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EXPRESSION OF ANTI-APOPTOTHIC PROTEIN SURVIVIN AND GENE *BIRC5* IN PRIMARY BREAST CARCINOMA AS A PROGNOSTIC MARKER FOR PROGRESSION OF DISEASE

Aim: *to study the expression of the anti-apoptotic survivin protein, as well as the expression of the BIRC5 gene of its encoding primary breast carcinoma as a potential predictive marker.* ***Object and methods:*** *using methods of immunohistochemistry and real-time polymerase chain reaction (PCR) 67 samples (biopsy) of the primary breast carcinoma were studied for the presence of expression of the survivin protein and the gene BIRC5.* ***Results:*** *expression of survivin was determined in 47 mammary carcinoma samples, which was 70.15%. Expression of survivin was most often determined in medium and high grade ductal carcinoma (G2–G3), and was associated with lymphovenous stromal invasion ($LVSI^+$). Expression of the survivin protein correlated with the expression of HER2-neu. In 59.6% of cases, survivin was expressed in tumors with a low Ki-67 index. Most often, survivin was expressed with luminal A and luminal B molecular-biological tumor subtype. Real-time PCR determined the expression of the BIRC5 gene in all 67 carcinoma samples. The level of normalized expression of the BIRC5 gene significantly moderately correlated with the expression of the own product of the survivin protein ($r = 0.704$, $p < 0.01$) and slightly correlated with the expression of the oncoprotein HER2-neu ($r = -0.285$, $p < 0.05$). The median follow-up was 40 months. The overall survival in the first group of patients without survivin expression in the primary tumor was 100%, the overall survival in the second group of patients with the presence of expression was 77.16 (74.6–80.1) ($p = 0.041$). Disease-free survival in the first group was 100%, in the second — 71.9 (70.1–74.2) ($p = 0.037$). The ratio of progression risks in the survivin expression group was 10.6 (95% confidence interval 0.85–132.2; $p = 0.041$).* ***Conclusion:*** *expression of the protein of survivin and its gene — BIRC5, is an independent adverse prognostic factor and can be used as a predictive marker.*

Survivin is a protein encoded in humans by the *BIRC5* gene, called the baculovirus protein [1, 2]. Survivin is a member of the apoptosis inhibitor protein family. The main function of the survivin protein is to inhibit caspase activation of apoptosis. The expression of *BIRC5* genes is high during prenatal development and with the development of most tumors, while its content is negligible in differentiated tissue [3]. The expression of the survivin gene is clearly regulated by the cell cycle. It has been established that expression of the *BIRC5* gene and the synthesis of survivin is associated with the p53 protein [4]. Experimental studies have shown that survivin inhibits apoptosis by stopping both the external and internal pathways by binding and inhibiting effector caspases 3 and 7 [5].

The expression of the survivin gene is particularly pronounced during fetal development, as well as in most types of tumor cells, and is rarely present in normal, benign adults.

Survivin protein can be regarded as an oncogene, since its overexpression in most cancer cells contributes to their resistance to apoptotic stimuli and chemotherapeutic methods of treatment, thus contributing to their continued survival and progression.

The aim is to study the expression of the anti-apoptotic protein of survivin, as well as the expression of the *BIRC5* gene on mRNA level in the primary breast carcinoma as a potential prognostic and predictive marker of disease progressing.

MATERIALS AND METHODS

The study included 67 samples (biopsies) of primary breast carcinoma obtained during surgery or trepan biopsy of a tumor in patients with verified resectable primary breast cancer of the pT1–4N3cM0 stage.

Along with standard immunohistochemistry (IHC), the evaluation of the expression of estrogen receptors and progesterone, as well as the epidermal growth factor receptor, the expression of the anti-apoptotic protein survivin was studied. Antibody Survivin Antibody Biotin conjugate («Pierce», France) was used, as well as a system for automatic visualization of BOND Polymer Detec-

tion Systems («Leica Microsystems», Germany). Staining was carried out automatically on the equipment immunity company «Leica Microsystems». Further study of microscopic preparations and their evaluation were carried out on «Leica microscopes».

