THERAPEUTIC PATHOMORPHOSIS IN MALIGNANT GLIOMA TISSUES AFTER PHOTODYNAMIC THERAPY WITH CHLORIN e6 (REPORTS OF TWO CLINICAL CASES)

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Abstract

In recent years, photodynamic therapy (PDT) has been increasingly introduced into the surgical practice of treating malignant neoplasms. In this publication, the authors show the appearance of therapeutic pathomorphosis *in vivo* in human malignant glioma cells after intraoperative photodynamic therapy. Tissue samples obtained 10–14 days after PDT revealed nuclear and cytoplasmic signs indicating apoptosis, necrosis, and autophagy. A decrease in the proliferative activity of glial tumor cells and their higher death count were detected.. Immunohistochemical analysis shows decreases expression of Ki-67 cell proliferation marker and decreased amount of transcription factor protein p53.

Keywords: photodynamic therapy, malignant gliomas, chlorin e6, therapeutic pathomorphosis, brain.

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ЛЕЧЕБНЫЙ ПАТОМОРФОЗ В ТКАНЯХ ЗЛОКАЧЕСТВЕННОЙ ГЛИОМЫ ПОСЛЕ ФОТОДИНАМИЧЕСКОЙ ТЕРАПИИ С ХЛОРИНОМ e6 (СООБЩЕНИЕ О ДВУХ КЛИНИЧЕСКИХ СЛУЧАЯХ)

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Резюме

Фотодинамическая терапия (ФДТ) в последние годы все более внедряется в хирургическую практику лечения злокачественных новообразований. В данной публикации авторами показано появление лечебного патоморфоза *in vivo* в клетках злокачественной глиомы человека после интраоперационной ФДТ. В образцах тканей, полученных через 10–14 дней после ФДТ, выявлены ядерные и цитоплазматические признаки, указывающие на апоптоз, некроз и аутофагию. Обнаружено снижение пролиферативной активности глиальных опухолевых клеток, увеличение числа случаев их гибели. По данным иммуногистохимии отмечено уменьшение экспрессии маркера клеточной пролиферации Ki-67 и снижение содержания белка транскрипционного фактора p53.

Ключевые слова: фотодинамическая терапия, злокачественные глиомы, хлорин е6, лечебный патоморфоз, головной мозг.

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Introduction

Currently, photodynamic therapy (PDT) is actively used to treat tumors of various locations. Its use in the treatment of brain tumors was first suggested by I. Diamond in 1972 [1], and as early as in 1980, C. Perria described the results of treatment of malignant gliomas (MG) with PDT [2]. Later, scientists performed a significant number of small-scale clinical studies to assess the safety and effectiveness of this technique in glioma therapy [3, 4].

The PDT mechanism is aimed at the destruction of tumor cells and vessels that have accumulated the introduced photosensitizer (FS) and are located in the bed of the resected tumor, which is done with laser irradiation at a certain wavelength. Upon being administered in blood, a PS product is accumulated in the metabolically active tumor tissue.

The effect on the tumor tissue induces a number of intracellular and tissue mechanisms, as a result of which the cell dies through apoptosis, necrosis, or autophagy. The response to photodynamic effects depends on the type of tumor cell, its genetic or metabolic potential, as well as on the total amount of energy delivered by irradiation, the types of PS used, and their intracellular localization. The initial site affected by PDT can determine which pathway of cell death is activated. It is assumed that the autophagy process is triggered when cells damaged due to PDT try to retain damaged proteins and then remove them from the cell. The mechanism of apoptosis is triggered in the case of sufficiently severe damage in the cell that cannot be repaired. The use of PDT at maximum radiation doses leads to necrosis, since proteins involved in autophagy and apoptosis can be quickly destroyed, and cell integrity can be lost. In addition, the closure of tumor-feeding vessels can lead to local depletion of nutrients and oxygen and cause secondary necrosis associated with PDT [5-7].

A number of authors suggest that the method of cell death observed in PDT depends on the location of the greatest accumulation of PS inside the cell. With PS is localized in mitochondria, PDT will result in the loss of membrane permeability and the release of proapoptotic mediators, while the damage to the endoplasmic reticulum releases cellular calcium deposits. Damage to the lysosomes in which PS has accumulated leads to activation of proteolytic enzymes during laser radiation. Lysosomes can also merge with autophagosomes, leading to activation of hydrolysis of damaged organelles and their recirculation by autophagy. When PS is localized in more than one organelle, several pathways can participate in cell death simultaneously [7].

