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ORIGINAL RESEARCH ARTICLE

Assessment of PD-L 1 expression in a tumor improves the accuracy of predicting the risk of regional breast cancer metastases

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ABSTRACT

Background: Improving the accuracy of axillary lymph node (ALN) status assessment and the search for new markers associated with the risk of breast cancer (BC) metastasis continues to be an urgent problem. **Aim:** To establish the prognostic significance of the expression of PD-L1 in a tumor for assessing the risk of BC metastasis in ALNs. **Materials and methods:** A retrospective, case–control cohort study included 158 patients aged 30 to 85 years with newly diagnosed BC. The material for the study was tumor samples obtained by trephine biopsy. The expression of PD-L1 in the tumor was studied on the invasive component of puncture biopsy specimens by immunohistochemistry using PD-L1 polyclonal antibodies. Statistical analysis was performed using Statistica 12.0 software. Receiver operating characteristic (ROC) curves for PD-L1 and Ki67 were constructed to discriminate cases with and without metastases in the ALNs. Univariate and multivariate analyses were performed to establish independent predictors associated with the risk of BC metastasis. A value of $p < 0.05$ was considered statistically significant. **Results:** According to the results, independent predictors of a high risk of BC regional metastasis were T2 (OR = 5.81, 95% CI = 1.75–19.35, p = 0.004) and T3-4 (OR = 43.07, 95% CI = 9.31–199.2, $p < 0.0001$) stages of BC, absence of an intraductal component (OR = 3.68, 95% CI = 1.32–10.33, $p = 0.013$), presence of lymphovascular invasion (OR = 3.32, 95% CI = 1.34–8.22, $p = 0.009$), luminal B HER2-positive (OR = 6.82, 95% CI = 1.13–42.26, $p = 0.036$) and triple negative (OR = 8.52, 95% CI = 1.12–64.89, $p = 0.038$) molecular biological subtypes of BC, and PD-L1 expression coefficient greater than 1.65 (OR = 6.39, 95% CI = 2.54–16.09, $p = 0.0001$). Based on the data obtained, an original noninvasive method for assessing a high risk of BC regional metastasis was developed, the sensitivity of which was 80.9%, the specificity - 82.6%, and the accuracy - 85.7%. The area under the curve (AUC) was 0.876 (95% CI = 0.818–0.924, $p < 0.0001$). **Conclusion:** The results of the study indicate that the assessment of PD-L1 expression in a tumor can improve the accuracy of predicting the risk of regional BC metastasis.

Keywords: breast cancer; axillary lymph nodes; risk of BC regional metastasis; PD-L1

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1. Introduction

Breast cancer (BC) ranks first in the world in terms of oncological morbidity and mortality among the female population[1]. Early diagnosis of the disease, accurate assessment of the BC prognosis and the risk of disease recurrence, as well as of the sensitivity of the tumor to the treatment provided, contribute to effective treatment and reduce social and economic losses from BC.

Currently, assessment of the status of axillary lymph nodes (ALNs) is the most valuable factor in the prognosis of the disease and the risk of BC recurrence[2,3]. In patients with early BC, sentinel lymph node (SLN) biopsy is the standard method for diagnosing metastases in the ALNs. However, this method has a number of problems

associated with a wide variety of techniques for performing the procedure and methods for detecting tumor cells in SLNs. For example, the frequency of false-negative results in SLN biopsy can reach up to 18.4% with some techniques[4]. At the same time, 30% to 70% of patients with metastases in the SLNs, according to the results of axillary lymph node dissection (ALND), did not have metastases in the ALNs[5]. Thus, improving the accuracy of ALN status assessment and the search for new markers associated with the risk of BC metastasis continues to be an urgent problem. We believe that one of these markers may be the expression of programmed cell death ligand-1 (PD-L1) in tumor cells, since this marker is known to be closely associated with tissue hypoxia, epithelial-mesenchymal transition (EMT), and BC metastasis[6–9].

