O.I. Kit, A.L. Bazaev, E.Yu. Zlatnik, I.A. Novikova, E.P. Ulianova, O.G. Shulgina, A.B. Sagakyants, E.N. Kolesnikov, V.S. Trifanov, S.S. Mezentsev, A.V. Snezhko, T.B. Katsieva

EXPRESSION OF SOME MOLECULAR AND BIOLOGICAL MARKERS IN DIFFERENT PERIODS OF PROGRESSION AFTER RADICAL SURGICAL TREATMENT OF ESOPHAGEAL CANCER

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Rostov, Russia: **Oleg I. KIT**, D.Med.Sc., Professor, Corresponding Member of RAS, General Director of Rostov Research Institute of Oncology, onko-sekretar@mail.ru, **Adlan L. BAZAEV**, post-graduate student, Rostov Research Institute of Oncology, tel. 89286146603, bazaev-adlan@mail.ru, **Elena Yu. ZLATNIK**, D.Med.Sc., Professor, Chief Researcher at Laboratory of Immunophenotyping of Tumors, Rostov Research Institute of Oncology, tel. 89612726968, elena-zlatnik@mail.ru, **Inna A. NOVIKOVA**, Cand.Med.Sc., Deputy General Director for Science, Rostov Research Institute of Oncology, tel. 89064162909, novikovainna@yahoo.com, **Elena P. ULIANOVA**, Researcher at Laboratory of Immunophenotyping of Tumors, Rostov Research Institute of Oncology, tel. 89064248556, uljanova_elena@lenta.ru, **Oksana G. SHULGINA**, Junior Researcher at Laboratory of Immunophenotyping of Tumors, Rostov Research Institute of Oncology, tel. 89185570986, ggaalliinnaa1@mail.ru, **Aleksandr B. SAGAKYANTS**, Cand.Biol.Sc., Associate Professor, Head of Laboratory of Immunophenotyping of Tumors, Rostov Research Institute of Oncology, tel. 89604458653, asagak@rambler.ru, **Evgeniy N. KOLESNIKOV**, Cand.Med.Sc., Head of Department of Abdominal Onkology No. 1, Rostov Research Institute of Oncology, tel. 89185538854, bony91@yandex.ru, **Vladimir S. TRIFANOV**, Cand.Med.Sc., Senior Researcher at Department of Abdominal Onkology No. 1, surgeon oncologist, Rostov Research Institute of Oncology, tel. 89185544312, Trifan1975@yandex.ru, **Stanislav S. MEZENTSEV**, Cand.Med.Sc., oncologist at Department of Abdominal Onkology No. 1, surgeon oncologist, Rostov Research Institute of Oncology, tel. 89185028118, stas@mezentsev.me, **Aleksandr V. SNEZHKO**, D.Med.Sc., Assistant at Department of Oncology, Rostov State Medical University, tel 89185131837, snezhko.sanya@yandex.ru, **Tanzila B. KATSIEVA**, Cand.Med.Sc., oncologist at Department of Abdominal Onkology No. 1, surgeon oncologist, Rostov Research Institute of Oncology, tel. 89895113131, t.kazieva.tao@yandex.ru.

30 patients with stage II and III of squamous cell carcinoma of the esophagus after surgical treatment were analyzed retrospectively. Prognostic significance of the expression of immunohistochemical markers p53, bcl-2, ki-67 and E-cadherin and tumor proliferative activity were studied. A significant decrease in the frequency of tumors expressing p53, as well as an increase in the number of tumors expressing bcl-2, was detected in the group of patients with an increase in the event-free period for up to 365-540 days. The mean numbers of bcl-2 positive cells in groups with progression from 181 to 364 days and from 365 to 540 days were 2.3 and 2.8 times higher than in patients with progression in 180 days and earlier. The ki-67 index in the latter was significantly higher than in groups with progression within 181-364 and 365-540 days.

An increased proliferative activity was registered only in patients with progression in 180 days and earlier. The E-cadherin expression in the group with progression in 365-540 days was significantly higher than in patients with progression in < 180 and 181-364 days.

