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Epithelial-mesenchymal transition markers, proliferation markers, and cytokine secretion in breast tissue in malignant and benign breast diseases

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ABSTRACT

Aim. To develop methodological grounds for assessing the probability of breast malignancy in patients with non-cancerous breast diseases (NCBD) by the following parameters: expression of markers of epithelial – mesenchymal transition (EMT) and proliferation and production of cytokines by samples of the breast tissue.

Materials and methods. In breast samples (BS) of patients with invasive carcinoma of no special type (ICNT) and patients with NCBD, immunohistochemistry was used to determine the expression of E-cadherin (CDH1), integrin β1 (CD29), type II collagen (CII), and proliferation of Ki-67. Using the enzyme-linked immunosorbent assay, concentrations of interleukin (IL)-2, IL-6, IL-8, IL-10, IL-17, IL-18, IL-1β, IL-1Ra, tumor necrosis factor (TNF)α, interferon (IFN)γ, granulocyte colony-stimulating factor (G-CSF), granulocyte – macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF)-A, and monocyte chemoattractant protein (MCP)-1 were determined in the supernatant of the cultured breast tissue samples.

Results. It was shown that ICNT and NCBD differ in the expression of E-cadherin, CD29, Ki-67, and the production of IL-2, IL-4, IL-6, IL-17, IL-18, IL-1Ra, TNFα, IFNγ, and MCP-1.

The ROC analysis found that the models characterizing the differences between the ICNT and NCBD samples were formed by the parameters of CD29 and Ki-67 expression and IL-17, IL-18, TNFα, VEGF-A, and MCP-1 production. The neural network analysis revealed that CD29, IL-1Ra, TNFα, and VEGF-A had the greatest normalized importance for assessing the differences between the ICNT and NCBD samples. Clustering of the combined database of patients with NCBD and ICNT by the expression of E-cadherin, CD29, Ki-67 and by the production of IL-17, IL-18, TNFα, MCP-1, and VEGF-A resulted in a cluster which includes the parameters of 94.1% of patients with NCBD. The parameters of less than 10% of patients with NCBD who fell into other clusters practically coincided with the studied parameters of the ICNT group, which suggests that these patients may form a risk group with the malignancy probability of more than 90%.

Conclusion. The data obtained made it possible to develop methodological grounds for assessing the likelihood of breast malignancy in patients with NCBD.

Keywords: non-cancerous breast diseases, invasive carcinoma of no special type, proliferation marker, markers of epithelial – mesenchymal transition, cytokines

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Маркеры эпителиально-мезенхимального перехода, пролиферации и продукция цитокинов в ткани молочной железы при злокачественных и незлокачественных заболеваниях молочной железы

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РЕЗЮМЕ

Цель. На основе изучения экспрессии маркеров пролиферации, эпителиально-мезенхимального перехода (ЭМП) и цитокинового профиля суперталантов образцов ткани молочной железы (МЖ) при раке МЖ и незлокачественных заболеваниях (НЗМЖ) разработать методологические основы оценки вероятности малигнизации МЖ при НЗМЖ.

Материалы и методы. В образцах МЖ больных с инвазивной карциномой неспецифического типа (ИКНТ) и пациентов с НЗМЖ иммуногистохимическим методом определяли экспрессию Е-кадгерина (CDH1), интегрина β1 (CD29), коллагена II типа (CII) и маркера пролиферации Ki-67. С помощью иммуноферментного анализа в супернатанте культивируемых образцов МЖ определяли концентрацию интерлейкина (IL) 2, IL-6, IL-8, IL-10, IL-17, IL-18, IL-1β, IL-1Ra, фактора некроза опухоли-альфа (TNFα), гамма-интерферона (IFNγ), гранулоцитарного колониестимулирующего фактора, гранулоцитарно-макрофагального колониестимулирующего фактора, фактора роста эндотелия сосудов (VEGF-A) и моноцитарного хемотаксического белка 1 (MCP-1).

