods were used. The FibrOut™ system was used in 7 samples. In all cases, in addition to the detachment of fibroblasts, loss of adhesion and death of tumor cells were also observed, which may have been due to their low confluence (no more than 20 %) on the surface of the culture vial. Using systems for targeted elimination of fibroblasts, it is necessary to take into account their possible toxic effect on tumor cells and strictly maintaining the concentration, select safe dosages and incubation time. Magnetic separation of fibroblasts using Anti-Fibroblast MicroBeads was used in 7 cases. After magnetic separation, viable tumor cells were preserved, which quickly attached to the surface of the vial and then actively proliferated. However, the removal of fibroblasts was incomplete, and after a while their growth was observed again, which required repeated separations. When using cold trypsinization (6 samples), the least complete removal of fibroblasts from the surface of the vial was observed, since the exposure time with trypsin was strictly limited due to the high probability of loss of tumor cells. To maintain a low fibroblast content, it was necessary to repeat the cold trypsinization procedure, which was eventually carried out more often than the procedure of magnetic separation of fibroblasts. Comparative characteristics of methods for removing fibroblasts from primary lung cancer culture are presented in Table 1.

According to the results of the study, 25 primary lung cancer cell cultures were obtained, of which only 15 passed the zero passage and were cryopreserved at passages 1, 2 and 4. The cells of the successful lines were spread out in a monolayer (confluence 100 %) along the bottom of the vials, had a predominantly polygonal shape corresponding to epithelial morphology, single mitoses were observed (Fig. 2E). In the remaining lines, gradual degradation of cells was observed for 21 days: vacuolization and detachment from the adhesive surface (Fig. 2F).

DISCUSSION

The process of creating primary cell cultures from lung tumors is accompanied by some difficulties, compared with other types of epithelial tissues, and the probability of successful release of primary lung cancer cell cultures is no more than 40 % [21], which corresponds to our experience. Other authors note the multinucleation of cells and vacuolization of the cytoplasm [22] gradual degradation of tumor cells during long-term cultivation [19].

In this work, we used the methods of enzymatic dissociation with collagenase I as the most effective for obtaining primary cell lines [19]. Enzyme cocktails are actively used by researchers to produce spheroids and organoids of lung cancer that have similar properties and genetic mutations as the original tumor [22]. Collagenase I gradually cleaves collagen fibrils, while new sections of the fiber become available for interaction with the enzyme. Degradation of collagen and extracellular matrix leads to the release of cells from tumor tissue [23]. In our work, we also confirmed that incubating a lung cancer sample with collagenase for 1 hour does not inhibit other cells, preserves their viability and adhesive and proliferative ability.

Table 1. Comparison of the fibroblasts extraction methods from primary lung cancer cells culture

	FibrOut™	Anti-Fibroblast MicroBeads	Cold trypsinization
Reduction of the proportion of fibroblasts, %	100 %	80 %	40 %
Effect on the viability of tumor cells	It has a toxic effect on tumor cells. It is necessary to select the optimal cultivation time	Shows no effect on the viability of tumor cells	Shows no effect on the viability of tumor cells
The optimal frequency of the procedure to achieve the effect	Cultivation in conjunction with the FibrOut™ system for 3–5 days. A repeat of the procedure is not required	For complete separation of tumor cells and fibroblasts, it is necessary to carry out 2–3 magnetic separation procedures	For complete elimination of fibroblasts, 3–4 procedures should be performed

Explants are an effective model for drug testing for many nosologies of solid cancer [24]. In particular, in the work of Karekla et al. explants of non-small cell lung cancer were used as an ex vivo model to evaluate the response to cisplatin therapy [14]. In our work, long-term cultivation was assumed, during which, as we believed, tumor cells would begin to exit the explant, which was observed in our practice earlier when working with glial tumors [25] and prostate cancer, when the migration of tumor cells to plastic occurred within a week [26]. However, in the present study, in all cases, prolonged cultivation did not lead to the release of a sufficient number of tumor cells from the explant to the culture plastic. What biological features of lung cancer underlie the low mobility of tumor cells in primary culture have yet to be established.

An important aspect of working with tumor material obtained from lung tissue is the removal of fibroblasts, which are present in large numbers in this type of tissue. It is known that fibroblasts are a source of a number of signaling influences regulating the proliferation and mobility of tumor cells both in situ [27] and in culture [28] Despite the fact that fibroblasts can support the growth of tumor cells in culture [29], their excessive proliferation leads to a restriction of the proliferation of tumor cells, since fibroblasts divide faster *in vitro* and gradually displace tumor cells from the vial. In this regard, the tactics of regular "thinning" of fibroblasts using the methods of cold trypsinization and magnetic cell sorting, which we implemented in our work, is the most successful for samples with a small number of tumor cells, since it allows us to preserve the signaling functions of fibroblasts while restraining their excessive reproduction. However, at later stages, when the tumor cells have reached the cell density necessary for successful cultivation, as well as in samples with the number of tumor cells initially sufficient to create the necessary contact interaction that supports their viability and proliferation, it may be recommended to switch to more radical methods of fibroblast removal, for example, the use of commercial FibrOut™ (CHI) systems Scientific, USA) or Genetecin™ (Thermo Fisher Scientific, USA), designed to target the suppression of this type of cells.

Finally, the decisive factor determining the success of the cultivation of primary cell lines is the choice of nutrient medium. In our work, we did not set the goal of selecting the optimal environment for the introduction of lung cancer cells into the culture, but used ready-made commercial solutions and the experience of other authors. At the first passage, we used the Primary Cancer Culture System (PromoCell®, Germany) environment for primary cell lines with the manufacturer's additives. The medium used by us at a later stage of cultivation was prepared on the basis of DMEM nutrient medium, one of the most common and widely used media for the cultivation of various tumor cells, including those with a high proliferation rate [30]. The medium also included fetal bovine serum (10 %), a mixture of insulin-transferrin-sodium selenite (1 %), 10 ng/ml FGF-2, 10 ng/ml EGF and a solution of essential amino acids (1 %). Serum, essential amino acids and the addition of insulin-transferrin-selenite are essential components for maintaining the vital activity of cells undergoing adaptation to *in vitro* conditions [31; 32].

The presence of growth factors, such as fibroblast growth factor and epidermal growth factor, is necessary for selective stimulation of the division of malignant tumor cells, the addition of these factors to the culture medium is due to their small concentrations in serum and is a commonly used technique to increase the efficiency of obtaining primary lung cancer cell lines. At the same time, the concentration of growth factors in the cultivation medium in various studies ranges from 10 ng/ml [16] to 20 ng/ml [32].

CONCLUSION

The result of the work that has been carried out were 15 primary lung cancer cell lines obtained. The most effective method of obtaining a primary lung cancer culture was enzymatic dissociation with collagenase I, followed by removal of fibroblasts using magnetic separation. The obtained cell lines can be used to solve a wide range of research tasks in the field of experimental oncology. Primary lung cancer cell cultures, unlike immortalized cell lines undergoing genetic aberrations with an increase in the number of passages, have biological characteristics of tumor cells similar or identical to those *in vivo*, which makes them ideal models for preclinical studies of new drugs, research of biological features of lung cancer, creation of xenographic models, etc.

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ORIGINAL ARTICLE

CHANGES IN PATHOPHYSIOLOGY OF TUMOR GROWTH AND FUNCTIONAL ACTIVITY OF THE HYPOTHALAMIC-PITUITARY-THYROID AXIS IN RATS OF BOTH SEXES WITH THE DEVELOPMENT OF GUERIN'S CARCINOMA ON THE BACKGROUND OF HYPOTHYROIDISM

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ABSTRACT

Purpose of the study. Was to analyze changes in pathophysiological parameters of transplantable tumor growth and functional activity of the hypothalamic-pituitary-thyroid axis (HPT) in rats of both sexes with Guerin's carcinoma in presence of induced hypothyroidism.

Materials and methods. The dynamics of tumor growth and average life span were assessed in white alley rats of both sexes with Guerins carcinoma transplanted subcutaneously on the background of thyreostatic induced hypothyroidism. RIA (radioimmune assay) and ELISA (enzyme-linked immunosorbent assay) methods were used to determine levels of thyroid hormones in the blood and thyroid and tumor samples, and thyrotropin-releasing hormone (TRH) in the hypothalamus, as well as TSH in the pituitary gland. The experiment included 2 control groups: animals of both sexes with hypothyroidism (control group 1, number of rodents = 15) and animals with subcutaneously transplanted Guerin's carcinoma without hypothyroidism (control group 2, number of rodents = 15).

Results. Hypothyroidism in female rats inhibited the tumor growth and improved median survival by 1.8 times ($p < 0.05$). No such effect was observed in males of the main group. Levels of regulatory peptides of the hypothalamus and pituitary gland declined in females of the main group, while levels of TSH in the pituitary gland in males increased, despite a decrease in TRH by 3.5 times. TSH levels decreased in the thyroid and blood of animals of both sexes; however, a decrease in levels of total and free circulating thyroxine (T4 and FT4) by 1.6 times and by 2.8 times was found in the tumor, respectively; samples of Guerin's carcinoma in males of the main group remained saturated with T4 and FT4 as well as and in control group rodents without induced hypothyroidism.

Conclusions. The gender differences in the pathophysiology of the tumor development in presence of hypothyroidism, as well as changes in the functional activity of the HPT axis in experimental animals revealed in this study can probably be associated with sex hormones, which requires further study of the hypothalamic-pituitary-gonadal (HPG) axis and steroid hormones in peripheral organs and tumor samples.

Keywords:
Guerin's carcinoma, hypothyroidism, hypothalamic-pituitary-thyroid axis, thyroid hormones

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ОРИГИНАЛЬНАЯ СТАТЬЯ

ИЗМЕНЕНИЕ ПАТОФИЗИОЛОГИИ РОСТА ОПУХОЛИ И ФУНКЦИОНАЛЬНОЙ АКТИВНОСТИ ГИПОТАЛАМО-ГИПОФИЗАРНО-ТИРЕОИДНОЙ ОСИ У КРЫС ОБОЕГО ПОЛА ПРИ РАЗВИТИИ КАРЦИНОМЫ ГЕРЕНА НА ФОНЕ ГИПОТИРЕОЗА

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РЕЗЮМЕ

Цель исследования. Изучение изменения патофизиологических параметров роста перевивной опухоли и функциональной активности гипоталамо-гипофизарно-тиреоидной оси (ГГТ) у крыс обоего пола с карциномой Герена на фоне индуцированного гипотиреоза.

Материалы и методы. У белых беспородных крыс обоего пола с подкожно перевитой карциномой Герена на фоне индуцированного тиреостатиком гипотиреоза (основная группа) изучали динамику роста опухоли и среднюю продолжительность жизни, радиоиммунным и иммуноферментным методами (РИА и ИФА) определяли содержание тиреоидных гормонов в крови, щитовидной железе и в образцах опухоли, тиреотропного рилизинг гормона в гипоталамусе и тиреотропного гормона (ТТГ) в гипофизе. В эксперименте использовали 2 контрольные группы: животные обоего пола с гипотиреозом – контрольная группа № 1 (по 15 животных) и контрольная группа № 2 – самостоятельный рост опухоли – подкожная перевивка карциномы Герена (по 15 животных).

Результаты. У самок крыс гипотиреоз вызвал торможение роста перевивной опухоли и увеличение средней продолжительности жизни в 1,8 раза ($p < 0,05$). У самцов основной группы подобного эффекта не наблюдали. У самок основной группы установлено снижение уровня регуляторных пептидов гипоталамуса и гипофиза, тогда как у самцов уровень ТТГ в гипофизе повышался несмотря на снижение ТГ-рилизинга в 3,5 раза. В щитовидной железе и крови у животных обоего пола установлено снижение содержания ТГ, однако в опухоли установлено падение уровня общего и свободного тироксина (Т4 и FT4) в 1,6 раза и в 2,8 раза соответственно, образцы карциномы Герена у самцов основной группы оставались насыщенными Т4 и FT4 также, как и в контрольной, у животных без индуцированного гипотиреоза.

Заключение. Выявленные в настоящем исследовании половые различия в патофизиологии течения злокачественного процесса на фоне гипотиреоза, а также изменения функциональной активности ГГТ оси у экспериментальных животных, вероятно, могут быть связаны с половыми гормонами, что требует дальнейшего исследования гипоталамо-гипофизарно-гонадной (ГГГ) оси и показателей стероидных гормонов в периферических органах и образцах опухоли.

Ключевые слова:
карцинома Герена, гипотиреоз, гипоталамо-гипофизарно-тиреоидная ось, тиреоидные гормоны

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INTRODUCTION

Thyroid hormones (TH) are one of the factors that have the greatest impact on the human body, and play a key regulatory role in many physiological processes, including cell growth, differentiation and metabolism [1; 2]. The synthesis and release of TH is strictly controlled by the hypothalamus– pituitary-thyroid axis (HPT axis). In response to various physiological and environmental stimuli, the neurons of the small-cell paraventricular nucleus secrete thyrotropin-releasing hormone (TG-releasing), which stimulates the anterior pituitary gland to produce thyroid-stimulating hormone (TSH). TSH, in turn, regulates all stages of growth and thyroid function. On the other hand, the products of TG-releasing and TTHare subjected to negative control with the help of TH [2].

The thyroid gland produces two main hormones: L-thyroxine (T4) and L-triiodothyronine (T3). T4 is the predominant form (more than 80 %) secreted by the gland and circulating, while T3 is considered the most active form because it binds with a much higher affinity with nuclear receptors [2; 3]. Free triiodothyronine and consolidated thyroxine (FT3 and FT4) enter cells through transmembrane carrier proteins and the most well-known mechanisms of their action are based on the regulation of transcription mediated by nuclear receptors. However, some cellular activities are initiated by THon the plasma membrane and are designated as "non-genomic" [2].

The HPT axis interacts with other regulatory axes – hypothalamic-pituitary-gonadal and adrenal. Thus, TG-releasing hormone of the hypothalamus is able to stimulate the release of prolactin, and TH hormones are able to regulate the mammotropic effects of prolactin and affect the metabolism of sex steroids [4], on the other hand, the activity of deiodinases is under the endocrine influence of sex steroids and prolactin [5].

Hypothyroidism refers to a pathological condition of thyroid hormone deficiency. The lack of adequate therapy can lead to serious adverse health consequences and, ultimately, to death [6]. Hypothyroidism is a common disease affecting about 5 % of the population, and is more common in women [7].

Due to the pleiotropic effect of thyroid hormones, hypothyroidism can also affect the course of other diseases. The mechanism of the relationship between thyroid dysfunction of both hypo- and hyperthyroidism and the risk of cancer remains unclear, and the data of epidemiological and experimental studies are quite contradictory [8; 9]. Some studies have found that women with hypothyroidism are at a higher risk of breast cancer than women without hypothyroidism, and taking levothyroxine may reduce the risk of breast cancer in women with hypothyroidism [10]. Other studies have reported that higher levels of TSH in the blood, as a biomarker of hypothyroidism, are associated with a reduced risk of breast cancer [11]. While Khan S. R. et al. In his studies, he did not reveal a link between hypothyroidism and breast cancer (BC) [12]. Wang Y. et al. It has been reported that high serum TSH levels improve the results of treatment of head and neck cancer, glioma and breast cancer, but are associated with poor results of treatment of renal cell carcinoma [13].

At the same time, experimental data have shown that T4 and T3 have proliferative and anti-apoptotic effects on breast cancer tumor cells, regulating gene expression and stimulating estrogen-like effects [9; 14]. The role of TH in the pathophysiological mechanism of proliferation and differentiation in malignant tumor cells remains controversial, however, it is known that their elevated level may correlate with a worse prognosis of the course of the disease [15].

The increased risk of malignant neoplasms of various localizations under the influence of taking thyroid hormones for the treatment of hypothyroidism can be explained by the fact that TH increases the activation of mitochondrial function responsible for the overproduction of ROS. An increased level of ROS may be associated with increased oxidative stress in the body and the further development of cancer. Previous studies have shown that oxidative stress can disrupt cell function and, consequently, leads to a number of chronic disease conditions, including cancer and autoimmune diseases [16].

However, since the mechanisms explaining the link between thyroid dysfunction and cancer risk have not been fully determined, a new approach is needed to further study the causal relationship between them. An experimental animal model, taking into account gender, may be the best option for determining a possible biological mechanism.

The purpose of the study was to study changes in the pathophysiological parameters of the growth of

the transplant tumor and the functional activity of the hypothalamic-pituitary-thyroid axis (HPT) in rats of both sexes with Guerin carcinoma on the background of induced hypothyroidism.

MATERIALS AND METHODS

The experiment was performed on white mongrel rats of both sexes weighing 150–180 g. The animals were obtained from the Research Center for Biomedical Technologies of the FMBA (Andreevka branch, Moscow region). Laboratory animals were kept under natural lighting conditions with free access to water and food. Work with animals was carried out in accordance with the rules of the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" (Directive 2010/63/EU), as well as in accordance with the "International Recommendations on conducting Biomedical research using animals" and the order of the Ministry of Health of the Russian Federation dated June 19, 2003 No. 267 "On approval of the rules of laboratory practices". Manipulations with animals were performed in the box in compliance with the generally accepted rules of asepsis and antiseptics.

The study used a culture of Guerin's carcinoma obtained from the N. N. Blokhin National Medical Research Centre of Oncology. The material for transplantation was obtained from donor rats on the 12th-16th day of tumor growth. Transplantation of Guerin's carcinoma to animals was carried out by standard injection of 0.5ml tumor suspension under the skin of the right scapula in proportions of 1:10 in saline solution.

White mongrel rats of both sexes for 30 days received the pharmacopoeial thyrostatic drug mercazolil (Akrikhin Russia) (active ingredient Thiamazole) at a daily dose of 2.5 mg/100 g of weight (the total dose was 75 mg/100 g of weight). The animals did not refuse to eat, gained weight, deterioration of the appearance of the skin and hair, lethargy and drowsiness were registered. Hypothyroidism in animals was confirmed by determining the serum content of total thyroxine and thyroid-stimulating hormone by radioimmune assay (RIA) using standard kits (Immunotech, Czech Republic) after 30 days of taking thyrostatica. Animals of each sex were divided into the following groups:

The main group, after receiving persistent hypothyroidism to study its effect on the growth of malignant tumors, groups of animals of both sexes (15 females and 15 males) were subcutaneously transplanted with Guerin's carcinoma

Control group No. 1 animals of both sexes with hypothyroidism (n = 15 females and n = 15 males

Control group No. 2 animals of both sizes (n = 15 females and n = 15 males) with subcutaneous grafting of Guerin's carcinoma in the same dose and volume as in animals from the main groups.

