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Comparison of the Clinicopathologic Features and T-Cell Infiltration of B7-H3 and B7-H4 Expression in Triple-negative Breast Cancer Subtypes

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Abstract: Previously we revealed an upregulated expression of B7-H3 and B7-H4 mRNA and protein in breast cancer, including triple-negative breast cancer (TNBC). However, little is known regarding the clinical impact and value of B7-H3 and B7-H4 in TNBC subtypes. Thus, this study evaluated the clinicopathologic effects of B7-H3 and B7-H4 mRNA and protein expression according to the TNBC subtypes. RNAscope in situ hybridization and immunohistochemistry of B7-H3 and B7-H4 was done for 186 TNBC samples using tissue microarray. Immunohistochemistry was also performed for TNBC molecular subtype-surrogate markers, CD3, and CD8. TNBCs were classified into basal-like (BL) (64.5%), luminal androgen receptor (10.8%), and unclassifiable (24.7%) subtypes. Tumor B7-H4 mRNA expression was associated with younger age at the initial diagnosis and with molecular TNBC subtypes. Expression of B7-H3 mRNA and protein in the tumor cells was negatively correlated with $CD3^+$ and $CD8^+$ T-cell infiltration density in the tumor and/or stromal region of TNBCs and their subtypes. High stromal B7-H3 mRNA expression was associated with poor disease-free and overall survival in the TNBCs and with overall survival in the unclassifiable subtype. Stromal B7-H3 mRNA expression was independently associated with overall survival and disease-free survival in the TNBCs and BL subtype, respectively. Our results indicate the importance of the stromal expression of B7-H3 mRNA as a prognostic factor in the TNBCs and BL subtype. The inverse relationship between B7-H3 expression and $CD3^+$ and $CD8^+$ T-lymphocyte infiltration represents a promising target for immunotherapy for the TNBCs, especially the BL subtype.

Key Words: breast cancer, tripe-negative, molecular subtypes, B7-H3, B7-H4

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Breast cancer remains the leading cause of cancer deaths in women worldwide including Korea.[1] Triple-negative breast cancer (TNBC) is a breast cancer subtype that lacks expression of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 and accounts for 10% to 15% of all breast cancers.[1,2] The management of TNBC is still challenging because of its aggressive behavior and limited treatment options.[3]

Recently, the manipulation of the immune system represents an attractive strategy for treatment of cancer including TNBC.[4,5] Immune checkpoints are modulators of costimulatory or coinhibitory signals of the adaptive immune system that affect antitumor immunity.[4] T cells are the main effector cells in antitumor immune response, whereas B7 family checkpoints are important for regulating T-cell responses.[6]

As T-cell coinhibitory signals, the interaction between programmed death 1 (PD-1) on T cells and its ligand PD-L1 (also known as B7-H1) on cancer cells prevents a comprehensive antitumor immune response.[7,8] Cancer immunotherapy using immune checkpoint inhibitors against PD-1/PD-L1 is being extensively explored in TNBC, and clinical trials have achieved notable benefits.[5,9,10] The Food and Drug Administration (FDA) has recently approved the use of atezolizumab, a monoclonal antibody drug targeting PD-L1, plus chemotherapy for treating patients with PD-L1-positive, advanced TNBC based on findings of the IMpassion130 clinical trial.[11]

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Despite increased toxicity and cost, the combination approach shows improved efficacy, depending on the complexity of controlling the tumor-host interactions and their non-reliance on a single regulatory pathway. Numerous clinical trials are ongoing for combinations of immune checkpoint inhibitors and other treatment modalities or drugs targeting other immune checkpoints in advanced cancers including TNBC.[5,9,10]

Other B7 immunoregulatory families also play major roles in tumor evasion from immunosurveillance and are new promising therapeutic targets for developing immunotherapy. B7-H3 and B7-H4, coregulatory molecules in the B7 family, inhibit T-cell proliferation and downregulate T-cell response, respectively.[12,13] B7-H3 and B7-H4 molecules have been associated with antitumor immunity and cancer development in breast cancer, including TNBC.[14–20]

Since TNBC is a heterogeneous disease, it has been further stratified into several molecular subtypes to discover therapeutic targets. Many molecular and immunohistochemical subclassifications have been proposed in the past decade to define more homogeneous TNBC subtypes.[21–24] These subtypes of TNBCs could be used in identifying personalized treatment strategies. Our previous study revealed the increased expression levels of B7-H3 and B7-H4 mRNA and protein in breast cancer, including TNBC.[25] However, little is known regarding the clinical impact and prognostic or predictive value of B7-H3 and B7-H4 in TNBC subtypes.

In this study, we evaluated the clinicopathologic effects of B7-H3 and B7-H4 mRNA and protein expression according to TNBC subtypes, using RNAscope in situ hybridization (ISH) and immunohistochemistry (IHC) in 186 patients with TNBC through constructing a tissue microarray (TMA). TNBCs were categorized into three subtypes—basal-like (BL), luminal androgen receptor (LAR), and unclassifiable type (UN)—based on IHC analysis for molecular subtype-surrogate markers [cytokeratin 5/6 (CK5/6), CK14, epidermal growth factor receptor (EGFR), and androgen receptor (AR)]. In addition, immunohistochemical staining was performed for CD3 and CD8 to investigate the roles of B7-H3 and B7-H4 in regulating T-cell infiltration.

