УДК 616.33-006.6-036-008.851:577.1
https://doi.org: 10.20538/1682-0363-2020-2-20-27

Structural and functional indicators of erythrocyte membranes in gastric cancer patients with different histotypes of the tumor and stages of the malignant process

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ABSTRACT

The aim was to study the structural and functional parameters of erythrocyte membranes in the blood of patients with gastric cancer (GC) – adenocarcinoma, depending on its grade, signet ring cell carcinoma (SRCC), and combined gastric lesions (CGL).

Materials and methods. The membrane fluidity in the area of the lipid bilayer and protein-lipid contacts, the polarity of the lipid bilayer and the immersion of proteins in the lipid matrix of the membrane in red blood cells were evaluated by fluorimetry using the hydrophobic pyrene-based probe. The study included 86 patients with GC divided into six groups: well- and moderately-differentiated adenocarcinoma (G1-2); poorly-differentiated adenocarcinoma (G3); SRCC; CGL and two groups of patients with a component of undifferentiated cancer: G4 + SRCC and G4 + G2-3. The results of the study were also analyzed in patients with serosal invasion and the spread to adjacent structures (T4 according to the TNM classification of malignant tumors) and in patients with stage IV disease.

Results. In all groups of GC patients, an increase in the membrane fluidity was observed. It was more pronounced in the zone of protein-lipid contacts, but it was also observed in the lipid bilayer. The membrane fluidity increased together with the grade of adenocarcinoma and was maximal when there were undifferentiated cells in stomach tumors, reaching 93.8% in the zone of protein-lipid contacts and 54.1% in the lipid bilayer, compared with healthy people (20 donors). An increase in the polarity of the lipid phase was also observed; it was most pronounced (by 7–8%, $p = 0.001$ 0.003) in adenocarcinoma patients with undifferentiated cells and with stage IV disease. A change in the immersion of proteins in the lipid matrix of erythrocytes was less characteristic of GC, compared with other cancers (breast, lung tumors, gynecological oncopathology, etc.).

Conclusions. Changes in the structural and functional properties of erythrocyte membranes reflect the state of the disease in patients with gastric cancer and may be important for predicting the course of the disease and the success of treatment.

Key words: gastric cancer, various tumor histotypes and cancer stages, erythrocyte membrane, fluidity, polarity, protein immersion, pyrene-based fluorescent probe.

Conflict of interest. The authors declare no obvious or potential conflicts of interest related to the publication of this article.

Source of financing. This work was carried out as part of the state assignment of Rostov Research Institute of Oncology.

Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study protocol was approved by the Ethics Council of Rostov Research Institute of Oncology (Protocol No. 11/1, dated 03.11.2016).

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For citation: Goroshinskaya I.A., Nemashkalova L.A., Frantsiyants E.M., Surikova E.I., Medvedeva D.E., Maslov A.A. Structural and functional indicators of erythrocyte membranes in gastric cancer patients with different histotypes of the tumor and stages of the malignant process. *Bulletin of Siberian Medicine.* 2020; 19 (2): 20-27. https://doi.org: 10.20538/1682-0363-2020-2-20-27.

Структурно-функциональные показатели мембран эритроцитов крови больных раком желудка с разным гистотипом опухоли и стадией злокачественного процесса

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РЕЗЮМЕ

Цель. Изучить структурно-функциональные показатели мембран эритроцитов в крови больных раком желудка (РЖ) при аденокарциноме, в зависимости от степени ее дифференцировки, при перстневидноклеточном раке (ПКР) и сочетанном поражении желудка (СПЖ).

Материалы и методы. Оценивали текучесть мембран в области липидного бислоя и белок-липидных контактов, полярность липидного бислоя и погруженность белков в липидный матрикс мембраны в эритроцитах крови с использованием гидрофобного зонда пирена флуориметрическим методом. В исследование было включено 86 больных РЖ, в зависимости от гистотипа разделенных на шесть групп: G1-2, G3, ПКР, СПЖ, G4 + ПКР и G4 + G2-3. Отдельно были проанализированы результаты исследования у больных с прорастанием опухоли в серозную оболочку и распространением на соседние структуры (T4 по системе классификации TNM) и у больных, находившихся в IV стадии.