Intact animals of both sexes (10 pieces each).

Animals with tumor growth – control group No. 2 and the main group were decapitated after 14 days of growth of Guerin's carcinoma. Animals of control group No. 1 and intact rats were decapitated at the same time as the main group. In the blood and 10 % of tumor homogenates, perifocal zone, thyroid gland and 1 % of pituitary homogenates, the level of thyroid hormones and TSH was determined using standard kits, in 1 % of hypothalamus homogenates, the level of TG-releasing was determined by the enzyme immunoassay (ELISA) method.

In addition, in 60 rats of both sexes (15 animals each), the dynamics of tumor growth and average life expectancy were studied with the growth of Guerin's carcinoma in its own variant and against the background of induced hypothyroidism.

Statistical analysis of the results was carried out using the Statistica 10.0 software package. The data obtained were analyzed for compliance with the normal distribution law using the Shapiro-Wilk criterion (for small samples). Quantitative data in the groups were compared using the Student and Mann-Whitney t-test. The data of the tables are presented in the form of M ± m, where M is the arithmetic mean, m is the standard error of the mean, $p < 0.05$ was taken as the level of statistical significance. The obtained results were statistically processed in compliance with the general recommendations for medical research.

RESEARCH RESULTS

The features of tumor growth during standard grafting of Guerin's carcinoma and grafting on the background of hypothyroidism in rats of both sexes are presented in Tables 1 and 2. Subcutaneous tumor in female rats of the main group started being deter-

mined 4 days after grafting, the tumor was grafted in 80 % of females, whereas in 20 % of female rats with hypothyroidism, Guerin's carcinoma did not reproduce. In the control group, the tumor was also detected after 4 days, the transferability was 100 %. In the females of the main group, at all stages of tumor measurement, the average volume was less than in the control group: 1.3 times after 4 days, 1.4

Table 1. Dynamics of tumor growth and survival in female rats with Guerin's carcinoma

Study timeline	Main group Hypothyroidism + Guerin's carcinoma (Tumor V, cm^3)	Control group Guerin's carcinoma (Tumor V, cm^3)
4 days	0.12 ± 0.01[1]	0.16 ± 0.06
7 days	2.28 ± 0.53[1]	3.18 ± 0.33
10 days	13.21 ± 0.93[1]	18.40 ± 2.42
14 days	27.28 ± 1.62[1]	44.76 ± 3.98
18 days	55.94 ± 5.4[1]	70.30 ± 4.78
21 days	75.73 ± 6.88[1]	107.96 ± 9.01
Baldness on the skin appearance	From day 11	Not detected
Necrotic areas appeared on the skin	Not detected	From day 14
Average life span (days)	29.3 ± 1.2[1]	18.2 ± 1.4
First lethal outcome in group	Day 24	Day 13
Last lethal outcome in group	Day 33	Day 26

Note: 1 – statistically significant differences compared to the indicators in animals of the control group $p < 0.05$

Table 2. Dynamics of tumor growth and survival in male rats with Guerin's carcinoma on the background of hypothyroidism

Study timeline	Main group Hypothyroidism + Guerin's carcinoma (Tumor V, cm^3)	Control group Guerin's carcinoma (Tumor V, cm^3)
4 days	0.04 ± 0.004[1]	0.50 ± 0.04
7 days	0.5 ± 0.002[1]	3.82 ± 0.27
10 days	7.94 ± 0.80[1]	14.74 ± 1.15
14 days	15.61 ± 1.40[1]	40.68 ± 3.81
18 days	44.90 ± 3.74	52.84 ± 5.48
21 days	72.93 ± 7.09	77.50 ± 6.25
Necrotic areas appeared on the skin	Not detected	From day 7
Average life span (days)	23.7 ± 2.1	20.0 ± 1.3
First lethal outcome in group	Day 20	Day 14
Last lethal outcome in group	Day 25	Day 24

Note: 1 – statistically significant differences compared to the indicators in animals of the control group $p < 0.05$.

times after 7 and 10 days, 1.5 times after 14 days, 1.3 times after 18 days and 1.4 times after 21 days ($p < 0.05$). At the same time, the survival rate of female rats of the main group was 1.6 times ($p < 0.05$) higher compared to rats of the control group. The first death of animals of the main group occurred 24 days later, 11 days later, compared with the first death of animals in the control group.

The study of the growth dynamics of Guerin's transfused carcinoma against the background of hypothyroidism in male rats is presented in Table 2. Subcutaneous tumor in male rats of the main group and control group in 100 % of cases began to be determined 4 days after the transfusion. In males of the main group, compared with the indicators in control animals, the average tumor volume at the stages of the experiment from day 4 to 14 was less: after 4 days by 13.3 times, after 7 days by 7.5 times, after 10 days by 1.9 times, after 14 days by 2.6 times ($p < 0.05$). However, after 18 days and after 21 days, there were no significant differences in tumor volumes. The average life expectancy of the animals had no significant differences compared to those of the males of the control group.

Taking into account the sex differences in the effect of hypothyroidism on the growth of Guerin's transfused carcinoma, we further conducted studies of the level of TH, as well as the main regulators of the HPT axis in blood and tissues. Indicators of the content of TH and TSH in the blood of rats of control and main groups are presented in Table 3.

It was found that in female and male rats with induced hypothyroidism (control group 1), the blood level of total T4 was lower than in intact animals by 7.3 times and 2 times, respectively, and TSH was increased by 1.6 times and 1.5 times. In addition, the level of total T3 was reduced by 1.3 times in male rats. At the same time, we have not established a change in the blood content of free forms of thyroid hormones in animals of both sexes.

It turned out that the growth of Guerin's carcinoma (control group 2 animals) also affected the content of thyroid hormones in the blood. Thus, in female rats with tumor growth, compared with the indicators in intact animals, the level of not only total T4 decreased by 2.0 times, but also free forms of thyroxine and triiodothyronine (FT4 and FT3) by an average of 1.4 times, despite the 1.6-fold increased content of total T3 and the absence of changes in the concentration of TSH.

In males of control group 2, a decrease in both general forms of TG was found: T4 by 4.7 times, T3 by 2.8 times, but also free forms – FT4 by 2 times

Table 3. The level of serum thyroid hormones in rats of both sexes

Группы	FT4 pMol/L	FT3 pMol/L	T4 pMol/L	T3 pMol/L	ТТГ mcIU/ml
Female					
Intact	15.73 ± 0.37	5.85 ± 0.14	61.29 ± 1.33	1.07 ± 0.06	0.085 ± 0.0015
Control 1	19.35 ± 1.20	5.82 ± 0.17	8.45 ± 0.28[1]	0.95 ± 0.07	0.14 ± 0.006[1]
Control 2	11.25 ± 0.14[1]	4.17 ± 0.11	28.12 ± 0.79[1]	1.76 ± 0.08[1]	0.07 ± 0.004[2]
Main group	8.85 ± 0.34[1,2,3]	3.5 ± 0.13[1,2]	26.70 ± 0.92[1,2]	0.55 ± 0.02[1,2,3]	0.07 ± 0.003[2]
Male					
Intact	20.11 ± 0.90	5.83 ± 0.30	75.77 ± 1.15	1.46 ± 0.07	0.083 ± 0.003
Control 1	17.18 ± 0.18	6.15 ± 0.26	38.51 ± 0.70[1]	1.11 ± 0.06[1]	0.122 ± 0.004[1]
Control 2	9.86 ± 0.44[1,2]	4.32 ± 0.19[1,2]	15.98 ± 0.64[1,2]	0.72 ± 0.03[1,2]	0.140 ± 0.005[1]
Main group	5.72 ± 0.35[1,2,3]	1.85 ± 0.08[1,2,3]	11.32 ± 0.48[1,2,3]	0.52 ± 0.03[1,2,3]	0.310 ± 0.011[1,2,3]

Note: significant differences compared to: 1 – with intact animals of the corresponding sex; 2 – with control group 1; 3 – with control group 2 ($p < 0.05$).

and FT3 by 1.3 times, against the background of an increased level of TSH by 1.8 times.

In the females of the main group, the level of common forms of thyroid hormones in the blood was lower than in intact animals T4 2.3 times and T3 1.9 times. The concentration of FT4 and FT3 was also 1.8 times and 1.7 times lower than normal, respectively. At the same time, the T4 content was 3.2 times higher than in animals with independent hypothyroidism, but had no significant differences from the indicators in females in control 2, and the T3 concentration was 1.7 times lower compared to the indicators of control group 1 and 3.2 times lower compared to control group 2. The level of FT4 in the blood of the females of the main group was 2.2 times lower than with hypothyroidism, and 1.3 times lower than with independent tumor growth, while the concentration of FT3 in the blood was 1.7 times lower than with hypothyroidism and had no significant differences from the indicators with independent growth of Guerin's carcinoma. The TSH level in the blood of the females of the main group was 2 times lower than in animals with hypothyroidism and did not differ from the indicators in intact animals and animals with independent tumor growth.

In males of the main group, the concentration of T4 in the blood was 6.7 times lower than in intact animals, compared with hypothyroidism by 3.4 times, compared with independent tumor growth by 1.4 times. The T3 content was also lower compared to the indicators: intact animals by 2.8 times; males with hypothyroidism – by 2.1 times, with the level of animals with tumor growth by 1.4 times. As for free forms, their content in the blood of males of the main group was lower than in intact animals and in control groups No. 1 and No. 2: FT4 3.5 times, 3 times and 1.7 times, respectively, and FT3 3.2 times, 3.3 times and 2.3 times, respectively. The TSH level in males of the main group exceeded the indicators in intact animals by 3.9 times, control 1 by 2.6 times, control 2 by 2.2 times.

Further, a study was conducted of the central links of the regulation of the HPT axis, namely, the level of TG-releasing in the hypothalamus and TSH in the pituitary gland in animals of the main and control groups (Table 4).

It was found that in female rats of both the main and control groups No. 1 and No. 2, the content of TG-releasing in the hypothalamus was lower than in intact females by 2.9 times, 2.1 times and 2.2 times, respectively. It should be noted that in animals of control group 1, the concentration of TG-releasing was lower than in the main and control group No. 2 by an average of 1.4 times. Only female rats of the main group showed a decrease in the content of TSH in the pituitary gland by an average of 1.4 times compared with the intact and control group animals.

In male rats, the level of TG-releasing in the hypothalamus was lower than normal, and TSH in the pituitary gland was higher, only in animals with independent hypothyroidism and combined with tumor growth – in control group 1 and the main group – 2.2 times and 3.5 times, respectively, and 1.4 times and 1.2 times, respectively. With the independent growth of Guerin's carcinoma, no significant differences in the content of these regulatory peptides were revealed.

Table 4. The content of TSH-releasing hormone in the hypothalamus and TSH in the pituitary gland of rats with the growth of Guerin's carcinoma on the background of hypothyroidism

Groups	Female		Male	
Indicators	TH-releasing, Hypothalamus (pg/gt)	TSH pituitary (mIU/gt)	TH-releasing, Hypothalamus (pg/gt)	TSH pituitary (mIU/gt)
Intact	42.57 ± 2.24	0.28 ± 0.015	30.7 ± 1.78	0.25 ± 0.015
Control 1	14.6 ± 0.56[1,3]	0.28 ± 0.019	14.2 ± 0.64[1,3]	0.35 ± 0.012[1,3]
Control 2	20.5 ± 1.21[1,2]	0.29 ± 0.017	36.1 ± 1.22[2]	0.27 ± 0.016[2]
Main	19.26 ± 1.10[1,2]	0.20 ± 0.01[1,2,3]	8.7 ± 0.47[1,2,3]	0.31 ± 0.026[1]

Note: significant differences compared to: 1 – with intact animals of the corresponding sex; 2 – with control group 1; 3 – with control group 2 ($p < 0.05$).

Table 5. Thyroid hormone and TSH levels in the thyroid gland in rats

Группы	FT4 pM/gt	FT3 pM/gt	T4 pM/gt	T3 pM/gt	ТТГ mclU/gt
Female					
Intact	37.52 ± 2.17	45.94 ± 1.68	29.50 ± 0.97	3.59 ± 0.11	1.97 ± 0.05
Control group 1	52.95 ± 1.23[1]	91.86 ± 1.52[1]	1.22 ± 0.05[1,3]	0.07 ± 0.004[1,3]	1.57 ± 0.07
Control grou 2	61.31 ± 0.66[1]	119.61 ± 12.18[1]	9.57 ± 0.51[1,2]	1.43 ± 0.06[1,2]	1.63 ± 0.058
Main group	31.90 ± 0.65[1,2,3]	24.41 ± 0.62[1,2,3]	3.19 ± 0.17[1,2,3]	0.72 ± 0.04[1,2,3]	5.71 ± 0.13[1,2,3]
Male					
Intact	23.66 ± 0.49	21.79 ± 0.68	26.61 ± 1.06	5.37 ± 0.28	0.26 ± 0.01
Control group 1	14.21 ± 0.83[1,3]	7.94 ± 0.33[1]	9.55 ± 0.45[1,3]	0.59 ± 0.03[1,3]	1.43 ± 0.05[1,3]
Control grou 2	59.89 ± 1.14[1,2]	9.03 ± 0.37[1]	91.90 ± 1.89[1,2]	7.41 ± 0.36[1,2]	0.20 ± 0.07[1,2]
Main group	7.99 ± 0.96[1,2,3]	2.18 ± 0.14[1,2,3]	9.19 ± 0.23[1,3]	1.21 ± 0.08[1,2,3]	0.15 ± 0.004[1,2,3]

Note: significant differences compared to: 1 – with intact animals of the corresponding sex; 2 – with control group 1; 3 – with control group 2 ($p < 0.05$).

Table 6. The level of thyroid hormones and TSH in the tumor and perifocal zone in rats with Guerin's carcinoma and hypothyroidism+Guerin's carcinoma

Groups	FT4 pM/gt	FT3 pM/gt	T4 pM/gt	T3 pM/gt
Female				
Control tumor	4.85 ± 0.23	3.88 ± 0.20	25.32 ± 0.41	0.52 ± 0.02
Control group p/zone	3.12 ± 0.16	1.18 ± 0.06	14.40 ± 0.29	0.48 ± 0.02
Main group tumor	1.73 ± 0.03[1]	2.40 ± 0.07[1]	16.11 ± 0.32[1]	0.59 ± 0.02
Main group p/zone	3.32 ± 0.11	1.30 ± 0.07	20.91 ± 0.42[2]	0.55 ± 0.02
Male				
Control tumor	1.47 ± 0.08	4.17 ± 0.14	23.83 ± 0.69	0.53 ± 0.03
Control group p/zone	4.57 ± 0.10	2.17 ± 0.055	19.61 ± 0.56	0.51 ± 0.01
Main group tumor	1.73 ± 0.06	3.12 ± 0.13[1]	20.33 ± 0.54	0.52 ± 0.03
Main group p/zone	1.41 ± 0.04[2]	0.27 ± 0.01[2]	28.20 ± 0.88[2]	0.52 ± 0.24

Note: significant differences compared to: 1 – with the tumor of the control group of the corresponding sex; 2 – with the perifocal zone of the control group of the corresponding sex ($p < 0.05$).

Next, the level of thyroid hormones and TSH in the peripheral organ – the thyroid gland was studied (Table 5).

It was found that in female rats of the control and main groups, a decrease in the level of T4 and T3 was found in the thyroid gland in varying degrees of severity, against the background of normal TSH content in the control groups and an increase of 2.9 times in the main group. The maximum decrease in the level of common forms of T4 and T3 in the thyroid gland – by 24.2 times and 51.3 times was found in control group No. 1, in female rats of control group 2 and the main group, the level of T4 and T3 was lower than the values of intact animals by 3.1 and 2.6 times and by 9.5 and 5.1 times, respectively.

Concentrations of free forms FT4 and FT3 in the thyroid gland in female rats of control group 1, despite the low level of common forms, were increased by 1.4 times and 2 times, respectively, compared with intact animals. In control group No. 2, the level of FT4 and FT3 in the thyroid gland was 1.6 times and 2.6 times higher than normal. In the main group, the FT4 content had no significant differences from the indicators in intact animals, and FT3 in the thyroid gland of females was 1.9 times lower.

In male rats with hypothyroidism independent and combined with tumor growth in the thyroid gland, a reduced content of common and free forms of thyroid hormones was also noted. Thus, the level of T4 in males of control group 1 and the main group was lower than in intact animals by an average of 2.8 times, T3 by 8.5 times and 4.3 times, respectively; the concentration of FT4 is 1.7 times and 3 times lower, respectively, and FT3 by 2.7 times and 10.3 times, respectively. With the independent growth of Guerin's carcinoma, on the contrary, the level of T4 and T3 was 3.5 times and 1.4 times higher than normal, respectively, and FT4 2.5 times. Only the FT3 content in male rats with tumor growth turned out to be 2.4 times lower than in intact animals. In males with hypothyroidism, the level of TSH in the thyroid gland was 5.4 times higher than in intact animals, whereas in the main group, the concentration of TSH, on the contrary, was 1.7 times lower.

Next, the level of thyroid hormones in the tumor and its perifocal zone was studied in animals with independent growth of Guerin's carcinoma and combined with hypothyroidism (Table 6).

It was found that in the females of the main group in the tumor, the level of total T4 was 1.6 times lower, and FT4 was 2.8 times lower, and FT3 was 1.6 times lower, compared with the samples of independently growing Guerin carcinoma. At the same time, there were no significant differences in the level of total T3 in the control and main groups.

In the perifocal zone in the females of the main group, only the level of T4 was 1.5 times higher than in the animals of the control group, and the content of T3, FT3 and FT4 had no significant differences from the indicators in the perifocal zone of the females of the control group.

In male rats of the main group, compared with the control group, the content of T4, T3 and FT4 in the tumor samples had no significant differences, and only FT3 was reduced by 1.3 times. In the perifocal zone in the males of the main group, the T4 level was 1.4 times higher, T3 had no significant differences from the indicators in the control group, while FT4 was 2.7 times lower and FT3 was 8 times lower compared to the control group.