Along with IHC, the expression of the anti-apoptotic protein of survivin in carcinoma tissue was investigated in real-time using the reverse transcriptase polymerase chain reaction (RT-PCR) for normalized expression of the *BIRC5* gene encoding the survivin protein. The tumor sample obtained in the course of a biopsy or surgical intervention in a volume of up to 5 mm^3 was frozen, then crushed and lysed. The selection of mRNA from lysed cells was carried out in accordance with the instructions of the manufacturer of the RNA isolation kits («SIVital», Belarus). Using reverse transcription technology, cDNA was synthesized, which was subsequently used to analyze the expression of the *BIRC5* gene in RT-PCR. The calculation of the normalized expression was made relative to the expression level of the reference housekeeping gene *c-ABL*.

Statistical processing of the data was carried out in accordance with modern requirements for conducting biomedical research. Quality indicators are represented by absolute and relative values. Quantitative attributes that do not obey the normal distribution law are presented as a median (Me), interquartile range (LQ/UQ), minimum and maximum values (min, max). As a communication measure for quantitative characteristics that do not obey the normal distribution law, the Spearman correlation coefficient was calculated. In all cases, differences were considered statistically significant at a significance level of $p < 0.05$. All values of p were two-sided. In the long term, overall survival (OS) rates and disease-free survival (DFS) rates were estimated. Cox analysis performed.

Statistical processing of the results was performed using SPSS Statistics 10.0.

RESULTS AND DISCUSSION

Given the fact that currently there are no immunohistochemical criteria for assessing the expression level for survivin, such as, for example, for the epidermal growth factor receptor Her2-neu, the expression of the targeted antigen was evaluated on the yes/no principle, i.e. there is expression of this protein in cells or not.

The expression of survivin was determined in 47 samples of breast carcinoma, which amounted to 70.15%.

The clinical and anatomical characteristics of the tumor process, as well as the pathological characteristics of primary carcinoma samples, in which the expression of the anti-apoptotic protein of survivin was determined by IHC, are presented in Tables 1 and 2, respectively.

When analyzing the clinical and anatomical characteristics of the tumor and the expression of survivin in the tumor tissue, no statistically significant dependencies or correlations were found.

Of greatest interest is the study of expression of survivin in comparison with the known prognostic markers detected in tumor tissue, mainly such as the histological type of tumor, molecular biological subtype of the tumor, degree of differentiation, lymphovascular stromal invasion, index of proliferative activity, etc. (Table 2).

Table 1

Clinical and anatomical characteristics of primary breast carcinomas expressing survivin (n = 47)

Clinical characteristics of the tumor			
Clinical sign	Variant	Value	
		n	%
Side	Left	25	53.2
	Right	22	46.8
Localization	Central	3	6.4
	Upper inner quadrant	8	17.0
	Lower inner quadrant	3	6.4
	Upper outer quadrant	26	55.3
	Lower outer quadrant	4	8.5
	Multicentric growth	3	6.4
T	1	21	44.7
	2	24	51.1
	3	2	4.3
N	0	20	42.6
	1	20	42.6
	2	2	4.3
	3	5	10.6
Stage	I	12	25.5
	IIA	16	34.0
	IIB	12	25.5
	IIIA	2	4.3
	IIIC	5	10.6

Table 2

Pathological characteristics of primary breast carcinomas expressing survivin (n = 47)

Pathological characteristics of the tumor			
Sign	Variant	Value	
		n	%
Morphological structure	Tubular carcinoma	1	2.1
	Medullary carcinoma	1	2.1
	Mucinous carcinoma	2	4.3
	Non-specific carcinoma	1	2.1
	Lobular carcinoma	8	17.0
	Ductal carcinoma	34	72.3
Grade	G1	2	4.3
	G2	23	48.9
	G3	22	46.8
Lymphovenous stromal invasion	$LVSI^+$	41	87.2
	$LVSI^-$	6	12.8
Morphological structure	Luminal A	24	51.1
	Luminal B $HER2^-$	11	23.4
	Luminal B $HER2^+$	5	10.6
	Hyper HER2 expressing	3	6.4
	Triply negative (basal)	4	8.5
Ki-67	> 15%	19	40.4
	< 15%	28	59.6

From the data presented in Table 2, it can be seen that the expression of the anti-apoptotic protein survivin was determined most often in duct carcinoma of medium and high malignancy, mainly combined with lymphovenous stromal invasion.

It should be noted that the expression of the survivin protein correlated with the expression of the epidermal growth factor receptor HER2-neu. Survivin was most often expressed in non-expressing HER2-neu tumors ($r = 0.113$, $p > 0.05$).

