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PROGNOSTIC ROLE OF RESIDUAL TUMOR FEATURES IN HER2 NEGATIVE BREAST CANCER

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The aim **of the study was to examine the prognostic value of immunobiological markers (tumor-infiltrating lymphocytes (TILs) and their subpopulations) in residual tumor after neoadjuvant chemotherapy (NACT) completion in patients with triple negative (TNBC) and luminal B HER2-neu negative breast cancer (LBBC).** ***Materials and Methods*****: The analysis of the treatment results of 59 patients with TNBC and 56 patients with LBBC with stage IIB–IIIB who received NACT was performed. The levels of TILs and their subpopulations ($FOXP3^+$, $CD4^+$, $CD8^+$) in patients at the time of diagnosis in core-needle biopsy material and in residual tumor in postoperative material were studied by immunohistochemical method.** ***Results*****: The risk of recurrence in patients with LBBC who received NACT before surgery is associated mainly with 4 factors: $FOXP3^+$ lymphocytes, Ki-67 index in residual tumor, the number of affected axillary lymph nodes after NACT and viable residual tumor volume. Analysis of the treatment outcome in patients with TNBC revealed that the lack of pathologic complete response (pCR) after NACT increases the risk of disease recurrence by 2.9 times, hazard ratio (HR) = 2.9 (95% confidence interval (CI) 1.4–6.1; $p = 0.005$) compared with patients in which pCR was achieved after NACT. It was also found that the presence of residual tumor in patients with TNBC after NACT increases the risk of death from this disease by 2.7 times (95% CI 1.0–7.1; $p = 0.05$). Increased intratumoral and stromal $CD8^+$ lymphocyte counts in the residual tumor after NACT significantly reduces the risk of death from TNBC, HR = 0.6 (95% CI 0.5–0.9; $p = 0.01$) and HR = 0.6 (95% CI 0.4–0.9; $p = 0.008$), respectively. Increase in intratumoral $CD4^+$ lymphocytes in residual tumor in the non-pCR group reduces by half the risk of death from TNBC, HR = 0.5 (95% CI 0.3–1.0; $p = 0.05$).** ***Conclusion*****: The results of our study indicate a favorable prognostic value of TILS in residual tumor in TNBC. It is also reasonable to include the determination of the level of $FOXP3^+$ lymphocytes in the residual tumor in the standard algorithms for stratification of risk groups.**

Key Words: **triple-negative breast cancer, luminal B breast cancer, neoadjuvant chemotherapy, residual tumor, tumor-infiltrating lymphocytes, $CD4^+$, $CD8^+$, $FOXP3^+$ lymphocytes, clinical prognosis.**

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In recent years, breast cancer (BC) remains the most common malignancy in women in Ukraine. BC occupies the first position in the female mortality rates causing 32.5% of all cancer-related deaths in Ukraine [1].

For many years, neoadjuvant chemotherapy (NACT) has been a standard approach for the treatment of prognostically unfavorable BC types. One of the main tasks of NACT is to achieve pathologic complete response (pCR). It is a known fact that pCR can be considered a surrogate marker of survival for prognostically unfavorable BC subtypes. Numerous international multicenter randomized trials have shown a direct correlation between overall survival (OS) and pCR achievement in patients with different molecular BC types who received NACT [2–5]. The strongest relationship between pCR and survival rates was found in patients with aggressive BC subtypes (triple negative, poorly differentiated hormone-dependent tumors and hormone-independent tumors with HER2-neu expression) [2–4].

In recent years, the scientific literature has been actively discussing the prognostic and predictive role of tumor-infiltrating lymphocytes (TILs) in BC [6–10]. Researchers have evaluated the role of TILs as a predictive factor in the effectiveness of neoadjuvant chemotherapy in BC [8–10]. The degree of intratumoral lymphocyte infiltration was estimated as an independent predictor of achieving pCR to NACT ($p = 0.001$) in different BC subtypes [9].