DISCUSSION

Our studies have shown that the growth of subcutaneously transplanted Guerin's carcinoma against the background of hypothyroidism had sexual specificity: in females, the tumor was transplanted only in 80 % of cases, there was inhibition of tumor growth, the life expectancy of animals increased. In male rats, Guerin's carcinoma was subcutaneously transferred, as in the control group in 100 % of cases, some slowing of tumor growth was detected up to 14 days, but then the tumor volume increased, and life expectancy did not have significant differences from the indicators in the males of the control group. We assume that a significant increase in the average life expectancy in female rats of the main group compared with the control group may indicate a decrease in the aggressiveness of the course of the disease and be associated with a change in the functional activity of the HPT axis, both central regulatory links and peripheral, as well as directly local TG content in tumor samples. At the same time, the main group of males is of particular interest, in which, despite the presence of hypothyroidism, there was no change in the average life expectancy of animals. In connection with such sexual differences

in the course of the malignant process against the background of comorbid pathology, of course, first of all it is worth paying attention to the differences in the functioning of the HPT axis of animals of control groups No. 1.

Experimental hypothyroidism induced in rats of both sexes and confirmed by blood test results, with elevated TSH levels and reduced T4, was also accompanied by a decrease in the hypothalamus content of TG-releasing, without change in females, but an increase in males in the pituitary gland of TSH. At the same time, in the thyroid gland of animals of both sexes, a decrease in general forms of TG and free forms of TG was revealed only in males. That is, we can note the sexual differences in the functioning of the pituitary gland and thyroid gland in response to the effects of thyrostatics. It is known that hypothyroidism affects the female part of the population to a greater extent [17], it is possible that the experimental increase in the level of TSH in the pituitary gland in males in response to low concentrations of TG-releasing in the hypothalamus indicates a greater resistance of the male body to the effects of thyrostatics. Literature data indicate that central hypothyroidism is quite rare and equally affects both sexes, is more often associated with disorders of the pituitary gland than with the hypothalamus, but often includes both [18].

Since in our study it was found that in females, unlike males, hypothyroidism had an inhibitory effect on the growth of subcutaneously transplanted Guerin's carcinoma, in order to consider the possible mechanisms of the sexual specificity of the functioning of the HPT axis in animals with the growth of a malignant tumor against the background of hypothyroidism, it is of particular interest to analyze how the independent growth of Guerin's carcinoma affected the factors HPT axis.

Literature data indicate that under the influence of many factors, including starvation, trauma, myocardial infarction, infection, surgery, inflammation, etc. in patients with normal thyroid function, a number of thyroid hormone level disorders occur, a decrease in TG levels is noted in the blood serum, without an increase in TSH levels, aggravated with increasing severity and duration of the disease. This condition is called euthyroid weakness syndrome, euthyroid disease syndrome or low3/low4 syndrome. At the same time, a violation of thyroid hormone levels is secondary to various clinical diseases due to normal primary thyroid function [19; 20].

At the same time, the functioning of the HPT axis during the growth of malignant tumors has its own characteristics. Patients with cancer of various localizations are characterized by multidirectional changes in thyroid hormones in the blood, controlled or not controlled by TSH [21; 22]. The specificity of changes in the thyroid hormonal background in cancer patients may also be associated with the presence or absence of metastases [23] or comorbid diseases [24]. In addition, experimental studies have shown that functional changes in the HPT axis have sexual specificity. Thus, in an experiment with transfused melanoma B16/F10 mice of the C57Bl6 line, deep thyroid hypofunction with loss of pituitary control in males and normal production of common forms of TG, with a decrease in free forms of hormone in females, was revealed [25–27].

In the present study, we found that in response to the independent growth of the tumor, only female rats in the hypothalamus had a reduced level of TG-releasing, whereas in males it remained within the normal range. At the same time, in animals of both sexes, the content of TSH in the pituitary gland did not change. That is, in this case, only females were characterized by the involvement of the central link of regulation in the change in the functional activity of the HPT axis. As a result, in the thyroid gland of female tumor carriers, a decrease in the level of common, but an increase in the content of free forms of TG was revealed, whereas in males, on the contrary, an increase in the general forms of TG and FT4, but a decrease in FT3.

Despite the sex differences in the hormonal saturation of the thyroid gland, in the blood of males with Guerin's carcinoma, a decrease in the level of both general and free forms of TG was revealed, accompanied by a high content of TSH, which corresponds to clinical hypothyroidism. In females with Guerin carcinoma in the blood, against the background of normal TSH content, low T4, FT4 and FT3 values were determined, which corresponds to lowT3/lowT4 syndrome.

It is known that thyroid insufficiency syndrome is associated with systemic changes in the immune and endocrine systems. In the modern literature it is reported that its specific mechanisms mainly include changes in the metabolism of thyroid hor-

mones, secretion of TSH, protein binding TG in serum, transmembrane transport of thyroid hormones, nuclear receptors. Consequently, in low FT3 syndrome, various factors lead to an abnormal response of the body and disorders of metabolism, regulation, transmembrane transport and binding of thyroid hormone receptors [20]. Most of the literature shows that the hypothyroid state correlates with the severity of the disease, and a decrease in FT3 levels can be used as a prognostic marker of an unfavorable course of the disease [28].

Thus, we found that the studied control groups – hypothyroidism and the independent growth of Guerin's carcinoma, had a sexual specificity of changes in the activity of the HPT axis links. In response to tumor growth, the level of TG-releasing in the hypothalamus decreased only in female rats, while hypothyroidism caused a decrease in the content of the regulatory peptide in animals of both sexes. Only in males, hypothyroidism caused multidirectional changes in the level of regulatory peptides in the hypothalamus and pituitary gland (TG-releasing and TSH). Only in males, the growth of Guerin's carcinoma caused the accumulation of common forms of TG in the thyroid gland, against the background of low blood content. Therefore, of particular interest was the study of the growth of a malignant tumor against the background of hypothyroidism in animals of both sexes – the main group of our study.

In the main group, the sex specificity of changes in the activity of the HPT axis links was revealed, which was probably one of the reasons for slowing tumor growth only in females, unlike males, since it is known that thyroid hormones play a key role in the proliferation and differentiation of solid tumors [9].

In female rats of the main group, inhibition of the growth of Guerin's carcinoma on the background of hypothyroidism was revealed with a decrease in the level of not only TG-releasing in the hypothalamus, but also TSH in the pituitary gland against the background of low content of common forms of TG and FT3 both in the peripheral organ and in the blood. It should be noted that the content of TSH in the thyroid gland is sharply increased, but without entering the blood.

In males of the main group, there was no inhibition of tumor growth, similar to females, while in males, against the background of a decrease in the level of TG-releasing in the hypothalamus, the level of TSH in the pituitary gland even increased. Judging by the indicators of TG and TSH in the blood of males of the main group, thyroid dysfunction by the type of hypothyroidism was aggravated, since the level of TSH in the blood increased compared to the control groups, and the content of TG decreased even more, in addition, the thyroid gland had minimal, compared with the control groups, indicators of free forms of TG and reduced, as with independent hypothyroidism, the level of T4.

There are experimental studies showing that induced hypothyroidism in rats reduces the incidence of breast cancer and tumor volume, and also increases the latent period of tumor development, but when these rats were treated with thyroxine, the anti-cancer protective effects of hypothyroidism were reversed [29].

We suggest that conflicting clinical data on the role of thyroid dysfunction on the risk of cancer of various localizations may be related to sexual specificity, as well as hormonal dependence of the tumor, which may be determined by histological structure, degree of differentiation, as well as polymorphism of various genes and many other factors. In this regard, the local saturation of thyroid hormones of the tumor and its perifocal zone, depending on the state of the HPT axis, is of interest.

In our study, it was found that in animals with independent growth of Guerin's carcinoma in tumor samples, the content of T4, T3 and FT3 in females and males was the same, and the average life expectancy did not differ. At the same time, tumor samples in males of the main group, with the growth of Guerin's carcinoma on the background of hypothyroidism, were also saturated with T4, T3 and FT4, as well as samples of the control group, whereas in females with Guerin's carcinoma on the background of hypothyroidism, a decrease in the level of T4, FT4 and FT3 was found in the tumor.

CONCLUSION

Thus, the sexual specificity of the development of the malignant process against the background of hypothyroidism was revealed, which manifested itself in the following: in female non-linear white rats with hypothyroidism, the increase in the volume of tumor nodes of Guerin's carcinoma, transplanted

subcutaneously, developed more slowly than in the control group, and the life expectancy of the animals was 1.6 times longer. In males of non-linear white rats with hypothyroidism, the increase in the volume of tumor nodes of Guerin's carcinoma, transplanted subcutaneously, did not develop evenly, in terms of up to 14 days slower, but then did not differ from the indicators of the control groups, while the life expectancy did not have significant differences. The sex differences revealed in this study during the malignant process against the background of hypothyroidism, as well as changes in the functional activity of the HPT axis in experimental animals, can probably be associated with sex hormones, which requires further investigation of the HPT axis and indicators of steroid hormones in peripheral organs and tumor samples.

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ORIGINAL ARTICLE

DYNAMICS OF CHANGES IN EXPRESSION OF VEGF NEOANGIOGENIC FACTOR IN TUMOR TISSUE BIOPTATES IN PATIENTS WITH SQUAMOUS CELL CARCINOMA OF ORAL MUCOSA RECEIVING CETUXIMAB TREATMENT AND CHEMOTHERAPY

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ABSTRACT

Purpose of the study. An analysis of changes in the expression of the VEGF neoangiogenic factor in the tumor tissue of patients with squamous cell carcinoma of the oral mucosa receiving targeted therapy with cetuximab and chemotherapy.
Patients and methods. We performed an immunohistochemical study of tumor samples obtained from 60 patients with squamous cell carcinoma of the oral mucosa T3-4N0-1M0. The main group comprised 30 patients who received therapy with cisplatin and fluoruracil plus cetuximab. The control group included 30 patients receiving standard chemotherapy without targeted therapy. Each group was divided into two subgroups with different treatment efficacy: patients sensitive to treatment (n = 17 in the group with cetuximab and n = 12 in the group without cetuximab) and resistant to treatment (n = 13 in the group with targeted therapy and n = 18 in the group with standard chemotherapy).
Results. Quantification of the VEGF expression demonstrated minimal numbers of vessels stained positively for this marker in the field of view in patients of the main group sensitive to chemotherapy and cetuximab. The value was 5.3 times lower than initial values, and 4.3 times lower than in the subgroup of patients resistant to the treatment (the data were statistically significant, p = 0.0132 and p = 0.0455, respectively). In the control group, patients who were sensitive to the treatment showed 1.4 times lower values than initially (p = 0.921), and patients who were resistant to the treatment had 1.1 times lower values than initial values (p = 0.936). The data were not statistically significant.
Conclusions. The study showed that the number of microvessels in patients resistant to chemotherapy and cetuximab was 4.3 times higher than in patients with effective targeted therapy (p = 0.0455). The differences in the control group were not statistically significant.

Keywords:
oral squamous cell cancer, sensitivity, resistance, VEGF, neoangiogenesis, targeted therapy, cetuximab

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ОРИГИНАЛЬНАЯ СТАТЬЯ

ДИНАМИКА ИЗМЕНЕНИЯ ЭКСПРЕССИИ ФАКТОРА НЕОАНГИОГЕНЕЗА VEGF В БИОПТАТАХ ОПУХОЛЕВОЙ ТКАНИ У БОЛЬНЫХ ПЛОСКОКЛЕТОЧНЫМ РАКОМ СЛИЗИСТОЙ ОБОЛОЧКИ ПОЛОСТИ РТА ПРИ ПРОВЕДЕНИИ ТЕРАПИИ ЦЕТУКСИМАБОМ И ХИМИОТЕРАПИИ

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РЕЗЮМЕ

Цель исследования. Изучить изменение экспрессии фактора неоангиогенеза VEGF в ткани опухоли при проведении таргетной терапии цетуксимабом и химиотерапии у больных плоскоклеточным раком слизистой оболочки полости рта.
Пациенты и методы. Было проведено иммуногистохимическое исследование образцов опухолевой ткани, полученных от 60 больных с плоскоклеточным раком слизистой оболочки полости рта T3-4N0-1M0. Основную группу составили 30 пациентов, которым была проведена лекарственная терапия препаратами цисплатин и фторурацил с добавлением цетуксимаба. Контрольную группу также составили 30 больных, которые подвергались стандартной химиотерапии без таргетной терапии. По степени эффективности каждая из исследуемых групп была поделена на две подгруппы: по чувствительности к лечению (n = 17 в группе с цетуксимабом и n = 12 в группе без цетуксимаба) и по резистентности к лечению (n = 13 в группе с таргетной терапией и n = 18 в группе со стандартной химиотерапией).
Результаты. При количественной оценке экспрессии VEGF было выявлено, что минимальное количество сосудов, эндотелий которых окрашен данным маркером в поле зрения, наблюдалось у пациентов основной группы с чувствительностью к ХТ и цетуксимабу. Данный показатель был ниже в 5,3 раза по сравнению с исходными значениями и в 4,3 раза по сравнению с подгруппой пациентов, у которых наблюдалась резистентность к данному лечению (данные статистически значимы p = 0,0132 и p = 0,0455, соответственно). Что касается контрольной группы, то было отмечено, что при чувствительности к проводимому лечению значения были в 1,4 раза ниже исходных цифр (p = 0,921), а при резистентности – в 1,1 раза ниже относительно исходных цифр (p = 0,936). Данные оказались статистически не значимы.
Заключение. В ходе исследования нами выявлено, что при наличии резистентности к химиотерапии и цетуксимабу количество сосудов в микроциркуляторном русле было в 4,3 раза выше по сравнению с больными, у которых наблюдался эффект от таргетной терапии (p = 0,0455). В контрольной группе значения оказались статистически незначимыми.

Ключевые слова:
плоскоклеточный рак слизистой оболочки полости рта, чувствительность, резистентность, VEGF, неоангиогенез, таргетная терапия, цетуксимаб

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RELEVANCE

Oncological diseases are one of the causes of mortality and disability worldwide.

Head and neck cancer ranks sixth in prevalence among all malignant neoplasms worldwide. Cancer of the oral mucosa is one of the urgent problems in oncology. About 630,000 new cases are diagnosed every year, and more than 350,000 patients with this pathology die every year. More than 90 % of head and neck cancers are squamous cell carcinoma, which mainly occurs in the oral cavity and oropharynx [1; 2].

Squamous cell carcinoma of the oral mucosa is characterized by an aggressive course, early metastasis and accounts for 90–95 % of all malignant neoplasms of the oral cavity [3]. Most of them are tumors of the tongue and the bottom of the oral cavity. Even after radical surgical intervention followed by adjuvant chemoradiotherapy, the survival rate of such patients remains extremely low due to the development of early recurrence and regional metastasis [4–6].

Vascular endothelial growth factor (VEGF) is a signaling protein best known for its role in the development of a pathological vascular network. It is a key mediator of angiogenesis (formation of new blood vessels) and binds two VEGF receptors (VEGF-1 receptor and VEGF-2 receptor), which are expressed on vascular endothelial cells. The production of VEGF and other growth factors by the tumor leads to an "angiogenic switch", where a new vascular network is formed inside and around the tumor, which allows it to grow exponentially [7; 8]. Therefore, it is very important to understand the basic cell biology of such tumors.

The term angiogenesis was first applied in 1971 by Folkman. The researchers reported that tumors can grow, forming new blood vessels from the existing vascular system, and that angiogenesis is closely related not only to tumors, but also to various other diseases, such as proliferative retinopathy, etc. [9; 10]. Various numerous interactions occur between cells, mediated by autocrine pathways that contribute to neoangiogenesis, uncontrolled tumor proliferation and metastasis [11].

It is known that cetuximab in malignant tumors is able to block not only the EGFR signaling pathway, but also indirectly affect the secretion of VEGF-A, thereby suppressing neoangiogenesis

Vascular endothelial growth factor (VEGF) is overexpressed in squamous cell carcinoma of the oral cavity and is in the focus of attention when creating new targeted drugs that are under development because increased cell proliferation and a rich vascular network are directly involved in the progression of the tumor.

The complexity of mechanisms in the development and progression of a malignant tumor requires a different approach to improve diagnosis, therapeutic decision-making and monitoring of the disease in personalized oncology. Although survival rates for squamous cell carcinoma of the oral cavity have improved over the past two decades, the prognosis still remains unfavorable compared to the development of therapy and the success achieved for other types of malignant tumors. Prognostic factors are numerous, and their interactions are complex and still unclear [12–14].

The main achievements in the treatment of oncological diseases are mainly associated with the

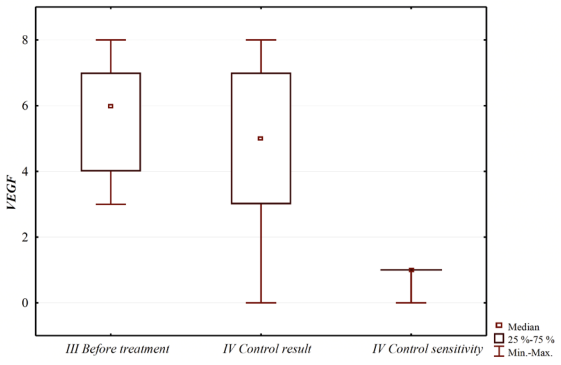

Fig. 1. Box-and-whiskers plot of VEGF expression in tumor cell vessels in patients of the main group.

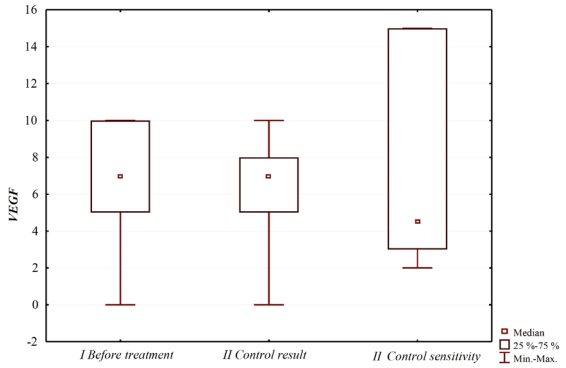

Fig. 2. Box-and-whiskers plot of VEGF expression in tumor cell vessels in patients of the control group.

appearance of molecular-targeted drugs, namely, acting on various molecules, cyclin-dependent kinases, VEGF growth factors, epidermal growth factor EGF, as well as on molecules suppressing apaptosis bcl-2, P53 cell cycle regulators, etc.

