Immunohistochemical Analysis of Mammary Epithelial Cells at Tumoral Growth in Republic of Sakha (Yakutia)

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ABSTRACT

The analysis of histological examination results of surgical material of breast cancer from 294 women with subsequent immunohistochemical determination of estrogen (ER) - and progesterone (PgR) - receptors, proliferative activity (Ki-67), expression of the mutant suppressor gene (p53) and gene-inhibitor apoptosis (bcl-2) was carried out.The obtained data testify that IHC-research of the tumor progression markers is a defining part in the forecast of course, optimization of therapeutic approaches with an individualization of chemo-, hormonal and beam therapy of mammary cancer, the decision of a question of medicamentous and/or surgical shutdown of the ovaries, based on the research results of the biological activity markers of the tumor.

Keywords: breast, cancer, immunohistochemistry.

Relevance. Currently one of the most urgent problems not only in oncology but also health care in general is the prevention of breast cancer (BC), due to the rapid, steady growth and widespread incidence of this form of cancer, which became on top of the structure of morbidity for women with malignant neoplasms [1].

Immunohistochemistry (IHC) is the method of pinpointing the precise localization of particular cell or tissue antigen, allowing carrying out the immunoassay of tissue sections while maintaining the cell morphology. Thus, the most important biological characteristics of tumors having a value in the forecast of disease include proliferative activity (PA) (antigen Ki-67) of tumor cells, their differentiation level, hormone receptor status, intensity of apoptosis (as markers of bcl-2 and p53), which are determined by immunohistochemical methods [2].

Despite the widespread adoption of immunohistochemical techniques in the diagnosis of cancer, it should be noted that in this area theknowledge about the changes in expression of certain markers of proliferation, differentiation and cell death is still being accumulated. Most of the studies evaluated the expression of a limited number of markers, which does not allow us to identify the most informative predictors of malignancy amplification.It is also important to note the need for diagnostic examinations considering ethnicity, since

It is also important to point out, that there is a need of diagnostic examinations considering ethnicity, since ethnic and genetic factors play significant role for many types of cancers, including breast cancer [3].

The **objective of research** is to examine expression patterns of molecular biologicalmarkers for breast cancer, depending on age and ethnicity on the example of Republic of Sakha (Yakutia).

MATERIALS AND METHODS

In our research work the material from 294 women who were held surgical treatment and / or puncturing biopsy in Yakutsk Republican Oncology Center and 2nd surgical department of the Clinical Center SBD Sakha (Yakutia) "Republican Hospital № 1 - National Medical Center" was investigated. In our research, as indigenous were considered Yakuts, Evens and Evenki people, as non-indigenous - all persons of other nationalities, who arrived at different times from regions of Russia and CIS countries. There were 118 (40.1%) of indigenous women and 176 (59.9%) women of non-indigenous nationalities. All studies were performed with the approval of the Local Committee on Biomedical Ethics "Yakut Scientific Center of complex medical problems" SB RAMS.

Histological processing of the material was carried out according to conventional techniques. Immunohistochemical study was performed on serial paraffin sections., Staining was performed by indirect immunoperoxidase method after unmasking the antigenic determinants.Sections were incubated for 40 - 60 min with primary monoclonal antibodies to the antigens of estrogen (ER), progesterone (PgR), Ki-67, p53, bcl-2 (used RTU-ER-6F11, RTU-PGR-312, RTU-Ki-67 -MM1, RTU-p53-DO7, RTU-bcl-2/100/D5, «Novocastra», UK). Staining was performed according to the manufacturer's instructions.

When evaluating the results of the research for the ER and PgR were performed determination of the fraction of stained cells in points [4,5]: 0 - total absence of nuclear staining, 1 - 10% of the cells in the investigated material have nuclear staining, 2 - 1/3 of the cells with nuclear staining, 3 - from 1/3 to ½ of the cells with nuclear staining, 4 –from ½ to 2/3 of the cells with nuclear staining, 5 – from 2\3 to 100% of the cells with nuclear staining.Determination of antibody expression intensity was also performed in points: 0 - complete absence of expression in the nuclei of tumor cells, 1 - weak nuclear staining, 2 - mild nuclear staining, 3 - strong nuclear staining. Upon receiving the total result which is less than or equal to 2, the reaction was considered negative (-), 3 or more - a positive (+).

Index of Ki-67 positive cells (proliferative activity - PA) was determined by analysis of at least 100 nuclei by the following formula:

$$PA = \frac{\text{"the number of Ki} - 67 \text{ positive cells x 100"}}{\text{"the total cell number"}}$$

Low PA corresponded to Ki-67 index of less than or equal to 15%; high PA - with Ki-67 index of more than 15%.

Low expression of p53 was detected in the presence of expression of less than 25% of the cells; high expression of p53 – in the presence of expression of more than 25% of tumor cell nuclei (nuclear stain).

Low expression of bcl-2 was detected in the presence ofexpression of less than 25%; High expression of bcl-2 - in the presence of expressionof more than 25% of tumor cells (cytoplasmic staining).

