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Expression of cytotoxic T lymphocyte-associated antigen 4, CD44, and E-cadherin in the microenvironment of breast carcinomas

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SUMMARY

OBJECTIVE: The expression of cytotoxic T lymphocyte-associated antigen 4, E-cadherin, and CD44 in the area of tumor budding was investigated in breast carcinomas in our study.

METHODS: Tumor budding was counted at the invasive margins in 179 breast carcinomas. To understand the microenvironment of tumor budding, we examined the expression status of the immune checkpoint molecules such as cytotoxic T lymphocyte-associated antigen 4, E-cadherin, and CD44.

RESULTS: Tumors were separated into low (≤5) and high tumor budding groups (>5) based on the median budding number. Lymphovascular, perineural invasion, and the number of metastatic lymph nodes were significantly higher in high-grade budding tumors (p=0.001, p<0.001, and p=0.019, respectively). Tumor-infiltrating lymphocytes were significantly higher in tumors without tumor buddings (p<0.001). When the number of budding increases by one unit, overall survival decreases by 1.07 times (p=0.013). Also, it increases the risk of progression by 1.06 times (p=0.048). In high tumor budding groups, the cytotoxic T lymphocyte-associated antigen 4 staining percentage of lymphocytes was significantly higher (p=0.026). With each increase in the number of buds, an increase in the percentage of cytotoxic T lymphocyte-associated antigen 4 staining was seen in lymphocytes in the microenvironment of TB (p=0.034).

CONCLUSION: Tumor budding could predict poor prognosis in breast carcinomas, and anti-cytotoxic T lymphocyte-associated antigen 4 immunotherapies may be beneficial in patients with high tumor budding tumors.

KEYWORDS: Breast neoplasms. CD44 protein, mouse. CTLA-4 antigen. E-cadherin. Tumor microenvironment.

INTRODUCTION

TB is considered the histological reflection of epithelial-mesenchymal transition (EMT)[1]. Loss of E-cadherin expression in the EMT area disrupts cell-cell interaction and causes an increase in the invasion capacity of the tumor[2,3]. CD44, a cell surface transmembrane glycoprotein, plays an important role in tumor invasion, metastasis, and EMT[4,5]. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), one of the immune checkpoint molecules, is a receptor that plays an important role in the regulation of T cell activation and the maintenance of self-tolerance[6]. It contributes to escape from immune surveillance by suppressing the immune response against the tumor. This may facilitate TB.

We aimed to reveal TB in breast carcinomas (BCs), the relationship between TB and the microenvironment, and clinicopathological prognostic factors. Treatments targeting immune checkpoints, such as CTLA-4, may be a patient-specific treatment option for patients with BCs.

METHODS

Definition and assessment of tumor-budding and tumor-infiltrating lymphocytes

From 2011 to 2018, 179 cases operated in our hospital were evaluated retrospectively. The definition of "isolated single cancer cell or cluster of less than 5 cancer cells" was accepted for TB. TB evaluation was performed at 200× magnification (BX51, 200×, field size 0.95 mm^2) in the most invasive area. Tumor ınfiltrating lymphocyte (TIL) was evaluated using the method proposed by the International TILs Working Group 2014 in BC[7].

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Immunohistochemical staining and evaluation

The sections of 162 cases with TB were stained with antibodies, including mouse anti-human E-cadherin (NCH-38, ready-to-use kit, Dako, California), anti-human CD44 (MRQ-13, 1:100, Cell Marque California), mouse anti-human CTLA-4 (F8, 1:100, Santa Cruz, Texas), and mouse anti-human Ki67 (MIB-1, 1:200, DakoCytomation).

E-cadherin and CD44 were evaluated in tumor and TB areas with 200× magnification. Membranous staining of 90% and above for E-cadherin and 10% and above staining for CD44 was considered positive (Figure 1).

CTLA-4 was evaluated at 400× magnification in the buds and lymphocytes in the bud microenvironment. Both staining percentages and staining intensity were evaluated. CTLA-4 was divided into four groups according to the staining intensity. If there was no cytoplasmic-membranous staining, the score was 0. Weak staining was scored as 1 point, moderate staining as 2 points, and strong staining as 3 points. Those with no staining and mild staining (scores 0 and 1) were included in the negative group and those with moderate and strong staining (scores 2 and 3) in the positive group (Figure 2).