MATERIALS AND METHODS

Patients and Tissues

This study included patients with TNBC who underwent surgical treatment at Chonnam National University Hwasun Hospital from January 2013 to December 2017. TNBC was defined as lacking expression of estrogen receptor and progesterone receptor (< 1% by IHC) and human epidermal growth factor receptor 2 (evaluated by IHC and/or by sliver ISH).[26–28]

The density of stromal tumor infiltrating lymphocytes (TILs) was recorded as the percentage of tumor stroma region that contained lymphocytic infiltrates without direct contact with carcinoma cells based on the recommendation by the International TIL Working Group in 2014.[29] The stromal TILs were scored as 0 (no TILs), 1 (< 5% stromal region with TILs), 2 (5% to 50% stromal region with TILs), and 3 (> 50% stromal region with TILs).

Under the medical insurance program controlled by the Ministry of Health and Welfare of Korea, the patients received standard radiation therapy and/or adjuvant chemotherapy after surgery. Patients who received neoadjuvant chemotherapy before surgical treatment were excluded. The medical records of patients were reviewed to obtain clinical data—patient age at initial diagnosis, local recurrence, distant metastases, and patient survival.

Construction of TMA

One hundred eighty-six TNBC cases were selected based on the availability of formalin-fixed-paraffin-embedded samples. To construct TMA, 2 replicate 2 mm cores were taken from each donor block. Serial sections of TMA blocks were provided by Chonnam National University Hwasun Hospital Biobank, a member of the Korea Biobank Network.

B7-H3 and B7-H4 mRNA and Protein Expression in TNBC

RNAscope ISH Assay

B7-H3 and B7-H4 mRNA expression was determined using ISH with RNAscope formalin-fixed-paraffin-embedded assay (Advanced Cell Diagnostics, Hayward, CA), as described previously.[25,30] RNAscope probes for B7-H3 (cat# 430411); B7-H4 (cat# 418081); peptidylprolyl isomerase B (PPIB), a positive control; and bacterial dihydrodipicolinate reductase (DapB), a negative control were used. RNAScope 2.0 HD Reagent Kit-Brown (Advanced Cell Diagnostics) was used as a detection reagent.

B7-H3 and B7-H4 mRNA expression was evaluated within the tumor area. Tumor area was defined as the area occupied by tumor cells (tumor) and their associated intratumoral and contiguous peritumoral stroma (stromal region).[29] RNAscope amplification was scored as recommended by the manufacturer: score 0 = no staining or <1 dot/10 cells; score 1 = 1 to 3 dots/cell; score 2 = 4 to 10 dots/cell; score 3 = > 10 dots/cell, with <10% of positive cells having dot clusters visible at 20×; and score 4 = > 10 dots/cell, with > 10% of positive cells having dot clusters at ×20.[25,30] Expression level of B7-H3 and B7-H4 mRNA was classified as low (score 0 to 2) or high (score 3 and 4).[25]

Immunohistochemistry

B7-H3 and B7-H4 immunohistochemical staining was performed using a Bond-max Autostainer (Leica Microsystems, Bannockburn, IL) as described in previous studies.[25,30] Monoclonal B7-H3 (6A1, 1 : 800 dilution; Thermo Fisher Scientific, Rockford, IL) and B7-H4 (D1M81, 1 : 200 dilution; Cell Signaling Technology, Danvers, MA) primary antibodies were used. B7-H3 and B7-H4 immunoreactivity was also evaluated in both tumor and stromal cells and was scored semiquantitatively based on the intensity of staining and the percentage of

stained cells. The intensity scores (0 = no staining; 1 = light staining; 2 = moderate staining; and 3 = strong staining) and percentage scores (0 = < 5%; 1 = 5% to 25%; 2 = 25% to 50%; 3 = > 50%) were multiplied, and the results were classified as low (score, 0 to 4) or high (score > 4) expression level for statistical analysis.[25,30]

Expression of TNBC Molecular Subtype-surrogate Markers, CD3, and CD8

Serial sections of TMA blocks were used for immunohistochemical staining for AR (AR441, 1 : 100 dilution; Dako, Carpinteria, CA), CK5/6 (D5/16B4, 1 : 50 dilution; Dako), CK14 (LL002, 1 : 50 dilution; Novocastra, Newcastle, UK), EGFR (C6, ready to use; Roche, Rotkreuz, Switzerland), CD3 (F7.2.38, 1 : 50 dilution; Dako), and CD8 (C8/144B, 1 : 100 dilution; Dako).