Interesting is the information obtained about the relationship between the expression of suvivin and the proliferative activity index Ki-67. For example, in 59.6%

of cases, survivin was expressed in tumors with a low Ki-67 index — < 15%.

Despite the fact that the data are not statistically significant with respect to the expression of HER2-neu, or Ki-67 ($p > 0.05$), and the correlation dependence is weak, this fact may indicate that the tumors expressing survivin are in a particular state, in the stage of not active proliferation, but in a state of inhibition of apoptosis.

A qualitative assessment of the expression of the protein survivin also has a certain value in terms of understanding the function of this protein and possible potential mechanisms of influence on this signaling pathway.

Thus, in IHC, it was found that the survivin protein was expressed predominantly in the cytoplasm of the tumor cells of the primary breast carcinoma (Fig. 1).

It should be noted that not all the tumor expresses this protein, and expression is observed in some cases in separate areas, which may indicate the heterogeneity of the tumor cells within the tumor itself. The heterogeneity of tumor cells within the tumor itself is one of the important moments in the pathogenesis and development of tumor resistance to the therapy being carried out. The different phenotype of tumor cells in the primary tumor and, mainly, the presence of cells at the stage of electromagnetic field and stem clones determine the prognosis of the course of the tumor process.

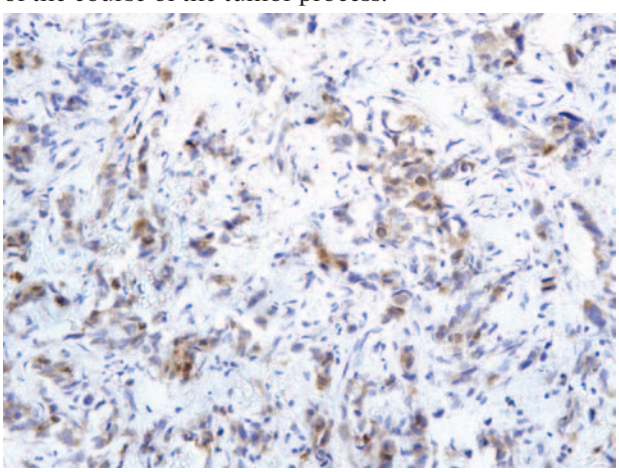


Fig. 1. Determined IHC expression of the survivin protein as a function of the molecular-biological subtype of breast carcinoma. Increase × 200

The expression of the survivin gene *BIRC5* in 67 samples of primary carcinoma of the mammary gland was also investigated using RT-PCR. In all tumor tissue samples, expression of the *BIRC5* gene was determined, however, the levels of the calculated normalized expression relative to the expression level of the reference *c-ABL* gene varied over a wide range (Table 3, Fig. 2). The results of the correlation analysis are shown in Table 4.

When analyzing the presented data, it was found that the level of normalized expression of the *BIRC5* gene was significantly moderately correlated with the expression of the own product of the survivin protein ($r = 0.704$, $p < 0.01$) and slightly negatively correlated with the expression of the HER2-neu oncoprotein ($r = –0.285$, $p < 0.05$).

Table 3

Indicators of normalized expression of the gene *BIRC5* in primary breast carcinoma (RT-PCR)

Gen	Value				
	med	min	max	LQ	UQ
BIRC5	1.49	0.015	30.40	0.662	13.5

LQ – lower quartile; UQ – upper quartile.

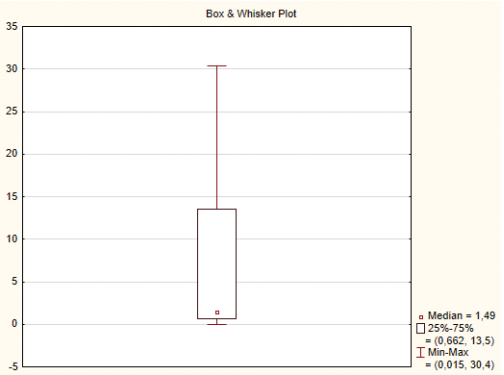


Fig. 2. Indicators of normalized expression of the *BIRC5* gene in primary breast carcinoma (RT-PCR, n = 67)

Table 4

Correlation of normalized expression of the gene *BIRC5* and other molecular-biological and pathological-anatomical characteristics of the primary breast carcinoma