Результаты. Во всех группах больных РЖ установлено увеличение текучести мембран, более выраженное в зоне белок-липидных контактов, но наблюдавшееся и в липидном бислое. При этом текучесть возрастала по мере снижения степени дифференцировки аденокарциномы и была максимальной при наличии в опухоли желудка недифференцированных клеток: выше, чем в группе здоровых, на 93,8% в зоне белок-липидных контактов и на 54,1% в липидном бислое. Наблюдалось также повышение полярности липидной фазы, наиболее выраженное (на 7–8%, $p = 0{,}002–0{,}003$) у больных аденокарциномой с наличием недифференцированных клеток и при IV стадии процесса. Изменение погруженности белков в липидный матрикс эритроцитов было менее характерно для РЖ по сравнению с другими раками (молочной железы, легкого, онкогинекологическая патология).

Заключение. Изменение структурно-функциональных свойств мембран эритроцитов отражает состояние процесса у больных раком желудка и может иметь значение для прогнозирования течения заболевания и успешности лечения.

Ключевые слова: рак желудка, различные гистотипы опухоли и стадии процесса, мембраны эритроцитов, текучесть, полярность, погруженность белков, флуоресцентный зонд пирен.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Работа выполнена в рамках госзадания Ростовского научно-исследовательского онкологического института.

Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Протокол исследования одобрен советом по этике ФГБУ «Ростовский научно-исследовательский онкологический институт» (протокол № 11/1 от 03.11.2016).

Для цитирования: Горошинская И.А., Немашкалова Л.А., Франциянц Е.М., Сурикова Е.И., Медведева Д.Е., Маслов А.А. Структурно-функциональные показатели мембран эритроцитов крови больных раком желудка с разным гистотипом опухоли и стадией злокачественного процесса. *Бюллетень сибирской медицины.* 2020; 19 (2): 20-27. https://doi.org: 10.20538/1682-0363-2020-2-20-27.

INTRODUCTION

According to the data of modern lipidomics, membrane lipids play an important role in the implementation of many cell functions and are involved in the development of a number of pathologies, including cancer [1]. The revealed compositional features of various membranes (outer and inner layers of the outer membrane, organelle membrane) of tumor cells in different types and stages of cancer open up opportunities for new strategies in treatment and prevention of cancer [2]. Among the nonspecific disorders of homeostasis that develop following the effect of a tumor on the body are changes in the structural and functional properties of the membranes of peripheral blood cells, primarily erythrocytes [3, 4]. In diseases of various origin, erythrocytes undergo both specific and nonspecific structural, functional, and metabolic rearrangements; therefore, when studying various physiological processes and pathological conditions, they are considered as a universal cellular model reflecting changes in the body [5]. It is assumed that the study of the functional state of erythrocyte membranes in cancer patients is very important for early diagnosis of the disease and monitoring the success of treatment [6]. The authors came to this conclusion after studying the dielectric constant determined by structural changes in the membranes, in erythrocytes of 28 healthy donors and 62 patients with breast and lung cancer.

Studying membranes with fluorescent probes provides important information about the structural state of membranes. The fluorescence parameters of the probe introduced into the membrane depend on the physicochemical properties of its direct microenvironment in the membranes, such as fluidity, polarity of the medium, proximity of charged groups, the presence of various acceptor molecules, electron excitation energy, and diffusion of fluorescence quenchers [7, 8]. The use of a hydrophobic pyrene-based probe allows to evaluate the fluidity of blood cell membranes in the zones of protein-lipid contacts and the lipid bilayer, the polarity and immersion of proteins in the lipid matrix. Fluidity is a complex indicator that reflects both the structure and the basic properties of the lipid component of membranes and zones of protein-lipid interactions; it plays a key role in the regulation of all processes occurring in cell membranes. Fluidity characterizes not only the state of membranes, but also their ability to react to changes in the body in response to development of the malignant process and effects of chemotherapy [1, 9–11]. It has been proven that induction of apoptosis by many antitumor agents is associated with a change in the fluidity of tumor cell membranes under their influence [12]. The polarity of the lipid bilayer reflects the state of the hydrophobic phase of the membrane and the level of hydrophilic clusters formed by hydroperoxides in it. Assessment of the immersion of proteins in the lipid matrix allows to judge about the association of proteins with membranes, as well as about the oligomerization of membrane proteins which may result from their oxidative modification [8].