AR immunostaining was evaluated according to the Allred score,[26] and tumors were considered AR-positive when the total score was ≥ 2. Immunohistochemical staining results for CK5/6, CK14, and EGFR were considered positive when > 1% tumor cells were stained.[23,31] The molecular subtypes of TNBCs were defined according to the immunohistochemical results as follows: (1) LAR, AR-positive; (2) BL, CK5/6-positive and/or CK14-positive and/or EGFR-positive; (3) UN, tumors with none of the above features.[23,32]

The density of $CD3^+$ and $CD8^+$ T-cell infiltration were evaluated in tumor and stroma region with the highest infiltration. The number of $CD3^+$ and $CD8^+$ T cells was counted in 3 high-power fields (hpfs), and the average value was recorded. The densities were grouped with a 4-tiered scoring system (group 0, ≤ 5 cells/3 hpfs; group 1, 6 to 50 cells/3 hpfs; group 2, 51 to 99 cells/3 hpfs; and group 3, ≥ 100 cells per 3 hpfs) and were classified as low (group 0 to 1) or high (group 2 to 3).[25,30]

Statistical Analysis

The SPSS 25.0 software (SPSS INC., Chicago, IL) was used for statistical analysis. χ^2 or the Fisher exact test was used to compare categorical nominal variables. The agreement between mRNA ISH and IHC data for B7-H3 and B7-H4 expression was analyzed using the κ statistic method. The Spearman correlation was used to evaluate the association between variables. Overall survival and disease-free survival were plotted using the Kaplan-Meier method with the log-rank test. Multivariate survival analysis was performed using the Cox regression model. A *P*-value of <0.05 was considered statistically significant in all comparisons.

Compliance with Ethical Standards

This retrospective study used archived materials and did not affect patient care. Therefore, it was approved by the hospital Institutional Review Board without informed consent from patients (CNUHH-2019-096).

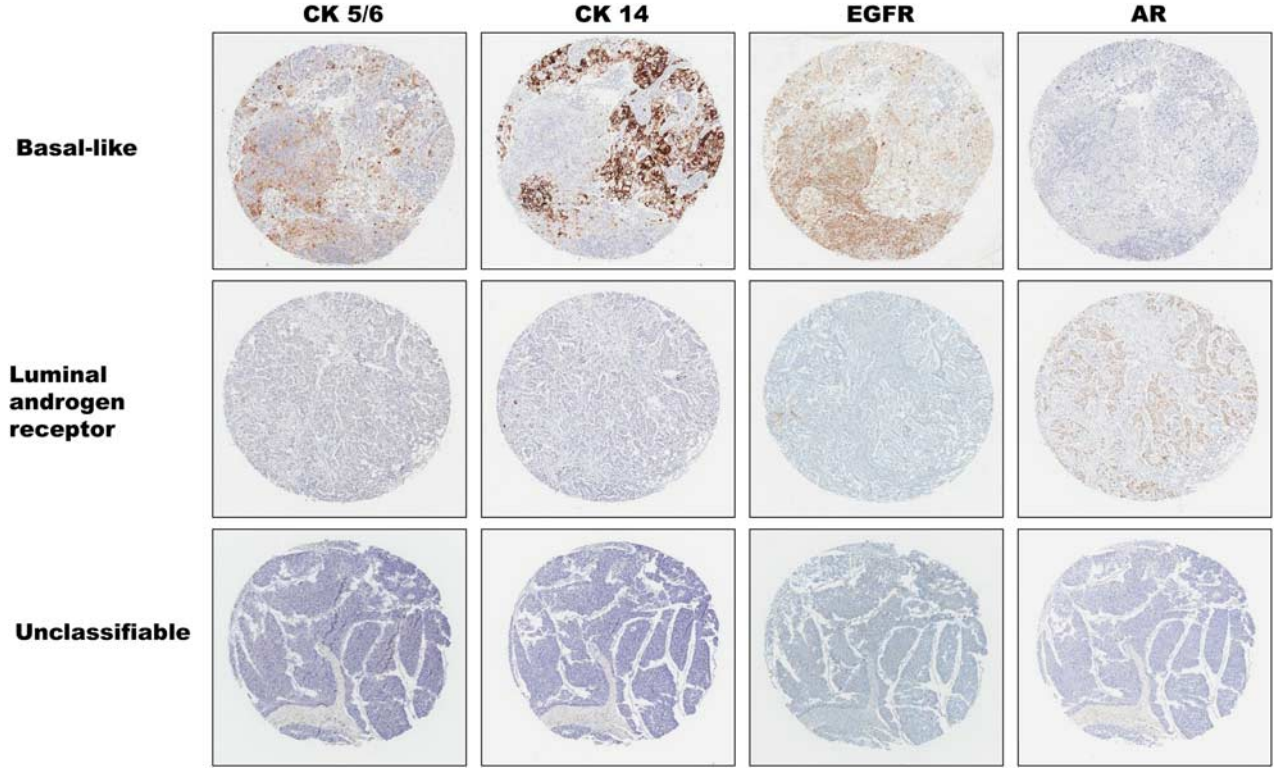

FIGURE 1. Representative cases of triple-negative breast cancer basal-like, luminal androgen receptor, and unclassifiable subtypes based on immunohistochemical staining of cytokeratin (CK) 5/6, CK14, epidermal growth factor receptor (EGFR), and androgen receptor (AR).