Criteria	The value of the Spearman correlation coefficient and statistical significance	
	r	p-value
Expression of Ki-67 (IHC)	–0.038913	0.754
Expression of HER2-neu (IHC)	–0.285245	0.019
Expression of survivin (IHC)	0.704108	0.000
Expression of ER (IHC)	–0.149550	0.227
Expression of PgR (IHC)	–0.031379	0.800
BIRC5 and HER2-neu (PCR)	–0.153308	0.215
T (tumor size)	0.129916	0.294
N (nodules number)	0.218674	0.075
G (grade)	0.004557	0.970
LVSI	0.018127	0.884

An important component of this study is the determination of the expression value of the anti-apoptotic protein survivin as a prognostic and predictive factor in the treatment of primary non-metastatic breast cancer. The analysis was carried out depending on the presence of expression of the determined IHC in the primary tumor and its absence. Despite the small sample, interesting data were obtained. For example, no patient with no expression of survivin died or progressed during the analyzed period of time. On the contrary, the presence of expression of anti-apoptotic survivin had a negative effect on indicators of OS and DFS. Indicators of differences in OS and DFS are statistically significant ($p = 0.041$ and $p = 0.037$, respectively) (Tables 5, 6, Fig. 3, 4).

As a result of a multivariate analysis using a regression model proportional to Cox risks and determining risk ratios, it was found that factors such as the presence of expression of the anti-apoptotic protein survivin in tumor tissue affect the DFS (Table 7).

Table 5

OS of patients in group 1, depending from the presence or absence of expression of survivin in the primary tumor (n = 67)

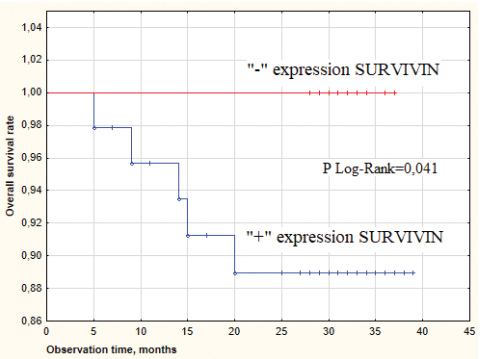
Category	OS, % (95% CI)		
	1-year old	2-year old	3-year old
The presence of expression survivin	93.61 (91.1–96.3)	85.88 (82.1–88.2)	77.16 (74.6–80.1)
Lack of expression survivin	100	100	100

Tables 5, 6, 7: CI – confidence interval.

Table 6

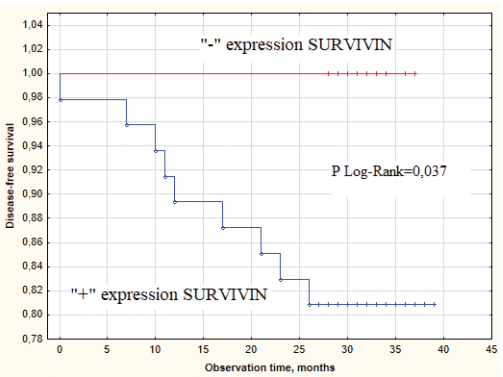
DFS of patients in group 1, depending on the presence or absence of expression of survivin in primary tumor (n = 67)

Category	DFS, % (95% CI)		
	1-year old	2-year old	3-year old
The presence of expression survivin	90.49 (89.5–93.1)	81.07 (79.2–83.1)	71.9 (70.1–74.2)
Lack of expression survivin	100	100	100



--------- «–» expression ---------- «+» expression

Fig. 3. Comparison of the OS of patients with the presence and absence of expression of survivin in the primary tumor (n = 67)



--------- «–» expression ---------- «+» expression

Fig. 4. Comparison of DFS of patients with the presence and absence of expression of survivin in the primary tumor (n = 67)

Table 7

The results of multivariate analysis

	Statistical data (progression risk)							
	β	SE	e (β)	Wald test	p-value	Risk ratio	CI	
							LL	UL
The expression of survivin in the primary tumor	2.364	1.285	4.884	3.380	0.041	10.634	0.85	132.2

LL – lower limit; UL – upper limit.

I. Tamm, *et al.* [5] showed that survivin levels were elevated in all 60 different human tumor lines. In the literature, there is evidence that inactivation of survivin in cancer cells helps to stop the formation of microtubules, which leads to polyploidy, as well as to large-scale apoptosis [6]. Another important function of survivin is to regulate the level of p53. It is known that a feature of most survivin overexpression tumors is the complete loss of wild-type p53 [7]. At the same time, the studies of A. Mirza, *et al.* [7] prove that there is a relationship between survivin and p53. Wild type p53 downregulated survivin expression at the mRNA level in both lung cancer cells and breast cancer cells.