When analyzing the immune infiltrate in primary and residual breast tumors, researchers focus on the actual level of TILs and their subpopulations: $CD4^+$, $CD8^+$ and $FOXP3^+$ lymphocytes [11–13]. Studies on the immunogenicity of BC, the effect of cytostatic therapy on the immune microenvironment of the tumor and the prognostic factors of the disease are relevant for the selection of patients for personalized treatment (immunotherapy) and the possible de-escalation of adjuvant treatment.

The aim of the study was to examine the prognostic value of immunobiological markers (TILs and their subpopulations) in residual tumor after NACT in patients with triple negative (TNBC) and luminal B HER2-neu negative BC (LBBC).

MATERIALS AND METHODS

The prognostic role of immune biomarkers was assessed in 115 patients with BC of stages IIB-IIIB (TNBC, n = 59; LBBC, n = 56). NACT in all patients was performed using anthracycline-containing regimens.

All patients signed an informed consent on participation in the study. This study was approved by the Ethics Committee of the National Cancer Institute of the Ministry of Health of Ukraine.

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Abbreviations used: BC – breast cancer; CI – confidence interval; HR – hazard ratio; IHC – immunohistochemical; LBBC – luminal B HER2-neu negative BC; NACT – neoadjuvant polychemotherapy; OS – overall survival; pCR – pathologic complete response; RFS – relapse-free survival; TILs – tumor-infiltrating lymphocytes; TNBC – triple negative BC; VRTV – viable residual tumor volume.

Morphological and immunohistochemical (IHC) studies were performed in the Department of Pathology of the National Cancer Institute. TILs were assessed according to the recommendations proposed by the International Working Group on TILs evaluation in BC [14]. Stromal TILs and FOXP3$^+$, stromal and intratumoral CD4$^+$, CD8$^+$ were evaluated. Lymphocytic infiltration of tumor tissue was assessed by evaluation of hematoxylin and eosin-stained tumor sections in 10 fields of view, and reported as percentage: no infiltration (0), mild infiltration (+) < 10%, intermediate (++) — 10–30% and high (+++) — > 30%. IHC study was performed on deparaffinized sections prepared from paraffin blocks of surgically removed tumors using antibodies: monoclonal mouse anti-human CD4, clone MT310, monoclonal mouse anti-human CD8 clone C8/144B, monoclonal mouse anti-human Ki-67, clone MIB-1 (Dako, Denmark), anti-FOXP3 clone 5H5L12 (Invitrogen, USA). For detection of IHC reaction, EnVision™ FLEX imaging system (Dako, Denmark) was used. The results of the IHC reaction were assessed by a semi-quantitative method, by counting the number of positively stained cells, i.e. by labeling index (expressed in percent).

pCR was determined by the absence of infiltrative cancer cells in breast tissue and lymph nodes and the size of the affected lymph nodes [15]. Evaluation of therapeutic pathomorphism was performed on the basis of determining the viable residual tumor volume (VRTV), according to the Miller — Payne classification of the presence of cancer *in situ*, as well as the number and size of affected lymph nodes [15].

The statistical analysis of the primary data was performed using the methods of descriptive statistics and univariate and multivariate Cox proportional hazards model, the significance of data differences was assessed by Fisher coefficient.

RESULTS

To assess the impact of residual tumor characteristics on overall survival (OS) and relapse-free survival (RFS), an analysis of the treatment outcomes of 56 patients treated with the radical program for locally advanced LBBC of stage IIB–IIIB was performed.

The study of postoperative material revealed no association between the therapeutic pathomorphism degree (TPMD), Ki-67 index and the TIL value in the residual tumor with the risk of death from LBBC. In addition, univariate Cox proportional-hazards model did not reveal an independent prognostic value of individual subpopulations of lymphoid cells (CD4$^+$, CD8$^+$ and FOXP3) in the residual tumor after NACT in patients with locally advanced LBBC.