RESULTS

TNBC Subtypes and B7-H3 and B7-H4 mRNA and Protein Expression

Of 186 TNBC cases, 29 (15.6%), 67 (36.0%), 113 (60.8%), and 20 (10.8%) cases were positive for CK5/6, CK14, EGFR, and AR, respectively. BL subtype was defined as positive for at least one of the basal markers (CK5/6, CK14, and EGFR). Seventy-six (40.9%) cases were positive for only 1 basal marker, and 17 (9.1%) cases were positive for all 3 basal markers. On the basis of the immunohistochemical evaluation, TNBCs were classified into the BL (n = 120, 64.5%), LAR (n = 20, 10.8%), and UN (n = 46, 24.7%) subtypes (Fig. 1). The baseline characteristics of the 186 patients according to TNBC subtypes are summarized in Table 1. BL and UN subtypes were associated with younger age at the initial diagnosis, when compared with the LAR subtype ($P < 0.01$). BL and UN subtypes had higher stromal TIL density than the LAR subtype, but it was not statistically significant ($P = 0.099$).

To investigate the expression and location of B7-H3 and B7-H4 mRNA and protein in TNBCs, the 186 TNBC cases were analyzed using ISH and IHC. B7-H3 mRNA was highly expressed in tumor cells and at low levels in the stromal region (Figs. 2A–D). In stromal region, B7-H3 mRNA was mainly expressed in the endothelial cells. In contrast, B7-H4 mRNA expression was observed only in tumor cells (Figs. 3A–C). B7-H3 and B7-H4 protein expression was observed in the cytoplasm and cell membrane (Figs. 2E–H, Figs. 3D–F). The staining patterns of B7-H3 and B7-H4 in IHC analysis were concordant with those in mRNA ISH. Although B7-H3 protein expression level was higher in tumor cells of TNBCs, it was expressed by both tumor and stromal cells (Figs. 2E–H). Vascular endothelial cells were the main stromal cells that expressed B7-H3 protein. B7-H4 protein was expressed in tumor cells; however, its expression in the stromal region was negative or weak (Figs. 3D–F). Among the 186 cases, mRNA ISH was interpretable in 175 and 174 cases for B7-H3 and B7-H4, respectively. Immunostaining data were available for 176 cases for both B7-H3 and B7-H4. For B7-H3, tumor mRNA and protein were highly expressed in 49 of 175 (28.0%) and 121 of 176 (68.8) cases; stromal mRNA and protein were highly expressed in 22 of 175 (12.6%) and 43 of 176 (24.4%) cases. For B7-H4, tumor mRNA and protein were highly expressed in 92 of 174 (52.9%) and 66 of 176 (37.5%) cases; however, high stromal B7-H4 mRNA and protein expression levels were not observed. We identified a positive correlation between ISH and immunohistochemical staining scores of B7-H3 mRNA and protein in the tumor and stromal regions of TNBCs ($r = 0.649$, $P < 0.001$ in tumor; $r = 0.370$, $P < 0.001$ in stroma) and between B7-H4 mRNA and protein expression in the tumor region of TNBCs ($r = 0.756$, $P < 0.001$). The concordance between B7-H3 mRNA ISH and protein IHC and those of B7-H4 is summarized in Table 2. Among these comparisons, the highest concordance (82.8%) was demonstrated between tumor B7-H4 mRNA ISH and protein IHC ($\kappa = 0.658$, $P < 0.001$).

TABLE 1. Clinicopathologic Characteristic According to Triple-negative Breast Cancer Subtypes

Variables	Basal-like [N (%)]	Luminal Androgen Receptor [N (%)]	Unclassifiable [N (%)]	P*
Age (y)				< 0.01
≤ 52	66 (55.0)	4 (20.0)	27 (58.7)	
> 52	54 (45.0)	16 (80.0)	19 (41.3)	
Histopathologic type				NS
Invasive carcinoma, NOS	106 (88.3)	20 (100)	39 (84.8)	
Other type	14 (11.7)	0	7 (15.2)	
Stromal TILs density score				NS
1	68 (56.7)	17 (85.0)	23 (50.0)	
2	43 (35.8)	3 (15.0)	18 (39.1)	
3	9 (7.5)	0	5 (10.9)	
Tumor size (cm)				NS
≤ 2	49 (40.8)	12 (60.0)	20 (43.5)	
2-5	68 (56.7)	8 (40.0)	22 (47.8)	
> 5	3 (2.5)	0	4 (8.7)	
Number of nodal metastasis				NS
0	88 (73.3)	13 (65.0)	34 (73.9)	
1-3	24 (20.0)	2 (10.0)	10 (21.7)	
4-9	5 (4.2)	2 (10.0)	2 (4.3)	
≥ 10	3 (2.5)	3 (15.0)	0	
Histologic grade				NS
1	3 (2.5)	1 (5.0)	2 (4.3)	
2	17 (14.2)	3 (15.0)	6 (13.0)	
3	100 (83.3)	16 (80.0)	38 (82.7)	
Stage				NS
I	43 (35.8)	8 (40.0)	20 (43.5)	
II	67 (55.8)	7 (35.0)	23 (50.0)	
III	10 (8.4)	5 (25.0)	3 (6.5)	
Recur or distant metastatic relapse				NS
No	109 (90.8)	18 (90.0)	39 (84.8)	
Yes	11 (9.2)	2 (10.0)	7 (15.2)	
Death				NS
No	114 (95.0)	19 (95.0)	41 (89.1)	
Yes	6 (5.0)	1 (5.0)	5 (10.9)	