Результаты. Показано, что ИКНТ и ДЗМЖ отличаются по экспрессии Е-кадгерина, CD29, Ki-67 и продукции IL-2, IL-4, IL-6, IL-17, IL-18, IL-1Ra, TNFα, IFNγ, MCP-1. При помощи ROC-анализа установлено, что модели, характеризующие различия между образцами ИКНТ и ДЗМЖ, формируются по параметрам экспрессии CD29, Ki-67 и продукции IL-17, IL-18, TNFα, VEGF-A и MCP-1. При помощи нейросетевого анализа выявлено, что наибольшую «нормализованную важность» для оценки различий образцов ИКНТ и ДЗМЖ имеют параметры CD29, IL-1Ra, TNFα и VEGF-A. При кластеризации объединенной базы данных пациентов с ДЗМЖ и ИКНТ по экспрессии Е-кадгерина, CD29, Ki-67 и по показателям продукции IL-17, IL-18, TNFα, MCP-1 и VEGF-A формируется кластер, в который входят показатели 94,1% пациентов с ДЗМЖ. Параметры менее 10% пациентов с ДЗМЖ, попавших в другие кластеры, практически совпадали с исследованными параметрами ИКНТ. Это дает основание предположить, что эти пациенты могут составить группу риска с вероятностной малигнизацией более 90%.

Заключение. Полученные данные позволили сформировать методологическую основу для оценки вероятности малигнизации МЖ у пациентов с ДЗМЖ.

Ключевые слова: незлокачественные заболевания молочной железы, инвазивная карцинома неспецифического типа, маркер пролиферации, маркеры эпителиально-мезенхимального перехода, цитокины

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

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Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Исследование одобрено этическим комитетом Института молекулярной биологии и биофизики Федерального исследовательского центра фундаментальной и трансляционной медицины (протокол № 2016-3 от 15.03.2016).

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INTRODUCTION

It is known that the pathogenetic background for the development of cancer may be non-cancerous breast diseases (NCBD) [1, 2]. According to the classification of the International Agency for Research on Cancer, ductal carcinoma *in situ* is included in the group of precancerous breast lesions [3]. However, according to the literature, precancerous breast lesions encompass sclerosing adenosis [4, 5], radial scar [6], and intraductal proliferative lesions that increase the risk of developing breast cancer from 1.27 to 10.35 times, depending on the form of pathology [7–10]. These data determine the relevance of research aimed at searching for new markers to detect precancerous changes in the breast tissue, which may reflect the mechanisms of breast tissue malignancy in NCBD.

One of the processes characterizing the onset of malignant transformation is epithelial – mesenchymal transition (EMT) [11, 12]. It is known that EMT is characterized by activation of the expression of mesenchymal markers, such as integrin β1 (CD29) and type II collagen (CII), as well as by a decrease in the expression of E-cadherin (CDH1) [13–17]. The most widely used marker of cell proliferation in breast cancer is Ki-67 due to its reliable correlation with the proliferative activity of cancer cells [18].

Detection of EMT in the breast tissue can be considered as the first sign of developing cellular atypia, and the expression of a number of molecules associated with EMT can be seen as a marker indicating the onset of malignant transformation in NCBD, induced by a number of inflammatory mediators, including cytokines [12, 13]. In turn, production of cytokines that stimulate EMT can be caused by activation of certain signaling pathways in cells. Thus, induction of EMT under the effect of interleukin (IL)- β1 and TNFα is due to activation of the NF-kB signaling pathway [14]. These data indicate that malignancy of the breast tissue may depend not only on EMT, but also on specific changes in the cytokine profile of the tumor that determine a tumor microenvironment, which includes various immunocompetent cells, fibroblasts, fibrocytes, epithelial cells, and other cells that produce various cytokines. Some of the cytokines, which are produced by cells of the tumor microenvironment, facilitate progression of breast cancer [19, 20]. However, the role of cytokines in the formation of cellular atypia and breast malignancies has not yet been sufficiently studied for them to be considered as markers indicating a high risk of malignancy.