2. Materials and methods

2.1. Patients

A retrospective, case–control cohort study included 173 patients aged 30 to 85 years with newly diagnosed BC. All patients signed informed consent to participate in the clinical study. Ethical approval was received from the Ethics Committee of the Orenburg State Medical University (protocol No. 311 dated 13 January 2023). To establish the diagnosis, all patients underwent a standard clinical and instrumental examination, which included bilateral mammography, ultrasound examination of the mammary glands and ALNs, core biopsy of the tumor with morphological and immunohistochemical examination of the obtained material, radiography of chest organs, ultrasound examination of the liver and pelvic organs, and osteoscintigraphy. Some patients additionally underwent computed tomography of the abdominal and thoracic organs to exclude metastatic lesions of the liver and lungs (n = 104). The BC stage was determined according to the TNM classification of malignant tumors[10].

The exclusion criteria were severe comorbidities, severe allergic processes or autoimmune diseases, taking glucocorticoids or nonsteroidal anti-inflammatory drugs, and the presence of a second tumor of another localization. The clinical and pathological data of the patients included in the study are presented in **Table 1**.

Table 1. Characteristics of breast cancer patients.

Clinical and morphological BC characteristics	Number of patients (n)	(%)
Histology		
Ductal (nonspecific)	120	69.4%
Lobular	45	20.6%
Others (mucinous, mixed, intraductal)	8	4.6%
Grade (G)		
G1	12	6.9%
G2	93	53.8%
G3	68	39.3%
Estrogen receptor (ER) status		
Negative (0%)	33	19.1%
Positive (⩾1%)	140	80.9%
Progesterone receptor (PR) status		
Negative (0%)	58	33.6%
Positive (⩾1%)	115	66.5%
Ki67		
Low (<20%)	33	19.1%
High (>20%)	140	80.9%

Table 1. (*Continued*).

Clinical and morphological BC characteristics	Number of patients (n)	(%)
HER2-status		
Negative	134	77.5%
Positive	39	22.5%
Molecular biological subtype		
Luminal A	26	15.0%
Luminal B HER2-negative	85	49.2%
Luminal B HER2 positive	28	16.2%
HER2 positive	12	6.9%
Triple negative	22	12.7%
T stage		
T1	39	22.5%
T2	88	50.9%
T3	5	2.9%
T4	41	23.7%
N stage		
N0	83	48.0%
N1	35	20.2%
N2	35	20.2%
N3	20	11.6%
Stage		
Ia	31	17.9%
Ib	3	1.7%
IIa	43	24.9%
IIb	22	12.7%
IIIa	24	13.9%
IIIb	22	12.7%
IIIc	15	8.7%
IV	13	7.5%

2.2. Immunohistochemical study

The material for the study was tumor samples obtained by trephine biopsy. The expression of PD-L1 in the tumor was studied on the invasive component of puncture biopsy specimens by immunohistochemistry using PD-L1 polyclonal antibodies (dilution 30 μg/mL, Cloud-Clone Corp., China), as described previously[11]. Histological specimens were examined by light microscopy (a Levenhuk D740T digital microscope connected to a 5.1 MP camera, Russia). All samples were examined by two investigators (EZ and MS) without knowledge of the clinical and pathological data of the patients. PD-L1 expression was evaluated in 5 fields of view at a magnification of 400×. The intensity of staining was scored as follows: no specific staining, 1 point; weak staining, 2 points; pronounced staining, 3 points (**Figure 1**). The PD-L1 expression coefficient was calculated using the following formula: the proportion of cells without expression × 1 + the proportion of cells with weak expression × 2 + the proportion of cells with pronounced expression × 3/5[12].