Keywords: esophageal cancer, immunohistochemical markers, proliferative activity, p53, bcl-2, ki-67, E-cadherin, progression.

Esophageal cancer is one of the most aggressive malignant diseases of the gastrointestinal tract significantly affecting the quality of life of the patient with high rates of poor outcome [3, 10]. Tumors of epithelial origin, mainly squamous cell carcinoma and adenocarcinoma, are the most common. According to the International Agency for Research on Cancer (IARC), EC incidence and mortality in 2008 were 49.2 and 34.3, respectively, per 100 000 per year [12].

High expression of p53 mutant protein is known to predict poor survival in cancer of the stomach, lung, and other cancers [4]. However, the predictor role of p53 for EC remains a debatable [14]. In 2015, British journal of cancer published a meta-analysis review of 11 large-scale studies on the role of p53 in EC over the past 20 years, in order to identify potential biomarkers that are sensitive and predictive in EC. As a result, only three studies including 268 patients showed a connection between the poor prognosis and high expression of p53. The results of other studies contained conflicting data, most of which showed that the prognostic role of this marker was not obvious [13].

Among other markers considered to be oncogenes, the bcl-2 gene involved in the negative apoptosis regulation can be marked. Some authors regard high expression of bcl-2 as a negative prognostic factor due to the fact that it contributes to the survival of tumor cells [6, 8]. Other researchers showed high expression of bcl-2 to be a prognostically favorable factor [5, 9].

Analysis of proliferative activity (PA) of a tumor is the most important characteristic of cancer, which allows characterizing its biological behavior. It is estimated by the ki-67 index, as only dividing cells detect antibodies to ki-67 [7].

Disorders of intercellular adhesion associated with pathological changes in the membrane protein E-cadherin are one of the factors contributing to the metastasis and progression of malignant tumors [1]. Studies of E-cadherin expression in lung, bladder and gastric cancers showed the significance of its determination. Studies have shown that E-cadherin has the properties of a tumor growth suppressor and its invasion [2]. An imbalance in the cadherin system, in particular inhibition of E-cadherin expression and overexpression of N-cadherin, is considered an important component of the epithelial-mesenchymal transition process.

Thus, these IHC markers allow characterization of the processes of adhesion, apoptosis and proliferation of tumor cells, which seems significant for assessing the potential of tumor progression. That is why we selected these markers and studied their expression in EC cells

in different periods of the disease progression after radical surgical treatment.

The purpose of the study was a comparative assessment of the expression of p53, bcl-2, ki-67, E-cadherin IHC markers in the tumor tissue of EC patients at different progression periods after surgical treatment.

Material and methods. The study included 30 patients diagnosed with stage II (n=15) and III (n=15) middle and lower thoracic esophageal squamous cell carcinoma undergoing treatment in Rostov Research Institute of Oncology. All patients received Lewis surgery with standard bizonal lymph node dissection. No special antitumor therapy was performed until progression was detected. Median age of patients was 57.83 years. All patients developed progression within 1.5 years (up to 540 days). Depending on the time to progression, the patients were divided into 3 groups: up to 180 days; from 181 to 364; from 365 to 540 days. In 11 of 30 cases (36.7%), progression occurred within 180 days; in 9 (30%) - from 181 to 364; in 10 (33.3%) - from 365 to 540 days. The median event-free survival was 9 months.

The IHC study was performed on 3-4 μm sections from paraffin blocks prepared using the Thermo Scientific Microm HM 325 rotary microtome (Great Britain). Automated immunohistochemical staining was carried out in the Thermo Scientific 480S IHC stainer. The following monoclonal antibodies were used for the study: to p53 (clone DO-7, DAKO, 1:100 dilution), bcl-2 (clone Sp 66, DAKO, 1:100 dilution), ki-67 (clone H3060, Spring Biosience, 1:200 dilution), E-cadherin (clone EP700Y, Thermo scientific, 1:100 dilution). Dewaxing and dehydration stages were performed according to the standard scheme. The PT-LinkThermo was used for antigen unmasking. The Reveal Polyvalent HRP-DAB Detection System (Spring Bioscience) was used for imaging. The cut-off point for positive tumor assessment was 25% for both nuclear staining for p53 and cytoplasm staining for bcl-2. Proliferative activity (PA) was calculated by counting the proportion of positively stained cells to the ki-67 marker per 100 tumor cells. The ki-67 index was determined by the formula: proliferative activity = the number of positively stained cells to the tumor marker ki-67 x 100/total number of cells. Statistical analysis of the results was processed using the Statistica 8.0 program [9], and the Student's t-test was calculated.