Cetuximab is a monoclonal antibody of immunoglobulin G1 that binds the epidermal growth factor receptor (EGFR), which is activated in about 90 % of patients with squamous cell carcinoma of the head and neck. Monoclonal antibodies against EGFR, such as cetuximab, compete with natural ligands, thereby preventing their binding to the receptor and, consequently, blocking the induction of cell growth signals and inhibiting the RAS signaling pathway and activation of ERK. Cetuximab binds to the extracellular domain of EGFR with higher affinity than natural ligands, blocking the tyrosine kinase-dependent signal transmission pathway induced by activation of the intracellular domain. Consequently, the antitumor effect of cetuximab is partly due to direct oncogenic signaling stress, which blocks cell survival, induces apoptosis and reduces the production of matrix metalloproteinase and vascular endothelial growth factor [15–17]. Understanding the molecular basis of the carcinogenesis of oral tumors and identifying potential molecular markers that may affect the prognosis and survival of patients with oral cancer is an urgent task of modern oncology. Neoangiogenesis stimulates tumor growth and promotes the occurrence of relapses and metastases due to a violation of the balance of proangiogenic and antiangiogenic factors. Vascular endothelial growth factor (VEGF) is the main regulator of angiogenesis, activating pro-angiogenic signaling pathways and regulating the formation of new blood vessels by binding to its main receptor. Angiogenesis plays a key role in the

Table 1. Expression with the VEGF medium in tumor cells in the studied groups (average numbers of vessels in a field of view)

Marker of angiogenesis	Main group (*n* = 30)		
	Initial values (*n* = 30)	Sensitivity (*n* = 17)	Resistance (*n* = 13)
VEGF	5.86 ± 0.72	1.1 ± 0.28* **↓ * *p* = 0.0132 ** *p* = 0.0455	4.8 ± 1.3
	Main group (*n* = 30)		
	Initial values (*n* = 30)	Sensitivity (*n* = 12)	Resistance (*n* = 18)
VEGF	6.5 ± 1.52	4.5 ± 1.71	6.1 ± 1.5

Note: * – statistically significant differences related to background values,
** – statistically significant differences related to subgroup with resistance.

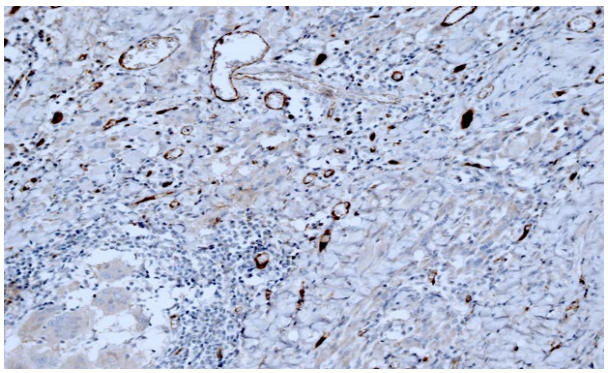

Fig. 3. VEGF-stained vessels in tumor cells of patients in the studied groups. Magnification × 200.

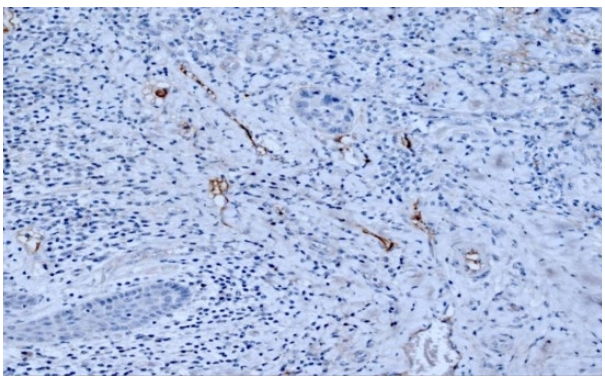

Fig. 4. Solitary VEGF-stained vessels in tumor cells of patients in the studied groups. Magnification × 200.

progression of the disease and mediates resistance to treatment. Thus, understanding neoangiogenesis, especially the VEGF pathway, is essential for risk stratification in patients with oral cancer and the development of new therapeutic targets [18].

The purpose of the study was to investigate changes in the expression of the neoangiogenesis VEGF in tumor tissue during targeted therapy with cetuximab and chemotherapy in patients with squamous cell carcinoma of the oral mucosa

PATIENTS AND METHODS

The study analyzed data on 60 patients with cancer of the oral mucosa, with the degree of prevalence of the tumor process T3-4N0-1M0. The surveyed groups were dominated by males (in the main group there were 22 men (73.3 %) and 8 women (26.7 %); in the control group – 19 men (63.3 %) and 11 (36.7 %) women). Most of the patients had moderate-grade squamous cell carcinoma (76.6 % in the main 23 patients) and 83.3 % in 25 control patients). The average age of patients in the main group was 62 years, and in the control group – 60 years. According to the stage of the tumor process in the main group, 17 patients were with stage III of the disease and 13 patients with stage IV; in the control group, the figures were distributed as follows: 15 patients with stage III and 15 patients with stage IV of the disease. Thus, we see that the patients in both groups were comparable in gender, age, degree of differentiation of the tumor, stage of the disease. The main group consisted of 30 patients who underwent chemotherapy with platinum cisplatin and fluorouracil with targeted EGFR blocker therapy: cisplatin 100 mg/m^2, intravenously, day 1, 5-fluorouracil 1000 mg/m^2/day, intravenously, 96-hour continuous infusion in combination with targeted therapy (cetuximab 400 mg/m^2 on day 1 in a loading dose, then 250 mg/m^2 on days 8 and 15). The control group also included 30 patients who underwent courses of standard chemotherapy without cetuximab – cisplatin 100 mg/m^2, intravenously, day 1, 5-fluorouracil 1000 mg/m^2/day, intravenously with 96-hour continuous infusion in 1–4 days. According to the degree of effectiveness, both the main and control groups were divided into two subgroups: with sensitivity and resistance. In the main group, the effect of treatment with cetuximab was observed in 17 patients, and resistance in 13 people. In the control group, 12 people were sensitive to treatment, and 18 were resistant to chemotherapy, respectively [19].

A solution of 10 % neutral buffered formalin was used to fix the material. Next, standard wiring and paraffinization of the fabric was performed. The next step was to prepare sections 3–5 microns thick, which were stained with hematoxylin and eosin (Accu-Cut SRM 200, Sakura (Japan)). The slices were applied to highly adhesive glasses and dried vertically in a thermostat at 37 °C overnight or at 60 °C for 1 hour. IHC was performed on sections from paraffin blocks intended for standard morphological examination. Primary monoclonal mouse and rabbit antibodies were used in the work. The degree of VEGF expression was determined by counting the number of vessels in each field of vision using 40 lenses (antibody VG1 Diagnostic BioSystems, dilution 1:200, 10 mMTris, buffer for "unmasking antigens" – 1 mMEDTA (pH 9.0). The calculation was carried out in 10 fields of view, then the average amount in the preparation was calculated [19].

The Statistica 10.0 application program was used to process statistical data [20]. The studied data were checked for compliance with the normal distribution according to the Shapiro-Wilk criterion. The data of the tables are presented in the form of M ± m, where M is the arithmetic mean, m is the standard error of the mean, $p \leq 0.05$ was taken as the level of reliability or statistical significance. If the distribution turned out to be far from normal, the comparison of groups was carried out using the nonparametric Mann-Whitney criterion (U-criterion) [19].

RESEARCH RESULTS AND DISCUSSION

Tumor biopsy was performed in all patients prior to treatment with further immunohistochemical examination of tumor tissue biopsies. During the processing of the obtained results, no fundamental differences in the initial values were revealed between the groups, and therefore, when describing the results, it was decided not to divide the background values of the studied markers into groups of patients depending on sensitivity, but to consider them as background in a single group. The analysis of the results of treatment of patients according to the response to the treatment according to the RECIST 1.1 criteria was carried out and compared with the VEGF level for each patient.

In the main group after cetuximab therapy, in the presence of efficacy from the treatment, the vascular spread was from 0 to 1, in the group without cetuximab, in the presence of sensitivity, these figures ranged from 2 to 15 vessels. In the absence of effectiveness from cetuximab, the spread of the number of vessels was up to 0 to 8 vessels in the field of view, in the group where cetuximab was not used – from 0 to 10 vessels [19].

The data is graphically shown in figures 1 and 2.

The average number of vessels whose endothelium is stained with the VEGF marker in the field of view is shown in the table.

When quantifying VEGF expression, it was revealed that in the main group of patients who underwent chemotherapy with targeted therapy with cetuximab in the presence of sensitivity to the treatment, there was a minimum number of vessels whose endothelium was stained with a VEGF marker. Compared with the initial values, this indicator was 5.3 times lower. And in the presence of resistance to chemotherapy and cetuximab, this indicator was 4.3 times lower than the baseline values (the data are statistically significant $p = 0.0132$ and $p = 0.0455$, respectively). In the chemotherapy group without cetuximab, the following indicators were noted: in the presence of sensitivity to chemotherapy, the minimum number of vessels stained with VEGF was 1.4 times lower relative to the initial figures ($p = 0.921$), and in the presence of resistance in this group – 1.1 times ($p = 0.936$). The data were not statistically significant [19].

When comparing the data, we obtained the following results: in the main group where chemotherapy with targeted therapy with cetuximab was used, in the presence of sensitivity to treatment, the number of vessels stained with VEGF marker in tumor cells was 4.1 times statistically lower compared to the control group of patients who also had sensitivity to the treatment ($p = 0.0035$). When resistance to chemotherapy and cetuximab appeared in the main group, the number of vessels stained with VEGF marker in tumor cells was 1.3 times lower compared to patients who also had resistance to the treatment ($p = 0.3699$) [19].

A statistical analysis of the effect of targeted therapy (comparison of control and main groups) revealed a statistically significant effect of cetuximab on this marker ($p = 0.028$).

Variants of VEGF expression in patients' tumor cells are shown in Figures 3 and 4.

Vascular endothelial growth factor (VEGF) and its receptors play an important role in both physiological and pathological angiogenesis. VEGF-A is widely expressed in almost all malignant tumors and is considered the most important factor in tumor angiogenesis. VEGF-A signaling also plays an important role in the development of diseases associated with angiogenesis, especially in malignant neoplasms.

VEGF causes deep angiogenesis during tumor formation. In this study, we tracked how the density of vessels stained with VEGF marker changed in the tumor cells of patients of the studied groups. (moved from the beginning of this section according to the reviewer's comment)

Multiple growth factors/cytokines and their signaling receptors often coexist in the same tumor microenvironment and collectively modulate tumor growth, invasiveness and metastasis. Among all known angiogenic factors, vascular endothelial growth factor A (VEGF-A), which modulates angiogenesis, vascular permeability, vascular survival and vascular remodeling, is probably the best characterized.

Transcription of the VEGF gene is activated under hypoxia by factor HIF1a (Hypoxia-inducible factor 1a). Hypoxia is one of the main causes of VEGF signaling activation in tissues. VEGF increases the level of VEGFR2 receptor expression by endotheliocytes of tumor microvessels, which activates cell growth and proliferation of endothelial cells [20]. Recent advances in molecular biology have revealed multiple gene changes in carcinogenesis in oral cancer that cause aberrant expression and function of proteins in a number of cellular processes, including angiogenesis.

Angiogenesis – the formation of new vessels from pre-existing ones, is crucial for tumor growth, invasion and metastasis of solid tumors. As the tumor grows, the cells in the tumor mass are deprived of oxygen due to their distance from the nearest blood vessels. The generation of a hypoxic state in tumors induces the production of vascular endothelial growth factor VEGF, a key mediator of angiogenesis. VEGF is overexpressed in a large number of human carcinomas, including squamous cell carcinoma of the head and neck, in particular in cancer of the oral mucosa [21; 22].

Cancer metastasis is the cause of mortality in cancer patients and involves complex interactions

modulated by various factors and cytokines between malignant cells and host cells. Vascular structures in solid tumors are crucial for intravasation of tumor cells into the bloodstream [23].

Although genetic changes in malignant cells determine the internal characteristics of invasiveness, cellular and molecular components of the host may play a predominant role in the invasion and metastasis of cancer [24].

For example, the vascular network of a tumor is necessary for tumor growth and metastasis, and blocking tumor angiogenesis is successfully used to treat cancer in animals and humans.

The subsequent formation of metastatic niches and the repeated growth of metastatic nodes to clinically detectable masses depend on angiogenesis and vascular remodeling. Tumors often express angiogenic factors at high levels, causing neovascularization.

A search for relevant studies was conducted in electronic databases. A meta-analysis of studies was conducted in which the relationship between overexpression of VEGF and survival of patients with oral cancer was quantified. Survival data were quantified. The results of these studies suggest that overexpression of VEGF has an adverse effect on overall survival and progression-free survival in patients with squamous cell carcinoma of the oral cavity, in patients with adenocystic cancer and mucoepidermoid cancer of the salivary glands. No significant heterogeneity was observed in all studies. Overexpression of VEGF indicates an unfavorable prognosis for patients with squamous cell carcinoma of the oral cavity, adenocystic and mucoepidermoid cancer of the salivary glands [25; 26]. In the era of personalized medicine and the treatment of malignant neoplasms based on the identification of biomarkers, it is important to find those therapeutic targets that need to be influenced to achieve maximum response to therapy, especially for squamous cell carcinoma of the oral cavity, since this pathology is very heterogeneous and poorly studied.

CONCLUSION

Based on the immunohistochemical study of tumor tissue biopsies, it can be concluded that in the presence of resistance to cetuximab, the number of vessels stained with the VEGF marker in the microcircular bed increases. Statistically significant results were obtained demonstrating the relationship between the degree of VEGF expression and the response to therapy with certain drugs. The data obtained are of particular clinical interest and can be used to predict the results of treatment in patients with squamous cell carcinoma of the oral cavity.

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ORIGINAL ARTICLE

EXPRESSION PROFILE OF IMMUNOPHENOTYPIC MARKER MOLECULES ON B-LYMPHOCYTES IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA AT THE STAGES OF IMMUNOCHEMOTHERAPY

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ABSTRACT

Purpose of the study. To study the expression of immunophenotypic marker molecules on B-lymphocytes of patients with chronic lymphocytic leukemia at the stages of immunochemotherapy while monitoring minimal residual disease.
Patients and methods. 20 patients with CLL were examined, who in the period 2019–2022 underwent 6 courses of immunochemotherapy (ICT) in the RB/FCR mode at the National Medical Research Centre for Oncology, Rostov-on-Don. Before, after 3, 6 courses of ICT, bone marrow immunophenotyping was performed by flow cytometry. The data is evaluated in Statistica 13.0.
Results. Before treatment, 3 groups of patients were identified depending on the expression of prognostic markers (CD38, ZAP-70, CD11c, CD25, FMC7). I (2 people) – without expression of CD38, ZAP-70, CD11c, CD25, FMC7 on tumor B-lymphocytes. II (14 people) – with variable expression of CD25, CD38 (0.4–47.6 % and 0.0–57.5 %, respectively), lack of expression of ZAP-70, CD11c, FMC7. III (4 people) – with high expression of CD38 (57.5–69.2 %), ZAP-70 (36.6–48.3 %), CD11c (20.0–96.5 %), CD25 (64.9–92.7 %), FMC7 (13.6–88.6 %). After the 3rd course of ICT, the minimum residual disease (MRD): 0 % in group I, 0.48 ± 0.13 % in group II, 33.5 ± 7.84 % in group III. After the 6th course of ICT MRD: 0 % in group I, 0.42 ± 0.09 % in group II, 33.2 ± 8.07 % in group III. The expression of immunophenotypic markers in groups II and III remained unchanged after 3, 6 courses of ICT. According to the criteria for assessing the response to therapy (IWCLL, 2018), patients of groups I, II after the 6th course of ICT have complete remission, 3 patients of group III have partial remission, 1 patient has stabilization of the process. Preliminary data have been obtained indicating that the absence or increased expression of CD38, CD25, ZAP-70, CD11c, FMC7 on B-lymphocytes of CLL patients before treatment may predetermine the hematological response to therapy according to RB/FCR regimens.
Conclusion. Initially, increased expression of all prognostic antigens simultaneously: CD38, CD25, ZAP-70, CD11c, FMC7 on the tumor population of B-lymphocytes in patients with CLL is associated with an unsatisfactory response to treatment, which seems promising from the point of view of studying the effect of the analyzed marker molecules on achieving a hematological response at the stages of immunochemotherapy.

Keywords:
chronic lymphocytic leukemia, flow cytometry, minimal residual disease, immunophenotypic markers, immunochemotherapy

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ОРИГИНАЛЬНАЯ СТАТЬЯ

ПРОФИЛЬ ЭКСПРЕССИИ ИММУНОФЕНОТИПИЧЕСКИХ МАРКЕРНЫХ МОЛЕКУЛ НА В-ЛИМФОЦИТАХ У БОЛЬНЫХ ХРОНИЧЕСКИМ ЛИМФОЛЕЙКОЗОМ НА ЭТАПАХ ИММУНОХИМИОТЕРАПИИ

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РЕЗЮМЕ

Цель исследования. Изучить экспрессию иммунофенотипических маркерных молекул на В-лимфоцитах больных хроническим лимфолейкозом на этапах иммунохимиотерапии при мониторинге минимальной остаточной болезни.
Пациенты и методы. Обследованы 20 больных ХЛЛ, которым в период 2019–2022 гг. проведено 6 курсов иммунохимиотерапии (ИХТ) в режиме RB/FCR в НМИЦ онкологии г. Ростова-на-Дону. До лечения, после 3, 6 курсов ИХТ выполнялось иммунофенотипирование костного мозга методом проточной цитофлюориметрии. Данные оценены в Statistica 13.0.
Результаты. До лечения в зависимости от экспрессии прогностических маркеров (CD38, ZAP-70, CD11c, CD25, FMC7) выделены 3 группы больных. I (2 чел.) – без экспрессии CD38, ZAP-70, CD11c, CD25, FMC7 на опухолевых В-лимфоцитах. II (14 чел.) – с вариабельной экспрессией CD25, CD38 (0,4–47,6 % и 0,0–57,5 %, соответственно), отсутствием экспрессии ZAP-70, CD11c, FMC7. III (4 чел.) – с высокой экспрессией CD38 (57,5–69,2 %), ZAP-70 (36,6–48,3 %), CD11c (20,0–96,5 %), CD25 (64,9–92,7 %), FMC7 (13,6–88,6 %). После 3 курса ИХТ минимальная остаточная болезнь (МОБ): в I группе 0 %, во II-й 0,48 ± 0,13 %, в III-й 33,5 ± 7,84 %. После 6 курса ИХТ МОБ: в I группе 0 %, во II-й 0,42 ± 0,09 %, в III-й 33,2 ± 8,07 %. Экспрессия иммунофенотипических маркеров в II, III группах без изменений после 3,6 курсов ИХТ. Согласно критериям оценки ответа на терапию (IWCLL, 2018 г.) у пациентов I, II групп после 6 курса ИХТ полная ремиссия, у 3-х пациентов III группы частичная ремиссия, у 1 больного стабилизация процесса. Получены предварительные данные, указывающие на то, что отсутствие или повышенный уровень экспрессии CD38, CD25, ZAP-70, CD11c, FMC7 на В-лимфоцитах больных ХЛЛ до лечения могут предопределять гематологический ответ на терапию по схемам RB/FCR.
Заключение. Исходно повышенная экспрессия одновременно всех прогностических антигенов: CD38, CD25, ZAP-70, CD11c, FMC7 на опухолевой популяции В-лимфоцитов больных ХЛЛ ассоциируется с неудовлетворительным ответом на лечение, что представляется перспективным с точки зрения изучения влияния анализируемых маркерных молекул на достижение гематологического ответа на этапах иммунохимиотерапии.