*Analyzed by χ^2 test.

N indicates number; NOS, not otherwise specified; NS, not significant; TILs, tumor infiltrating lymphocytes.

Relationships Between B7-H3 and B7-H4 mRNA and Protein Expression and Clinicopathologic Features of TNBCs

The associations between B7-H3 and B7-H4 mRNA and protein expression and clinicopathologic variables in patients with TNBCs are shown in Supplementary Table 1 (Supplemental Digital Content 1, http://links.lww.com/AIMM/A329). Tumor B7-H4 mRNA expression tended to be associated with younger age at the initial diagnosis and molecular TNBC subtypes ($P < 0.05$, both). Specifically, tumor B7-H4 mRNA expression level was significantly higher in the BL subtype than in the UN subtype ($P < 0.05$). High stromal B7-H3 mRNA expression level was more frequently observed in death patients than in those alive ($P < 0.05$). Other clinicopathologic characteristics were not associated with B7-H3 and B7-H4 expression. For subtype analysis,

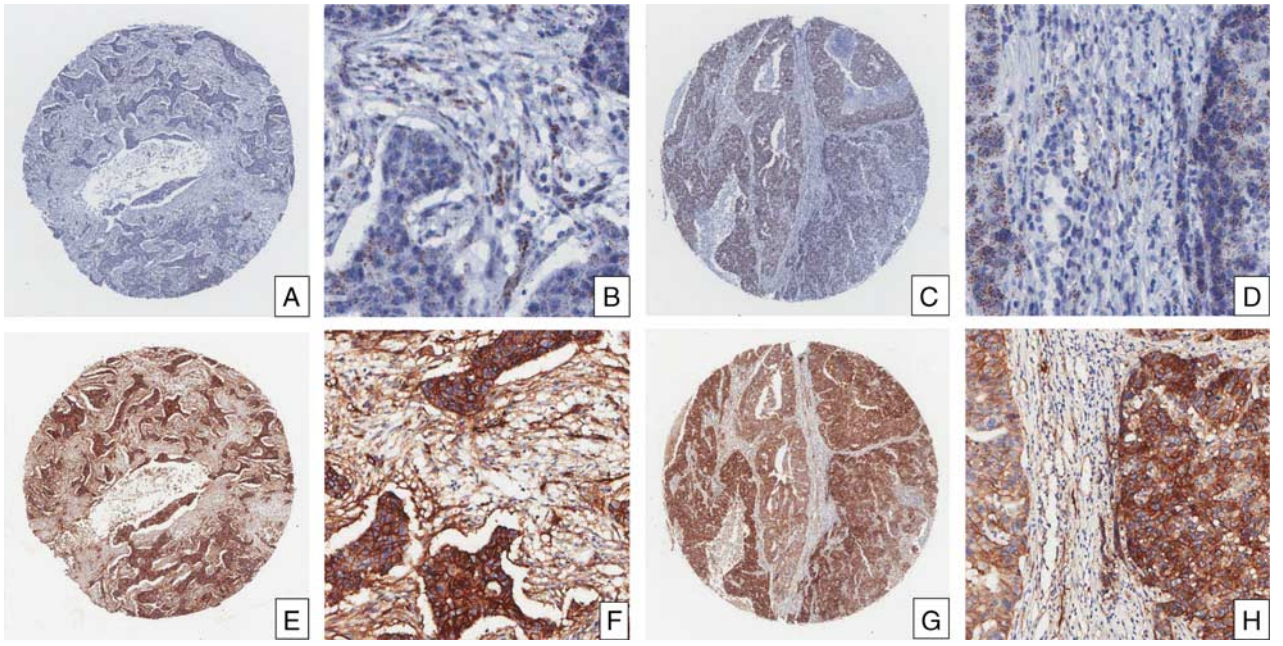

FIGURE 2. Expression and localization of B7-H3 mRNA (A–D) and B7-H3 protein (E–H) in 2 cases of triple-negative breast cancers. B7-H3 mRNA and protein expression levels are higher in tumor cells than in adjacent stromal tissues. Vascular endothelial cells are the main stromal cells showing high B7-H3 mRNA and protein expression levels. A, C, E, G ×2; B, D ×400; F, H ×200. full color online

tumor B7-H4 mRNA expression was associated with younger age in the BL subtype; stromal B7-H3 mRNA expression was associated with relapse in the LAR subtype ($P < 0.05$) and with younger age and survival of patients in the UN subtype ($P < 0.05$ and <0.01, respectively).