Results and discussion. The results were expressed in a comparative analysis of the frequency of expression and average values of indicators depending on the time to EC progression. An increased time interval to disease progression was accompanied by an increase in the frequency of expression of bcl-2+ and a decrease in p53+ (table 1). No correlation was observed between the disease stage and event-free period.

Table 1 demonstrates that longer relapse-free survival was accompanied by decreased rates of tumors expressing p53, with their minimal number (1.5 times lower compared with patients who did not survive six months without progression) in patients with the longest event-free period (365-540 days).

decrease in the proportion of p53 positive tumor cells was detected as the time to progression increased. With an increase in the time to progression to 540 days, the number of p53 positive cells was 2.2 times lower than in patients with progression within 180 days from the surgery, and 1.5 times lower than in cancer progression from 181 to 364 days. The differences were statistically significant in all cases ($p<0.05$).

While expression of p53+ positive cells decreased significantly with longer time to progression, expression of bcl-2 showed the opposite tendency. The average number of bcl-2 positive cells in tumors was 2.3 times higher in progression

Table 1

Frequency of expression of IHC markers (p53 and bcl-2) depending on the time to progression, absolute count (%)

Time to progression, days	Markers (IHC)			
	p53+	p53-	bcl-2+	bcl-2-
< 180	9 (81.8)	2 (18.2)	7 (63.6)	4 (36.4)
from 181 to 364	6 (75)	2 (25)	6 (75)	2 (25)
from 365 to 540	6 (54.5)	5 (45.5)	10 (90.9)	1 (9.1)

In these patients, the maximum rate of positive bcl-2 expression was found – 1.4 ($p<0.05$) and 1.2 ($p<0.05$) times higher than in groups with progression-free periods of <180 and 181-364 days, respectively (Table 1, Figure 1).

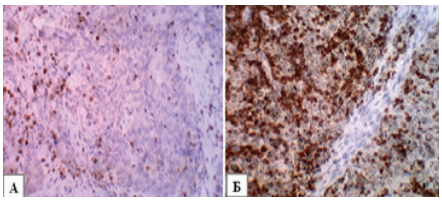

Fig. 1. Squamous cell carcinoma of the esophagus: А - 360 days to progression. Low expression of bcl-2; Б - 540 days to progression. High expression of bcl-2. 400x magnification

Table 2 presents the results of a comparative analysis of average expression of IHC markers. A statistically significant

from 181 to 364 days, compared to patients with progression within 180 days. When progression-free period lasted for up to 540 days, the value increased by up to 2.8 times (differences were statistically significant, $p<0.05$).

The maximal index of proliferation of tumor cells determined by ki-67+ was detected in tumors with the shortest time to progression. It was 3.1 and 2.5 times higher, compared to the values in groups with progression in 181-364 and 365-540 days.

No statistically significant differences in proliferation indices determined by ki-67+ cell proportion were observed in patients with progression within 181-364 and 365-540 days. Therefore, this indicator can be considered as a marker of early (up to six months after surgery) progression of esophageal cancer.

Assessment of the proliferative potential of tumors in the same patients also re-

Table 2

Expression of studied markers in patients with different periods to progression, %

Time to progression, days	Expression of IHC markers			
	p53	bcl-2	ki-67	E-cadherin
< 180	60.9±7.4	21.4±3.9	49.7±8.6	65.5±4.6
from 181 to 364	41.9±6.6°↓	48.9±4.6°↑	15.9±4.8°↓	66.3±8.8
from 365 to 540	28.2±4.2°↓	59.5±4.8°↑	19.7±4.9°↓	89.1±4.9°↑°°↑

Note. ° – differences from values < 180 days; °°– differences from values 181–364 days. Differences were statistically significant in both cases ($p<0.05$).