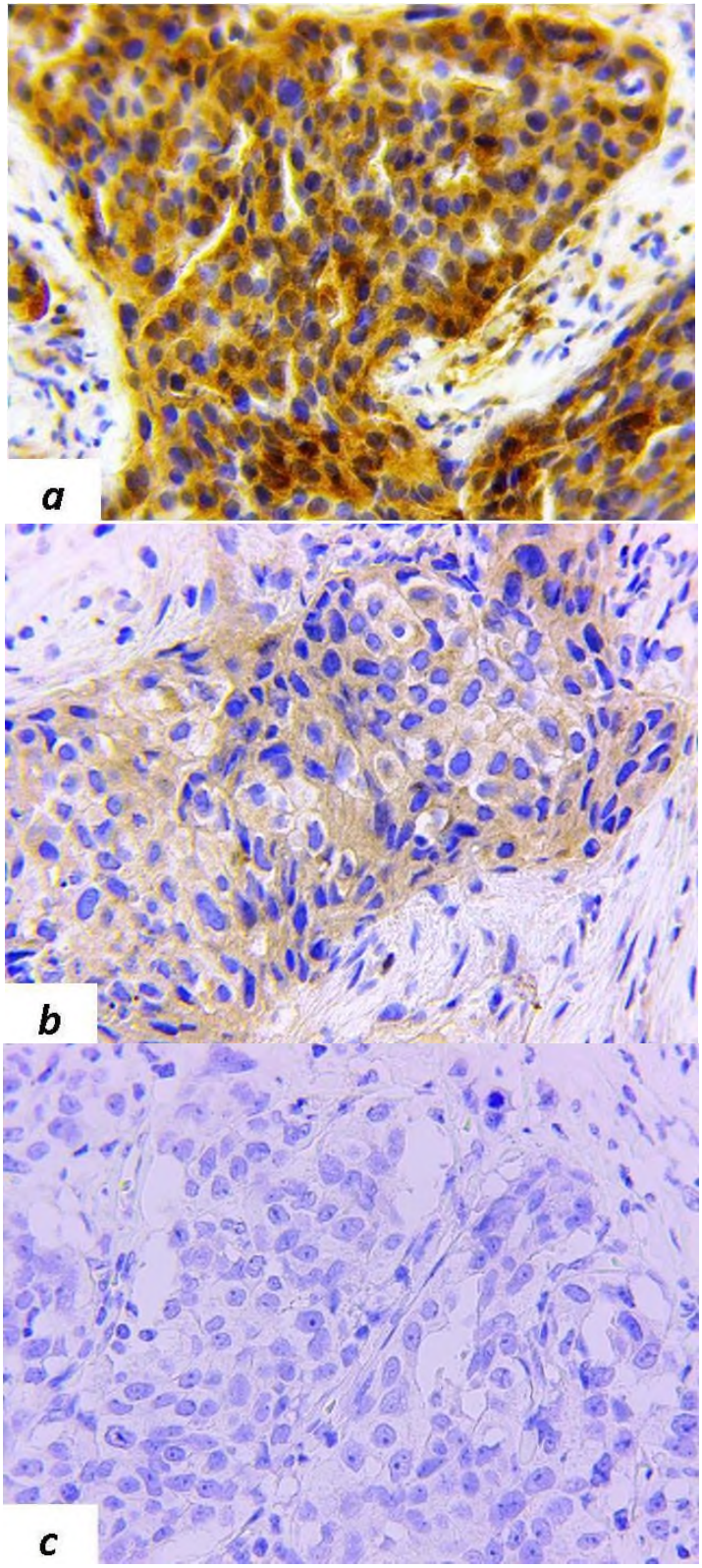

Figure 1. Severity of PD-L1 expression in tumor tissue: **(a)** pronounced expression; **(b)** weak expression; **(c)** lack of expression. IHC staining with antibodies against PD-L1, 400×.

2.3. Statistical analysis

Statistical analysis was performed using Statistica 12.0 software. To discriminate cases with the absence and presence of metastases in the ALNs, receiver operating characteristic (ROC) curves for PD-L1 and Ki67 were constructed. The best threshold values (cutoffs) were determined by the highest Youden index (J =

sensitivity + specificity – 1). The effectiveness of the predictive models was evaluated by the area under the curve (AUC). Univariate and multivariate analyses were performed to establish independent predictors associated with the risk of BC metastasis in the ALNs. A value of $p < 0.05$ was considered statistically significant.