Additional mechanisms of the innate immune system trigger antiblastomic immunity in areas that are outside the PDT exposure zone. In some cases, PDT stimulates the immune system through several mechanisms, for example, by damaging cells through a combination of cytostatic mechanisms [7] and endogenous intracellular molecules known as Damage Associated Molecular Patterns (DAMPs) [8]. DAMPs that increase the immune system's sensitivity to tumor cells include Calreticulin, phosphatidylserine, adenosine triphosphate, peroxyredoxin 1, HMGB1, BCL-2, and annexin A1 [5, 9].

Among the many cytokines that can be activated by PDT, special attention is given to interleukin-1 (IL-1) and interleukin 6 (IL-6), serving as chemoattractants for various forms of immunocytes, including neutrophils, phagocytes, and lymphocytes [10]. Neutrophil penetration into cells that accumulated PS and were irradiated occurs within a few minutes after the PDT session, which increases the level of IL-1 and IL-6 in addition to the appearance of E-selectin in the cells of the perifocal tumoral inflammation zone. Some authors describe the significance of neutrophils in mediating PDT-induced cytotoxicity. In addition, activation of granulocyte-macrophage colony-stimulating factor (GM-CSF) leads to an increase in the number of neutrophils in tissues, potentiating the effectiveness of PDT. Cecic I. et al. showed the importance of the presence of anaphylatoxin C3a together with PDT-induced neutrophilic leukocytosis [11]. An inhibition of substances occurs in the cellular microenvironment, namely, of vasoendothelial growth factor, cyclooxygenase type 2, metalloproteinases of type 2 and type 9, Survivin apoptosis inhibitor, heat shock protein HSP-90, etc. In addition, PDT-induced release of other heat shock family proteins, such as HSP-47, 60, and 90, leads to increased sensitivity of the antigen-presenting complex to tumor cells [8]. The inhibition stops pathological angiogenesis and tumor cell proliferation. HSP70 binds to antigen-presenting cells (APCs) and facilitates antigen presentation, leading to dendritic cell maturation and activation of CD8 and cytostatic T-lymphocytes [12].

A PS fixed on the cell and exposed to laser radiation also activates non-immune mechanisms of antiblastoma resistance, such as carcinolytic cells (phagocytes, natural killers, cytotoxic T-lymphocytes), tumor necrosis factor-alpha, allogeneic inhibition factor and destruction of xenogenous cells (contact inhibition factors that inhibit the taxis and proliferation of tumor cells), alpha-lipoproteins of the surrounding tissue.

The outcome of these reactions is hypoxia, inflammation, and oxidative stress, leading to apoptotic and aseptic tumor necrosis. Secondary decrease in the synthesis of macroergic compounds in secondary tumor tissue hypoxia caused by the reduction of cytochrome C3, a lower ATP/ADP, and a reduction of NADN and NAD^+, causing a sharp decline in the respiratory capacity of mitochondria [13].

Activation of the lysosomal enzymes of the tumor cell causes intracellular catabolism of proteins and lipids, re-

sulting in the accumulation of underoxidized metabolic products, such as beta-oxybutyric acid and acetoacetic acid. There is a decrease in the activity of antioxidant systems of the glial tumor cell mainly due to the level of activity of superoxide dismutase and glutathione peroxidase, which triggers the activation of free radical oxidation and leads to the destruction of the cytoplasmic membranes of the tumor cell, mitochondria, lysosomes, as well as endoplasmic reticulum membranes, and the disruption of the cell's transport systems.

Activation of intracellular processes triggers a caspase cascade of complement activation with the formation of apoptosomal intracellular bodies. Caspases, through a series of biochemical reactions, activate the p53 protein, which suppresses the growth of tumor cells in the G1 phase. The point of application of PDT is also the endothelium of blood vessels and the system of macrophage cells, after irradiation of which the production of inflammatory mediators and cytokines (lymphokines, thromboxanes, prostoglandins, etc.) occurs, creating the vascular component for tumor stroma destruction [14].

It is extremely difficult to assess therapeutic pathomorphosis *in vivo* after PDT performed 7-14 days after surgery in patients with malignant glial tumors due to the intracranial location of the neoplasm. For its assessment, repeated surgical intervention is required in order to collect biopsy material, which is only possible subject to clinical indications. At the moment, there is almost no information in the literature concerning these data. The available scientific publications and reports are mainly based on the results of study of histological material obtained on models of glial tumors in animals, or on the analysis of glial tumor tissues *in vitro*, without blood flow and the microenvironment, with impaired biochemical and biophysical characteristics of the tissue, 6-72 hours after surgery [6, 15-20]. As a result, the data obtained are far from the changes that occur in real practice conditions.