Statistical analysis was performed using the statistical package SPSS STATISTICS 17.0 (SPSS Inc.).

RESULTS AND DISCUSSION

The average age of the operated women was 54,2 ± 12,1 years, most were women over 50 years old - 96 women (32.6%), 60 - 69 years old - 64 women (21.8%), in ages of 40 - 49 years - 60 women (20.4%), and under 39 years old - 37 women (12.6%), and over 70 years old - 37 women (12.6%) (Table 1).

Table 1

Distribution of material by age and ethnicity

Age group	Indigenous		Non-indigenous		In total	
	abs. / rel.	the average age	number abs. / rel.	the average age	number abs. / rel.	the average age
under 39 years old	15/12,7	33,7±5,3	22/12,5	33,8±3,6	37/12,6	33,7±4,3
40 – 49 years old	28/23,7	45,3±2,9	32/18,2	45,0±2,8	60/20,4	45,1±2,9
50 – 59 years old	31/26,3	54,8±2,7	65/37,0	54,1±2,9	96/32,6	54,3±2,9
60 – 69 years old	25/21,2	63,4±2,6	39/22,1	62,9±3,1	64/21,8	63,1±2,9
over 70 years old	19/16,1	74,2±2,9	18/10,2	73,3±3,3	37/12,6	73,8±3,2
Total number:	118/100	54,8±12,9	176/100	53,8±11,5	294/100	54,2±12,1

It should be noted that the breast cancer, which is traditionally considered as a disease of women older than 50 years, today became much "younger" - there are cases of disease of women of forty and thirty years old, and even women in their twenties. [6] This tendency was observed in our research. For example, there are twice more women aged over 50 years than those under 50 years (197 versus 97), the largest number recorded is significantly more frequent in the age group of 50 - 59 years old (96 cases or 32.6% of total surveyed) . In second place in frequency of our research is the age group of 40 - 49 years old (60/20, 4%).Also, studies were conducted in women aged under 39 years (37/12, 6%) and women aged over 70 years (37/126%). These results are comparable to those of developed countries, where about 75% of cases of breast cancer occur among women in postmenopause[7].

To determine ER and PgRimmunohistochemical examine was carried out (Fig. 1, 2) and 4 groups with various combinations of steroid receptors in tumor cells were allocated: ER + / PGR-(7,8%), ER-/PGR + (5,4%), ER -/PGR- (35%), ER + / PGR + (51,7%).

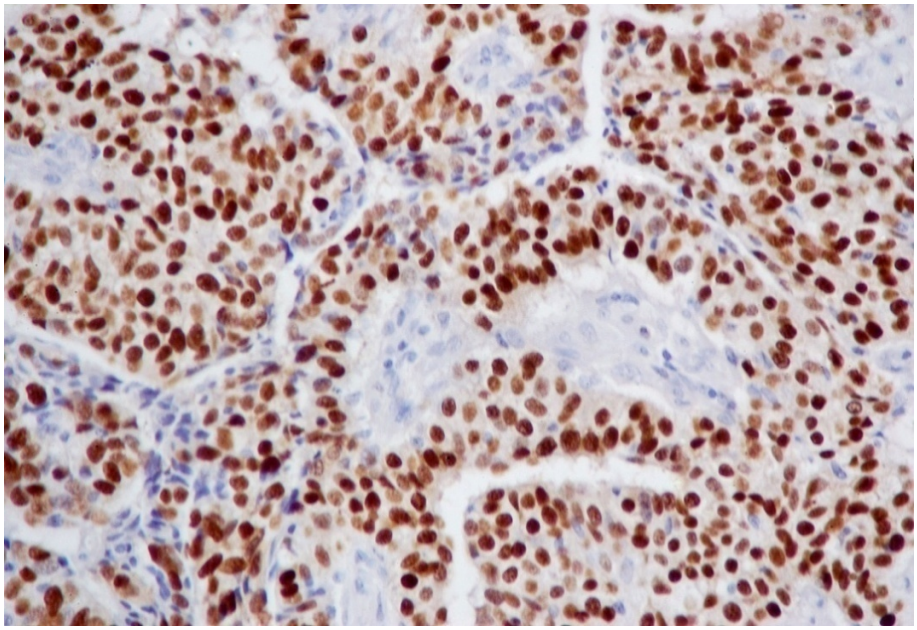

Fig. 1. Infiltrative ductal breast cancer.x200.

The immunohistochemical reaction with the monoclonal antibody to ER.

Proportion of stained cells in scores = 5 (from 2/3 to 100% of the cells with nuclear staining), the intensity of expression in scores = 3 (strong nuclear staining), the sum score = 8. Result: ER-positive breast cancer.