Ключевые слова:
хронический лимфолейкоз, проточная цитометрия, минимальная остаточная болезнь, иммунофенотипические маркеры, иммунохимиотерапия

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RELEVANCE

Chronic lymphocytic leukemia (CLL) is a tumor of lymphoid tissue from mature (peripheral) B cells, characterized by damage to the bone marrow and lymph nodes. People over the age of 50 get sick, mostly. CLL is detected randomly, progresses slowly and often proceeds without pronounced symptoms for a long time [1; 2]. According to a number of studies [3; 4], CLL is classified as an extremely heterogeneous disease, the nature of the course of which varies from indolent to aggressive, and the prognosis in the same patient can change significantly over time [5]. Tumor B lymphocytes in CLL express antigens – CD19, CD5, CD23, CD20 (weak), CD22 (weak), CD43 [6; 7].

The assessment of the response to therapy is carried out according to the updated criteria of the International Working Group on CLL (IWCLL, 2018), according to which complete remission, partial remission, stabilization or progression of the disease is established. Parameters characterizing tumor mass (lymphadenopathy, hepatomegaly, splenomegaly, blood lymphocyte level, bone marrow infiltration, constitutional symptoms) and indicators characterizing bone marrow function (platelet, hemoglobin, neutrophil levels) are evaluated [8]. The most important stage in the final assessment of the effect of immunochemotherapy used in the treatment of CLL is the determination of minimal residual disease (MRD), which IWCLL is also included in the criteria for evaluating the response to therapy [8]. Minimal residual disease is characterized by the presence of a population of tumor cells in patients in complete remission, which cannot be detected by cytological method, but can be determined by highly sensitive methods of PCR and multicolored flow cytometry. Most prognostic schemes are based on the assessment of MRD in the blood and/or bone marrow, which allows us to reliably confirm MRD-negative complete remission. Thus, the content of the residual population of CLL cells in the blood or bone marrow above 1 % foreshadows an early relapse and may serve as a basis for changing therapy. The MRD content in the range from 0.90 % to 0.01 % characterizes a group of patients with a median disease progression-free survival (IBD) of about 3 years, which gives reason to consider the possibility of maintenance therapy. MRD values below 0.01 % indicate a high probability of long-term remission (> 5 years) [9; 10]. Gabor Kovacs et al. [11] analyzed the prognostic significance of MRD in comparison with the clinical response to therapy. It has been shown that achieving MRD negativity is as important as the clinical response to evaluate the effectiveness of therapy.

In the last decade, an improved understanding of the pathogenesis of CLL and the active introduction of the flow cytometry method into clinical practice has made it possible to use the expression of a number of immunophenotypic markers as prognostic indicators. In particular, activation antigens CD38 and CD25, ZAP70 protein, myelomonocytic antigens CD11c and CD11b, FMC7 (antigen of mature B-lymphocytes), absence of CD23 antigen or its weak expression on the surface of lymphocytes, etc. [10]. It was also found that CD38 expression on more than 20 % of CD19+/CD5+ cells is associated with a poor prognosis, and patients with immunophenotypically immature CD38+ CLL respond poorly to long-term multimode chemotherapy and, therefore, have a short life expectancy [12]. In almost 50 % of CLL patients, the expression of CD25 activation antigen on lymphoid cells is noted, which is considered a marker of tumor lymphocytes in hairy cell leukemia and is associated with an unfavorable prognosis of the disease [12]. The expression of ZAP-70 ≥ 20 % on tumor B-lymphocytes is considered as a risk factor for the progression and development of Richter syndrome [12]. In 26.7 % of patients with CLL, expression of the myelomonocytic antigen CD11c is observed, which is associated with a short doubling time of the number of lymphocytes in peripheral blood (< 12 months) [13]. The absence of CD23 antigen or its weak expression on the surface of lymphocytes, along with the simultaneous detection of positive expression by FMC7 (antigen of mature B-lymphocytes), is also associated with a poor prognosis [14].

Analysis of the literature data shows that the search for markers that allow predicting the course of the tumor process and the response of CLL patients to therapy at the stages of diagnosis and monitoring of MRD does not lose its relevance.

Purpose of the study: to study the expression profile of immunophenotypic marker molecules on B-lymphocytes in patients with chronic lymphocytic leukemia at the stages of immunochemotherapy while monitoring minimal residual disease.

PATIENTS AND METHODS

The study included 20 patients (13 men, 7 women), median age 66.4 ± 1.9 years with chronic lymphocytic leukemia (CLL) in stage C according to Binet, who had not previously received specific therapy. In the period from 2019 to 2022, 6 courses of antitumor drug therapy in RB or FCR mode were conducted in the Department of Oncogematology National Medical Research Centre for Oncology in Rostov-on-Don. At the stages before, after 3 and 6 courses of ICT, bone marrow immunophenotyping was performed by 10-color flow cytometry (Navios 10/3, Beckman Coulter, USA). The studies were carried out in native bone marrow cells in a solution of anticoagulant K2 EDTA. The study of the primary immunophenotype of B lymphocytes and prognostic marker molecules was performed using a panel of monoclonal antibodies labeled with various fluorochromes: CD45 (PB), CD19 (ECD, PC7), CD5 (PC7, APC), CD10 (PE), CD11c (PE), CD20 (PC7), CD22 (PE), CD23 (PE), CD25 (PC5), CD38 (FITC, PC7), CD43 (APC-A750), and FMC7 (FITC), ZAP-70 (PE), CD3 (PC7), kappa (FITC), lambda (PE). MRD was evaluated, taking into account that the residual population of CLL cells in the bone marrow < 0.01 % (0 %) is estimated as MRD negative status [9]. The results of flow cytometry were analyzed using the Kaluza v2.1 software (Beckman Coulter, USA). The collection of clinical information, biological material, sample preparation, quality control of biological samples, storage, as well as compliance with legal norms and rules related to patient confidentiality were carried out according to the developed algorithms of actions of departments of research and clinical groups National Medical Research Centre for Oncology [15]. The data obtained are evaluated in the Statistica 13.0 program, the results are presented taking into account the average values (M), the errors of the averages (m).

RESEARCH RESULTS AND DISCUSSION

Prior to treatment (MRD day 0), according to the results of flow cytofluorometry, all patients showed high expression of markers characteristic of CLL– CD5, CD23, CD20, CD22, CD43 (Fig. 1). Depending on the expression profile of prognostic markers such as CD38, ZAP-70, CD11c, CD25, FMC7, 3 groups of patients were identified (Table 1). Group I (2 people) was characterized by the absence of expression of CD38, ZAP-70, CD11c and CD25 markers, the level of FMC7 expression did not exceed 0.2 %. In group II (14 people), variable expression of CD25 and CD38 was noted, respectively 0.4 % – 47.6 % and 0.0 % – 57.5 %, the absence of expression of ZAP-70, CD11c, FMC7 was not more than 1.6 %. In group III (4 people), high expression of all studied prognostic markers was found: CD38 (57.5 % – 69.2 %), ZAP-70 (36,6 % – 48,3 %), CD11c (20.0 % – 96.5 %), CD25 (64.9 % – 92.7 %), FMC7 (13.6 % – 88.6 %) (Fig. 1, 2). There were no differences in age and gender between the groups.

After 3 courses of ICT, a decrease in the number of tumor B-lymphocytes was noted in all groups, but with varying degrees of intensity. Thus, patients of group I had MRD – negative status: no CLL cells were detected in the bone marrow (Table 2). In group II, a significant decrease in the amount of MRD (0.48 ± 0.13 %) was noted in comparison with the data before treatment (86.2 ± 1.43) ($p < 0.05$), at this stage attention was paid a decrease in CD38 expression on CLL cells to < 3 %, the expression of other prognostic markers remained unchanged in comparison with the level before treatment. In group III, after 3 courses of ICT, the residual population of CLL cells (MRD) averaged 33.5 ± 7.84 %, while the expression profile of all prognostic markers detected at the onset of the disease remained unchanged.

After 6 courses of ICT, attention was drawn to the absence of statistically significant differences in MRD values in all 3 groups in comparison with the data after 3 courses of ICT. The amount of MRD in group I was 0 %, in group II – 0.42 ± 0.09 %, in group III – 33.2 ± 8.07 % (Table 2). The expression profile of prognostic markers remained unchanged: the absence of expression in group I, variable CD25 expression, decreased CD38 expression after the 3rd course of ICT and the absence of ZAP expression-70, CD11c and FMC7 in group II, high expression of all analyzed markers – CD25, CD38, ZAP-70, CD11c and FMC7 in group III. The lack of dynamics in the values of MRD and expression of immunophenotypic markers after 6 courses of ICT may be the basis for evaluating the effect of treatment by IFT already at intermediate stages, that is, after the 4th and/or 5th courses of therapy in order to revise treatment regimens, which in our opinion requires further research.

At the same time, the differences in the expression profile of marker molecules on B-lymphocytes

established by us before the start of ICT in patients with CLL are of undoubted interest. Thus, a number of authors are conducting studies to assess the effect of CD38, ZAP-70, CD11c, CD25, FMC7 expression on the results of specific therapy, but these data are contradictory. It is known that in CLL, the positivity of CD38, which is a transmembrane glycoprotein, is a poor prognostic marker associated with resistance to treatment [16; 17]. It is assumed that CD38 expression of more than 20 % is associated with damage to the lymph nodes, liver, as well as with the aggressive course of the disease [17]. According to other data, CD38 positivity in the late stages is associated with eventless and overall survival, and in the early stages is a poor prognostic factor for overall survival [17].

According to our data, patients of group II (remission) had initially high levels of expression of 2 markers – CD38 and CD25. After ICT, there was a significant decrease in CD38 expression. And in group III (stabilization), the outcome showed high expression of all markers – CD38, ZAP-70, CD11c CD25, FMC7 and no change in the expression profile after treatment.

The results of the study of FMC7 expression are consistent with the literature data. It was shown that patients with FMC7 expression below 30 % needed treatment more often, in contrast to patients with expression over 30 % [18]. In our study, on the contrary, the absence of FMC7 expression was noted in groups I and II of patients with a positive response to RB and FCR treatment, while in group III there was a high expression of FMC7, which is consistent with the data of Choi Y. et al. (2021), who received similar results to ours [19].

Further, CD11c positivity is defined as aberrant expression in CLL [20; 21]. Clinical data and prognostic significance of CD11c in CLL are limited. A study by Umit E. G. (2017) revealed a significant relationship between CD11c positivity (≥ 20 %) and time to treatment [22]. According to our data, CD11c positivity was noted in group III patients and varies from 20.0 to 96.5 %.

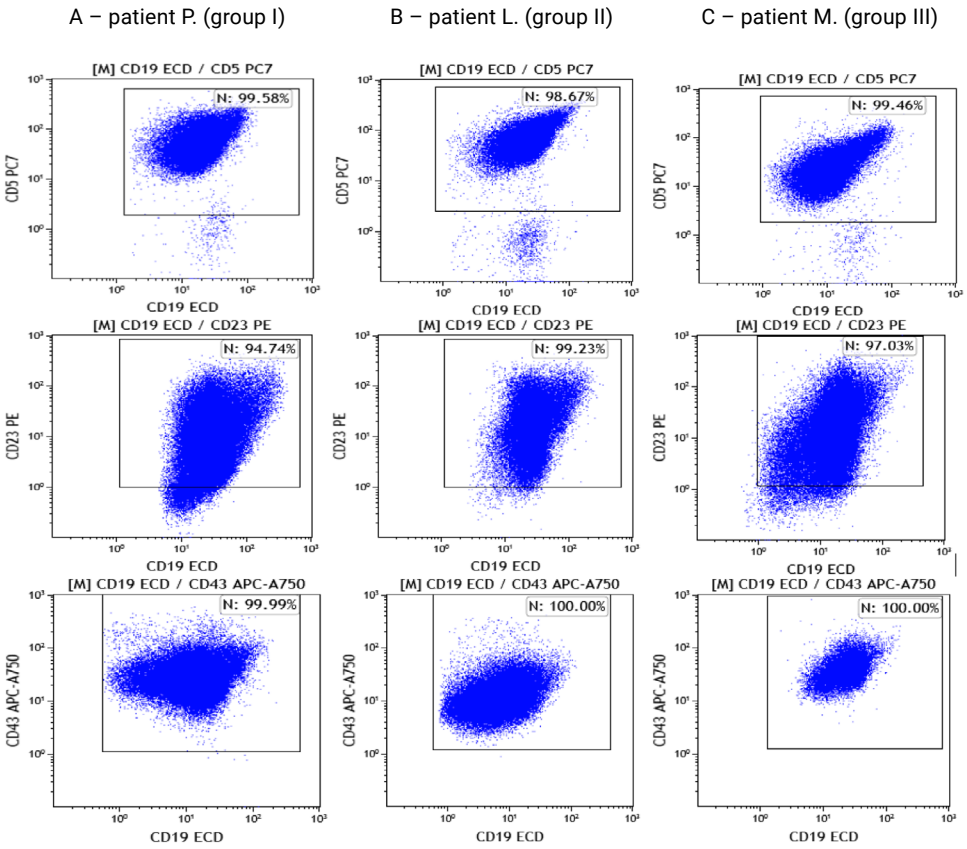

Fig. 1. Results of immunophenotyping of the bone marrow of patients with CLL by flow cytometry before treatment. Dot graphs of B-CLL-specific coexpression of CD molecules. The population of tumor B-lymphocytes is highlighted in blue: A – patient P. (group I), B – patient L. (group II), C – patient M. (group III).

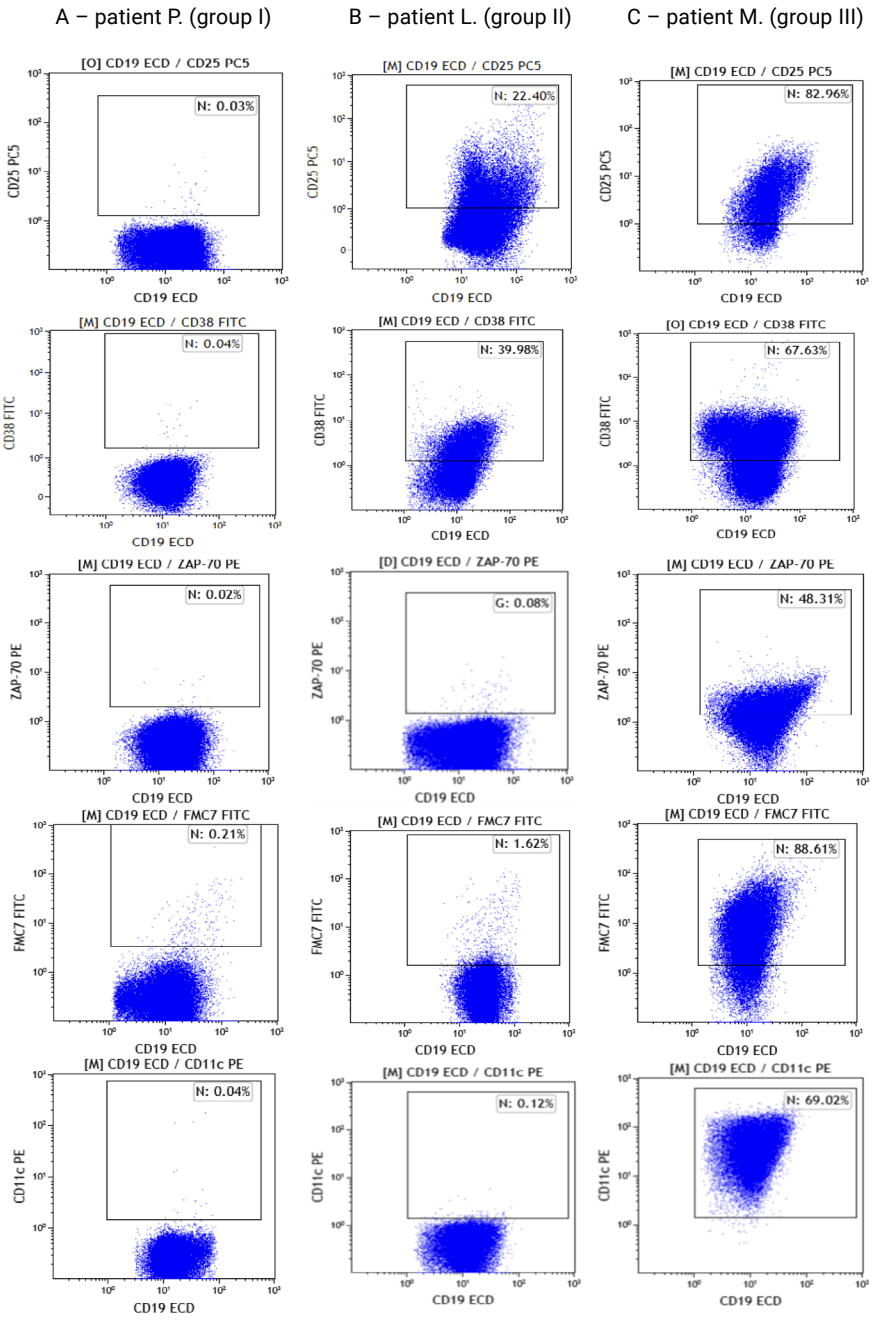

Fig. 2. Results of immunophenotyping of the bone marrow of patients with CLL by flow cytometry before treatment. Dot graphs of the expression of prognostic marker molecules. The population of tumor B-lymphocytes is highlighted in blue: A – patient P. (group I), B – patient L. (group II), C – patient M. (group III).