The above-described results of studies conducted on various experimental models of glial neoplasms demonstrate that changes in tumor cells caused by PDT show signs of their death along the pathway of apoptosis, and less often by necrosis and autophagy [15-23]. There is evidence of the possibility of activation of PS in tumor cells located at a distance from the main focus or lying in the perifocal zone, since the penetration of radiated light into the brain tissue and tumors is limited by their physical and chemical properties. However, this is sufficient for a significant volumetric effect on the tissue achieved with the use of various diffusers to irradiate the entire cavity [4, 24-27].

Materials and methods

97 patients with supratentorial glial tumors were treated at Polenov Russian Neurosurgical Institute from 2004 to 2016.

According to the WHO classification, Grade IV was diagnosed in 49 (50.5%) patients, Grade III in 30 (31%), and Grade II in 18 (18.5%). Among patients with a Grade IV tumor, 48 had a morphological diagnosis of glioblastoma, and one of gliosarcoma. In the group of patients with Grade III tumors, anaplastic astrocytomas prevailed, while oligoastrocytomas and oligodendrogliomas were less common. The most common astrocytic tumors of Grade II were fibrillar-protoplasmic astrocytomas.

The patients underwent a complex treatment that included surgical removal of the tumor, intraoperative fluorescence diagnostics and PDT with Photoditazin, a second-generation chlorin e6 preparation (OOO VETA-GRAND, Russia, registration certificate No. ЛС 001246 dated 18.05.2012) [24, 25].

The degree of the totality of tumor removal was assessed with brain MRI (CT) data in the first 72 hours after surgery. In the vast majority of patients, a total (52 patients, 53.63%) or subtotal (33 patients, 34.0%) tumor removal was achieved. In 12 (12.37%) patients, the tumor was removed partially.

The choice and prescription of further adjuvant radiation or chemotherapy depended on the histological structure of the tumor.

An analysis of the effectiveness of PDT based on the results of morphological examination of biopsies obtained intraoperatively in 2 (4.1%) patients with Grade IV glial brain tumors of supratentorial localization was performed.

The methods of intraoperative photodynamic therapy

During the patient's stay on the operating table, after introductory anesthesia and 1.5 – 2 hours before the intended removal of the tumor, Photoditazine, a drug with chloride e6 as the active substance, diluted in 200 ml of saline solution in the dose of 1 mg per 1 kilo of body mass, was administered intravenously. The vial with the diluted agent was enclosed in a light-proof material. Photoditazine selectively accumulated in glioma tissue, while its concentration in normal brain tissue remained minimal, which allowed for determination of the tumor sites by the characteristic red fluorescence of chlorines (Fig. 1).

To perform fluorescence diagnostics in blue light, a fluorescent extension (LOMO, Saint Petersburg, Russia) was connected to the surgical microscope (LEICA OHS-1, Leica Microsystems, Germany). The fluorescent pattern with a high color contrast allowed to differentiate the tumor tissue that had accumulated Photoditazine from the intact tissue. At the same time, unaffected normal brain matter was also visible. The tissue with red fluorescence was gradually removed with due account for the functional and anatomical and physiological features of the tumor localization.

After removal, the neoplasms achieved thorough he-

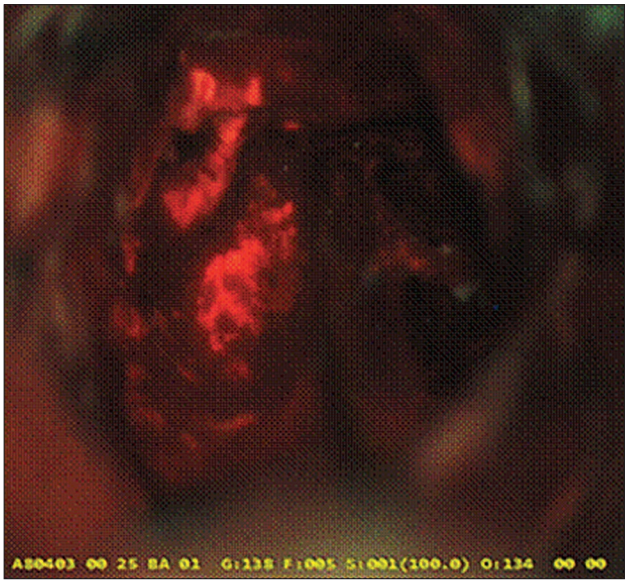

Рис. 1. Флуоресценция фотодитазина в глиобластоме
Fig. 1. Fluorescence of Fotoditazin in glioblastoma