Statistics

The Mann-Whitney U test was used for comparing two independent groups. The Kruskall-Wallis test was used for comparing more than two independent groups. For comparisons between categorical variables, the Pearson χ^2 test was used in 2×2 tables, and Fisher's exact test was used in cross tables. Immunohistochemical staining differences between tumor and TB were compared with categories, groupings, and the McNemar test. For statistical significance, type 1 error level is used as 5%. In the survival analysis, the Kaplan-Meier analysis used the log-rank test for the comparison of survival curves.

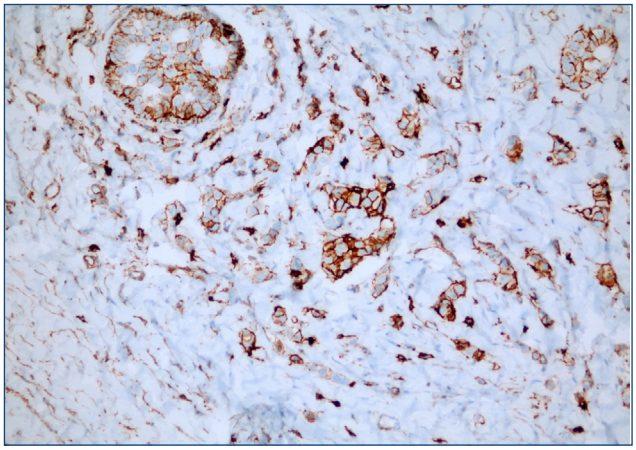

Figure 1. CD44 positivity in the tumor (CD44, 200´ magnification).

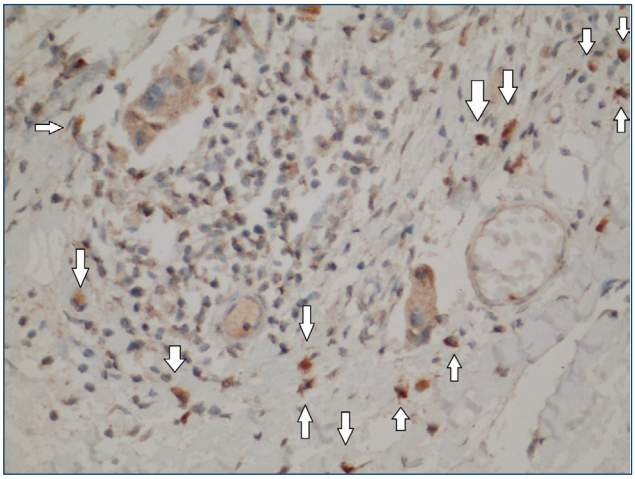

Figure 2. Cytotoxic T lymphocyte-associated antigen 4 evaluation according to the percentage of staining in lymphocytes around the tumor bud 13% (cytotoxic T lymphocyte-associated antigen 4, 400´ magnification).

The cutoff value was considered the median value. The significance level was considered $p<0.05$ in the statistical analysis.

Ethical approval

Our study's ethics committee approval was obtained from the University of Health Science Bagcılar Training and Research Hospital, non-interventional clinical research ethics committee chairmanship. According to the Declaration of Helsinki and the ethical standards of the institutional research committee, the study was conducted.

RESULTS

General characteristics and findings in cases with high and low buds

TB was not observed in 17 of the patients. Of the patients, 64 (35.7%) were at pT1, 93 (52%) at pT2, and 22 (12.3%) at pT3. A total of 13 (7.3%) were in Grade 1, 66 (36.9%) in Grade 2, and 100 (55.8%) in Grade 3.

The number of TB ranges from 0 to 35. The average number of buds was determined as 6±5.1. The value "5," which is the median of the bud numbers, was determined as the cut-off score. A total of 96 cases (53.6%) with ≤5 buds were categorized as low TB, and 83 cases (46.4%) with >5 buds were divided into high TB (Table 1).