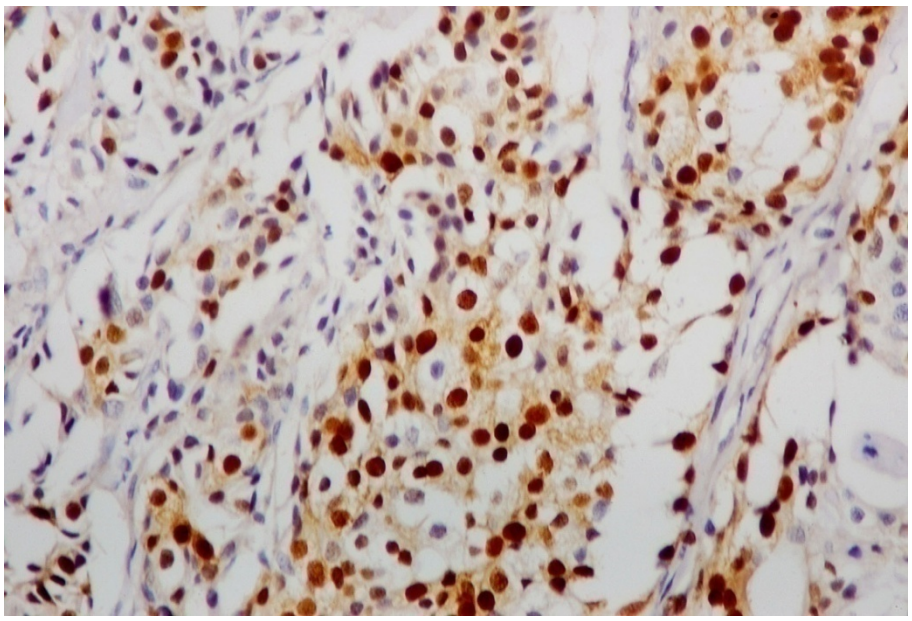

Fig. 2. Infiltrative ductal breast cancer x200.

Immunohistochemical reaction with monoclonal antibodies to PgR.

Proportion of stained cells in scores 4 = (½ to 2/3 of the cells with nuclear staining), the intensity of expression in scores = 3 (strong nuclear staining), the sum score = 7. Result: PgR-positive breast cancer.

Collectively receptor-positive tumor cells in breast cancer were significantly higher than the receptor-negative ($p < 0.05$) - 191 cases (64.9%) of the total number of studies: aged under 50 years - 60 cases (31, 4%), over 50 years - 131 cases (68.6%). Analysis of the dynamics of change of the receptor status of breast cancer according to age revealed no statistically significant differences ($p = 0.14$), but set a trend toward increased frequency (percentage) receptor-positive breast cancer with increasing the age (Fig. 3). Thus, in a group under 39 years old, they made 56.7% and in the group over 70 years old - 75.6%. At the same time, in both groups receptor-positive breast cancer profile prevailed (correspondingly 48.3 and 54% of cases in indigenous and non-residents); receptor-negative breast cancer profile was less frequent (correspondingly 38.1 and 32.9% of cases).

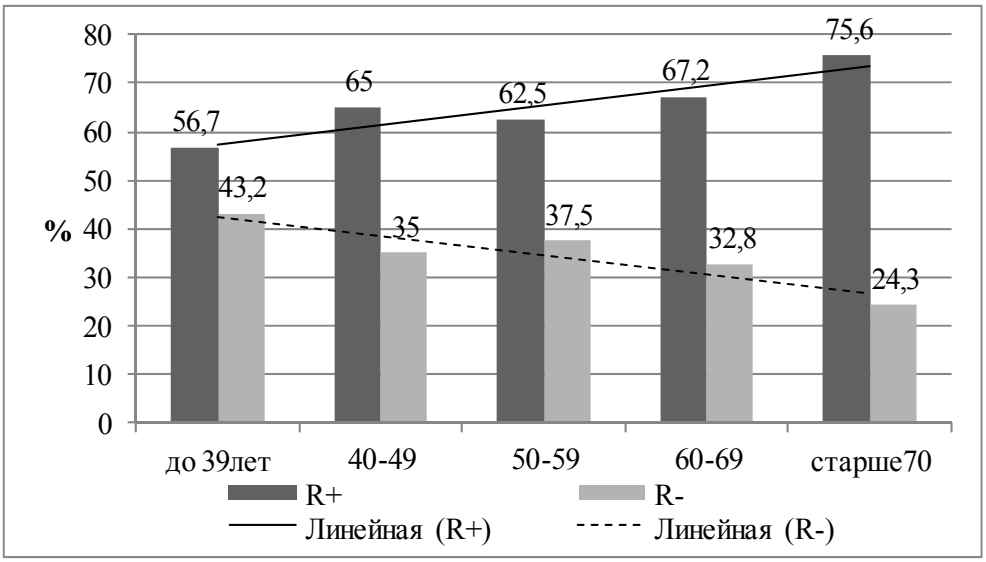

Figure 3. Dynamics of changes in receptor status of breast cancer according to age.

The connection between the age of patients and the expression of ER ($p = 0.03$). If under the age of 39 years, the average total score estimates of the proportion of positive cells and the intensity level of ER expression was equal to 3.2, the following age groups indicated a steady rise in these indicators up to 3.7 points at patients with breast cancer who were older than 70 years. In assessing the changes in the level of expression of PgR similar linear relationship is not detected ($p = 0.54$).