3. Results

To determine the optimal cutoff points for the Ki67 index and the PD-L1 expression coefficient, which discriminate between cases with the absence (N0) and presence (N1-3) of BC metastases in the ALNs, an ROC analysis was performed (**Figure 2**).

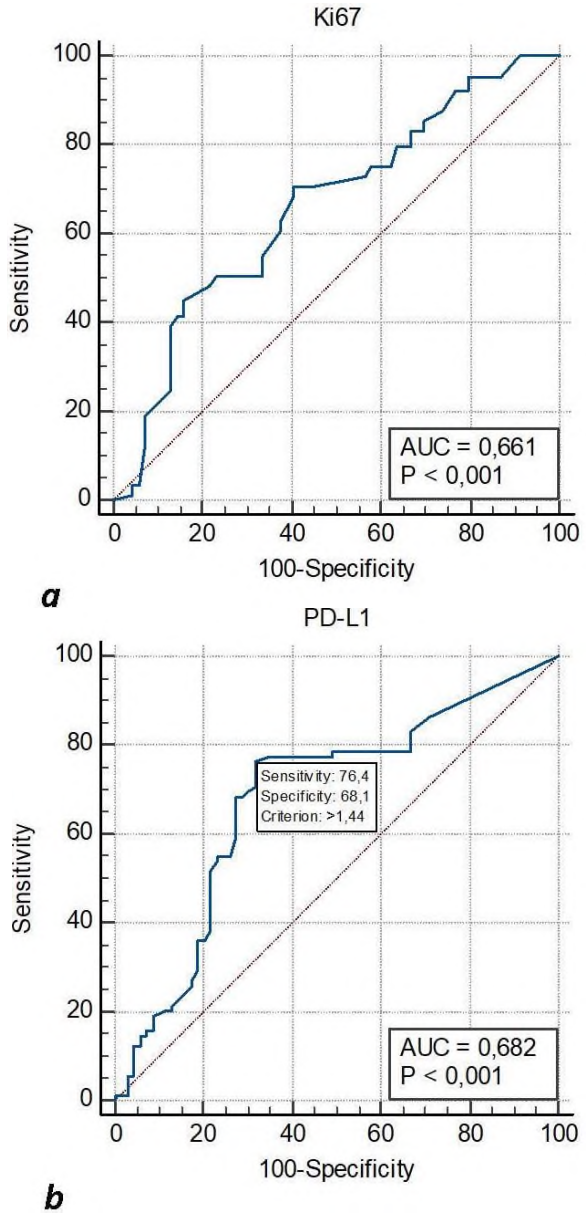

Figure 2. ROC curves for PD-L1 and Ki67: **(a)** ROC curve for Ki67 (AUC = 0.661; 95% CI = 0.582-0.735; $p < 0.001$); **(b)** ROC curve for PD-L1 (AUC = 0.682; 95% CI = 0.599-0.765, $p < 0.001$).

It was established that the optimal threshold values that distinguish between cases without metastases and with metastases of BC in the ALNs were 1.65 for PD-L1 and 38% for the Ki67 index. The areas under the ROC curves (AUCs) were 0.682 (95% CI = 0.599-0.765) for PD-L1 and 0.661 (95% CI = 0.582-0.735) for

Ki67. Thus, the Ki67 index and PD-L1 expression in tumor tissue were associated with the risk of regional BC metastasis.

To establish independent factors associated with the risk of regional BC metastasis, univariate and multivariate analyses were performed. The data are presented in **Table 2**.

Table 2. Univariate and multivariate analyses.