Survival analysis

Progression was observed in 35 (19.6%) of the cases. Of the 35 patients with progression, 19 of them died from the disease.

Table 1. Significant results of tumor budding.

	Tumor budding present	No tumor budding	p-value	High-tumor budding	Low-tumor budding	p-value
Grade						
G1	10 (6.2%)	3 (17.65%)	0.155	5 (6%)	8 (8.3%)	0.798
G2	62 (38.3%)	4 (23.52%)		32 (38.6%)	34 (35.4%)	
G3	90 (55.5%)	10 (58.83%)		46 (55.4%)	54 (56.30%)	
Mean tumor size	3.1±2.2 cm	2.1±1.8 cm	0.075	3.3±2.2	2.8±2.1	0.133
Mean number of metastatic lymph nodes	3.9±6.6	0.3±0.6	0.024*	4.8±7.9	2.6±4.4	0.019*
N stage						
N0	58 (35.8%)	13 (76.5%)	0.006*	23 (27.7%)	48 (50%)	0.008*
N1	49 (30.2%)	4 (23.5%)		26 (31.3%)	27 (28.1%)	
N2	33 (20.4%)	0 (0%)		22 (26.5%)	11 (11.5%)	
N3	22 (13.6%)	0 (0%)		12 (14.5%)	10 (10.4%)	
Lymphovascular invasion						
Negative	99 (61.1%)	3 (17.6%)	0.001*	24 (28.9%)	53 (55.2%)	0.001*
Positive	63 (38.9%)	14 (82.4%)		59 (71.1%)	43 (44.8%)	
Molecular subgroup						
Luminal	97 (59.9%)	5 (29.4%)	0.031*	52 (62.7%)	50 (52.1%)	0.114
Nonluminal	65 (40.1%)	12 (70.6%)		31 (37.3%)	46 (47.9%)	
Tumor-infiltrating lymphocytes						
Low	101 (62.3%)	7 (41.2%)	0.01*	53 (63.9%)	54 (56.2%)	0.354
Intermediate	39 (24.1%)	4 (23.5%)		20 (24.1%)	23 (24%)	
High	22 (13.6%)	6 (35.3%)		10 (12%)	19 (19.8%)	

*Indicate statistical significance at the p<0.05 level.

One unit increase in the number of buds increases the risk of progression 1.06 times (1.00–1.13, p=0.048). Also, one unit increase in the number of buds decreases the overall survival (OS) by 1.07 times (1.01–1.12, p=0.013).

In a multivariate analysis including bud number, tumor size, Ki-67 groups, pT, pN, molecular groups, PR, necrosis, LVI, PNI, and neoadjuvant therapy, the number of buds independently affected disease-free survival (DFS).

There was no significant difference in 5-year OS and DFS between cases with and without TB and between high- and low-bud groups (p>0.05).

E-cadherin, CD44, and cytotoxic T lymphocyte-associated antigen 4 expression in budding cells

In the IHC study conducted in 162 cases with TB, loss of staining with E-cadherin was detected in 21 (13%) of the tumors and in 131 (81%) of the tumor buddings (p=0.7, p>0.9).

CD44 was stained in 50 (63%) of the low-bud tumors. CD44 staining percentage was significantly higher in low-bud tumors (p=0.026). CD44 was stained in 88% (54.3%) of TB. There was no significant difference between the two groups in terms of CD44 staining of TB (p=0.3).

The percentage of CTLA-4 staining of lymphocytes in the microenvironment of TB ranged from 0 to 100, with an average of 12±12.643%. While the percentage of CTLA-4 in the lymphocytes in the microenvironment of cases with high buds was found to be 13.82% on average, it was observed to be 10.48% in those with low buds. The percentage of CTLA-4 in lymphocytes in the bud microenvironment was found to be significantly higher in the high-bud group compared to the low-bud group (p=0.026). Each increase in the number of buds correlates with an increase in the percentage of CTLA-4 staining in lymphocytes in the tumor microenvironment (rho=0.17, p=0.034).

According to the staining intensity score, 62 (38.3%) of the buds were stained and 100 (61.7%) were not stained with CTLA-4. As homogeneous staining was observed in all of the stained TB, the staining percentage was accepted as 100%. There was no significant difference between the bud groups and lymphocytes in the bud microenvironment in terms of CTLA-4 staining intensity (p>0.05).