The aim of this study was to assess the structural and functional parameters of erythrocyte membranes in the blood of patients with gastric cancer – adenocarcinoma, depending on its grade, signet ring cell carcinoma, and combined gastric lesions.

MATERIALS AND METHODS

The study included 86 patients with gastric cancer who had not previously received treatment. The average age of the patients was 62 years (61.9 ± 1.2). Depending on the histotype of the tumor, the patients were divided into six groups: 1) well- and moderately-differentiated adenocarcinoma (G1-2) – 24 people, 16 men and 8 women; 2) low-differentiated adenocarcinoma (G3) – 15 patients, 10 men and 5 women; 3) signet ring cell carcinoma (SRCC) – 19 people, 10 men and 9 women; 4) combined gastric lesions (CGL) – adenocarcinoma with the presence of signet ring cell fragments – 15 patients, 14 men and 1 woman; 5) patients with undifferentiated cells and signet ring cell fragments (G4 + SRCC) – 5 people, 3 men and 2 women; 6) patients with undifferentiated cells in gastric adenocarcinoma (G4 + G2–3) – 8 people, 6 men and 2 women. In the G1-2 group, moderately differentiated adenocarcinoma was detected in 92% of cases. In addition, the results of the study were separately analyzed in patients with serosal invasion and the spread to adjacent structures (T4 according to the TNM classification of malignant tumors) and in patients with stage IV cancer. All studied indicators in the blood of patients were compared with the corresponding values in a group of healthy men and women of comparable age without cancer (the donor group consisted of 20 people). All patients gave an informed consent for the use of biological material for scientific research.

Red blood cells were obtained by centrifuging heparinized blood for 15 min at 1 500 rpm, followed by three times washing them with physiological saline, buffered with phosphate buffer (pH 7.4), and centrifuging at 3 000 rpm for 10 min, at 4 °C. In the red blood

cells of patients with gastric cancer, the structural and functional parameters of the membranes were evaluated using a pyrene-based fluorescence probe (C16H10, M = 202.3; Serva, USA), determining the fluidity of the membranes in the region of the lipid bilayer and protein-lipid contacts, the polarity of the lipid bilayer and the protein immersion into the lipid matrix of the membrane according to the method of Yu.A. Vladimirova and G.E. Dobretsova (1980) [7] on the Fluorat-02-Panorama spectrofluorimeter (Russia). The method for determining the fluidity of membranes is based on the ability of a pyrene-based fluorescent probe to excimerize in a non-polar medium. The fluidity of the lipid bilayer of membranes is directly proportional to the excimerization coefficient of pyrene Fe / Fm, determined by the ratio of the fluorescence peak of the pyrene excimer Fe (in relative fluorescence units at emission wavelength λemi. = 470 nm) to the fluorescence peak of the pyrene monomer Fm (in relative fluorescence units at λemi. = 393 nm) with excitation wavelength $\lambda_{exc.}$ = 334 nm. The fluidity of protein-lipid contacts was determined by the ratio of the fluorescence intensity of the pyrene excimer to the pyrene monomer at $\lambda_{exc.}$ = 282 nm. The method is based on the ability of pyrene to intercept the energy of absorbed light from aromatic protein residues within a distance called the Förster radius. The polarity of the lipid phase of the membranes was evaluated by the ratio of the fluorescence intensities of the two monomeric forms F_{372} / F_{393} in the thin structure of the pyrene spectrum at $\lambda_{exc.}$ = 334 nm. The degree of immersion of proteins in the lipid bilayer was determined by quenching of the fluorescence of aromatic amino acid residues (tyrosine and tryptophan) following non-radiative energy transfer to the pyrene molecule. To measure protein immersion, the erythrocyte suspension was fluorimetric at $\lambda_{exc.}$ = 282 nm and λemi. = 330 nm. Then, after incubation with pyrene for 1 minute, the fluorescence intensity of the sample was measured again. The height of the fluorescence peak in this case (F) was less than in the first measurement (F_0) due to quenching of tryptophan fluorescence by pyrene. We determined the efficiency of energy transfer from tryptophanyls to pyrene that was equal to the value of $F_0 - F$.

Statistical processing of the results was carried out using the Statistica 6.0 software package and Student's t-test for two independent samples. The differences were considered statistically significant at $p < 0.05$, and at $0.1 > p > 0.05$ a trend towards statistical significance was observed. The samples were preliminarily checked for compliance with the normal distribution according to the Shapiro – Wilk W-test and Kolmogorov – Smirnov criterion. The data were presented as $M \pm m$, where M is the sample mean, and m is the error of the mean.