By the statistical analysis of the data according to ethnicity we have revealed differences among the indigenous women and women of non-indigenous nationalities. There was a tendency to a slight increase in the expression level of ER of indigenous women with the age, while increase of the expression level of ER of women of non-indigenous nationalities was statistically significant ($r = 0,20$, $p = 0,01$).Other authors have also noteddifferences in the content of hormones in the blood of women of different ethnic groups in Siberia, in particular, higher levels

of estradiol of indigenous (Tyva, Hakass, Buryat) at a lower the incidence ofbreast cancer [8]. A comparison of the degree of malignancy of tumors with the presence of ER and PgR (Fig. 4).

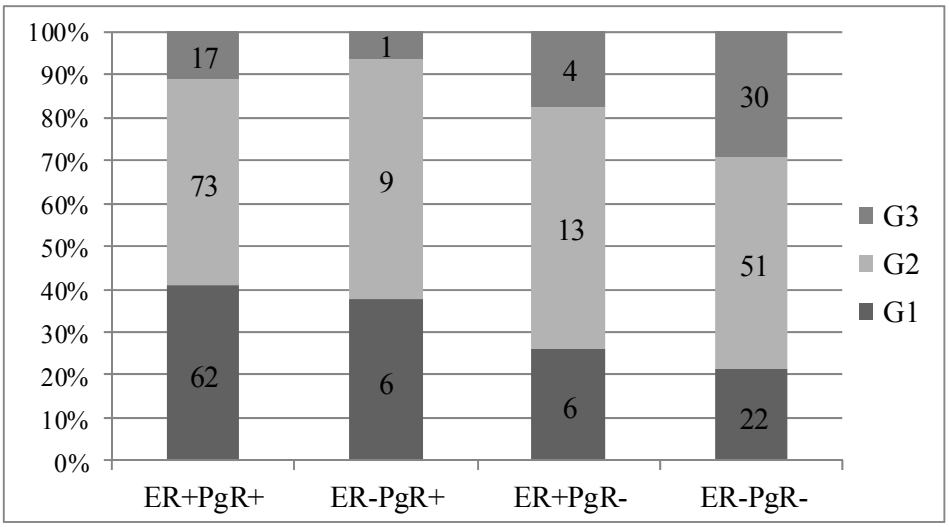

Fig. 4. Distribution of degrees of malignancy (by Elston& Ellis) at various hormone profile of breast cancer

The obtained data demonstrate certain dependence of histological grade of breast cancer on receptor profile of tumor cells ($\chi 2 = 20.40$, df = 6, p = 0.002). Thus, G1 at the receptor-positive tumors was detected 2 times more frequently (40.8% of the tumors in this group) than in the receptor-negative (21.3%) and only the ER-positive tumors (26.1%) and PgR positive (37.5%).G2 was detected equally often in receptor-positive and receptor-negative breast cancer (48 and 49.5%, respectively), while it was the vast majority of tumors with only ER-positive (56.5%) and only PgR-positive (56.2%) profile. Though G3 in the general totality was diagnosed more than 2 times less than remaining degrees, it constituted 29.1% of tumors with a receptor-negative profile and 11.2% - with a positive profile and was found in only 17.4% with ER-positive, and 6.2% of the PgR-positive profile.

Thus, by increasing the degree of malignancy receptor-negative tumors are significantly more observed ($p < 0.05$), while receptor-positive rate decreases.

Proliferative activity of breast tumor cells was determined using a universal proliferation marker Ki-67, which is expressed in almost all phases of the mitotic cycle and reflects the value of the proliferative tumors pool [9]. In our research Ki-67 expression was detectedin 272 cases (92.5%) – less than 15% of tumor cells (low PA). We observed Ki-67 expression in 22 women (7.5%) – more than 15% of tumor cells (high PA) (Fig. 5-6).