Aim of the study: to develop methodological grounds for assessing the probability of breast malignancy in patients with NCBD by studying the expression of EMT and proliferation markers and cytokine profile in the supernatants of breast tissue samples in breast cancer (BC) and NCBD.

MATERIALS AND METHODS

The material of the study was samples of breast tumors obtained from 79 women who were treated at

the oncology department No. 1 of Novosibirsk City Hospital No. 1. Of them, 62 people had stage II invasive carcinoma of no special type (ICNT) and 17 people had NCBD, including 8 people with fibroadenoma, 6 people with fibrocystic breast disease, including fibroadenomatosis, 2 people with ductal hyperplasia with areas of sclerosing adenosis, and 1 person with focal fibrosis with microcalcifications. The average age of patients with ICNT was 53.9 ± 1.8 (23–76 years), with NCBD – 45.4 ± 5.1 (19–67 years). The exclusion criteria were signs of distant metastasis and concomitant hormonal, chronic, inflammatory, and infectious diseases.

The study and all research protocols were approved by the Ethics Committee at the Institute of Molecular Biology and Biophysics (Protocol No. 2016-3) of the Federal Research Center for Fundamental and Translational Medicine (Novosibirsk, Russia). All procedures performed in this study were carried out in accordance with the Declaration of Helsinki of 1964 and its subsequent amendments (Brazil, Fortaleza, 2013). Each patient was informed about the study, its aim, and methods. An informed consent to participate in the study and to use tumor samples was signed by each patient and verified by the attending physician.

Tumor samples (8 mm^3) obtained by trepanobiopsy were washed with the DMEM-F12 culture medium three times, then placed in a glass vial with 1 ml of the DMEM-F12 medium, and incubated for 72 hours at 37 °C. After incubation, the test samples were removed from the medium and fixed in a 10% neutral buffered formalin solution for further immunohistochemical and histopathological studies. Concentrations of IL-2, IL-6, IL-8, IL-10, IL-17, IL-18, IL-1β, IL-1Ra, tumor necrosis factor (TNF) α, interferon (IFN) γ, granulocyte colony-stimulating factor (G-CSF), granulocyte – macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF)-A, and monocyte chemoattractant protein (MCP)-1 were determined in the supernatant of the cultured breast tissue samples by enzyme-linked immunosorbent assay (ELISA) using reagent kits manufactured by Vector-Best JSC (Russia).

The tissue samples fixed in 10% neutral buffered formalin were dehydrated and embedded in paraffin. Dewaxing and rehydration of paraffin sections were carried out according to the standard xylene / ethanol protocol.

The expression levels of Ki-67, E-cadherin (CDH1), integrin β1 (CD29), and CII in the ICNT and NCBD samples were determined using monoclonal antibodies, such as anti-Ki-67 (Leica Biosystems, Inc.), anti-E-cadherin (BD Biosciences, USA), anti-CD29 (BD Transduction Laboratories, USA), and anti-CII (Santa Cruz Biotechnology, Inc.), and VECTASTAIN ABC detection systems (Vector Laboratories, PK-7200, USA) in accordance with the manufacturers' instructions. The sections were additionally stained with hematoxylin and eosin and mounted with Canada balsam. The expression of the studied markers was analyzed using the MICROMED-6 microscope, the DSM 510 digital camera, and the ImageJ 1.42g software (NIH, USA). For each patient, 10 microphotographs (taken at x40) were evaluated. The expression data for Ki-67, CDH1, CD29, and CII were presented as percentage (% of cells expressing the marker).

The level of statistical significance of differences between the groups was determined using the nonparametric Wilcoxon – Mann – Whitney test. The data were presented as the median and the interquartile range Me (Q_{25}; Q_{75}). The calculations were performed using the Statistica v. 7 software.