RESULTS

An examination of the state of erythrocyte membranes showed an increase in their fluidity in all groups of patients with gastric cancer, compared with the level both in the zone of protein-lipid contacts and in the lipid bilayer in the donors (table). Only in G1–2 patients fluidity was elevated only in the zone of protein-lipid contacts – by 31.1%. In patients with low-grade adenocarcinoma, this indicator in the zone of protein-lipid contacts increased by 52.3% compared to the donor level, in the lipid bilayer – by 36.6%, which was 30.2% higher than in G1–2 patients (p = 0.054). An increase in the adenocarcinoma extent (T4) did not significantly affect the level of fluidity – it was elevated in the zone of protein-lipid contacts by 38.5% ($p < 0.001$), and in the lipid bilayer there was a trend towards its increase by 19.5%. The most pronounced increase in erythrocyte membrane fluidity was detected in patients with adenocarcinoma with undifferentiated cells (G4 + G2-–3): by 93.8% in the zone of protein-lipid contacts and by 54.1% in the lipid bilayer in comparison with healthy people, which was statistically significantly higher than the level in G1–2 patients – by 47.8% and 46.9%, respectively ($p < 0.05$). In patients with SRCC and combined gastric lesions, fluidity was elevated in the zone of protein-lipid contacts by 36–38% and in the lipid bilayer by 17.2–21.5%. In the presence of undifferentiated cells in patients with SRCC, an increase in fluidity was 51.9% and 34%, respectively, as opposed to healthy people. The highest increase in fluidity of the lipid membrane bilayer was found in patients with stage IV disease – by 62.4%, while in the zone of protein-lipid contacts the indicator increased by 32.3%.

An increase in the fluidity of membranes in almost all groups was accompanied by an increase in the polarity of their lipid phase. A statistically significant increase in the polarity was detected in patients with moderately-differentiated adenocarcinoma – by 3.9%, with the advanced form of adenocarcinoma (T4) – by 4.6%, with combined lesions – by 5.1%. The greatest increase in polarity was recorded in patients with adenocarcinoma with the presence of undifferentiated cells – by 7.1% and with stage IV disease – by 7.8%.

Table

Structural and functional indicators of erythrocyte membranes in patients with different histotypes and stages of gastric cancer, relative fluorescence units, $M \pm m$

Groups of patients depending on histology and stage	Membrane fluidity in the zone of		Polarity of the membrane lipid phase	Protein immersion in the lipid matrix
	protein-lipid contacts	lipid bilayer		
Healthy people, $n = 20$	0.405 ± 0.010	0.303 ± 0.015	1.422 ± 0.013	0.203 ± 0.005
Adenocarcinoma G1–2, $n = 24$	0.531 ± 0.028 $p < 0.001$	0.318 ± 0.017 $p_{IVSt} = 0.011$	1.477 ± 0.017 $p = 0.013$	0.204 ± 0.012 $p_{IVSt} = 0.050$
Adenocarcinoma G3, $n = 15$	0.617 ± 0.057 $p < 0.001$	0.414 ± 0.053 $p = 0.017$ $p_1 = 0.054$	1.44 8 ± 0.024	0.199 ± 0.017 $p_{IV} = 0.082$
Signet ring cell carcinoma (SRCC), $n = 19$	0.551 ± 0.021 $p < 0.001$	0.355 ± 0.016 $p = 0.020$ $p_{IVSt} = 0.040$	1.454 ± 0.018 $p_{IVSt} = 0.078$	0.198 ± 0.017 $p_{IVSt} = 0.086$
Combined gastric lesions (G2–3+ SRCC), $n = 15$	0.559 ± 0.027 $p < 0.001$	0.368 ± 0.038 $p = 0.066$	1.494 ± 0.018 $p = 0.002$	0.231 ± 0.016 $p = 0.077$
Presence of undifferentiated cells and signet ring cell fragments (G4+ SRCC), $n = 5$	0.615 ± 0.08 $p < 0.001$	0.406 ± 0.107 $p = 0.071$	1.457 ± 0.024	0.219 ± 0.032
Presence of undifferentiated cells in adenocarcinoma (G4+G2–3), $n = 8$	0.785 ± 0.155 $p < 0.001$ $p_1 = 0.019$ $p_{IVSt} = 0.065$	0.467 ± 0.105 $p = 0.014$ $p_1 = 0.037$	1.523 ± 0.033 $p = 0.002$ $p_2 = 0.084$	0.171 ± 0.029 p = 0.035 $p_{IVSt} = 0.037$
Adenocarcinoma T4, $n = 17$	0.561 ± 0.035 $p < 0.001$	0.362 ± 0.033 $p = 0.074$	1.487 ± 0.021 $p = 0.010$	0.223 ± 0.019
IV stage, $n = 4$	0.536 ± 0.035 $p < 0.001$	0.492 ± 0.125 $p = 0.003$	1.533 ± 0.037 $p = 0.003$	0.274 ± 0.049 $p = 0.013$