Clinical and morphological BC characteristics	**Univariate analysis OR (95% CI)**	***p***	**Multivariate analysis OR (95% CI)**	***p***
Clinical form		**<0.0001***		>0.05
Nodal	1		1	
Diffuse	8.30 (3.09-22.66)		1.30 (0.47-3.12)	
T stage		**<0.0001***	-	-
T1	1		1	
T2	6.84 (2.40-19.53)		5.81 (1.75-19.35)	0.004*
T3-4	44.80 (11.84-169.5)		43.07 (9.31-199.2)	<0.0001*
Histology		**>0.05**	-	-
Ductal (nonspecific)	1			
Lobular	1.17 (0.56-2.43)			
Others	0.00 (0.00)			
Grade		**0.013***		>0.05
G1-G2	1		1	
G3	2.35 (1.20-4.61)		1.76 (0.81-3.85)	
Intraductal component		**0.042***		**0.013***
Present	1		1	
Absent	2.10 (1.03-4.31)		3.68 (1.32-10.33)	
Lymphovascular invasion		**0.015***		**0.009***
Absent	1		1	
Present	2.24 (1.17-4.30)		3.32 (1.34-8.22)	
Perineural invasion		**>0.05**	-	-
Absent	1			
Present	1.26 (0.62-2.58)			
Estrogen receptor (ER) status		**0.08**	-	-
Negative (0%)	1			
Positive (⩾1%)	0.45 (0.19-1.10)			
Progesterone receptor (PR) status		**0.002***		>0.05
Negative (0%)	1		1	
Positive (⩾1%)	0.31 (0.15-0.65)		0.62 (0.26-1.48)	
Ki67		**0.0019***		>0.05
⩽38%	1		1	
>38%	2.81 (1.47-5.37)		1.54 (0.60-3.95)	
HER2-status		**0.032***		>0.05
Negative	1		1	
Positive	2.43 (1.08-5.48)		1.32 (0.49-3.57)	

Table 2. (*Continued*).

Clinical and morphological BC characteristics	Univariate analysis OR (95% CI)	*p*	Multivariate analysis OR (95% CI)	*p*
Molecular biological subtype		**0.0079***		
Luminal A	1		1	
Luminal B HER2-negative	2.47 (0.91-6.64)		3.01 (0.65-13.86)	>0.05
Luminal B HER2 positive	6.53 (1.90-22.50)		6.82 (1.13-42.26)	0.036*
HER2 positive	5.33 (1.06-26.90)		3.21 (0.42-24.70)	>0.05
Triple negative	6.40 (1.65-24.77)		8.52 (1.12-64.89)	0.038*
PD-L1 expression coefficient		**<0.0001***	-	0.0001*
⩽1.65	1		1	
>1.65	5.25 (2.65-10.40)		6.39 (2.54-16.09)	

According to the results of a univariate analysis, the predictors of a risk of regional metastasis were a diffuse BC form, T-stage, grade 3, the absence of an intraductal component, the presence of lymphovascular invasion (LVI), a negative status of progesterone receptor (PR), a Ki67 index of more than 38%, HER2-positive and triple negative BC molecular biological subtypes, and a PD-L1 expression coefficient greater than 1.65. In turn, independent predictors of a high risk of BC regional metastasis according to multivariate analysis were T2 (OR = 5.81, 95% CI = 1.75-19.35, $p = 0.004$) and T3-4 (OR = 43.07, 95% CI = 9.31-199.2, $p < 0.0001$) stages, absence of an intraductal component (OR = 3.68, 95% CI = 1.32-10.33, $p = 0.013$), presence of LVI (OR = 3.32, 95% CI = 1.34-8.22, $p = 0.009$), luminal B HER2-positive (OR = 6.82, 95% CI = 1.13-42.26, $p = 0.036$) and triple negative (OR = 8.52, 95% CI = 1.12-64.89, $p = 0.038$) molecular biological subtypes of BC, and PD-L1 expression coefficient more than 1.65 (OR = 6.39, 95% CI = 2.54-16.09, $p = 0.0001$).