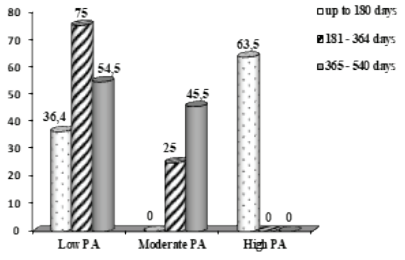

Figure 2. Proliferative activity of tumors (percentage) in dependence on time to progression in patients with stage II-III EC. The Y axis - %.

vealed a number of differences (Figure 2).

Tumors with high PA were found only in patients with an early period before progression (up to 180 days), while such activity was not detected in tumors of patients with progression from 181–364 and 365–540 days.

Tumors with low PA were most often observed in the group with progression in 181-364 days, and tumors with moderate PA were found in the group of 365–540 days. The number of tumors with moderate PA in the 365–540 day group was 1.8 times ($p<0.05$) higher than in the 181–364 day group. It should be noted that tumors with moderate PA were not detected in the group with progression in <180 days.

Patients in groups with progression within <180 and 181-364 days did not show statistically significant differences in the average values of E-cadherin expression. However, when the time to progression increased to 365–540 days, this indicator became 1.4 and 1.3 times higher, respectively (the differences were statistically significant, $p<0.05$); table 2, figure 3).

Conclusions. Immunohistochemical analysis allowed identification of some differences in occurrence rates and expression of molecular and biological markers p53, bcl-2, ki-67 and E-cadherin associated with the processes of proliferation, adhesion and apoptosis of tumor cells at different periods to the progression of esophageal cancer (<180 days; from 181 to 364; from 365 to 540 days). The results showed that the most favorable outcome of surgical treatment for esophageal cancer was characterized by

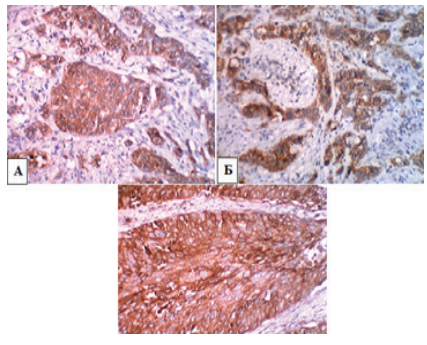

Fig. 3. Squamous cell carcinoma of the esophagus. Days to progression: А – 180, Б - 364 days, В – 540. Expression of E-cadherin. 400x magnification.

high expression of bcl-2 and E-cadherin with low proliferative activity of the tumor and low expression of p53, suggesting the potential use of these markers as prognostic ones along with well-known clinical prognosis factors.

References

1. Бабиченко И.И., Ковязин В.А. Новые методы иммуногистохимической диагностики опухолевого роста: учебное пособие. М.: РУДН, 2008. 109 с. [Babichenko II, Kovyazin VA. New methods of immunohistochemical diagnosis of tumor growth: study guide. Moscow: RUDN, 2008. 109 pp. (In Russ.)]

2. Делекторская В.В. Молекулярно-биологические маркеры метастазирования и прогноза при раке толстой кишки: дис. д-ра мед. наук: 14.00.14. М., 2007. 240 с. [Delektorskaya VV. Molecular and biological markers of metastasis and prognosis in colon cancer: dis. d-ra med. nauk: 14.00.14. Moscow, 2007. 240 pp. (In Russ.)]

3. Дулганов В.К., Дулганов П.К. О метастазировании рака пищевода. Материалы 5 Всероссийского съезда онкологов. Казань, 2000. Т. 2. С.72. [Dulganov VK, Dulganov PK. On esophageal cancer metastasis. Materials of V all-Russian congress of oncologists. Kazan, 2000. Vol. 2. P. 72. (In Russ.)]