Note. The data are presented as $M \pm m$, where M is the sample mean; m is the error of the mean. The statistical significance of the differences: p – relative to the group of healthy people, p_1 – relative to the group of patients with adenocarcinoma G1–2, p_2 – relative to the group of patients with adenocarcinoma G3, p_{IVSt} – relative to patients with stage IV disease.

Interestingly, in patients with gastric cancer, a statistically significant increase in protein immersion into the lipid matrix of erythrocyte membranes was detected only at stage IV disease (by 35%, $p = 0.015$), and there was also a trend towards a significant increase in patients with combined lesions (by 13.8%, $p = 0.077$). Patients with undifferentiated cells in gastric adenocarcinoma showed a decrease in protein immersion (by 15.8%, $p = 0.035$). According to the data obtained at Rostov Research Institute of Oncology earlier, this indicator turned out to be the most labile and drastically increased at most localizations of the malignant process; and its changes often correlated with the state of patients and the effectiveness of therapy. Significant changes in the immersion of proteins in the lipid matrix of blood cell membranes were detected in gynecological cancers, breast cancer, lung cancer, oropharyngeal cancer, and malignant brain lesions [13, 14]. In patients with breast cancer with high efficacy of chemotherapy, normalization of initially increased protein immersion in the lipid matrix of erythrocyte and lymphocyte membranes was observed in the absence of positive changes in patients with tumor stabilization [15]. Therefore, in gastric cancer, the change in protein immersion in the lipid matrix of erythrocyte membranes differed from what was observed in other cancers.

Separately, it is necessary to dwell on the indicators of erythrocyte membranes in patients with stage IV gastric lesions. In these patients, the increase in the fluidity was more pronounced in the lipid bilayer – 62.4%, than in the zone of protein-lipid contacts – 32.3%, while in all other groups of patients the increase in fluidity in the zone of protein-lipid contacts was more pronounced. The maximum increase in the fluidity of the lipid bilayer among all groups corresponded to the largest increase in the polarity of the lipid phase of the membranes and in the immersion of proteins in the lipid matrix. The fluidity of the lipid bilayer in patients with stage IV lesion was significantly higher than in patients with adenocarcinoma G1–2 and SRCC (54.7% and 38.6%, respectively). A trend towards statistical significance of higher levels of polarity in these patients relative to patients with SRCC (by 5.4%) and immersion relative to patients with

adenocarcinoma G1–2, G3, and SRCC (by 34.3%, 37.7%, and 38.4%, respectively) was revealed. Statistically significant excess of protein immersion was observed in patients with stage IV disease, as opposed to a reduced level of this indicator in patients with G4 + G2–3 (by 60.2%), with a trend towards a significant decrease in the fluidity of protein-lipid contacts (by 31.7%) relative to the same group.

DISCUSSION

According to the data obtained, the majority of examined patients with gastric cancer were characterized by an increase in the erythrocyte membrane fluidity which was more pronounced in the zone of protein-lipid contacts, but also observed in the lipid bilayer. The greatest changes were revealed in the presence of undifferentiated cells in stomach tumors. In this case, an increase in the fluidity of erythrocyte membranes was observed both in gastric adenocarcinoma and in SRCC. In adenocarcinoma, it was also accompanied by a more pronounced increase in the polarity of the lipid phase of the membranes. And only the G4 + G2–3 group of patients was characterized by a statistically significant decrease in the immersion of proteins in the membrane lipid matrix.