Correlation Between B7-H3 and B7-H4 mRNA and Protein Expression and Infiltration of $CD3^+$ and $CD8^+$ T Cells

When $CD3^+$ and $CD8^+$ T cells were analyzed for cases capable of analyzing B7-H3 and B7-H4 mRNA and protein expression, $CD3^+$ and $CD8^+$ T cells were observed in the stromal region of all cases (Fig. 4). The number of stromal $CD3^+$ and $CD8^+$ T cells per 3 hpfs was in the range 1 to 397 (mean ± SD: 95.9 ± 82.3) and 1 to 333 (mean ± SD: 59.6 ± 53.6), respectively. Meanwhile, $CD3^+$ and $CD8^+$ T cells were present in the tumor region of 153 and 152 cases, respectively (Fig. 4), and their numbers per 3 hpfs were in the range of 0 to 318 (mean ± SD: 25.1 ± 41.9) and 0 to 278 (mean ± SD: 20.0 ± 35.6), respectively.

On the basis of the cutoff point, high density $CD3^+$ and $CD8^+$ T-cell infiltrates in tumor and stromal area were found in 27 (15.3%) and 22 (12.5%) and 108 (61.4%) and 78 (44.3%) of 176 TNBC cases, respectively. Supplementary Table 2 (Supplemental Digital Content 2, http://links.lww.com/AIMM/A330) summarizes the correlation between B7-H3 and B7-H4 expression and $CD3^+$ and $CD8^+$ T-cell infiltration. High tumor B7-H3 mRNA expression level was associated with low tumor $CD3^+$, low stromal $CD3^+$, and low stromal $CD8^+$ T-cell infiltration in TNBCs. This correlation was also maintained in the analysis based on the subtype. High tumor B7-H3 mRNA expression level was associated with low stromal $CD3^+$ and low stromal $CD8^+$ T-cell infiltration in the BL, low stromal $CD3^+$ T-cell infiltration in the LAR, and low stromal $CD3^+$ and $CD8^+$ T-cell infiltration in the UN subtypes. High tumor B7-H3 protein expression level was also associated with low tumor $CD3^+$ and $CD8^+$ T-cell infiltration in TNBCs and the BL subtype. However, high stromal B7-H3 mRNA expression level in the UN subtype was associated with low stromal $CD3^+$ T-cell infiltration only. Tumor B7-H4 mRNA and protein expression did not correlate with $CD3^+$ and $CD8^+$ T-cell infiltration. Coexpression of tumor B7-H3 and B7-H4 expression did not increase the negative effect on $CD3^+$ and $CD8^+$ T-cell infiltration, when compared with expression of single protein.

Summary of Survival Analysis

Tumor size, lymph node status, stage, and $CD3^+$ and $CD8^+$ T-cell infiltration were significant risk factors affecting disease-free and/or overall survival of patients with TNBC in univariate analysis (Supplementary Table 3, Supplemental Digital Content 3, http://links.lww.com/AIMM/A331). When analyzing patients with TNBC as a whole and by subtypes, high stromal B7-H3 mRNA expression level was associated with poor disease-free and overall survival in the TNBCs ($P < 0.05$ and <0.01, respectively) and overall survival in the UN subtype ($P < 0.001$) (Supplementary Table 3, Supplemental Digital Content 3, http://links.lww.com/AIMM/A331, Figs. 5 and 6). Cox multivariate analysis demonstrated that stromal B7-H3 mRNA expression was independently associated with overall survival in the TNBCs and disease-free survival in the BL subtype, but not in the UN subtype (Supplementary Table 4, Supplemental Digital Content 4, http://links.lww.com/AIMM/A332, Tables 3 and 4).