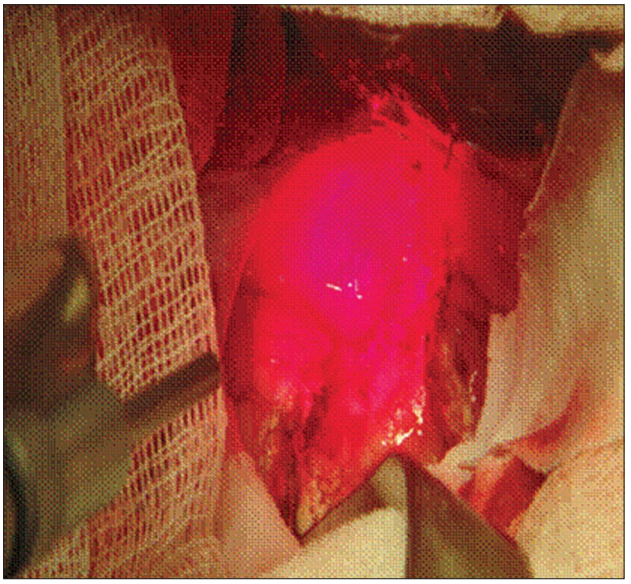

Рис. 2. Внешний вид операционного поля при проведении сеанса интраоперационной фотодинамической терапии
Fig. 2. A session of photodynamic therapy

mostasis in the perifocal zone. Then the distal end of a fiber instrument connected to a laser radiation source (Latus 2.5, Atkus, Russia) with a wavelength of 662 nm and a maximum power of 2.5 W was entered to reach the bed of the removed tumor, and laser irradiation was performed (Fig. 2). The duration of irradiation did not exceed 15-20 minutes. To prevent the risk of thermal damage to tissues during irradiation, the tumor bed was continuously irrigated with saline solution. The light dose was on average 180 J/cm^2. In the following 24 hours, the patient wore dark glasses to avoid direct sunlight on the retina to prevent its photodamage due to the presence of residual Photoditazine on it.

In all patients, the diagnosis was confirmed by histological examination of the surgical material in accordance with the WHO classification of CNS tumors. The material was fixed in 10% formalin, dehydrated in the standard way, and covered with paraffin. Sections with a thickness of 3-5 microns were made (Leica SM 2000R microtome, Leica Microsystems, Germany), stained with hematoxylin and eosin, and, if necessary, other types of staining were performed: van Gieson pycrofuxin, silver impregnation. A Leica 4000B laboratory microscope (Leica Microsystems, Germany) and a Leica DM 2500 laboratory microscope (Leica Microsystems, Germany) equipped with a digital camera and an adapted Adobe Photoshop CS 3 program were used for visualization and microphotography. The expression of p53 and Ki-67 (MIB-1) markers was determined by the immunohistochemical method.

Results

Two patients underwent repeated operations for the developed complications.

In the first observation, patient K., a 58-year-old male with glioblastoma of the right frontal lobe, was re-operated 10 days after PDT surgery in connection with the formation of an acute epidural hematoma in the area of surgery due to an injury sustained when he felt severe dizziness and fell down. In the postoperative period, the neurological status of the patient did not change.

During the first operation, photoditazine was administered intravenously at a dose of 1 mg per kilo of body mass 1.5 hours before the tumor was removed, to induce photodynamic effects. The PDT session was performed with a 1 cm long diffuser in continuous mode, an optical fiber diameter of 200 microns, an output optical power of 0.5 W, and an energy density of 180 J/cm^2. The duration of irradiation was 15 minutes.

In another observation, patient M., a 45-year-old male with glioblastoma of the left frontal lobe, was re-operated for osteomyelitis of the bone flap 2 weeks after PDT surgery. In the postoperative period, the patient's neurological status did not deteriorate.

To induce photodynamic effects during the first operation, Photoditazine was also administered intravenously at a dose of 1 mg per kilo of body mass 1.5 hours before the tumor removal. The duration of irradiation was 15 min. The irradiation was performed with a 1 cm long cone-shaped diffuser with a ball at the end, in continuous mode, with an optical fiber diameter of 200 microns,

and an output optical power of 0.5 W. The energy density was 180 J/cm^2.