The neural network analysis and ROC analysis of the obtained data were performed using the IBM SPSS software, v. 22. Normalized importance of various tumor sample characteristics for assessing the differences between the ICNT and NCBD samples was evaluated by the neural network analysis of the entire database, including parameters of ICNT and breast tissue in NCBD. The study used a neural network model generated on the basis of the Multilayer Perceptron model, with one hidden layer consisting of three hidden neurons.

The hidden layer activation function was hyperbolic tangent activation function, the output layer activation function was identity function. To verify the accuracy of the neural network analysis, the normalized importance of all model parameters was determined using two training methods – batch gradient descent method and interactive gradient descent method. The cluster analysis was performed using the Statistica v. 7 software.

RESULTS

Table 1 assesses the differences between the ICNT and NCBD samples by the expression of proliferation markers, EMT markers, and cytokine concentrations in the supernatant. Fig. 1 shows the ICNT and NCBD samples stained for Ki-67, E-cadherin, CII, and CD29.

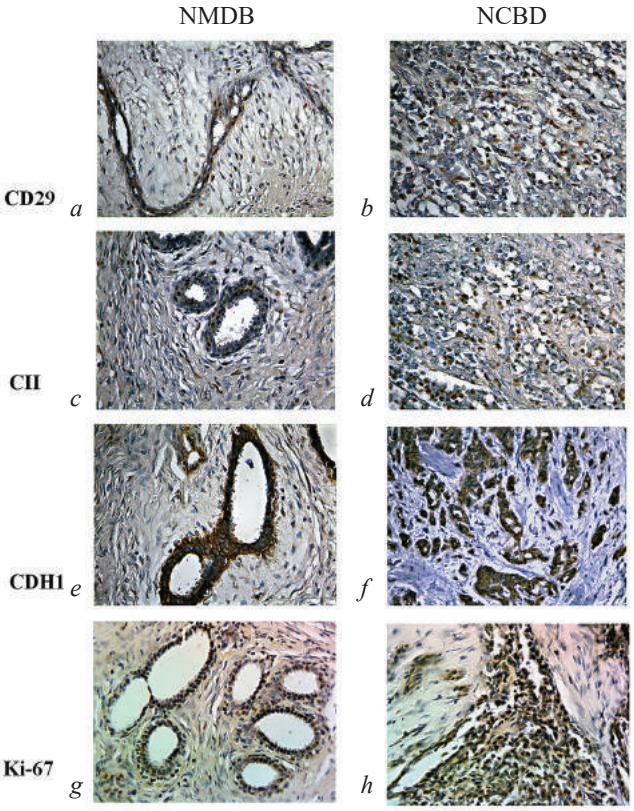

Fig. 1. Results of the immunohistochemical analysis of the tumor tissue: NCBD – non-cancerous breast disease (*a, c, e, g* – fibroadenoma); ICNT – invasive carcinoma of no special type (*b, d, f, h*). The brown – yellow coloration indicates the expression of EMT markers (CDH1 – E-cadherin; CD29 – integrin β1; CII – type II collagen) and the proliferation marker Ki-67. Counterstaining with hematoxylin and eosin, ×400

Ki-67 expression was 2.7 times higher in the ICNT samples compared to the NCBD samples. It was shown that the ICNT samples and the NMDB samples significantly differed in the expression of E-cadherin and CD29: the expression of E-cadherin was higher in the NCBD group than in the ICNT samples, while the expression of CD29 was higher in the ICNT samples than in the NCBD samples. There were no significant differences in the CII expression between the groups.

It was found that the ICNT and NCBD samples significantly differed in the production of IL-2, Il-6, Il-17, IL-18, IL-1Ra, TNFα, IFNγ, VEGF-A, and MCP-1. The concentration of IL-6 and MCP-1 in the breast tissue supernatant was higher in the NCBD samples than in the ICNT samples, and the concentration of IL-2, Il-17, IL-18, IL-1Ra, TNFα, IFNγ, and VEGF-A was higher in the ICNT samples compared to the NCBD samples.