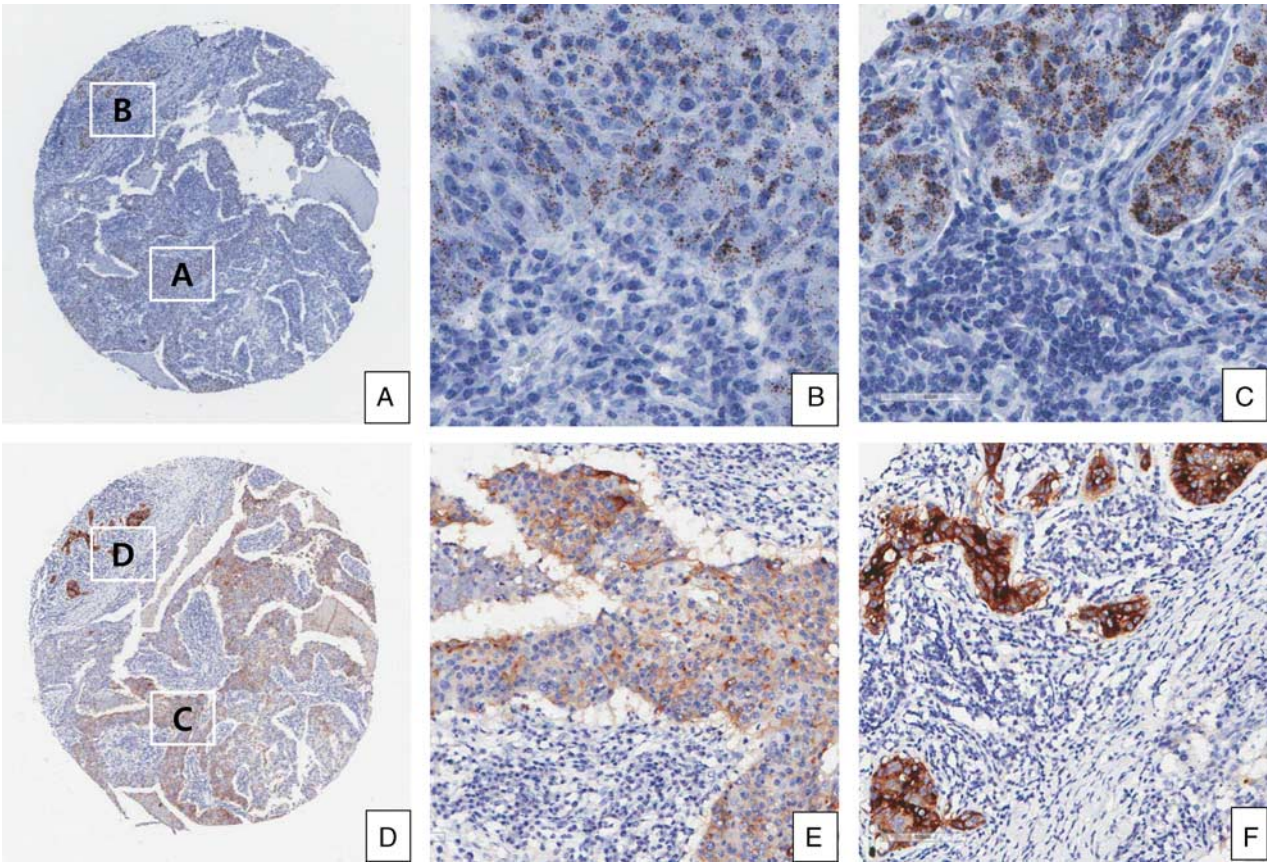

FIGURE 3. Expression and localization of B7-H4 mRNA (A–C), and B7-H4 protein (D–F) in a triple-negative breast cancer. B7-H4 mRNA and protein expression is observed only in cancer cells. Images B, E, C, and F are enlarged views of A–D, respectively. A, D ×2; B, C ×400; E, F ×200. full color online

DISCUSSION

The relationship between B7-H3 and B7-H4 expression in TNBC subtypes with respect to tumor immune surveillance and clinical outcomes is unknown. In this study, tumor expression of B7-H3 mRNA and protein was negatively correlated with $CD3^+$ and $CD8^+$ T-cell infiltration density in TNBCs and their subtypes. Moreover, stromal B7-H3 mRNA expression was independently associated with overall survival in the TNBCs and disease-free survival in the BL subtype.

Despite advances in breast cancer treatment, managing TNBC remains a challenge.[3] Since TNBC is a heterogeneous disease, considerable efforts have been made to categorize TNBC into subtypes that can be targeted in the clinic. On the basis of gene expression profiles, TNBC can be classified into 6 (BL 1, BL 2, immunomodulatory,

TABLE 2. Crosstabulation of B7-H3 and B7-H4 Status by mRNA RNAscope In Situ Hybridization and Protein Immunohistochemistry in Triple-negative Breast Cancers

	Immunohistochemistry					
RNAscope In Situ Hybridization	**Low**	**High**	**Total**	**Concordance**	**κ value**	***P***
B7-H3 tumor				58.9%	0.296	<0.001
Low	54	72	126			
High	0	49	49			
Total	54	121	175			
B7-H3 stromal				77.7%	0.280	<0.001
Low	123	30	153			
High	9	13	22			
Total	132	43	175			
B7-H4 tumor				82.8%	0.658	<0.001
Low	81	3	84			
High	27	63	90			
Total	108	66	174			