Based on the results obtained, a method has been developed for predicting the high risk of BC metastasis in ALNs. The estimated probability of ALN metastasis was calculated by summing the OR of each independent predictor. For example, for a patient with stage T2 (OR = 5.81), without an intraductal component (OR = 3.68) and LVI (OR = 1), with a triple negative BC molecular biological subtype (OR = 8.52) and a PD-L1 expression coefficient of more than 1.65 (OR = 6.39), the sum OR value was 25.4 (5.81 + 3.68 + 1 + 8.52 + 6.39). Based on these results, an ROC curve was constructed to distinguish between cases with and without BC metastases in the ALNs (**Figure 3**). The AUC was 0.876 (95% CI = 0.818-0.924, $p < 0.0001$). When the sum of ORs was ⩾ 15.63(cutoff), metastases in the ALNs were detected in 72 (85.71%) of 84 cases, while at values less than 15.63, metastases were detected in 17 (22.97%) of 74 cases. The sensitivity of the method was 80.9%, the specificity was 82.6%, and the accuracy was 85.7%.

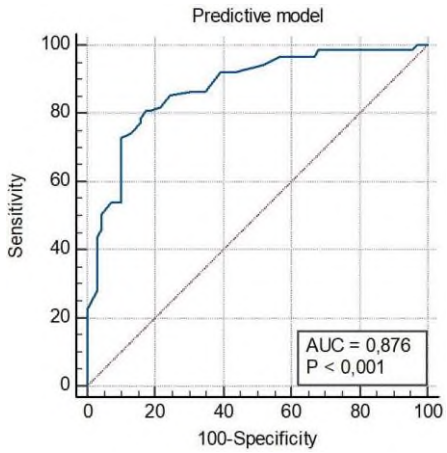

Figure 3. ROC curve predictive model (AUC = 0.876; 95% CI = 0.818-0.924, $p < 0.0001$).

4. Discussion

BC remains the most common type of cancer in women and one of the leading causes of cancer death worldwide[13]. Currently, the standard approach for BC staging is SLN biopsy. This procedure determines the need for ALND and subsequent BC treatment. Thus, in patients with early BC and negative SLNs, it is possible not to perform ALND without worsening the long-term results of treatment[14]. However, this invasive procedure has a number of disadvantages associated with a high frequency of false negative results, the lack of unified methods for detecting tumor cells in the SLN, and uncertain diagnostic value for neoadjuvant strategies[15]. Histological examination of SLNs can be performed intraoperatively on frozen sections or on permanent sections. Intraoperative pathologic assessment of SLNs requires skilled pathologists and equipment, which increases the surgical time and costs. Moreover, following the results of the Z0011 study, intraoperative frozen section analysis of SLNs in patients undergoing conservative breast surgery is considered inappropriate[16,17]. This is due to both the low sensitivity of the method (from 66.7% to 95.8%) and a wide range of false negative results (from 5.5% to 43%)[17,18]. For the study of permanent sections, its main disadvantage is the need to perform a repeat operation in patients with ALN metastases.

Another issue that continues to be discussed is the need to perform ALND in the presence of metastases in the SLN. This is because when 1-2 SLNs are affected, more than 70% of patients do not have metastases to non-SLNs[19]. Although various methods for assessing the status of non-SLNs have now been developed[15,20,21], this issue is still far from being resolved[22].

One more problem associated with assessing SLN status is that when planning neo-A-CT, this assessment is based on clinical and instrumental diagnostic methods, as well as the results of a cytological examination of lymph node fine-needle biopsy. Meanwhile, the accuracy of such an assessment is not high enough and is determined by the doctor's experience, the availability of modern equipment and other factors[23].

Thus, there remains an urgent need to search for new noninvasive technologies, preferably preoperative determination of ALN status and new markers associated with a high risk of BC metastasis.