In both cases, biopsies were taken from the bed of the removed tumor at the PDT site. The PDT-induced medical pathomorphosis was subject to pathomorphological and immunohistological evaluation. The tissue samples obtained after PDT were found to have nuclear and cytoplasmic signs indicating apoptosis, necrosis, and autophagy. Chromatin marginality along the intact nuclear membrane, chromatin condensation, swollen mitochondria with fragmentation of mitochondrial ridges, an increase in the number of cytoplasmic vacuoles, and membrane erosions were observed (Fig. 3, 4). In glial tumor biopsies, homogenization, loss of cell boundaries, the formation of ghost cells with pycnotic nuclei, perinuclear vacuolization and cytoplasm shrinkage, and pronounced vacuolar degeneration were observed. Individual cells in tumor nodules, mainly located at the borders, showed morphological changes characteristic of apoptosis: chromatin condensation, karyopyknozis, eosinophilic cytoplasm and increased nuclear-cytoplasmic ratio.

In the first case, against the background of PDT, pronounced fields of gliosis and necrosis, intracellular signs of chromatin condensation, cell fragmentation, apoptotic corpuscles, and the presence of phagosomes were observed. There were signs of typical dystrophic and alterative changes against the background of pronounced vascular disorders: stasis, sharp hyperemia, sludge phenomenon, microthrombosis, plasmarrhage and hemorrhage, and inflammatory infiltration.

In the second case, there was a marked increase in the number of cytoplasmic vacuoles, membrane erosion, fragmentation of mitochondrial ridges, karyopycnosis, the signs of autophagy, and the presence of "monster cells". The blood vessels were slit-shaped with partially formed thin walls and deformed lumen.

The foci of necrosis were surrounded by connective tissue (substitutive gliosis). The formation of lymphoplasmocytic infiltrates, as well as lymphocytic perivascular couplings, and the appearance of giant multinucleated and xanthomous cells, was observed around tumor cells groups. It should be particularly noted that the alterative changes in the tumor tissues had a gradient character, with the severity gradually decreasing from the center to the periphery.

The presence of therapeutic pathomorphosis after PDT was also observed in the immunohistochemical study of drugs, which revealed a decrease in the expression of Ki-67 cell proliferation marker from 31 to 7% in the first case and from 29 to 6% in the second case. There was a decrease in the protein content of transcription factor p53 after PDT from "+++" to "+" in both patients (Fig. 5, 6).

The catamnesis in the first patient after complex treatment with intraoperative PDT and subsequent radiation therapy (total boost dose: 95 Gy) and chemotherapy with Temozolomide (6 courses) was 19 months. In the second patient, after complex treatment with intraoperative PDT and subsequent radiation therapy (total boost dose = 60 Gy) and chemotherapy with Temozolomide (4 courses), the catamnesis was 17.5 months.

Discussion

High risks of continued tumor growth and low median survival in MG are the main reasons that adversely affect the outcome of treatment. Despite the high probability of tumor recurrence, many literature sources report that the delaying time to relapse and the median life expectancy of patients with MG depends on the degree of radicality of the surgical intervention. This pattern also remains true with repeated surgical interventions. However, it should be taken into account that glial tumors are characterized by invasive growth with the spread of tumor cells across perivascular spaces at a significant distance from the main tumor focus. The nature of glioma growth and the limited possibility of resection in functionally significant areas of the brain do not allow for the total removal of the tumor [3, 4, 24-26]. Therefore, the search for the latest treatment methods that achieve the maximum possible removal of tumor cells in the perifocal zone of the tumor and at a distance from the main focus remains an urgent problem. One of these methods is intraoperative PDT. The safety and effectiveness of this technique in the treatment of oncological diseases of different localities have been shown by a number of authors. In recent years, publications on the results of PDT use in patients with MG have become more frequent. However, reseach on the effectiveness of PDT in patients with MG based on the results of morphological studies is extremely rare. Those are mainly experimental models of cell strains and biopsy materials in animals with implanted human gliomas subjected to PDT.

The response of tumor tissues to intraoperative PDT is a complex of induced alterative/destructive changes in the glioma. Its assessment is of particular importance for determining the effectiveness of PDT, which occupies a crucial place in the treatment of MG.

In our study, the evaluation of the effectiveness of PDT was based not only on catamnesis data but also on the study of therapeutic pathomorphosis in the histological examination of tumor preparations before and after PDT.