Table 2 presents the results of the ROC analysis and the neural network analysis used to identify the differences between the ICNT and NCBD samples in the expression of proliferation and EMT markers and cytokine concentrations in the tumor tissue supernatant. The ROC analysis showing the quality of the models found that the best quality models characterizing the differences between the ICNT and NCBD samples were formed when CD29 and Ki-67 expression, as well as production of IL-17, IL-18, TNFα, VEGF-A, and MCP-1 were used as comparison parameters. According to the ROC analysis based on these parameters, the quality of models for detecting the differences between the ICNT and NCBD samples was good (AUC > 0.7) or very good (AUC > 0.8). The AUC values for CD29 and Ki-67, IL-17, IL-18, TNFα, VEGF-A, and MCP-1 were 0.750, 0.863, 0.732, 0.784, 0.722, 0.873, and 0.742, respectively (Table 2).

According to the data obtained using the neural network analysis, the highest normalized importance (more than 80%) in the neural network model used to detect the differences between the ICNT and NCBD samples was found for CD29 expression (100%), IL-1Ra production (> 90%), TNFα (> 90%), and VEGF-A (> 80 %). Relatively high normalized importance (more than 70%) in the neural network model used to identify the differences between the ICNT and NCBD samples was detected for E-cadherin and Ki-67 (Table 2). Table 2 shows that the neural network model training method (batch or interactive gradient descent method) did not have a significant impact on the results of the analysis for all variables.

Table 1

Expression of EMT-associated markers, Ki-67, and cytokine concentrations in the supernatant of ICNT and NCBD samples, *Me* (Q_{25}; Q_{75})

Parameter	Breast tissue samples		*p*
	ICNT	NCBD	
E-cadherin	64.2 (60.9; 91.7)	82.7 (79.7; 97.6)	0.020
CD29	19.6 (8.4; 19.7)	8.9 (1.3; 15.3)	0.001
CII	12.1 (6.5; 15.7)	10.5 (5.3; 14.9)	0.542
Ki-67	21.0 (12.0; 43.0)	8.3 (3.2; 19.8)	0.001
IL-2	2.8 (2.1; 5.4)	2.2 (2.1; 2.5)	0.005
IL-4	2.7 (1.7; 4.1)	3.2 (2.6; 4.4)	0.286
IL-6	297.8 (87.2; 482.7)	502.4 (279.6; 654.5)	0.027
IL-8	366.6 (203.1; 672.8)	378.7 (295.5; 1,360.0)	0.467
IL-10	6.1 (1.3; 11.8)	9.5 (1.7; 19.5)	0.404
IL-17	2.3 (1.0; 5.1)	6.0 (2.2; 7.4)	0.003
IL-18	42.4 (15.2; 180. 6)	5.0 (3.3; 26.5)	0.001
IL-1b	32.3 (14.7; 70.2)	17.0 (11.2; 38.8)	0.148
IL-1Ra	3,273.5 (2,172.6; 4,195.0)	2,070.6 (689.6; 3,003.2)	0.034
TNFα	2.9 (1.5; 5.2)	2.0 (1.1; 3.2)	0.046
IFN γ	11.5 (5.2; 26.0)	5.9 (2.0; 17.4)	0.027
G-CSF	61.1 (8.7; 424.6)	80.3 (41.1; 468.1)	0.745
GM-CSF	8.6 (3.1; 22.2)	3.2 (2.0; 13.4)	0.099
VEGF-A	1,359.2 (161.0; 2,144.0)	55.8 (18.4; 377.2)	0.001
MCP-1	560.9 (196.9; 1,556.0)	660.8 (259.6; 1,133.2)	0.046

Note: the expression of E-cadherin, CD29, and CII is presented as a percentage (% of expressing cells); cytokine values – in pg / ml.