4. Кит О.И., Водолажский Д.И., Базаев А.Л. и др. Молекулярные маркеры плоскоклеточного рака пищевода [Эл. ресурс]. Современные проблемы науки и образования. 2017;5:2. URL: https://science-education.ru/ru/article/view?id=26709. [Kit OI, Vodolazhsky DI, Bazaev AL et al. Molecular markers of esophageal squamous cell carcinoma. Sovremennye problemy nauki i obrazovaniya. 2017;5:2. (In Russ.)]

5. Новичкова О.Н., Яковлева Л.М., Новичков Е.В. Иммуногистохимическое исследование прогностических маркеров опухоли в рецидивирующих муцинозных карциномах яичников. Иммуноонкология. 2015;1:84-89. [Novichkova ON, Yakovleva LM, Novichkov EV. Immunohistochemical study of prognostic tumor markers in recurrent mucinous carcinoma of the ovaries. Immunoonkologiya. 2015;1:84-89. (In Russ.)]

6. Кит О.И., Ващенко Л.Н., Тодоров С.С., Ибрагимова Е.Л., Аушева Т.В. Способ прогнозирования рецидивирования сарком мягких тканей. Патент №2532332 Российская Федерация, МПК A61B. Опубл. 10.11.2014, Бюллетень № 31. [Kit OI, Vashchenko LN, Todorov SS, Ibragimova EL, Ausheva TV. Method to predict recurrence of soft tissue sarcoma. Patent №2532332 of the Russian Federation, МПК A61B. Published 10.11.2014, Bulletin № 31. (In Russ.)]

7. Смолякова Р.М., Машевский А.А., Кохнюк В.Т. и др. Прогностическая значимость экспрессии маркеров опухолевой пролиферации ki-67, p53 и bcl-2 у больных колоректальным раком и полипозом. Онкологический журнал. 2008;2(1):29-39. [Smolyakova RM, Meshevsky AA, Kokhnyuk VT et al. Prognostic significance of ki-67, p53 and bcl-2 expression in colon cancer and adenomas. Onkologicheskiy zhurnal. 2008;2(1):29-39. (In Russ.)]

8. Сакало А.В., Базалицкая С.В., Бондаренко Ю.М. и др. Прогностическое значение p53 и bcl-2 у больных герминогенными опухолями яичка в динамике прогрессирования заболевания и химиотерапевтического лечения. Урологія. 2012;16(1):68-72. [Sakalo AV, Bazalitskaya SV, Bondarenko YuM et al. Prognostic value of p53 and bcl-2 in patients with germ cell tumors of the testis in the dynamics of disease progression and chemotherapy. Urologiya. 2012;16(1):68-72. (In Russ.)]

9. Петров С.В., Райхлин Н.Т. (ред.). Руководство по иммуногистохимической диагностике опухолей человека. 4-е изд. Казань, 2012. 624 с. [Petrov SV, Raikhlin NT, eds. Guidance on the immunohistochemical diagnosis of human tumors. 4th ed. Kazan, 2012. 624 pp. (In Russ.)]

10. Черноусов А.Ф., Богопольский П.М., Курбанов Ф.С. Хирургия пищевода. М.: Медицина, 2000. 350 с. [Chernousov AF, Bogopolskiy PM, Kurbanov FS. Esophagus surgery. Moscow: Meditsina, 2000. 350 pp. (In Russ.)]

11. Borovikov V.P. STATISTICA: the art of data analysis on the computer. For professionals. SPb.: Piter, 2001.

12. Ferlay J., Shin H.R., Bray F. et al. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010; 127:2893–2917.

13. Murata A., Baba Y., Watanabe M. et al. p53 immunohistochemical expression and patient prognosis in esophageal squamous cell carcinoma. Med Oncol. 2013;30(4):728.

14. McCormick Matthews L.H., Noble F., Tod J. et al. Systematic review and meta-analysis of immunohistochemical prognostic biomarkers in resected oesophageal adenocarcinoma. Br J of Cancer. 2015; 113(1):107-118. DOI:10.1038/bjc.2015.179.