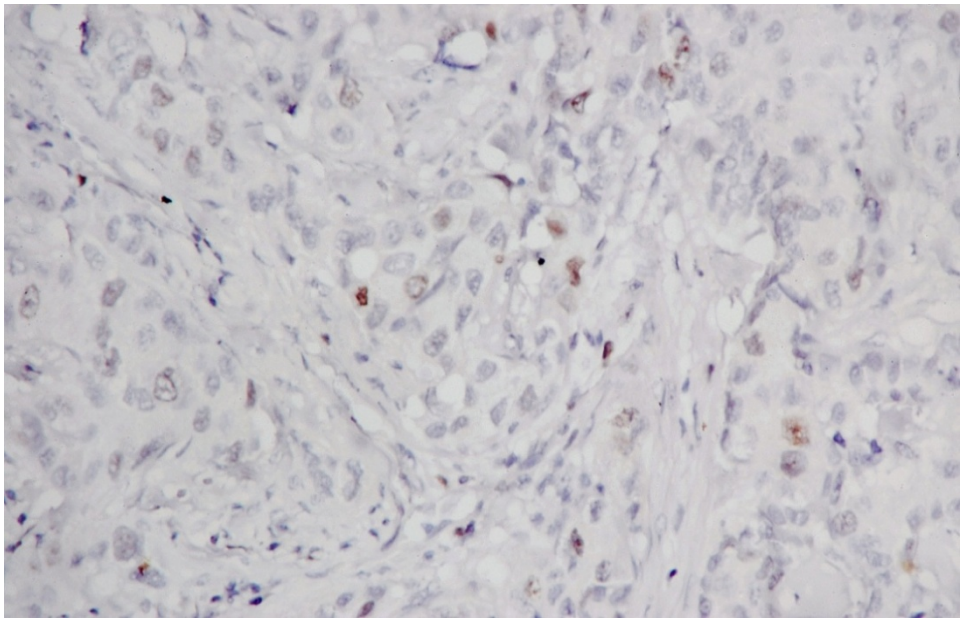

Fig. 5.Infiltrative ductal breast cancer X200.

The immunohistochemical reaction with the monoclonal antibody Ki-67.Expressionof 20%.

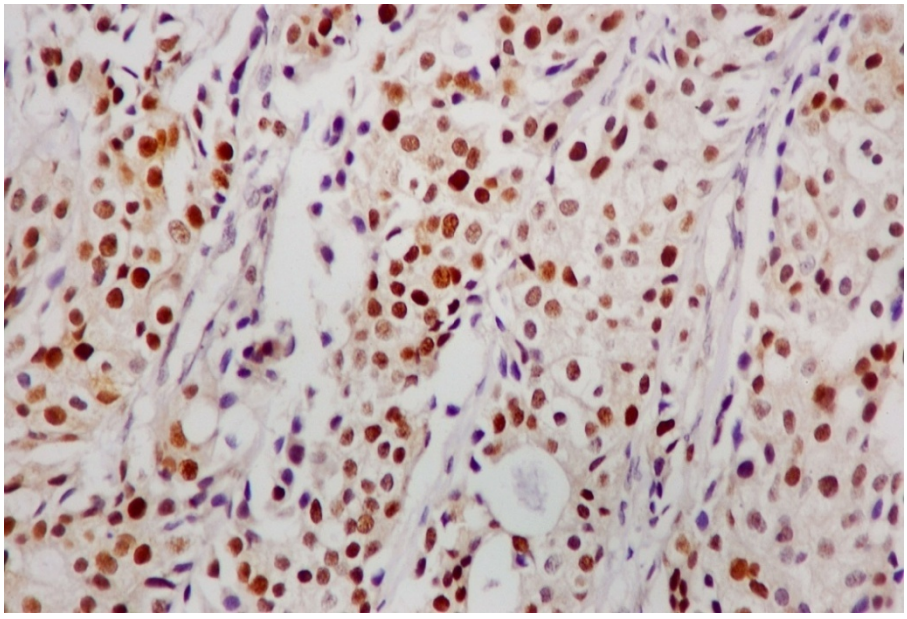

Fig. 6. Infiltrative ductal breast cancer x200.

The immunohistochemical reaction with the monoclonal antibody Ki-67.Expressionof 90%.

Analysis of the expression pattern of Ki-67, depending on the age revealed that the expression level of Ki-67 antigen was independent of age ($\chi 2=4.1$, df=4, p =0.39). High PA of tumor cells occurs in both elderly patients and younger aged ones. In aggregate indicators in groups of indigenous and non-indigenous population in breast cancer statistically significant correlations with PA ethnicity has not been found ($\chi 2=1.6$, df=1, p=0.20).Numerous studies have shown that PA of tumor cells in breast cancer is directly correlated with the histological degree of malignancy [10,11]. The results obtained in our study indicate that the expression of Ki-67 antigen significantly differ between the different histological variants of breast cancer ($\chi 2=3.58$, df=1, p=0.05): there was a tendency of increasing the histological degree of malignancy while increasing the expression of Ki-67 (Fig. 7).