The fluidity is known to characterize the ability of membranes to respond to disorders occurring in the body, including the development of a malignant process [16]. The reason for the increase in the fluidity of the lipid bilayer may be an increase in the free radical oxidation of membrane lipids, their interaction with lipid peroxidation products, and a change in the lipid composition of membranes [3, 17]. The main factors determining the fluidity of membranes include the content of cholesterol, which contributes to a more stringent ordering of the lipid bilayer, and the content of unsaturated lipids, which increase the fluidity and permeability of the membranes [2]. Since membrane lipids form the environment for the functioning of membrane proteins, a change in the structure of the lipid bilayer leads to a violation of the conformation of proteins associated with it. The intensification of free radical oxidation of membrane proteins leads to a change in their tertiary structure and an increase in protein aggregation or fragmentation, which contributes to an increase in fluidity in the zone of protein-lipid contacts, immersion of proteins in the lipid matrix and, as a result, a decrease in their functional capabilities, including the receptor apparatus [5]. It was shown that membrane areas (domains) enriched in cholesterol and sphingolipids, with epidermal growth factor receptors (EGFs), estrogens, and other receptors built into them, participate in cell proliferation, while a ceramide-enriched domain with built-in Fas receptors (CD95) and TNF-R1 triggers caspases and promotes apoptosis [2]. Changes in lipid domains and proteins within the domain can be involved in malignant transformation, uncontrolled growth, invasiveness and metastasis [18], and also affect the state of antioxidant and proteolytic systems that play an important role in cancer [19, 20].

The functional importance of changes in the protein component of membranes in malignant pathology is indirectly indicated by high frequency of increased fluidity of the membranes in the zone of protein-lipid contacts revealed in red blood cells of patients with gastric cancer. Our results on the intensification of lipid peroxidation in blood plasma and erythrocytes of patients with gastric cancer are consistent with the activation of free radical oxidation as one of the reasons for the increase in the fluidity of erythrocyte membranes. Herewith, the level of lipid peroxidation products and the degree of violation of conjugation of erythrocyte antioxidant enzymes increased with a decrease in the differentiation of adenocarcinoma, especially in the presence of undifferentiated cells in the tumor [21].

An analysis of the changes in the properties of membranes that occur in cancer led to the conclusion that an increase in the fluidity of the membranes of tumor cells promotes metastasis, and a decrease in the fluidity leads to the development of multidrug resistance [2]. Our data indicate that functionally significant structural features are characteristic not only for tumor cells. Changes in the fluidity, polarity of the lipid bilayer and the immersion of proteins in it also occur in erythrocyte membranes, depending on the histological characteristics of the tumor and types and stages of cancer. Thus, the results obtained in the study of erythrocyte membranes in gastric cancer are consistent with the opinion expressed by foreign researchers [2, 22, 23] that in-depth studies of cell membranes and their lipid composition, that is lipidomics of various types and stages of cancer, as well as modification of membrane components provide great opportunities for treatment and prevention of cancer and will be more often used in the coming years as markers of prognosis and progression in malignant pathologies.

CONCLUSION

An increase in the fluidity of erythrocyte membranes in the zone of protein-lipid contacts and lipid bilayer,

as a rule, is accompanied by a small, but statistically significant increase in the polarity of the lipid phase of the membranes. It is typical for patients with gastric cancer and increases with a decrease in the degree of tumor differentiation. The maximum increase in the fluidity and polarity of the lipid bilayer, accompanied by a significant increase in the immersion of proteins in the lipid matrix, was revealed in patients with stage IV tumors. The data obtained indicate that changes in the structural and, therefore, functional properties of membranes that are typical not only of tumor cells, but also of blood cells, can contribute to the development of the tumor process and can be used as prognostic markers of disease progression and treatment success.

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Authors contribution

Goroshinskaya I.A. – analysis and interpretation of the results and literature data, statistical processing of the results, drafting and design of the article. Nemashkalova L.A. – determination of membrane parameters, participation in the selection of literature. Frantsiyants E.M. – final approval of the manuscript for publication. Surikova E.I. – analysis of clinical indicators of patients for their division into groups. Medvedeva D.E. – collection of the material for research and provision of information on patients. Maslov A.A. – diagnosis, determination of a treatment plan for patients included in the study.

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Received 29.03.2019
Accepted 25.12.2020