It was found that the tissue and cellular targets of photoditazine PS are the vascular wall, plasma membrane of neoplastic tissue, and intracellular structures and mechanisms responsible for proliferation and biosynthesis processes. The intracellular arrangement of FS in various organoids (mitochondria, lysosomes, endoplasmic reticulum, cytoplasmic membrane, etc.) played an important role in the cell death mechanism. After PDT, the proliferative activity of tumor cells decreased. According to the immunohistochemical study, there was a decrease in the

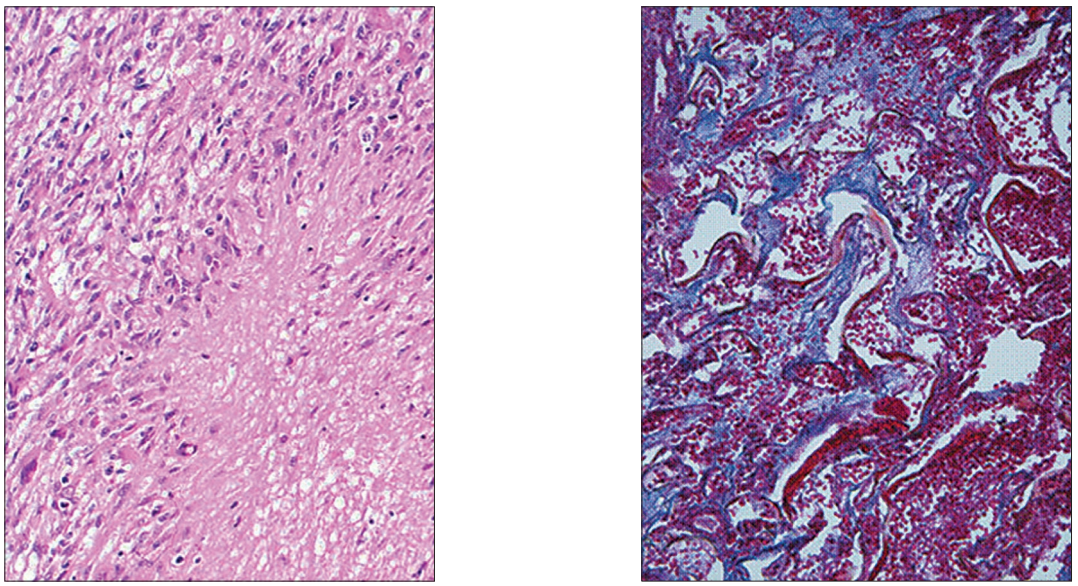

Рис. 3. Микрофотография препарата глиобластомы пациента К.:
a – до проведения фотодинамической терапии;
b – лечебный патоморфоз через 10 дней после проведения фотодинамической терапии. Окраска гематоксилином-эозином. Ув. х200.
Fig. 3. Micrograph of glioblastoma of patient K. (magnification x200, hematoxylin-eosin staining):
a – before photodynamic therapy;
b – therapeutic pathomorphosis 10 days after photodynamic therapy