Table 1. Expression of CD38, ZAP-70, CD11c, CD25, FMC7 on B-lymphocytes before treatment in the bone marrow of CLL patients

Patients groups	CD38, %	ZAP-70, %	CD11c, %	CD25, %	FMC7, %
I (*n* = 2)	0	0	0	0	0–0.2
II (*n* = 14)	0–57.5	0	0	0.4–47.6	0–1.6
III (*n* = 4)	57.5–69.2	36.6–48.3	20.0–96.5	69.4–92.7	13.6–88.6

Table 2. Quantitative characteristics of MRD in the bone marrow of CLL patients at the stages of ICT

Patients groups	Quantitative characterization of MRD at the stages of examination (% of the total residual population of CLL nucleated cells)		
	Day 0 (M ± m)	After 3 ICT courses (M ± m)	After 6 ICT courses (M ± m)
I (*n* = 2)	72.4 ± 1.21	0*,**	0*,**
II (*n* = 14)	86.2 ± 1.43	0.48 ± 0.13*,**	0.42 ± 0.09*,**
III (*n* = 4)	90.1 ± 1.60	33.5 ± 7.84*,**	33.2 ± 8.07*,**

Note: NC – nucleated cells, * – statistically significant differences from MRD "day 0" in its group ($p < 0.05$), ** – statistically significant differences from MRD I and II/III groups ($p < 0.05$).

The expression of the CD25 antigen (the α-chain of the IL-2 surface receptor) reflects the activated state of the tumor lymphocyte. Shvidel L. et al. (2012) found no evidence that this parameter alone can be used as a predictor of overall survival or time to first treatment [23]. In our studies, the increased expression of CD25 on tumor B lymphocytes in group III, along with CD38, ZAP-70, CD11c, FMC7, was accompanied by an unsatisfactory response to therapy.

Thus, preliminary data were obtained indicating that the absence or increased expression of prognostic markers CD38, CD25, ZAP-70, CD11c, FMC7 on B-lymphocytes in patients at the stage of diagnosis of CLL may predetermine the hematological response to therapy according to RB or FCR schemes.

CONCLUSION

Initially, increased expression of all prognostic antigens simultaneously: CD38, CD25, ZAP-70, CD11c, FMC7 on the tumor population of B-lymphocytes in patients with CLL is associated with an unsatisfactory response to treatment, which seems promising from the point of view of studying the effect of the analyzed marker molecules on achieving a hematological response at the stages of immunochemotherapy.

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Lysenko I. B. – consultation on a clinical cases, interpretation and analysis of data, editing of the article;
Konovalchik M. A. – writing the text of the manuscript, editing the article.

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ORIGINAL ARTICLE

LOCAL IMMUNITY FEATURES IN PATIENTS WITH NON-INVASIVE MUSCULAR BLADDER CANCER OF VARIOUS DEGREES OF MALIGNANCE

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ABSTRACT

Purpose of the study. To study the features of the local distribution of populations of immune system cells in patients with non-invasive muscular bladder cancer of various degrees of malignancy.

Materials and methods. The study included 51 patients with newly diagnosed non-muscle-invasive bladder cancer (papillary urothelial carcinoma) who received complex treatment and follow-up after 9 months at the oncourological department of the National Medical Research Center of Oncology. Patients were divided into two groups: group 1 – with a tumor of low malignant potential (Low grade – LG), *n* = 31; group 2 – with a tumor of high malignant potential (High grade – HG), *n* = 20. After 6–9 months, 24 patients were diagnosed with a relapse of the disease – in 48,4 % in patients of group 1 (*n* = 15) and in 45 % – in group 2 (*n* = 9). In cell suspensions obtained from the primary and recurrent tumors, as well as the perifocal zone, the relative number of populations of immunocompetent cells was estimated using flow cytometry. A comparison was made of the content of individual populations of lymphocytes in the tumor tissue, the perifocal zone of primary and recurrent lesions of various degrees of malignancy. Statistical processing was performed using Statistica 13.0.

Results. The development of a recurrent tumor of low malignant potential is accompanied by the involvement of cells of innate immunity (NK- and NKT-lymphocytes) into its microenvironment, which is associated with an imbalance in the number of main cells of adaptive immunity – a fairly pronounced decrease in the tumor of T-lymphocytes of the helper-inductor type was noted with a constant content cytotoxic T-lymphocytes, as well as the multidirectional nature of changes in DP- (decrease) and DN-lymphocytes (increase). A feature of the development of a recurrent tumor of high malignant potential is that it is accompanied by the involvement of innate immunity cells (NK- and NKT-lymphocytes) into its microenvironment, as well as multidirectional changes in DP- (decrease) and DN-lymphocytes (increase).

Conclusion. Studies of the population composition of tumors and their perifocal tissues of NMIBC revealed a number of features that are reflected in the redistribution of cytolytic cells, the formation of immunosuppressive conditions, which are reflected both in the manifestation of the biological properties of tumor cells and in changes in the cellular composition of bladder tissues involved in the process. development and progression of cancer.

Keywords:
bladder cancer, non-muscle-invasive bladder cancer, local cellular immunity, lymphocytes, relapse

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ОРИГИНАЛЬНАЯ СТАТЬЯ

ОСОБЕННОСТИ ЛОКАЛЬНОГО ИММУНИТЕТА У ПАЦИЕНТОВ С НЕИНВАЗИВНО-МЫШЕЧНЫМ РАКОМ МОЧЕВОГО ПУЗЫРЯ РАЗЛИЧНОЙ СТЕПЕНИ ЗЛОКАЧЕСТВЕННОСТИ

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РЕЗЮМЕ

Цель исследования. Изучить особенности локального распределения популяций клеток иммунной системы у пациентов с немышечно-инвазивным раком мочевого пузыря различной степени злокачественности.
Материалы и методы. В исследование были включены 51 пациент с впервые выявленным немышечно-инвазивным раком мочевого пузыря (гистологическая верификация папиллярной уротелиальной карциномы), после комплексного лечения и динамического наблюдения в течение 9 мес. в онкоурологическом отделении ФГБУ «НМИЦ онкологии» Минздрава России. Пациенты были распределены на две группы: 1 группа – с опухолью низкого злокачественного потенциала (Low grade – LG), *n* = 31; 2 группа – с опухолью высокого злокачественного потенциала (High grade – HG), *n* = 20. Через 6–9 мес. у 24 пациентов был диагностирован рецидив заболевания – в 48,4 % у пациентов 1 группы (*n* = 15) и в 45 % – 2 группы (*n* = 9). В клеточных суспензиях, полученных из первичной и рецидивной опухоли, а также перифокальной зоны с использованием проточной цитометрии, оценивали относительное содержание иммунокомпетентных клеток. Проводили сравнение содержания отдельных популяций лимфоцитов в ткани опухоли, перифокальной зоны первичных и рецидивных образований различной степени злокачественности. Статистическая обработка выполнялась с использованием Statistica 13.0.
Результаты. Развитие рецидивной опухоли низкого злокачественного потенциала сопровождается привлечением в её микроокружение клеток врожденного иммунитета (NK- и NKT-лимфоцитов), что сопряжено с дисбалансом в количестве основных клеток адаптивного иммунитета – отмечено достаточно выраженное снижение в опухоли Т-лимфоцитов хелперно-индукторного типа при неизменном содержании цитотоксических Т-лимфоцитов, а также разнонаправленном характере изменения ДП- (снижение) и ДН-лимфоцитов (увеличение). Особенностью развития рецидивной опухоли высокого злокачественного потенциала является то, что оно сопровождается привлечением в её микроокружение клеток врожденного иммунитета (NK- и NKT-лимфоцитов), а также разнонаправленным изменением ДП- (снижение) и ДН-лимфоцитов (увеличение).
Заключение. Проведенные исследования популяционного состава опухолей, так и их перифокальных тканей немышечно-инвазивного рака мочевого пузыря позволили выявить особенности, отражающиеся в перераспределении цитолитических клеток, формированием иммуносупрессивных условий, отражающихся как на проявлении биологических свойств опухолевых клеток, так и на изменении клеточного состава тканей мочевого пузыря, вовлеченного в процесс развития и прогрессирования онкологического заболевания.

Ключевые слова:
рак мочевого пузыря, немышечно-инвазивный рак мочевого пузыря, локальный клеточный иммунитет, лимфоциты, рецидив

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RELEVANCE

According to the latest GLOBOCAN data, malignant neoplasms (MN) of the bladder account for 3 % of all cancer diagnoses in the world, a high prevalence is observed in developed countries [1]. According to world statistics, it continues to occupy the 10th line in the structure of general oncological morbidity [2]. Almost 75 % of urothelial carcinomas are non–invasive carcinomas with a high frequency of recurrence of the disease after surgical treatment without infiltration of the bladder wall or distant metastases, the remaining 25 % are a muscle-invasive form of bladder cancer, which is highly invasive and with the presence of distant metastases [3]. Among patients with superficial papillary lesions, multiple relapses are usually observed, and only 10–30 % of them develop invasive tumors of a high degree of malignancy [4].

Diagnostic methods for assessing non-musculoskeletal invasive bladder cancer (NMIBC), as well as relapse and progression, have a number of disadvantages: low sensitivity and specificity, therefore, there is a need to study this direction for more thorough diagnosis and detection of malignant neoplasms (MN) at the early stages of the development of relapse of the disease for adequate and timely treatment and selection management tactics of patients [5].

Currently, it is generally accepted that the development of tumors of various localization accompanies a violation of the antigenic homeostasis of the human body, which, at certain stages of the development of the pathological process, naturally causes the activation of various effector mechanisms of innate and adaptive immunity. Further development of neoplasm is accompanied by its complex interaction with other anatomical and physiological structures of the body, through the implementation of a number of stages, the study of which in order to identify new diagnostic and prognostic markers is an urgent task of modern oncoimmunology [6].

It has been shown that the distribution pattern and density of immune cells in the tumor reflect the activity of the immune system against tumor cells. In this context, various populations of immune cells have been studied by Russian and foreign authors. The close interaction of microenvironment cells with each other and with tumor cells leads to a change in their phenotype, gene expression and changes in functional activity. Previous studies have revealed that the density of T-lymphocytic infiltration by CD3- , CD8- or CD45R0-positive lymphocytes has a high prognostic value for various tumors. So, Sharma P. and co-authors demonstrated the best relapse-free survival and overall survival in 69 patients with muscle-invasive bladder cancer (IMBC) with a high content of CD8+ lymphocytes [7]. In addition, it has been shown that high levels of intracellular infiltration by CD3- and CD8-positive lymphocytes suggest better overall survival results among patients with BC [8].

However, despite the available information on the study of the role of individual cells of the immune system in the development of tumors and, in particular, BC, much remains unclear, including the features of the distribution of immune cells (IC) between the tumor and its perifocal zone, which may contribute to the nature of the development of the pathological process and the effectiveness of the treatment methods used. In connection with the above, the study of the role of the local distribution of cells of the immune system is an urgent task to identify new potential markers of the development of BC and the likelihood of its recurrence.

The aim of the study was to study the features of the local distribution of immune system cell populations in patients with noninvasive muscle bladder cancer of various degrees of malignancy.

MATERIALS AND METHODS

The study examined tumor tissue samples from 51 patients with newly diagnosed NMIBC. After the complex treatment in the volume: transurethral resection of the bladder + adjuvant intravesical chemotherapy No. 6 (TUR + IUCT), all patients were dynamically monitored for 9 months after the complex treatment. According to the results of histological analysis, patients with papillary urothelial carcinoma were divided into two groups: group 1 – with a tumor of low malignant potential (Low grade – LG), n = 31; Group 2 – with a tumor of high malignant potential (High grade – HG), n = 20. Every 3 months, patients underwent a control examination, including a cystoscopic examination of the bladder, as a result of which, after 6–9 months, 24 patients were diagnosed with a relapse of the disease – in 48.4 % of patients of group 1 (n = 15) and in 45 % of group 2 (n = 9).

Written informed consent was received from all patients to participate in the study.

Complex treatment was recommended to all patients with newly diagnosed NMIBC as treatment: TUR + IUCT. Intraoperatively, the material was collected – fragments of the tumor (TF) and the perifocal zone (PZ), as well as a similar sampling was carried out in patients with a relapse of the disease, which was detected during 6–9 months of dynamic observation. The preparations were delivered to the laboratory, where they were subjected to mechanical crushing followed by tissue homogenization using a BD Medimachine homogenizer, USA (2 ml of Cell Wash buffer was added to the TF fragments, homogenized for 30 seconds, the cell suspension was filtered using 50 μm Medicons, USA). The cells were deposited in a refrigerated centrifuge Eppendorf Centrifuge 5702R (Eppendorf AG, Germany) at 250g for 5 minutes. After removal of the supraplastic fluid, the cells were resuspended in 100 μl of "Cell Wash" buffer.

The cell suspension was treated with a panel of monoclonal antibodies: CD3 FITC/CD15+56 PE/CD45 PerCP-Cy5.5/CD4 PE-Cy7/CD19 APC/CD8 APC–Cy7 in accordance with the manufacturer's instructions (BD, USA). The results were evaluated using a FacsCantoII flow cytometer (BD, USA). At least 100,000 cells were accumulated in each sample for data analysis. The relative (percentage) content of cells of the desired phenotype to the total number of living cells was estimated. The content of individual populations of lymphocytes was compared (total number of lymphocytes (CD45+ cells, Lymph); CD45+CD3+ cells (total CD3+ lymphocytes); CD45+CD3+CD4+ cells (helper T-lymphocytes (Th)); CD45+CD3+CD8+ cells (cytotoxic T-lymphocytes (CTL)); CD45+CD3+CD4+CD8+ cells (double positive lymphocytes, DP); CD45+CD3+CD4-CD8 cells (double negative lymphocytes, DN); CD45+CD16+CD56+ cells (NK-lymphocytes); CD45+CD3+CD16+CD56+ cells (NKT-lymphocytes); CD45+CD19+ cells (B lymphocytes)) in the tumor tissue, the perifocal zone of primary and recurrent formations of varying degrees of malignancy.

Statistical processing was carried out using the STATISTICA 13 package (StatSoft Inc., USA). The nature of the distribution of the obtained data was evaluated using the Shapiro-Wilk criterion. Since the obtained results of the evaluation of the determined parameters did not obey the law of normal distribution, they are presented in the form of median (Me) and interquartile range – 25 and 75 percentiles (Me [LQ; UQ]). The reliability of the differences was assessed using the nonparametric Mann-Whitney criterion. The results were considered statistically significant at $p < 0.05$.

RESEARCH RESULTS AND DISCUSSION

When analyzing the nature of the development of the tumor process, the likelihood of disease progression and the peculiarities of the local immunological status, a special role is assigned to identifying the distribution of individual populations of ICC between the tumor tissue itself and its perifocal zone.

The results of such a comparison in low-grade tumors are presented in Figures 1, 2, 3.

It can be seen from the presented results that, in comparison with the tumor in the perifocal zone, there is an increase in the content of a number of lymphocytes: CD4+, CD45+CD3+CD4+CD8+, CD45+CD3+CD4-CD8- and CD45+CD19+ cells, respectively, by 30 % (45.8 (41.9; 46.9) vs. 35.1 (33,7; 41,2), p = 0,048), 133 % (0,7 (0,49; 1,4) against 0.3 (0.2; 0.5), p = 0,041), 93 % (6,2 (5,9; 8,1) against 3.2 (2.4; 3.9), p = 0.042) and 85 % (10.9 (7.5; 14.3) vs. 5.9 (2.8; 7.3), p = 0.035). The tendency to decrease in the relative number of CD8+ T-lymphocytes and CD45+CD16+CD56+ in the CD is noteworthy, which indicates their accumulation in the tumor tissue (Fig. 1).

In the case of considering the features of the distribution of IC between primary TFand its PP in patients with subsequent relapse (a group of primary relapsing), in this case, an increase in lymphoid infiltration of PP compared with TFwas revealed, which is probably realized due to an increase in cells with potential cytolytic activity – CD8+, CD45+CD16+CD56+ lymphocytes (Fig. 2). These indicators were higher in the PP compared to the TF by 130 % (38.2 (24.2; 47.9) vs. 16.6 (8.8; 21.1), p = 0,037), 23 % (50.4 (53.9; 67.6) against 41.0 (37.2; 44.9), p = 0.048) and 316 % (20.8 (12.4; 25.8) vs. 5.0 (3.9; 7.3), p = 0.028). Against this background, a significant decrease in CD45+CD16+CD56+ and CD45+CD19+ cells in the PP with their probable accumulation in the tumor was revealed (Fig. 2). These indicators in the PP were lower than the values in the tumor by 54 % (3.5 (2.3; 4.5) vs. 7.6 (5.8; 10.2), p = 0.035) and 53 % (4.7 (3.9; 8.3) vs. 10.0 (7.9; 17.2), p = 0.042).

Probably, in conditions of increasing the likelihood of further relapse, there is a redistribution of IC between the tumor tissue and the perifocal zone with

an increase in the latter lymphocytic infiltration, CD8+ and CD45+CD3+CD16+CD56+, as well as a decrease in CD4+, CD45+CD19+ and CD45+CD16+CD56+ cells. At the same time, the multidirectional nature of the distribution of the effector cells of innate immunity is revealed – NK lymphocytes accumulate in the tumor, while NKT lymphocytes concentrate in the perifocal zone.