Table 2

Evaluation of the differences between the ICNT and NCBD samples by the expression of the EMT and proliferation markers and cytokine concentrations in the breast tissue supernatant using the ROC analysis and the neural network analysis

Parameter	Normalized importance of a parameter in the NN-model; batch gradient descent method	Normalized importance of a parameter in the NN-model; interactive gradient descent method	Area under the curve (AUC) in the ROC analysis
E-cadherin	79.4%	78.6%	0.549
CD29	100.0%	100.0%	0.750
CII	36.3%	38.0%	0.162
Ki-67	78.0%	77.7%	0.863
IL-2	53.9%	53.5%	0.642
IL-4	22.5%	34.4%	0.415
IL-6	47.5%	49.7%	0.280
IL-8	45.5%	50.4%	0.441
IL-10	33.6%	40.4%	0.433
IL-17	68.3%	68.5%	0.732
IL-18	34.3%	23.1%	0.784
IL-1β	53.8%	40.6%	0.616
IL-1Ra	90.7%	95.7%	0.668
TNFα	91.3%	95.2%	0.722
IFNγ	12.3%	12.0%	0.676
G-CSF	9.7%	9.7%	0.473
GM-CSF	76.7%	71.7%	0.459
VEGF-A	81.6%	82.8%	0.873
MCP-1	72.1%	69.0%	0.742

Note: the NN-model – the neural network model. The results of the NN analysis are presented in terms of normalized importance of each parameter (%). The results of the ROC analysis are presented in AUC values.

Using the cluster analysis, we assessed the probability of cluster formation from the parameters of patients with NCBD with account of only the parameters with the highest normalized importance in the neural network analysis and the greatest AUC in the ROC analysis: CD29, Ki-67, IL-17, IL-18, TNFα, MCP-1, and VEGF-A. It was shown that when clustering combined data of patients with ICNT (sample No. 1–62) and NCBD (sample No. 63–79) by the specified parameters of breast tissue samples, 4 clusters were formed at the Euclidean distance of 15. One of the clusters – cluster III – included parameters of more than 90% (94.12%) of patients with NCBD (Fig. 2).

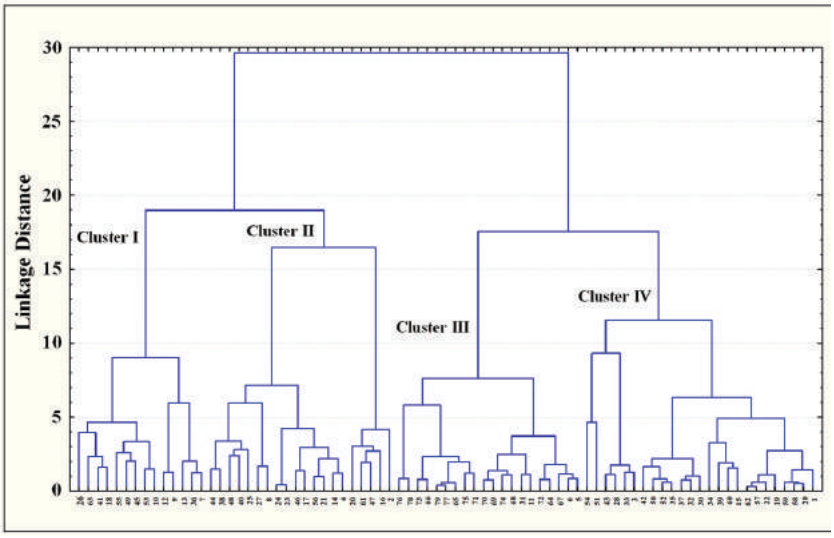

Fig. 2. Graphical representation of the results of the multidimensional cluster analysis of combined data from patients with ICNT (sample No. 1–62) and NCBD (sample No. 63–79). The dendrogram is constructed using the Ward's method. The horizontal axis represents encrypted numbers of samples obtained from different patients with ICNT (from 1 to 62) and NCBD (from 63 to 79), and the vertical axis represents the clustering distance (Euclidean distances). Clustering was performed on the basis of the simultaneous analysis of E-cadherin, CD29, and Ki-67 expression in the tumor samples and IL-17, IL-18, TNFα, MCP-1, and VEGF-A secretion by the tumor samples