DISCUSSION

TB is the histological reflection of a dynamic process that determines the potential of tumor invasion[2]. The increased migration and invasion capacity of budding cells facilitate the spread to lymphatics and lymph nodes. These results suggest that TB can be used as a parameter to predict possible lymph node metastasis and a poor prognostic factor in BCs. Studies also support that TB is a poor prognostic factor for survival independent of other prognostic parameters[8-11]. The loss or decrease of E-cadherin expression is by the interaction of signal pathways and transcription factors during EMT. It is considered that the separation of TB from the main tumor mass with loss of connections between cells, increased mobility, and invasion capacity is thought to represent EMT[3]. In our study, the loss of E-cadherin expression was determined as 81% in both high- and low-bud areas, and this indicates that E-cadherin decreases in the bud area regardless of the number of buds. As a result, the loss of E-cadherin seen in the bud areas in BCs supports EMT.

Molecular studies show that high CD44 expression is associated with cancer stem cell characteristics and EMT and demonstrated that it contributes to tumor invasion, metastasis, recurrence, and drug resistance[12-14]. Therefore, an increase in CD44 expression is expected in the TB area, which is thought to be the histological reflection of EMT and shows stem cell characteristics. Gurzu et al.[15] found an increase in CD44 staining in the bud area in their study on colorectal carcinomas[15]. Similarly, an increase in CD44v6 expression was observed in the budding area in the study of Masaki et al.[16]. In our study, CD44 staining was significantly higher in low-bud tumors, supporting studies showing good prognosis in CD44-positive tumors. However, no relationship was found with CD44 in bud groups. This suggests that the relationship between basic cell biology and clinical behavior is complex, and extensive studies are needed on CD44 expression in tumors and buds.

In our study, TILs were found to be significantly higher in tumors with no TB. It can be thought that the high immune response in the tumor stroma prevents the increase in the invasive potential of the tumor. Gujam et al.[8] found that high TB was associated with a lower inflammatory response, according to Klintrup-Makinen's grade[8]. In our study, although TIL was detected less frequently in high TB, no significant difference was found between them. According to the TIL evaluation recommended by ITILWG in breast cancers, we evaluated TIL in the entire tumor stroma[7]. However, evaluating only the invasive margin of stroma in the Klintrup-Makinen grading may have more clearly demonstrated the relationship between the number of TB and TIL in their study.

CTLA-4 is an immune checkpoint molecule that plays an important role in regulating T-cell activation and maintaining self-tolerance[17,18]. Paulsen et al.[19] evaluated CTLA-4 expression in tumoral cells in lymph node metastases in non-small cell lung carcinomas[19]. Yu et al. associated high CTLA-4 expression and low tumor CTLA-4 expression in lymphocytes in the interstitial area around the tumor with a good prognosis[20]. This finding suggests that EMT suppresses the antitumor immune response. In our study, the average CTLA-4 percentage in lymphocytes in the high budding area reflecting EMT was found to be significantly higher. It can be considered that patients with high-bud tumors may benefit greatly from anti-CTLA-4 antibodies.

Three main results were found in our study. First, in BCs, TB can be considered a poor prognostic factor alone as it predicts lymph node metastasis, LVI, and PNI. Second, the density of tumor-infiltrating lymphocytes may play a role in the prevention of TB by the antitumor immune response. Third, in tumors with high TB, significantly higher staining of CTLA-4 is observed in lymphocytes around the TB; thus, CTLA-4 may promote TB by inhibiting the antitumor immune response. If supported by comprehensive studies, it is thought that anti-CTLA-4 therapy may be beneficial in patients with high TB tumors.

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AUTHORS' CONTRIBUTIONS

TBS: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **TCS:** Conceptualization, Formal Analysis, Investigation, Validation, Writing – review & editing. **HEP:** Conceptualization, Formal Analysis, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing. **AM:** Data curation, Funding acquisition, Resources. **MT:** Resources. **ÇÖ:** Methodology, Supervision, Writing – review & editing.

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