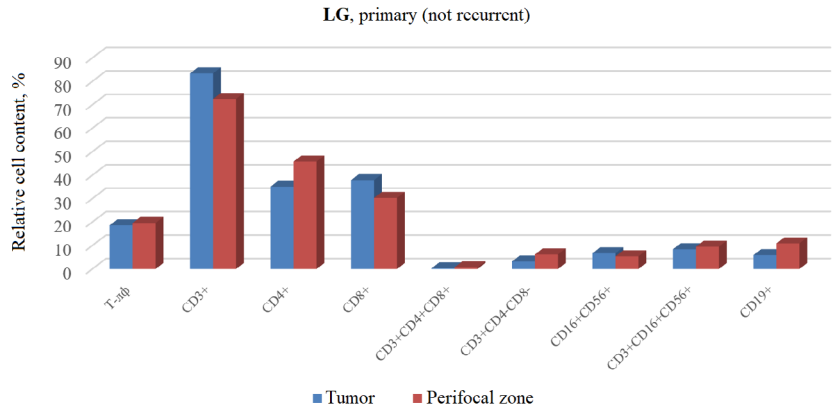

Fig. 1. Percentage of IC in the tumor and perifocal zone of patients with low-grade NMIBC, group 1 (LG) primary (non-recurrent).
Note: * – $p < 0.05$.

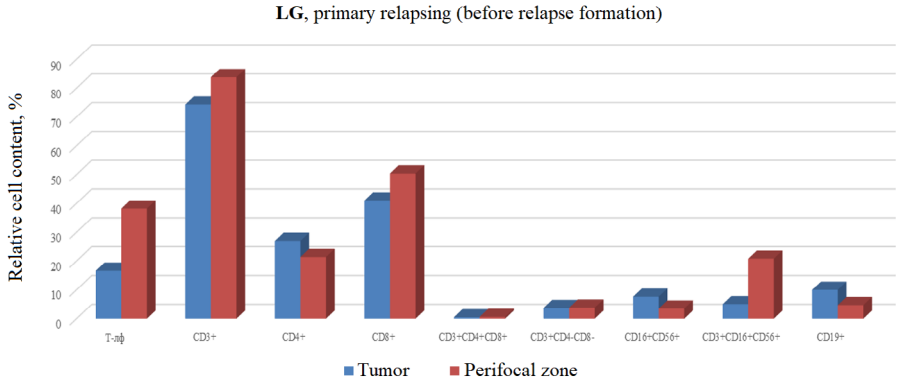

Fig. 2. Percentage of IC in the tumor and perifocal zone of patients with low-grade NMIBC, group 1 (LG) Primary relapsing (before relapse).
Note: * – $p < 0.05$.

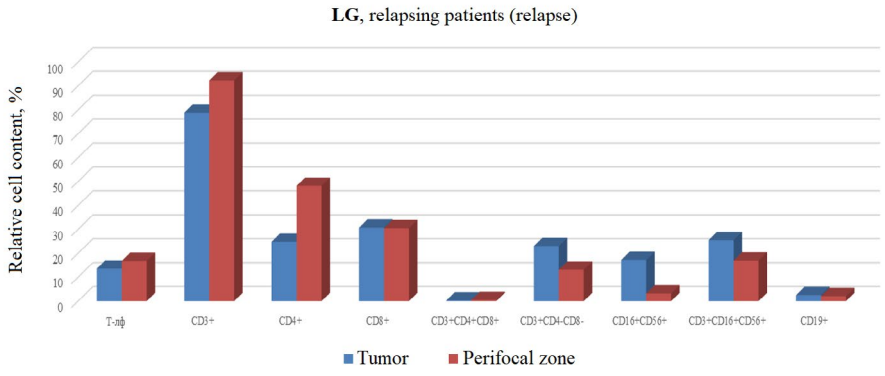

Fig. 3. Percentage of IC in the tumor and perifocal zone of patients with low-grade NMIBC, group 1 (LG) – relapsing patients (relapse).
Note: * – $p < 0.05$.

The analysis of the obtained results of determining the features of the distribution of IC in a recurrent tumor and its perifocal zone revealed the following (Fig. 3).

With the development of relapse, the accumulation of CD4+ and CD45+CD3+CD4+CD8+ cells in the tumor was observed, the content of which exceeded the values in the TF by 94 % 48.2 (40.3; 56) vs. 24.8 (16.1; 33.4), p = 0.021) and 67 % (0.5 (0.41; 0.75) vs. 0.3 (0.25; 0.35), p = 0.045). Against the background of the noted changes in the PD, a decrease in the number of CD45+CD3+CD4-CD8- (DN-lymphocytes) and effector cells of innate immunity – NK- and NKT-lymphocytes, which accumulate in the tumor, was found. The specified parameters in the PP were lower than in the TF by 43 % (13.1 (8; 18.2) versus 23.0 (18.5; 30), p = 0.046), 81 % (3.2 (2.6; 3.7) against 17.2 (10; 24.3), p = 0.033) and 33 % (17.0 (12; 21.9) vs. 25.5 (20.4; 30.5), p = 0.037).

Thus, the development of a recurrent tumor of low malignant potential is accompanied by the involvement of innate immunity cells (NK- and NKT-lymphocytes) into its microenvironment, which is associated with an imbalance in the number of main cells of adaptive immunity – a sufficiently pronounced decrease in helper-inductor type T-lymphocytes in the tumor was noted with a constant content of cytotoxic T-lymphocytes, as well as the multidirectional nature of the changes in DP- (decrease) and DN-lymphocytes (increase).

The results of comparing the distribution of IC populations between PP and TF in high-grade bladder tumors are presented in Figures 4, 5, 6.

From the presented results, it can be seen that compared with the tumor in the perifocal zone, there is an increase in the content of a number of lymphocytes: CD8+, CD45+CD3+CD4+CD8+, CD45+CD16+CD56+ and CD45+CD3+CD16+CD56+ cells, respectively, by 50 % (33.9 (27.1; 49.3) against 22.6 (16.4; 25.8), p = 0.026), 350 % (1.8 (1.4; 3.6) against 0.4 (0.25; 1.45), p = 0.008), 92 % (7.1 (4.9; 14.3) against 3.7 (2.6; 5.5), p = 0.031) and 134 % (13.8 (10.6; 20.3) vs. 5.9 (3.4; 6.6), p = 0.017). There was a decrease in the relative number of CD4+ and CD45+CD19+ cells in the PP, respectively, by 26 % (29.6 (16.8; 33.3) versus 39.8 (35.4; 47.9), p = 0.045) and 50 % (5.9 (2.5; 7.3) versus 11.7 (10.5; 16.4), p = 0.042), which indicates their accumulation in the tumor tissue (Fig. 4).

In the case of considering the features of the distribution of IC between the primary TF and its PP in patients with subsequent relapse (a group of primary relapsing), in this case, an increase in the infiltration of PP compared with the main populations of adaptive and innate immunity was revealed (Fig. 5). CD4+ content in the PP was statistically significant compared with the OP, CD8+ and CD45+CD3+CD4+CD8+ cells, as well as CD45+CD16+CD56+ and CD45+CD3+CD16+CD56+ cells, respectively, by 26 % (50.0 (49; 51.6) vs. 39.8 (30.6; 48.2), p = 0.037), 51 % (28.3 (27.9; 28.7) vs. 18.7 (12.7; 26.6), p = 0.046), 57 % (0.55 (0.52; 0.57) against 0.35 (0.23; 0.43), p = 0.047), as well as by 117 % (6.3 (4.8; 9.3) vs. 2.9 (2.1; 4.7), p = 0.041) and 52 % (6.4 (6.2; 8.6) vs. 4.2 (2.9; 5.1), p = 0.043). Against this background, a significant decrease in the content of B-lymphocytes in the PS with their probable accumulation in the tumor was revealed. These indicators in the PP were 46 % lower than the values in the tumor (11.4 (11.1; 19.3) vs. 21.0 (18.9; 30), p = 0.044).

Probably, in conditions of an increase in the likelihood of further relapse, there is a redistribution of IC between the tumor tissue and the perifocal zone with an increase in adaptive and innate immunity cells in the latter, potentially possessing cytolytic activity, as well as a decrease in the number of B lymphocytes.

The analysis of the obtained results of determining the features of the distribution of IC in a recurrent tumor and its perifocal zone revealed the following (Fig. 6).

With the development of relapse, CD45+CD3+CD4+CD8+ cells and CD45+CD19+ cells accumulated in the tumor, the content of which exceeded the values in the TF by 50 % (0.3 (0.25; 0.39) vs. 0.2 (0.1; 0.24), p = 0.047) and 396 % (27.3 (15.1; 32.4) vs. 5.5 (3.7; 6.05), p = 0.005). Against the background of the noted changes in the PD, a decrease in the number of total lymphoid infiltration, as well as CD45+CD3+CD4-CD8- (DN-lymphocytes) and effector cells of innate immunity – CD45+CD3+CD16+CD56+ (NKT-lymphocytes), which accumulate in the tumor, was found. The specified parameters in the PP were lower than in the TF by 54 % (20.8 (12.4; 29.2) vs. 45.2 (32; 48.7), p = 0.044), 88 % (1.5 (1.1; 3.5) against 12.2 (7.0; 15.3), p = 0.039) and 42 % (3.6 (2.1; 3.7) vs. 6.2 (3.6; 6.1), p = 0.042).

The data obtained earlier in our laboratory, which are also consistent with the results of similar studies, indicate that the immune microenvironment of tu-

mors largely contributes to their progression, among the variants of which are metastasis and recurrence [9]. Despite the fact that the latter is often regarded as a surgical problem arising due to the non-radicality of the operation, more and more data are accumulating indicating that a number of cellular factors, such as USC, as well as cells of the immune microenvironment, are involved in relapse. In particular, the review by Yan Chen et al. in 2022, which is a meta-analysis of recent studies on the role of the microenvironment in

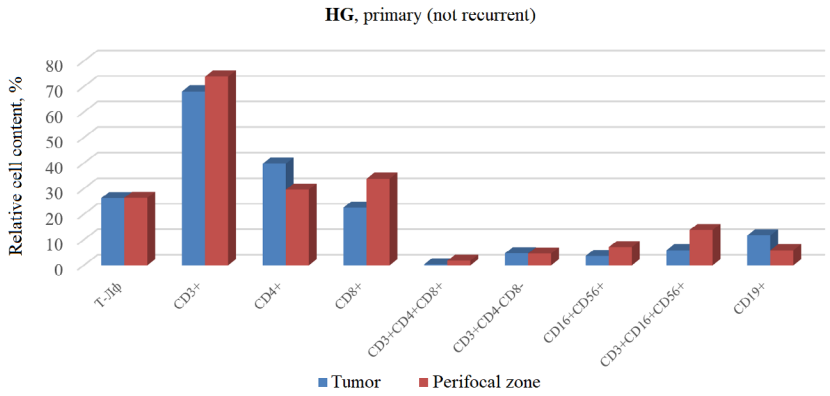

Fig. 4. The percentage of IC in the tumor and perifocal zone of patients with low-grade NMIRMP, group 2 (HG) primary (not recurrent). Note: * – $p < 0.05$.

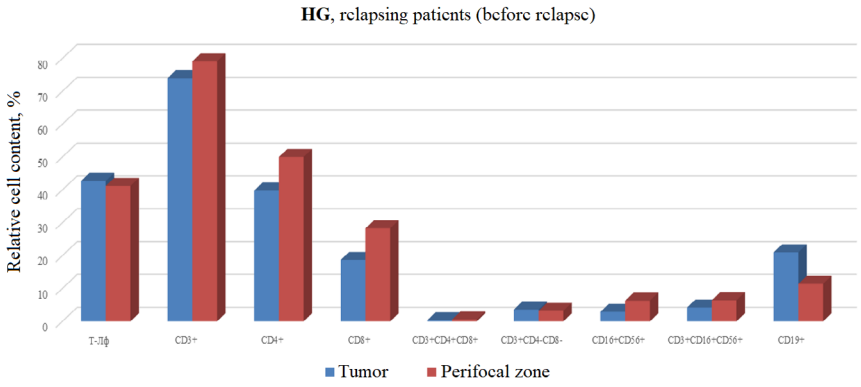

Fig. 5. Percentage of IC in the tumor and perifocal zone of patients with low-grade NMIBC, group 2 (HG) Primary relapsing (before relapse formation). Note: * – $p < 0.05$.

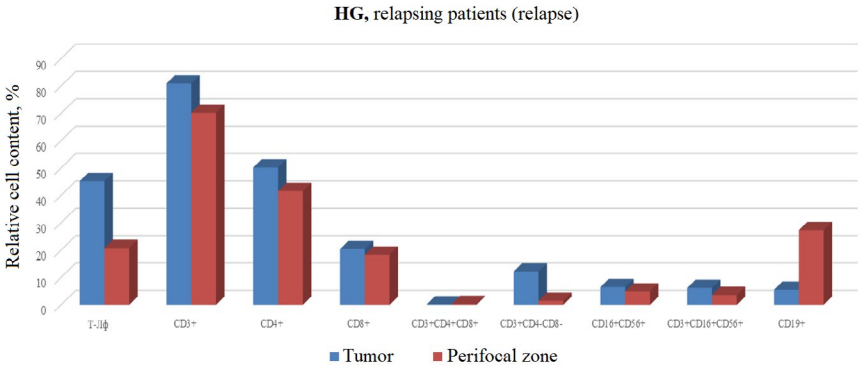

Fig. 6. Percentage of IC in the tumor and perifocal zone of patients with low-grade NMIBC, group 2 (HG) – relapsing patients (relapse). Note: * – $p < 0.05$.

the recurrence of gastric cancer, emphasizes the role of a high number of CD8+, CD4+Tm, NK lymphocytes, M1 macrophages, and a low content of M2 macrophages, Tregs lymphocytes, mast cells for long-term relapse-free survival of patients [10].

It is assumed that some minor subpopulations of T-lymphocytes, in particular, DP and DN, may play an important role in the process of relapse. According to the results of the study of the number of DP T-lymphocytes in the blood of patients with tumors of urological localization, including RMP, an increase in the level of these cells was found, and their heterogeneity was revealed, represented by CD4highCD8low and CD4+CD8 high DP subpopulations with that phenotype and related to Th2 [11].

DN T cells also attract a lot of attention. The review by Zhiheng Wu et al., 2022, summarizes reports on the multidirectional effects of these cells on tumor growth – from stimulation to antigen-independent cytolysis and the possibility of using them for adoptive immunotherapy [12]. At the same time, their phenotypic and functional heterogeneity is noted, as well as a change in their activity in the tumor microenvironment [13].

CONCLUSION

The conducted studies of the population composition of tumors and their perifocal tissues of NMIRMP revealed some features that are characterized by the development of a recurrent tumor of high malignant potential (HG) by the involvement of innate immunity cells (NK- and NKT-lymphocytes) in its microenvironment, as well as the multidirectional nature of changes in DP- (decrease) and DN-lymphocytes (increase). The emerging immunosuppressive conditions affect both the manifestation of the biological properties of tumor cells and the change in the cellular composition of bladder tissues involved in the development and progression of cancer.

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CLINICAL CASE REPORTS

SEQUENTIAL BRONCHOPLASTIC LOBECTOMIES IN COMPLEX TREATMENT FOR SYNCHRONOUS BILATERAL MULTIPLE PRIMARY NON-SMALL CELL LUNG CANCER: A RARE CLINICAL CASE

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ABSTRACT

Today, lung cancer (LC) occupies a special place in the oncological general morbidity among the male population both in Russia and in foreign countries. Despite modern diagnostic capabilities provided for modern physicians, steadily frequent cases of triggering and exclusion are more common in patients older than 60–65 years. Surgery is the main treatment for early-stage non-small cell lung cancer (NSCLC), but as the disease progresses, unfortunately, its effectiveness decreases. The strategy of diagnosing and treating patients with one NSCLC has been developed and worked out for a long time and does not cause any difficulties, but in the presence of two or more tumors, especially when they are located in both lungs, the correct choice of therapy is determined by many additional factors. This article describes the rare use of extended bronchoplastic upper lobectomy as a surgical component of the complex treatment of a patient with bilateral synchronous NSCLC. Based on our own observational data, it can be claimed that the use of modern therapeutic principles in combination with surgical intervention allows achieving satisfactory long-term results in the treatment of patients with primary multiple NSCLC.
The interest of the presented observation is based on the fact that it contains a description of a rare and unique application of sequential extended bronchoplastic upper lobectomy as a surgical component of the complex treatment of a patient with bilateral synchronous NSCLC, which we have not found analogues in the literature. We have shown that the consistent use of modern therapeutic modalities makes it possible to achieve satisfactory long-term results in the treatment of a locally advanced disease.

Keywords:
lung cancer, non-small cell lung cancer, primary multiple cancer, synchronous cancer, surgical treatment, multimodal approach

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КЛИНИЧЕСКОЕ НАБЛЮДЕНИЕ

ПОСЛЕДОВАТЕЛЬНЫЕ БРОНХОПЛАСТИЧЕСКИЕ ЛОБЭКТОМИИ В СТРУКТУРЕ КОМПЛЕКСНОГО ЛЕЧЕНИЯ СИНХРОННОГО ДВУХСТОРОННЕГО ПЕРВИЧНО-МНОЖЕСТВЕННОГО НЕМЕЛКОКЛЕТОЧНОГО РАКА ЛЕГКОГО: РЕДКОЕ КЛИНИЧЕСКОЕ НАБЛЮДЕНИЕ

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РЕЗЮМЕ

На сегодняшний день рак легкого (РЛ) занимает лидирующее место в структуре общей онкологической заболеваемости среди мужского населения как в России, так и на территории зарубежных стран. Несмотря на современные диагностические возможности, имеющиеся в арсенале у врачей, неуклонно растет показатель запущенности и смертности, а больше половины новых случаев данной патологии диагностируется у пациентов старше 60–65 лет. Хирургическое вмешательство является основным методом лечения ранних стадий немелкоклеточного рака легкого (НМРЛ), однако по мере прогрессирования заболевания, к сожалению, снижается эффективность его применения. Тактика диагностики и лечения пациентов с одним НМРЛ давно разработана и отработана, не вызывает никаких затруднений, а вот при наличии двух и более опухолей, особенно при их локализации в разных легких, правильный выбор терапии обусловлен множеством дополнительных факторов. Как правило, больные с местно-распространенным синхронным НМРЛ часто получают только консервативную терапию, а для тех пациентов, кто всё-таки подвергается хирургическому вмешательству, факторы прогноза клинического течения до сих пор непонятны. В данной статье приведено описание редкого применения расширенной бронхоспластической верхней лобэктомии в качестве хирургического компонента комплексного лечения больного двухсторонним синхронным НМРЛ. Основываясь на данных нашего собственного наблюдения, можно утверждать, что применение современных терапевтических принципов в комплексе с хирургическим вмешательством, позволяет добиться удовлетворительных отдаленных результатов лечения пациентов с первично-множенным НМРЛ.