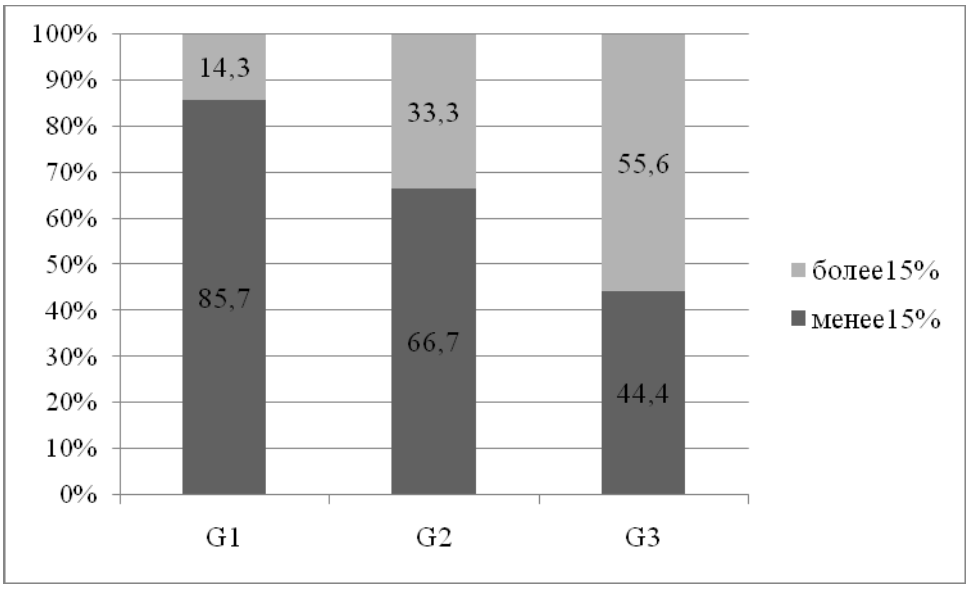

Fig. 7.Dependence of histological degree of malignancy on the level of Ki-67 expression.

These results do not contradict to the literature data on the Ki-67 antigen as a marker of adverse outcome of the disease [12,13,14]. Importantly, PA of tumor cells serves as an independent prognostic indicator of relapse, overall and disease-free survival [16], as well as a predictive factor in determining the sensitivity to radiotherapy and chemotherapy [17,18].Our analysis confirmed the dependence between the degree of histological malignancy and proliferative activity of tumors, which proves the necessity of accounting PA of tumor cells in the evaluation of malignant potential and prognosis of breast cancer.

In immunohistochemical study of mutant tumor suppressor gene p53 low expression (less than 25% of tumor cells) was observed in 197 (67.0%) cases, whereas high expression (more than 25% of tumor cells) - 97 (33 0%) cases (Fig. 8, 9).

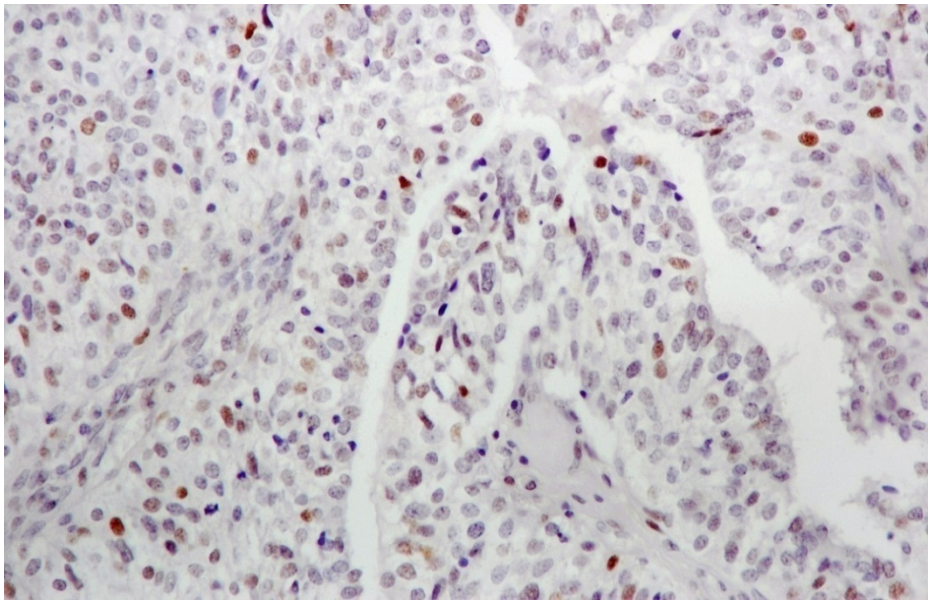

Fig. 8. Invasive breast cancer x200.

The immunohistochemical reaction with monoclonal antibodies to p53. Expression of 15%.

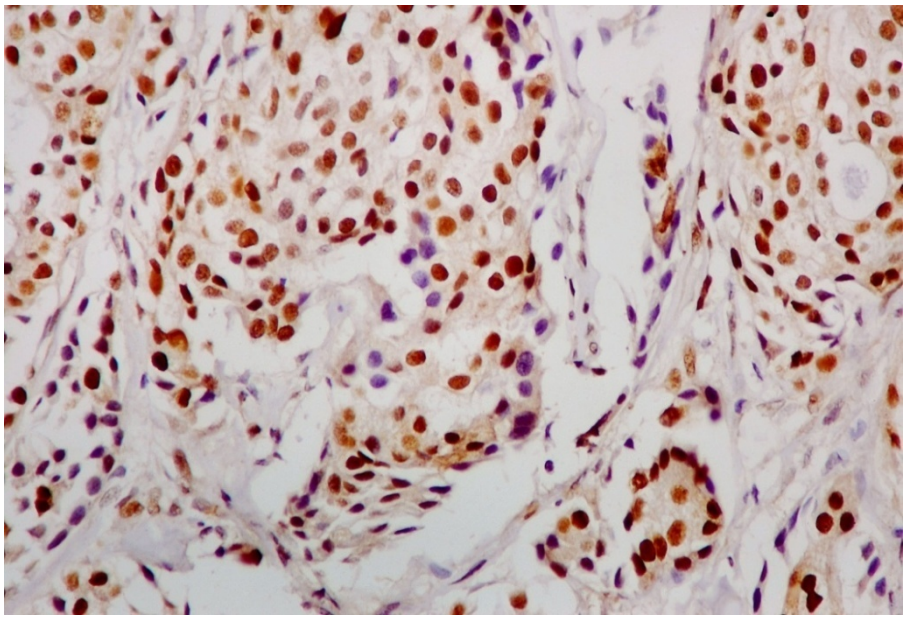

Fig. 9. Infiltrative ductal breast cancer X200.