Table 1 shows the results of the assessment of the impact of each of the risk factors on RFS of the patients. Therefore, we found no association between the TMPD and the TILs count in the residual tumor with the risk of metastasis in LBBS cases. No significant increase in the risk of metastasis ($p = 0.09$) in patients with LBBS with high levels of Ki-67 in the residual tumor after NACT has been observed as well.

It should be noted that the analysis of RFS of patients with LBBC using univariate Cox proportional hazards model did not reveal an independent prognostic value of CD4$^+$, CD8$^+$ lymphocytes in residual tumor. In contrary, there was found an increase in the risk of metastasis ($p = 0.03$) along with an increase in FOXP3$^+$ lymphocyte count in the residual tumor, hazard ratio (HR) = 1.18 (95% confidence interval (CI), 1.01–1.39). Thus, the independent prognostic value of FOXP3$^+$ lymphocytes in residual tumor after completion of NACT in patients with locally advanced BC was shown.

A multivariate Cox proportional hazards model was used to identify the minimum set of factors associated with RFS. In total, 19 indices were used: age of patients, menstrual function, stage of the disease, size of primary tumor (pT category according to TNM classification), presence or absence of metastases in axillary lymph nodes (pN category according to TNM classification), tumor differentiation grade, expression of estrogen receptors, and progesterone receptors, type of chemotherapy, VRTV, counts of TILs, stromal and intratumoral CD4$^+$, CD8$^+$ and stromal FOXP3 lymphocytes, Ki-67 labeling index in residual tumor. The stepwise selection method was used to select significant features (stepwise, inclusion threshold $p < 0.1$ and exclusion threshold $p > 0.3$).

During the selection process, 4 valuable variables were identified: the level of FOXP3$^+$ lymphocytes, the Ki-67 index in the residual tumor, VRTV, and the number of affected lymph nodes. The model for predicting RFS based on these variables was adequate ($c^2 = 20.0$ at 6 degrees of freedom, $p = 0.003$). Table 2 shows the results of the analysis of the independent influence of the variables on the RFS of patients under standardization for other risk factors included in the model.

The analysis showed that the risk of recurrence in patients with LBBC who received NACT before surgery is associated mainly with four factors: the level of FOXP3$^+$, Ki-67 index in residual tumor, the number of affected axillary lymph nodes after NACT, and VRTV. We found that in patients with locally advanced BC who received NACT the increased number of affected axillary lymph nodes from 10 and more (category pN3) increases ($p = 0.001$) the risk of BC recurrence HR = 8.9 (95% CI, 4.2–13.8), compared with patients who did not have metastases in regional lymph nodes (category pN0), with standardization for other risk factors. Also, it was found that in patients with high VRTV values after NACT, the probability of recurrence increases per each percent ($p = 0.02$), HR = 1.03 (95% CI, 1.00–1.06), with standardization for other risk factors. There was also an increasing risk of disease recurrence ($p = 0.01$) with increasing FOXP3 lymphocytes characteristic for the immunosuppressive microenvironment of the tumor after NACT, HR =

1.21 (95% CI, 1.08–1.46) per each percent, with standardization for other risk factors.

Given the different prognostic value of both the presence of residual tumor after NACT and immune infiltrate in different molecular BC types, the results of treatment of patients with TNBC were analyzed. The prognostic effect of pCR was initially assessed in 59 patients with TNBC who received NACT. Analysis of the results of treatment of these patients has shown that the lack of pCR after NACT increases the risk of disease recurrence by an average of 2.9 times, HR 2.9 (95% CI 1.4–6.1; $p = 0.005$) compared with patients in which pCR was achieved after NACT.

In addition, it was found that the presence of residual tumor in patients with TNBC after NACT increases the risk of death from this disease by 2.7 times (95% CI 1.0–7.1; $p = 0.05$).

Table 3 shows the data of univariate regression analysis of RFS depending on the immunobiological markers of residual tumor after NACT.

As shown in Table 3, the increase in intratumoral $CD4^+$ lymphocytes in the residual tumor after NACT reduces the risk of disease recurrence on average almost by 1.5 times, HR = 0.7 (95% CI, 0.5–1.0; $p = 0.048$) per each gradation.