Интерес представленного наблюдения заключается в том, что оно содержит описание редкого и уникального применения последовательной расширенных бронхопластических верхних лобэктомий в качестве хирургического компонента комплексного лечения больного двухсторонним синхронным НМРЛ, аналогов которого нами не было найдено в литературе. Нами показано, что последовательное применение современных терапевтических модальностей позволяет добиться удовлетворительных отдаленных результатов лечения местно-распространенного заболевания.

Ключевые слова:
рак легкого, немелкоклеточный рак легкого, первично-множественный рак, синхронный рак, хирургическое лечение, мультимодальный подход

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RELEVANCE

Lung cancer (LC) remains the main cause of cancer death among the male population, more than half of new cases of which are diagnosed in patients older than 60–65 years [1–3]. Acknowledging that the surgical method of treatment is the main one in the treatment of early stages of non-small cell lung cancer (NSCLC). However, as the process progresses, the risk of developing distant metastases increases and the effectiveness of the surgical method decreases. The principles of treatment of patients with one NSCLC have been developed for a long time and do not raise questions, and in the presence of two or more tumors, especially in two lungs, the correct choice of treatment depends on many factors. First of all, in the absence of extrathoracic metastases, the bilateral process can represent both independent primary tumors and intrapulmonary metastatic foci [4]. If in the first case the operation will benefit the patient, then in the second, unfortunately, it will not affect or may even negatively affect the outcome of the disease. The available data in the literature indicate the complexity of the issue under consideration. In a number of studies, it was reported that there was no long-term survival of patients who underwent surgical interventions, which is why there was an opinion about the poor prognosis of multifocal tumors, regardless of the probability of different biological behavior of tumors [5]. The median overall survival of patients with synchronous primary multiple lung cancer in the early stages, in the presence of contraindications to surgical treatment as a result of multimodal conservative therapy, now reaches about 31 months [6].

The ability to distinguish primary multiple lung tumors in order to determine indications for radical surgical treatment is constantly being improved using molecular genetic studies [3]. The previously developed criteria for primary multiple tumors do not help patients with synchronous lung cancer: about 50.8–57.9 % of tumors have a similar morphological histotype, and the correct assessment of the involvement of mediastinal lymph nodes (N+) before surgery is difficult [7]. However, despite the uncertainty regarding management tactics in recent studies, long-term survival of patients with bilateral synchronous NSCLC after surgical treatment has been reported [8; 9].

In this article, we present a clinical observation of a patient with bilateral central primary multiple NSCLC who underwent complex therapy with a good long-term treatment result. Despite the initial prevalence of tumor processes, radical organ-preserving surgical treatment became the basis of success. In the available literature, we have found no reports of sequential performance of extended bronchoplastic upper lobectomy on both sides as part of the complex therapy of primary multiple NSCLC.

The purpose of the study was to report on the clinical case of a patient with bilateral central primary multiple NSCLC who underwent complex therapy with a good long-term treatment outcome.

CLINICAL CASE REPORT

Patient K., 61 y.o., admitted to the National Medical Research Centre for Oncology with complaints of dry cough and shortness of breath during physical activity. It is known from the anamnesis that he considers himself sick since March 2018, when he noticed a change in the nature of the cough, which became dry, tearing and especially disturbed the patient at night. After performing a computed tomography of the thoracic cavity organs (CT TCO) at the place of residence, central cancer of the upper lobe of the left lung was suspected, and therefore the patient independently contacted our center.

The CT scan of the chest from 04/19/2018: central peribronchial nodular cancer of the left lung 5.0 × 5.5 cm with lesions of the upper lobe bronchus and distal sections of the left main bronchus. Central peribronchial nodular cancer of the right lung 2.2 × 2.7 cm with lesions of the upper lobe bronchus. Hypoventilation and pulmonitis of the upper lobes of both lungs. Lymph nodes were determined anteriorly from the aortic arch 2.2 cm, retrocaval 1.3 cm (Fig. 1).

On fibrobronchoscopy (FBS) from 04/28/2018: trachea and carina without features. On the right, the lumen in 3 is slit-like narrowed due to external pressure with signs of submucosal infiltration; on the left, the lumen of the upper lobar bronchus is blocked by an exophytic tumor by 4/5 (Fig. 2).

Histological conclusion: from B3 on the right, No. 40978-82/18 – foci of squamous cell carcinoma; from the upper lobar bronchus on the left, No. 40983-88/18 – foci of squamous cell carcinoma.

Based on the examination, the diagnosis was made: primary multiple synchronous cancer with damage to both lungs (classification of lung cancer according to the TNM system of the 7th revision):

1. Cancer of the left lung central peribrochial nodular form with lesions of the upper lobe and distal left main bronchus cT2N2M0, stage III, cl.gr.2.
2. Cancer of the right lung central peribrochial nodular form with lesions of the upper lobe bronchus cT1N2M0, stage IIIA, cl.gr.2. Cancer of the right lung central peribrochial nodular form with lesions of the upper lobe bronchus cT1N2M0, stage IIIA, cl.gr.2.

According to the recommendations of the council of the National Medical Research Centre for Oncology, taking into account the loco-regional prevalence of the tumor, the first stage of treatment was decided to conduct 3 courses of induction chemoimmunotherapy with a combination of cisplatin – 80 mg/m^2 and gemcitabine – 1600 mg/m^2 with recombinant tumor necrosis factor-thymosin alpha-1–150,000 IU/m^2, which were carried out in the period from 05/08/2018. to 07/26/2018 [10].

After completing the courses of induction chemotherapy, a control CT of TCO was performed 08/10/2018 (Fig. 3, 4). Its effectiveness was evaluated using the criteria of RECIST 1.1, which showed the presence of a partial response, and therefore a decision was made on surgical treatment.

Criteria for choosing the side of the lesion for the first stage of surgical treatment: taking into account the assumed greater prevalence of the tumor of the left lung (transition to the main bronchus, possible involvement of the left pulmonary artery in the tumor

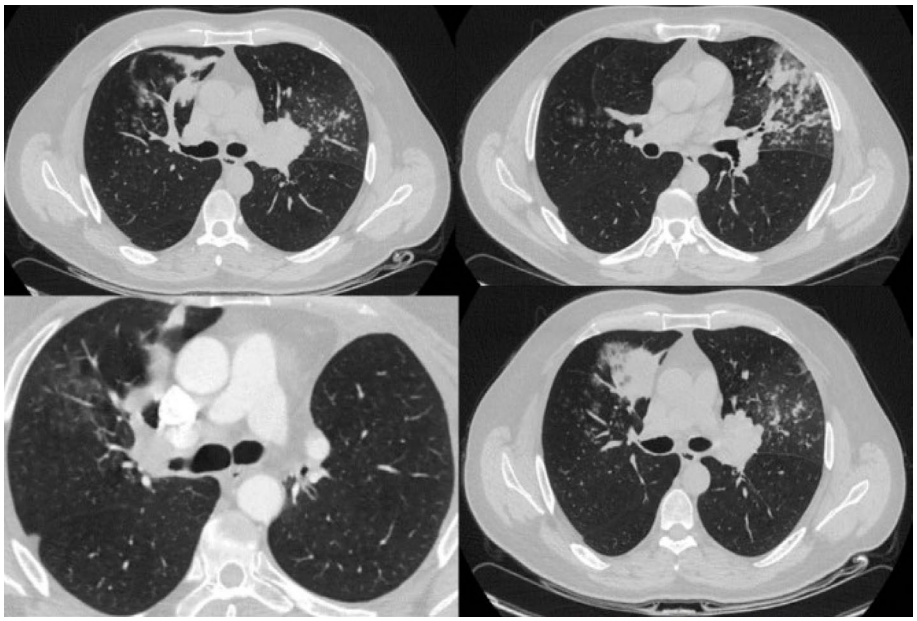

Fig. 1. CT of TCO from 04/19/2018 before the start of treatment.

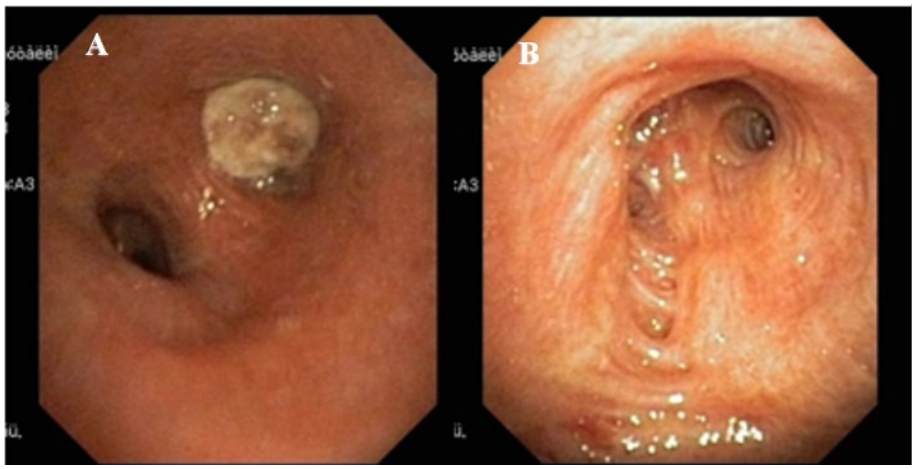

Fig. 2. FBS of 04/28/2018 before the start of treatment.

process, as well as the presence of complete atelectasis of the upper lobe), it was decided to perform the first stage of bronchoangioplastic upper lobectomy on the left.

On 09/03/2018, an extended upper bronchoplastic lobectomy was performed on the left with resection of three cartilaginous semicircles of the left main bronchus and lower lobar bronchus at the level of the mouth of B6 with the formation of a direct interbronchial anastomosis between the main and lower lobar bronchi "end to end". The postoperative period proceeded without complications. Postoperative histological analysis: highly differentiated squamous cell carcinoma with keratinization and foci of necrosis; along the line of bronchial resection at a distance of 2 cm from the visible boundaries of the tumor without signs of tumor growth; in 5 out of 6 bronchopulmonary lymph nodes, squamous cell carcinoma metastases; there are no metastases in the lymph nodes of the lung root, the "aortic window" and the tracheal bifurcation zone.

After 3 weeks, the patient underwent a control examination, including CT of TCO, FBS: without signs of progression, the anastomosis is stable, without signs of inflammation.

On 10/30/2018, an expanded bronchoplastic upper lobectomy was performed on the right with circular resection of two cartilaginous semicircles of the right main and one semicircle of the intermediate bronchi and the imposition of an end-to-end interbronchial anastomosis. The postoperative period proceeded without complications with early activation of the patient. Postoperative histological analysis: moderate differentiated squamous cell carcinoma with keratinization; bronchial resection lines without signs of tumor growth; in the lymph nodes of the root of the right lung, paratracheal metastases of squamous cell carcinoma on the right.

Thus, based on the results of morphological examination, the final diagnosis was established: primary multiple synchronous cancer with damage to both lungs:

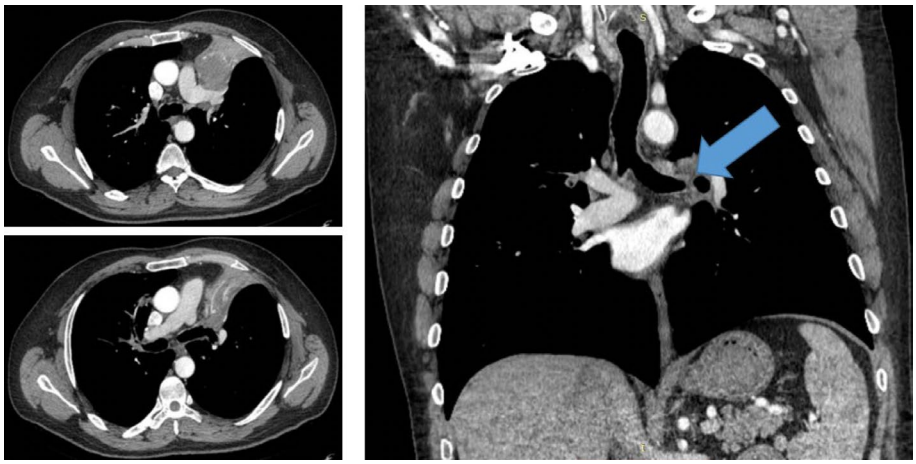

Fig. 3. CT of TCO from 08/10/2018 (after induction chemoimmunotherapy).

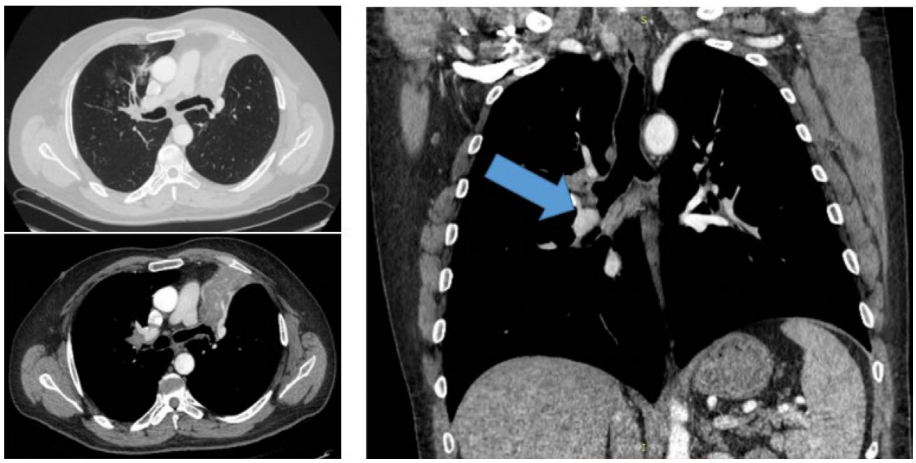

Fig. 4. CT of TCO from 08/10/2018 (after induction chemoimmunotherapy).

1. Cancer of the left lung central peribrochial nodular form with lesions of the upper lobe and distal left main bronchus pT2N1M0 G1R0, stage IIB cl.gr.2.
2. Cancer of the right lung central peribrochial nodular form with lesions of the upper lobe bronchus pT1N2M0G2R0, stage IIIA, cl.gr.2. Cancer of the right lung central peribrochial nodular form with lesions of the upper lobe bronchus pT1N2M0G2R0, stage IIIA, cl.gr.2.

By the decision of a consultation consisting of a thoracic oncologist, radiologist and chemotherapist and taking into account the effectiveness of induction chemotherapy, it is recommended to conduct 4 courses of adjuvant chemotherapy with a combination of carboplatin AUC 5–6 and gemcitabine 1000 mg/m^2 with an interval of 28 days.

From 11/24/2018 to 01/31/2019, the patient underwent 2 courses of adjuvant chemotherapy, which were accompanied by the development of an adverse toxic reaction in the form of grade 4 thrombocytopenia, which required transfusion of thromboconcentrate. From 03/01/2019 to 04/15/2019, 2 more courses of adjuvant chemotherapy were carried out, but with a 50 % reduction in the dose of gemcitabine.

After completion of chemotherapeutic treatment from 05/20/2019 to 06/14/2019 on the Novalis TX linear accelerator, Varian, by means of 7 static conformal fields using the IMRT mode, consolidating radiation therapy was performed on the lung root area and mediastinum ROD-2 Gr. to SOD = 46 Gr. From 07/25/2019 to 08/08/2019, 3D conformal IMRT radiation therapy was performed on the area of cervical-supraclavicular lymph nodes on both sides, ROD –3Gr. 5 fractions per week up to SOD = 39 isoGr. No radiation reactions were noted, the treatment was carried out satisfactorily.

The patient came for a follow-up examination in November 2020 on the FBS from 11/02/2020: condition after bronchoplastic upper lobectomy on the left and right. Anastomoses are consistent, without signs of inflammation and deformation. The lobular and segmental bronchi are freely passable on both sides.

02/02/2022 (40 months after surgery on the left lung, 39 months after surgery on the right lung), the patient underwent a control CT scan of the TCO: the condition is satisfactory, there are no complaints. There were no data for relapse of the disease.

DISCUSSION

The prognosis of the disease in patients after surgery for bilateral synchronous primary multiple NSCLC is quite favorable. According to the authors, the median overall survival in operations of any volume reached 52 months [5], and the 5-year survival rate was 38 % [8], which is several times higher compared to survival in stage IV NSCLC. Among all operations, as a rule, bilateral lobectomies are performed sequentially in approximately 1/3 of patients. After performing bilateral lobectomies or lobectomies with contralateral sublobar resections, the 3- and 5-year overall survival reaches 84.5 % and 75.0 %, respectively [9]. It was also found that the most important predictors of poor prognosis are: the degree of involvement of intra-thoracic lymph nodes N2 compared to N0 and N1 and unilateral localization of tumors compared to bilateral. The best survival rates of patients with bilateral NSCLC are due to a greater probability of true primary multiple lesion, taking into account the distance between the "tumor fields" without obvious signs of hematogenous metastasis. Morphological similarity of tumors as a prognostic factor is not associated with worse survival, on the contrary, a tendency to improve the survival rates of patients with tumors of the same histological structure was revealed.

In order to study the influence of these prognostic factors, Tanvetyanon and colleagues evaluated survival in 2 groups of patients, divided depending on the presence of risk factors such as gender, age, advanced stage of the disease and tumor localization. It was revealed that patients without risk factors had significantly better survival than patients with more than one adverse risk factor. The 5-year survival rate was 82 % for patients with absent risk factors compared to 43 % for those with present risk factors [5]. Since an equally favorable outcome will not affect all patients, established prognostic factors are necessary for making clinical decisions. The interest of our report lies in the fact that it contains a description of the rare use of extended bronchoplastic upper lobectomy as a surgical component of the complex treatment of a patient with bilateral synchronous NSCLC. Our experience shows that consistent application of modern therapeutic modalities allows us to achieve satisfactory long-term results of treatment of a locally advanced disease.

CONCLUSION

The interest of our observation lies in the fact that it contains a description of a rare and unique application of sequential expanded bronchoplastic upper lobectomy as a surgical component of complex treatment of a patient with bilateral synchronous NSCLC. It is shown that the consistent application of modern therapeutic modalities makes it possible to achieve satisfactory long-term results of treatment of a locally advanced disease.

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