The immunohistochemical reaction with monoclonal antibodies to p53. Expression of 100%.

In the analysis of this indicator based on ethnicity, we have established that indigenous women have statistically significant weak positive correlation ($r = 0,21$, $p = 0,02$) between age and the expression of p53. Low expression of p53 was observed in indigenous women aged 40 - 49 years (27.2%), while non-indigenous - aged 50 - 59 years (36.7%). In aggregate indicators in

both groups statistically significant age differences were not found ($\chi 2 = 1,8$, df = 4, p = 0.76). The positive relationship between the level of p53 expression and increased histological tumor grade was found ($\chi 2=7.9$, df =2, p=0.01), which proves the need to consider the severity of the process of cell apoptosis in evaluating the malignant potential of the tumor and prognosis of breast cancer (Fig. 10).

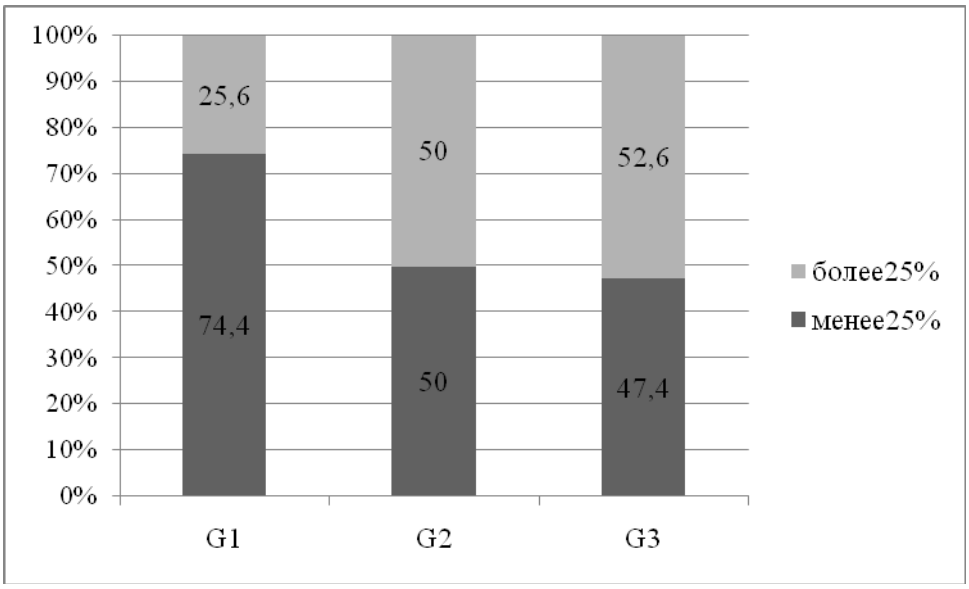

Fig. 10. Dependence of histological grade on the level of p53 expression.

By immunohistochemical study of the expression of anti-apoptotic tumor-suppressor gene bcl-2 we found low expression of bcl-2 in 142 cases (48.3%), high expression - in 152 cases (51.7%) (Figure 11).

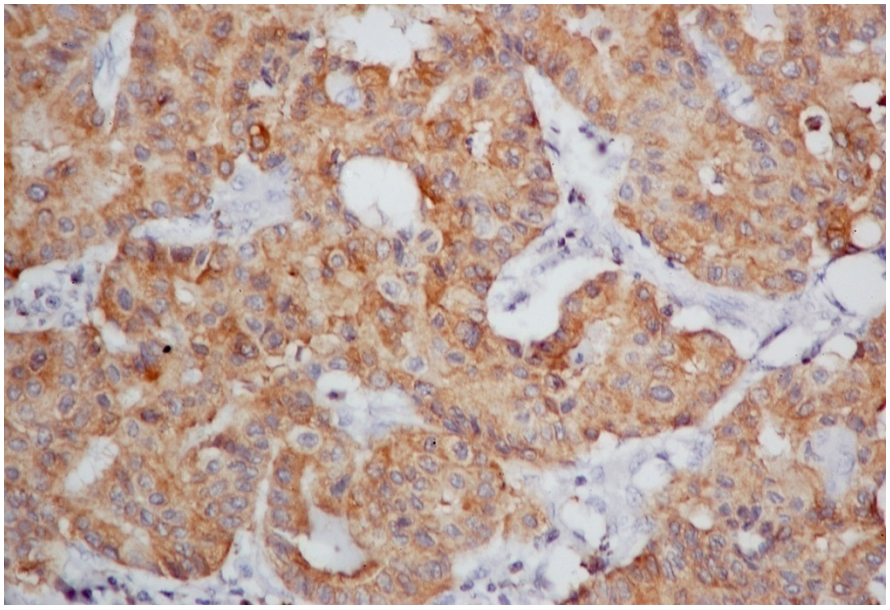

Fig. 11. Invasive papillary breast cancer x200.