Currently, various nomograms have been developed to predict the risk of regional BC metastasis using the clinical, morphological and molecular biological characteristics of BC[24], preoperative magnetic resonance imaging[25] and elastography[26] as well as various markers associated with the risk of BC metastasis, such as CA 15-3 and CEA[15]. Thus, according to data obtained by Ceylan et al.[24], tumor size >2 cm, HER2 positive status, LVI, palpable tumor, microcalcifications, multifocality or multicentricity, and axillary ultrasonographic findings were independent predictors of SLN metastasis. The AUC of the presented model was 0.870, and its sensitivity, specificity and accuracy were 84%, 90% and 87.4%, respectively[24]. However, it should be noted that the authors of this study did not provide data on what should be considered a cutoff that discriminates between cases with a high and low risk of BC metastases in the SLNs.

High sensitivity, accuracy and specificity were demonstrated by the method of preoperative magnetic resonance imaging based on radiomics[25]. This method allows us not only to predict the risk of BC metastasis in the ALNs and to estimate the number of SLNs with metastases but also to predict the relapse-free survival of patients with early BC. The AUC of this model ranged from 0.900 to 0.920. However, despite all the advantages of the method, the need for expensive equipment and experienced specialists seriously limits its widespread use.

Bae et al. developed a nomogram that takes into account the size of the SLNs and their mean stiffness and elasticity ratio determined using intraoperative ex vivo shear-wave elastography[26]. The presented nomogram showed good discriminatory ability: the AUC was 0.856 in the development cohort and 0.791 in the validation cohort. The main limitation of this model is associated with the need to perform intraoperative ultrasound examination of removed SLNs, the results of which may be influenced by the availability of

appropriate equipment and experienced specialists. In addition, as the authors of the study note, the question of the cutoff value of the nomogram for performing the intraoperative pathologic examination remains open.

We suggest that one of the promising markers associated with the risk of BC regional metastasis may be PD-L1 expression in the tumor. According to the literature, there is a close relationship between hypoxia, EMT, immunological tolerance and BC metastasis[7]. A number of experimental studies have shown that immunosuppression is associated with a decrease in the expression of epithelial proteins and an increase in the expression of mesenchymal proteins during EMT, as well as with an increase in tumor cell migration[7,27]. However, the results of clinical studies on PD-L1 expression in BC are extremely controversial. Thus, some authors provide evidence that high PD-L1 expression in tumor cells is associated with metastases of BC in the ALNs[28]; others, on the contrary, note the relationship between high PD-L1 expression and the absence of metastases in the ALNs[29]. Additionally, there are studies that did not reveal a relationship between the severity of PD-L1 expression and regional metastases of BC[30–32]. This may be due to the lack of a unified methodology for assessing PD-L1 expression, since the authors used different reagents and different criteria for assessing its expression, such as the types of cells being evaluated (tumor and stromal), the nature of cell staining (cytoplasmic, membrane), and different threshold values of PD-L1 expression[33,34].

In our study, independent predictors of a high risk of BC metastasis in the ALNs were a tumor size greater than 2 cm (T2-4), the absence of an intraductal component, the presence of LVI, luminal B HER2-positive and triple negative molecular biological subtypes, and a PD-L1 expression coefficient >1.65. These data are consistent with the results of studies that similarly noted the association of the risk of BC regional metastasis with tumor size[24,26,35,36] and LVI[24,35,36]. Some researchers have also noted an increased risk of regional metastasis in patients with HER2 positive status[24,35] and negative receptor status[35,36]. However, in our study, HER2-positive status and negative PR and estrogen receptor (ER) status (p = 0.08) were predictors of ALN metastasis risk only in univariate analysis. We believe that the main reason is that we included BC molecular biological subtypes in the multivariate analysis (multiple logistic regression analysis), which, as a result, leveled the prognostic significance of PR, ER and HER2 status, leaving only HER2 positive and triple negative molecular biological subtypes of BC as independent predictors. Regarding reducing the risk of regional metastasis in the presence of an intraductal component, it is difficult to say what this is related to. We found no studies that compared the risk of metastasis in patients with invasive BC with or without an intraductal component. We believe that additional research is required to answer this question.