The subcluster within cluster III, located at the Euclidean distance of 2.0, contained the parameters of three NCBD samples (sample No. 64, 67, 72) and two ICNT samples (sample No. 5, 6). The specified subcluster included samples from patients with NMDB with proliferative fibrocystic changes and atypical ductal hyperplasia (sample No. 64 and 72), as well as with fibroadenoma with severe ductal hyperplasia (sample No. 67) in the medical history. The parameters of one NCBD sample (assigned to other clusters) almost coincided with the parameters of ICNT. This sample was obtained from the patient diagnosed with fibroadenomatosis with pronounced proliferation (cluster I, sample No. 63).

DISCUSSION

The parameters of ICNT and NCBD samples obtained from different patients varied in terms of the expression of immunohistochemical markers of EMT and proliferation, as well as in cytokine production. In this regard, one of the main tasks was to develop a neural network model that would make it possible to predict and evaluate the probability of malignancy in non-cancerous diseases based on the assessment of EMT and proliferation markers and cytokine profile produced by tumor samples. It is known that if output parameters of a neural network model change, the importance of a particular tumor parameter also changes. The output of a neural network model may also depend on the way the model is trained. Therefore, when conducting the neural network analysis, we used two options for training the model.

With the help of the ROC analysis and neural network analysis, we found that some parameters of cytokine production by BS may have an even greater prognostic value for assessing the differences between malignant tumors and non-cancerous diseases than E-cadherin, CII, CD29, and Ki-67. Such cytokines include IL-17, IL-18, TNFα, MCP-1, and VEGF-A, as well as a number of others with a lower prognostic value. It was shown that when clustering the combined database of patients with NCBD and ICNT by a wide range of BS parameters, the expression of E-cadherin, CD29, and Ki-67 and the production of IL-17, IL-18,

TNFα, MCP-1, and VEGF-A allow to form a cluster which includes parameters of more than 90% of patients with NCBD. At the same time, the parameters of less than 10% of the NCBD samples that fell into other clusters practically coincided with the studied parameters of ICNT.

On the one hand, these data indicate that IL-17, IL-18, TNFα, MCP-1, and VEGF-A may play an important role in the formation of the microenvironment contributing to the onset of breast tissue malignancy in NCBD. On the other hand, at a certain level of their production, they can be considered as markers indicating the probability of malignancy in NCBD. According to the results of the study, patients with the following diagnoses were attributed to a group with a probable risk of malignancy in NCBD: fibroadenomatosis with pronounced proliferation, proliferative fibrocystic breast disease with atypical ductal hyperplasia, and fibroadenoma with pronounced ductal hyperplasia and with the presence of interductal proliferative lesions.

CONCLUSION

The data obtained make it possible to form a risk group of patients with NCBD with a probability of breast tissue malignancy of more than 90%. Thus, a more accurate prediction of probable malignancy in NCBD can be made taking into account not only the expression of E-cadherin, CII, CD29, and the proliferation marker Ki-67, but also the production of IL-17, IL-18, TNFα, MCP-1, and VEGF-A.

The data obtained can serve as methodological grounds for further study of cytokines that form the microenvironment in the breast tissue in non-cancerous diseases, which may contribute to breast tissue malignancy, and the level of cytokine production can serve as a marker for assessing the likelihood of this process.

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Autenshlyus A.I., Arkhipov S.A., Lyahovich V.V. – conception and design, analysis and interpretation of the data; justification of the manuscript and critical revision of the manuscript for important intellectual content; final approval of the manuscript for publication. Mikhaylova E.S., Arkhipova V.V., Proskura A.V., Varaksin N.A. – analysis and interpretation of the data; final approval of the manuscript for publication.

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