The immunohistochemical reaction with the monoclonal antibody bcl-2. Expression of 98%.

Data analysis based on ethnicity did not reveal differences in the frequency of cases with low and high expression of bcl-2 ($\chi 2$=0.23, df =1, p=0.64). Low expression of bcl-2 in indigenous and non- indigenous women most frequently was recorded at the age of 50 - 59 years (respectively 33.9 and 36.1%). High expression of bcl-2 was more common among indigenous women aged 60 - 69 years (27.1%), in non- indigenous women - aged 50 - 59 years (37.6%).

According to the literature, bcl-2 is a tumor-suppressor gene affecting cell death mechanisms and suppressing apoptosis [15]. According to many authors, the increased expression of bcl-2 is associated with increased survival of patients after adjuvant therapy. Perhaps this is due to the close relationship between the expression of bcl-2 and the expression of ER and PgR, and low PA. However, bcl-2 is not an independent prognostic factor [15]. We have established a negative correlation between the expression of bcl-2 antigen, and the degree of histological malignancy of tumor (r = -12.9, p=0.03), i.e. tumors with a high degree of malignancy were characterized by low expression of bcl-2, which describes by preserving the regulation of apoptosis in normal levels (Fig. 12).

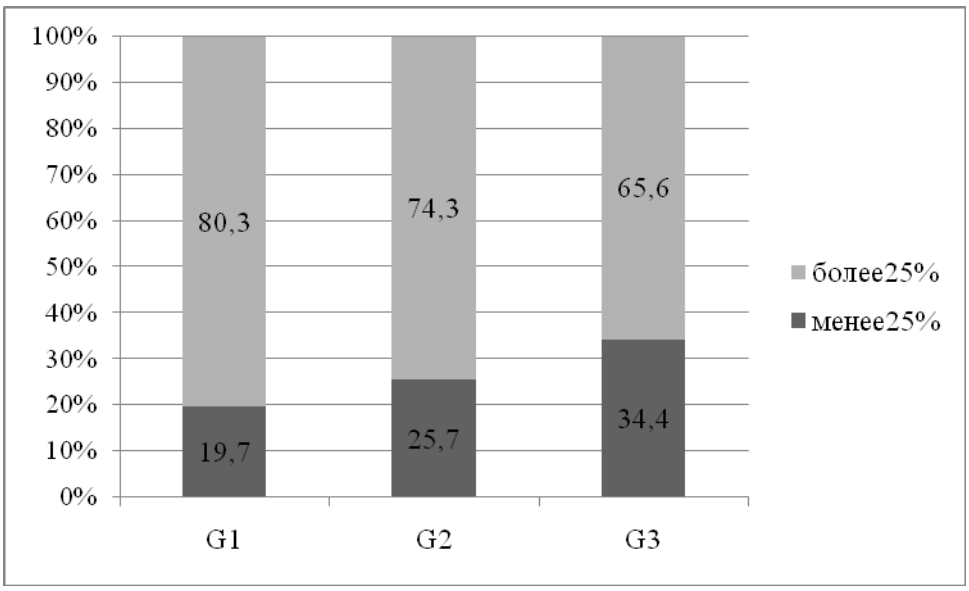

Fig. 12. Dependence of bcl-2 expression on the degree of malignancy.

Thus, the data of comparative immunohistochemical study of some molecular biological markers for breast cancer analysis of their expression levels showed:

- According to clinical and morphological analysis, breast cancer develops most often in representatives of non-indigenous nationalities of the Republic of Sakha (Yakutia) (60%) than those belonging to indigenous nationalities (40%). With the highest rate recorded in women of indigenous and non-indigenous nationalities in the age group 50 - 59 years (32.6%) and 60 - 69 years (21.8%).

- In breast cancer tumors with the presence of steroid hormone receptors are predominant (65%).

- With increasing age tumor cells of breast cancer the level of ER expression increases ($p = 0.03$).

- Tumors with a high degree of malignancy are characterized by low expression of estrogen receptors and progesterone receptors ($p < 0,05$).

- The tendency of tumor grade increasing by increasing the expression of p53 and Ki-67 is found.

- The level of expression of bcl-2 antigen and its prevalence is positively correlated with the presence of ER and PgR receptors on tumor cells and low-grade tumors.

- The most informative criteria of increasing breast cancers are the decrease in expression of estrogen and progesterone receptors, increased expression of Ki-67 and p53 and decreased expression of bcl-2.

- The most informative criterias of increasing of breast tumors malignancy are the decrease in expression of estrogen and progesterone receptors, increased expression of Ki-67 and p53 and decreased expression of bcl-2.

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