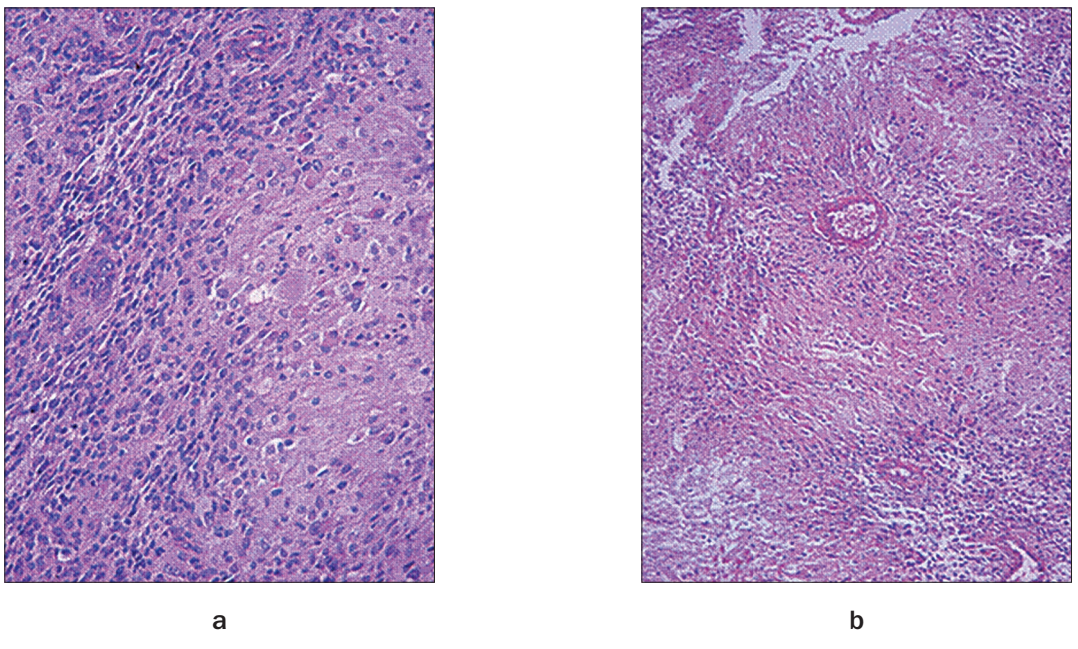

Рис. 4. Микрофотография препарата глиобластомы пациента М.:
a – до проведения фотодинамической терапии;
b – лечебный патоморфоз через 14 дней после фотодинамической терапии. Окраска гематоксилином-эозином. Ув. х200.
Fig. 4. Micrograph of glioblastoma of patient M. (Magnification x200, stained with hematoxylin-eosin):
a – before photodynamic therapy;
b – therapeutic pathomorphosis 14 days after photodynamic therapy

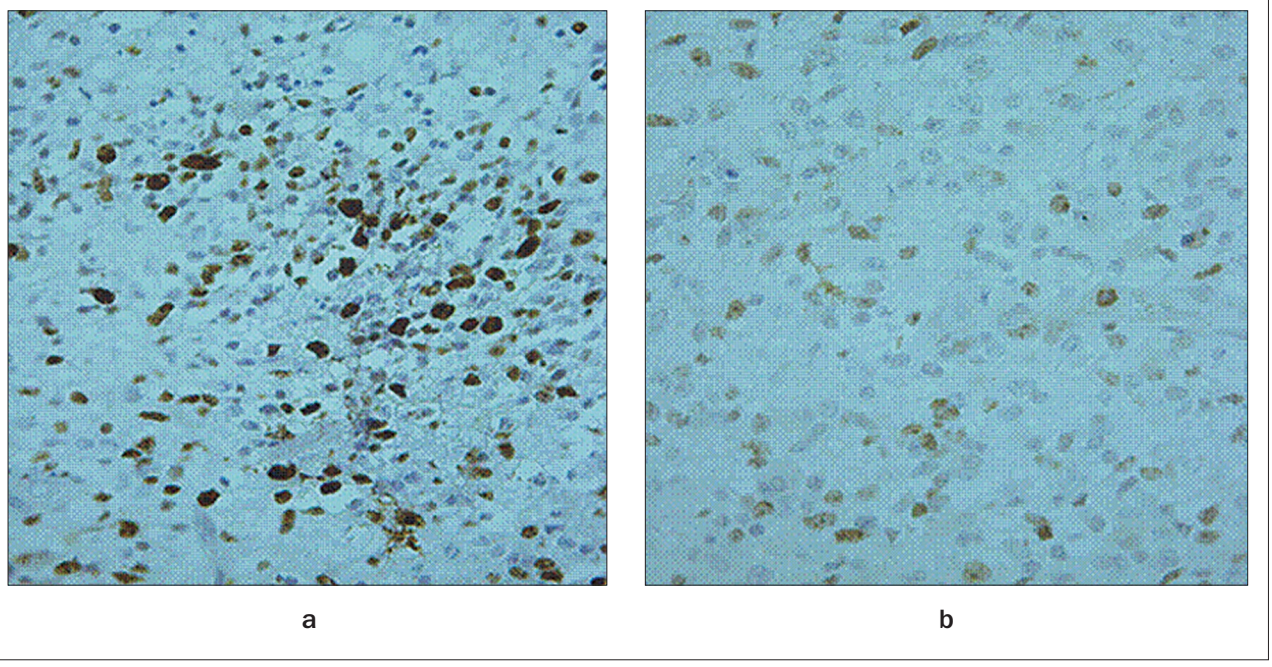

Рис. 5. Иммуногистохимия. Экспрессия Ki-67 в препарате глиобластомы пациента М.:
a – до проведения фотодинамической терапии;
b – лечебный патоморфоз через 14 дней после фотодинамической терапии. Ув. х400
Fig. 5. Immunohistochemistry. Expression of Ki-67 in glioblastoma of patient M. (magnification x400):
a – before photodynamic therapy;
b – therapeutic pathomorphosis 14 days after photodynamic therapy