Table 4 presents the data on analysis of OS depending on the immunobiological markers in residual tumor after NACT.

As shown in Table 4, an increase in intratumoral $CD4^+$ and $CD8^+$ lymphocytes in the residual tumor after NACT reduces the risk of death from TNBC, HR = 0.6 (95% CI, 0.3–0.9; $p = 0.03$) and HR = 0.7 (95% CI, 0.5–1.0; $p = 0.03$), respectively.

Given the important prognostic value of tumor response to treatment, we separately analyzed the prognostic value of immunobiological indices of BC after NACT in patients who achieved complete therapeutic pathomorphism and who did not have a pCR.

It was determined that in patients with pCR after NACT no independent prognostic effect of immunobiological parameters was found. In contrast, in patients with TNBC who did not achieve pCR (non-pCR group), an independent prognostic value of immunobiological markers was found (Table 5).

As can be seen from Table 5, an increase of intratumoral and stromal $CD8^+$ lymphocytes in the residual tumor after NACT significantly reduces the risk of death from TNBC, HR = 0.6 (95% CI, 0.5–0.9; $p = 0.01$) and HR 0.6 (95% CI, 0.4–0.9; $p = 0.008$), respectively. Increase in intratumoral $CD4^+$ lymphocytes in residual

Table 1. The relationship of the variables with the risk of disease recurrence in residual tumor of patients with LBBC after NACT (univariate Cox model)

Index	Model coefficient, b ± m	*p*	HR (95% CI)
VRTV	0.0020 ± 0.0075	0.77	1.01 (0.99–1.03)
TILs	0.08 ± 0.25	0.67	1.09 (0.75–1.96)
Stromal $CD4^+$	0.0020 ± 0.0080	0.80	1.00 (0.99–1.02)
Intratumoral $CD4^+$	–0.085 ± 0.080	0.23	0.92 (0.80–1.03)
Stromal $CD8^+$	0.0092 ± 0.0077	0.24	1.01 (0.99–1.02)
Intratumoral $CD8^+$	–0.003 ± 0.011	0.84	1.01 (0.99–1.02)
$FOXP3^+$	0.17 ± 0.08	0.03	1.18 (1.01–1.39)
Ki-67	0.0097 ± 0.0057	0.09	1.01 (1.00–1.02)

Table 2. Prediction of recurrence-free survival of patients with LBBC (four-variate Cox model)

Index		Model coefficient, b ± m	*p*	HR (95% CI)
N	1 *vs* 0	0.39 ± 0.85	0.80	1.19 (0.40–5.50)
	2 *vs* 0	0.73 ± 0.80	0.41	2.12 (0.41–9.98)
	3 *vs* 0	3.70 ± 1.10	0.001	8.90 (4.20–13.80)
VRTV		0.024 ± 0,015	0.02	1.04 (1.02–1.06)
$FOXP3^+$		0.19 ± 0,07	0.01	1.21 (1.08–1.46)
Ki-67		0.035 ± 0,011	0.007	1.03 (1.01–1.05)

Table 3. Relationship of the variables with the risk of disease recurrence in the patients with TNBC in residual tumor after NACT (univariate Cox models)

Index	Model coefficient, b ± m	*p*	HR (95% CI)
Stromal $CD4^+$	–0.19 ± 0.16	0.28	0.9 (0.6–1.1)
Intratumoral $CD4^+$	–0.39 ± 0.21	0.048	0.7 (0.5–1.0)
Stromal $CD8^+$	–0.24 ± 0.16	0.15	0.85 (0.6–1.1)
Intratumoral $CD8^+$	–0.14 ± 0.15	0.39	0.9 (0.7–1.2)
FOXP3+	–0.09 ± 0.16	0.91	0.9 (0.7–1.3)
Ki-67	0.0079 ± 0.0077	0.30	1.01 (0.99–1.02)

Table 4. Relationship of the variables with overall survival of the patients with TNBC in residual tumor after NACT (univariate Cox models)