One of the main results of our study was the development of a method for assessing the risk of BC regional metastasis. The estimated probability of ALN metastasis was calculated by summing the ORs of each independent predictor, and if the sum of the ORs was $\geqslant$15.63, then a high risk of ALN metastasis was predicted. The fundamental difference between the proposed method and similar prognostic models is that it takes into account the PD-L1 expression coefficient. The method is noninvasive and is characterized by high sensitivity, specificity and accuracy, which were 80.9%, 82.6% and 85.7%, respectively. The AUC for the presented model was 0.876. Considering that the method is performed on biopsy material, its results can influence not only the decision on the need for ALND and the advisability of performing an intraoperative study of SLNs on frozen sections, but also the determination of indications for neoadjuvant therapy. However, there is no doubt that further research is needed to clarify the prognostic value of the proposed method.

It is important to note that in this study we used polyclonal antibodies from Cloud-Clone Corp. (China). Unlike monoclonal antibodies, which predominantly stain tumor cell membranes, this polyclonal antibody interacts with several PD-L1 epitopes, which are the product of the CD274 gene (PDCD1L1). According to information posted on the Uniprot resource, this antibody can stain PD-L1 localized on the cell surface membrane, the membrane of circulating endosomes, and in the nucleoplasm[37]. It was this fact that was decisive when choosing the appropriate antibody for this study, since it is currently customary to distinguish

between membrane PD-L1, cytoplasmic PD-L1, nuclear PD-L1 and serum PD-L1[38–40]. It is believed that the intracellular distribution of PD-L1 is essential for both the prognosis of the disease and the effectiveness of drug and immunotherapy. At the same time, a number of studies have noted that intracellular expression (cytoplasmic and nuclear) of the marker is more associated with aggressive tumor behavior and poor prognosis of the disease than membrane expression[38,40,41].

5. Conclusion

Thus, as a result of the study, independent predictors of a high risk of BC regional metastasis were established, including a tumor size greater than 2 cm, the absence of an intraductal component, the presence of LVI, luminal B HER2-positive and triple negative molecular biological subtypes, and a PD-L1 expression coefficient ⩾15.63. The data obtained made it possible to develop a method for predicting the high risk of BC metastasis in ALNs, the effectiveness of which (AUC) was 0.876, the sensitivity - 80.9%, the specificity - 82.6% and the accuracy - 85.7%. We believe that the proposed method can complement standard clinical and instrumental studies to clarify the indications for ALND, determine the feasibility of performing an intraoperative study of SLNs on frozen sections, and be taken into account when determining indications for neoadjuvant therapy for BC patients. Considering that the possibility of monitoring patients with early breast cancer without performing SLN biopsy is currently being actively studied[42], our results may also contribute to the selection of patients in this group. At the same time, it should be noted that the proposed method has limitations associated with a small sample of patients and a single-center study. We believe that further studies of the role of PD-L1 expression in the tumors and its intracellular distribution in BC progression are promising.

Author contributions

Conceptualization, MS; methodology, EZ; software, EZ and DS; validation, EZ and DS; formal analysis, MS and EZ; investigation, EZ, MS, DS and AP; resources, EZ; data curation, EZ and DS; writing—original draft preparation, EZ and MS; writing—review and editing, EZ, MS, DS and AP; visualization, EZ; supervision, MS; project administration, MS and AP; funding acquisition, MS, EZ, DS and AP. All authors have read and agreed to the published version of the manuscript.

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Compliance with patients' rights and bioethics rules

The study was approved by the Ethics Committee of the Orenburg State Medical University (protocol No. 311 dated 13 January 2023). All patients signed an informed consent to participate in the clinical trial.

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Conflict of interest

The authors declare no conflict of interest.

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