When examining blood samples from cancer patients, scientists found antibodies that are specific for survivin and are absent in healthy people. Measuring the level of survivin-specific antibodies in a patient's blood is monitoring the development of a tumor, and determining the expression of survivin in all the most common malignant tumors (lung, colon, pancreas, prostate, breast cancer, soft tissue sarcoma, T-cell leukemia) risk of death [8]. In addition, studies have shown that breast cancer cells overexpressing the epidermal growth factor gene HER2-neu had higher levels of survivin, which correlated with increased resistance to taxane-induced apoptosis. At the same time, the combination of taxan with a survivin inhibitor resulted in increased apoptosis in *HER2*-overexpressing breast cancer cells compared with monotherapy [9, 10].

CONCLUSION

The obtained data on the study of the expression of the gene *BIRC5* and its protein product from the family of inhibitors of apoptosis of the protein survivin allow us to consider this gene and its product as one of the breast carcinoma tumor-specific markers. Its positive correlation with a known prognostic factor, such as the expression of the oncoprotein of the epidermal growth factor receptor HER2-neu ($r = -0.285$, $p < 0.05$), and the active transcription of the coding gene ($r = 0.704$, $p < 0.01$) allow to consider the survivin protein and the gene encoding it — *BIRC5*, as an unfavorable prognostic factor and use it as a predictive marker of the response to chemotherapy, including the target therapy.

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ЕКСПРЕСІЯ АНТИАПОПТОТИЧНОГО ПРОТЕЇНУ СЮРВІВІНУ ТА ЙОГО ГЕНА *BIRC5* У КЛІТИНАХ ПЕРВИННОЇ КАРЦИНОМИ МОЛОЧНОЇ ЗАЛОЗИ В ЯКОСТІ ПРОГНОСТИЧНОГО МАРКЕРА ПРОГРЕСУВАННЯ ЗАХВОРЮВАННЯ

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Резюме. ***Мета:*** *дослідити в якості потенційних прогностичних та предиктивних маркерів експресію антиапоптотичного протеїну сюрвівіну, а також гена BIRC5, який його кодує, у клітинах первинної карциноми молочної залози.* ***Об'єкт і методи:*** *експресію сюрвівіну та гена BIRC5 вивчали в клітинах 67 зразків (біоптатів) первинної карциноми молочної залози за допомогою імуногістохімічних методів та полімеразної ланцюгової реакції в режимі реального часу.* ***Результати:*** *експресія сюрвівіну була виявлена в клітинах 47 біоптатів пухлин (70,2%). Найчастіше — при протоковій карциномі середнього та високого ступеня злоякісності (G2–G3) у поєднанні з лімфовенозною стромальною інвазією. Часто експресію цього білка виявляли при люмінальному А та люмінальному Б молекулярно-біологічних підтипах пухлини. Відмічали кореляцію між експресією сюрвівіну та онкопротеїну HER2-neu. У 59,6% випадків сюрвівін експресувався в пухлинах з низьким показником Ki-67. Експресію гена BIRC5 визначали у всіх досліджених зразках. Спостерігали достовірну помірну позитивну кореляцію між показниками експресії гена BIRC5 та його продукту сюрвівіну ($r = 0,704$, $p < 0,01$) та слабку негативну кореляцію цього показника з експресією онкопротеїну HER2-neu ($r = -0,285$, $p < 0,05$). Медіана спостереження становила 40 міс. Загальна виживаність в групі пацієнтів без експресії сюрвівіну сягала 100%, за умови експресії — 77,16% ($p = 0,041$). Безрецидивна виживаність відповідно становила 100 і 71,9% ($p = 0,037$). Відношення ризиків прогресування у пацієнтів з експресією сюрвівіну — 10,6 (95% довірчий інтервал 0,85–132,2; $p = 0,041$).* ***Висновки:*** *експресію сюрвівіну та його гена BIRC5 у клітинах первинної карциноми молочної залози можна розглядати як незалежний несприятливий прогностичний фактор та використовувати в якості предиктивних маркерів.*

Ключові слова: рак молочної залози, сюрвівін, *BIRC5*, прогноз.

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