Index	Model coefficient, b ± m	*p*	HR (95% CI)
Stromal $CD4^+$	–0.31 ± 0.20	0.16	0.8 (0.5–1.1)
Intratumoral $CD4^+$	–0.58 ± 0.27	0.03	0.6 (0.3–0.9)
Stromal $CD8^+$	–0.31 ± 0.19	0.09	0.7 (0.5–1.1)
Intratumoral $CD8^+$	–0.38 ± 0.18	0.03	0.7 (0.5–1.0)
$FOXP3^+$	–0.25 ± 0.21	0.28	0.8 (0.5–1.2)
Ki-67	0.0016 ± 0.0009	0.23	1.01 (0.99–1.03)

Table 5. Relationship of the variables with overall survival of the patients with TNBC from non-pCR group (univariate Cox models)

Index	Model coefficient, b ± m	*p*	HR (95% CI)
Intratumoral $CD4^+$	–0.64 ± 0.33	0.09	0.6 (0.3–1.0)
Stromal $CD8^+$	–0.44 ± 0.18	0.01	0.6 (0.5–0.9)
Intratumoral $CD8^+$	–0.51 ± 0.19	0.008	0.6 (0.4–0.9)
Ki-67	0.0013 ± 0.0009	0.46	1.01 (0.99–1.03)
$FOXP3^+$	0.01 ± 0.16	0.74	1.03 (0.75–1.37)

tumors in the non-pCR group reduces by half the risk of death from TNBC, HR = 0.6 (95% CI, 0.3–1.0; p = 0.05).

DISCUSSION

Currently, there is no unambiguous opinion about the prognostic value of T-regulatory cells; however, there are data on their negative impact on the survival rates of patients with some cancer types, including BC [16]. An interesting fact is that cytotoxic agents can reduce the degree of infiltration of the tumor with FOXP3$^+$ regulatory cells, which correlates with a favorable prognosis [17].

We proved the independent prognostic value of FOXP3$^+$ lymphocytes in the residual tumor of patients with LBBC. Our results coincide with the data of various research groups. In particular, in a meta-analysis performed by Jiang *et al.* [18] in 8277 patients with BC, FOXP3$^+$ infiltration was shown to be significantly associated with low tumor differentiation grade (G3), (HR = 3.067); metastases to regional lymph nodes (HR = 1.305); as well as worse RFS rates (HR = 1.752), and OS (HR = 1.447). Some other studies have also found that the degree of FOXP3$^+$ infiltration is positively correlated with increased tumor malignancy and lack of estrogen receptor expression, and is associated with an unfavorable prognosis of BC patients [19, 20]. However, the latter statement is contradictory and is not confirmed by some authors [7, 21, 22]. In particular, there is evidence that in the estrogen-negative BC group, FOXP3$^+$ levels correlate with a positive prognosis as a marker of an active immune response [13, 21]. In addition, in a study of the prognostic value of FOXP3$^+$ lymphocytes in different molecular BC types, a team of Canadian researchers headed by Shuzen Liu proved that high levels of FOXP3$^+$ lymphocytes along with low CD8$^+$ levels in hormone-dependent BC [23] were associated with poor survival rates.

Using multivariate analysis, we revealed that in LBBC patients who received NACT a risk of metastasis was related to FOXP3 levels, Ki-67 index in residual tumor, and VRTV. However, other research groups did not confirm the prognostic value of FOXP3 regulatory lymphocytes in patients with different molecular BC types, so further study on this issue is required [24].

In our study, it was found that achieving a pCR of the tumor to NACT significantly increases the recurrence-free and OS in patients with TNBC, which is in agreement with the data of other researchers [2–5]. Also, we found that the presence of residual tumor in patients with TNBC after NACT significantly increases the risk of death from this disease. In patients with TNBC, who did not achieve pCR after NACT, an independent prognostic value of immunobiological tumor markers was found. The obtained data are in general consistent with the results of a retrospective study of Myashita *et al.* [25] who showed that the increase of CD8$^+$ counts in residual tumor correlated with an increased OS.