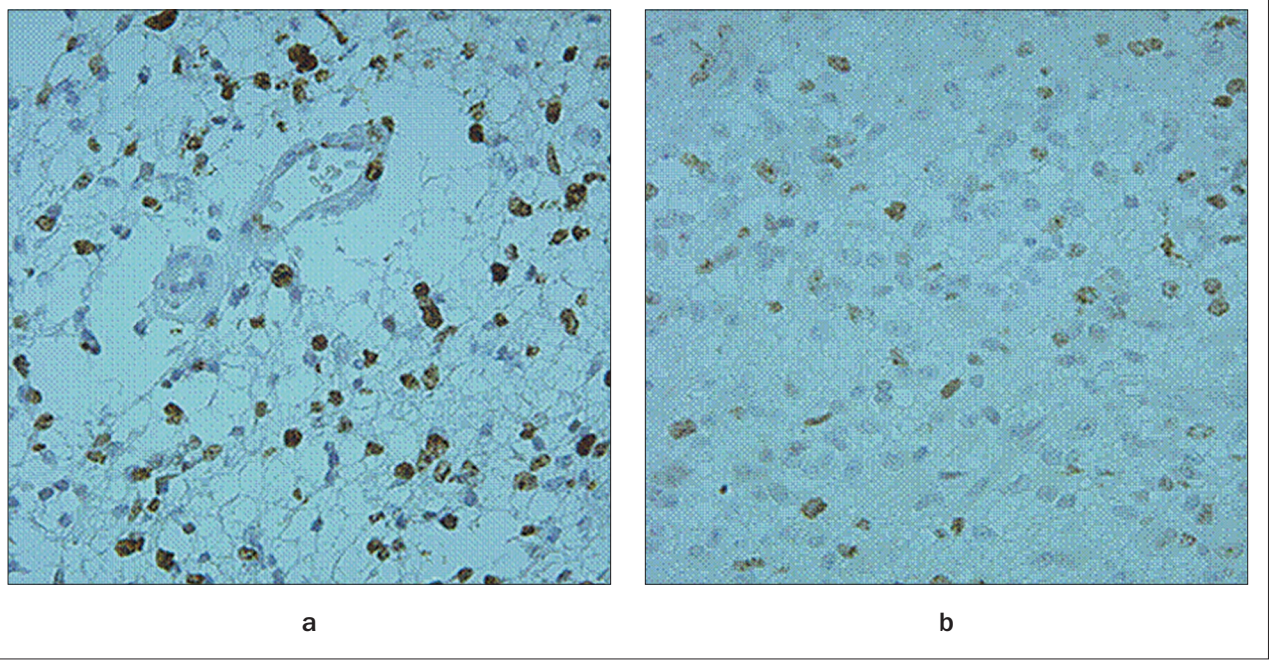

Рис. 6. Иммуногистохимия. Экспрессия p53 в препарате глиобластомы пациента М.:
a – до проведения фотодинамической терапии;
b – лечебный патоморфоз через 14 дней после фотодинамической терапии. Ув. х400.
Fig. 6. Immunohistochemistry. Expression of p53 in glioblastoma of patient M. (magnification x400):
a – before photodynamic therapy;
b – therapeutic pathomorphosis 14 days after photodynamic therapy

expression of the cell proliferation marker Ki-67 and the level of p53 transcription factor protein. Thus, a decrease in Ki-67 and p53 expression after PDT can be considered as a favorable predictor of therapy effectiveness.

The data obtained as a result of our study indicate morphological changes in glial tumor cells after PDT. However, the small scale of our clinical study does not allow us to draw unambiguous and reliable conclusions, so a further study of the issue is required.

Conclusion

The results of our work can testify to the effectiveness of PDT in the structure of complex treatment of patients with MG, as evidenced by the results of the application of the technique by many authors [3, 4, 7, 24-29].

The revealed morphological changes in glioblastoma tissues after PDT should be regarded as a manifestation of therapeutic pathomorphosis.

In the near future, PDT will definitely become the standard of treatment for patients with MG on a par with such methods as radiation and chemotherapy. Moreover, PDT has no systemic side effects on healthy tissues, such as those that occur after chemotherapy and radiation therapy, and belongs to the methods based on superselective action on tumor cells.

PDT is a promising and safe method that makes it possible to intraoperatively affect disseminated tumor cells lying in the perifocal zone and cause structural changes in those cells (therapeutic pathomorphosis), which determines the best long-term results of MG patients treatment. This technique should be used as part of comprehensive treatment in the surgery of glial brain tumors of supratentorial localization of varying degrees of malignancy. However, further clinical data is necessary to study the effectiveness and usefulness of PDT in MG patients.

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