Although immunobiological markers have shown prognostic value in many cancer types, including BC, even such an important prognostic factor as TILs is currently not included in standard algorithms for risk group stratification of BC patients and is not used in routine clinical practice. The composition of the immune infiltrate in TNBC accurately reflects the response of the immune system against malignant tumors, which is why it is an important prognostic parameter. Moreover, immune biomarkers are becoming important factors in selecting patients who may benefit from immunotherapy. It is also reasonable to include the determination of the level of FOXP3$^+$ lymphocytes in the residual tumor in the standard algorithms for stratification of risk groups, which include determination of the number of affected lymph nodes, Ki-67 and VRTV after NACT.

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ПРОГНОСТИЧНА РОЛЬ ХАРАКТЕРИСТИК РЕЗИДУАЛЬНОЇ ПУХЛИНИ У ПАЦІЄНТОК З HER2-НЕГАТИВНИМ РАКОМ ГРУДНОЇ ЗАЛОЗИ

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Метою дослідження було вивчення прогностичного значення імунобіологічних маркерів (пухлино-інфільтрувальних лімфоцитів (ПІЛ) та їх субпопуляцій) у резидуальній пухлині після завершення неоад'ювантної поліхіміотерапії (НПХТ) у пацієнток з тричі негативним (ТНРГЗ) та люмінальним В HER2-neu-негативним раком грудної залози (ЛВРГЗ). ***Матеріали і методи*:** Проведено аналіз результатів лікування 59 хворих на ТНРГЗ та 56 пацієнток з ЛВРГЗ IIB–IIIB стадій, які отримували НПХТ. Імуногістохімічним методом досліджено рівні ПІЛ та їх субпопуляцій ($FOXP3^+$, $CD4^+$, $CD8^+$) у хворих на момент встановлення діагнозу в матеріалі трепан-біопсії та у резидуальній пухлині в післяопераційному матеріалі. ***Результати*:** Визначено, що ризик рецидивів у хворих на ЛВРГЗ, які отримували НПХТ до операції, пов'язаний переважно з 4 факторами: рівнем $FOXP3^+$, індексом Ki-67 у резидуальній пухлині, кількістю уражених аксилярних лімфатичних вузлів після НПХТ та об'ємом життєздатної залишкової пухлини. У ході аналізу результатів лікування пацієнток з ТНРГЗ виявлено, що відсутність повної патологічної відповіді (ППВ) після НПХТ підвищує ризик рецидиву хвороби в середньому в 2,9 раза, відношення ризиків (ВР) = 2,9 (95% довірчий інтервал (ДІ) 1,4–6,1; p = 0,005) порівняно з хворими, у яких було досягнуто ППВ після НПХТ. Також було виявлено, що наявність резидуальної пухлини у хворих на ТНРГЗ після НПХТ підвищує ризик смерті від цього захворювання у 2,7 раза (95% ДІ 1,0–7,1; p = 0,05). Підвищення рівня інтратуморальних та стромальних $CD8^+$-лімфоцитів у резидуальній пухлині після НПХТ достовірно знижує ризик смерті внаслідок ТНРГЗ, ВР = 0,6 (95% ДІ 0,5–0,9; p = 0,01) та ВР = 0,6 (95% ДІ 0,4–0,9; p = 0,008) відповідно. Підвищення інтратуморальних $CD4^+$-лімфоцитів у резидуальній пухлині у групі відсутності ППВ знижує ризик смерті від ТНРГЗ у 2 рази, ВР = 0,5 (95% ДІ 0,3–1,0; p = 0,05).

***Ключові слова*:** тричі негативний рак грудної залози, рак грудної залози люмінального В підтипу, неоад'ювантна хіміотерапія, резидуальна пухлина, пухлино-інфільтруючі лімфоцити, $CD4^+$, $CD8^+$, $FOXP3^+$ лімфоцити, клінічний прогноз.