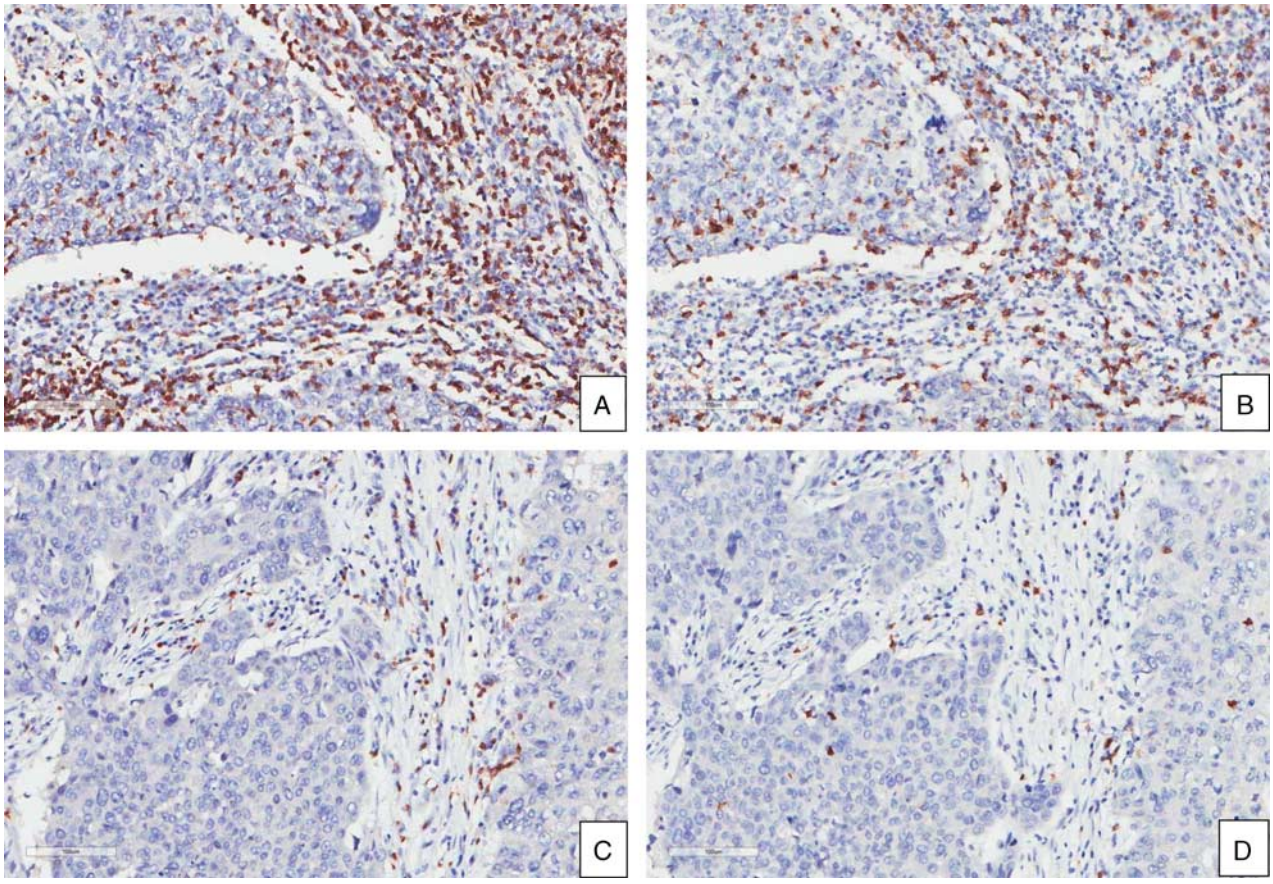

FIGURE 4. Immunohistochemical localization of $CD3^+$ (A, C) and $CD8^+$ (B, D) T cells in triple-negative breast cancers. $CD3^+$ and $CD8^+$ T cells are infiltrated in the tumor and stromal regions. A–D, ×200. full color online

mesenchymal, mesenchymal stem-like, and LAR types) or four (LAR, mesenchymal, BL immune-suppressed, and BL immune-activated) molecular subtypes.[21,22] Recently, Jiang et al classified TNBCs into four transcriptome-based subtypes replacing the BL immune-activated with immunomodulatory type.[24] Each TNBC subtype has a specific gene expression pattern and response to specific targeted therapy.[21,22,24]

From a practical standpoint, IHC surrogate panels could be a convenient alternative method for subtyping TNBCs. Kim et al subclassified TNBCs into 6 subtypes (LAR, AR-positive; mesenchymal, claudin-3-negative and/or E-cadherin-negative; BL, CK5/6-positive and/or EGFR-positive; mixed, tumors with features of 2 or 3 types; UN, tumors with none of the above features).[23] We excluded mesenchymal markers because no positive immunohistochemical markers can be classified as mesenchymal types. We used 3 basal-cell markers (CK5/6, CK14, and EGFR) and AR and categorized TNBCs into 3 subtypes: BL, LAR, and UN. Here, TNBCs were classified into BL (64.5%), LAR (10.8%), and UN (24.7%) subtypes. These findings are consistent with those of other studies.[23,31] In the report by Kim et al,[23] 22 cases (11.0%) of the LAR type were found in 200 TNBCs. In a study using 3 immunohistochemical basal makers (CK5, CK14, and EGFR), the BL type was observed in 63.6% of TNBCs.[31] Here, BL subtype was associated with younger age. Although it was not statistically significant, stromal TIL density was higher in the BL subtype than in the LAR subtype. TNBC subtypes did not significantly affect prognosis. This observation is consistent with that of a previous study.[23]

Immunotherapy has become an attractive strategy for TNBC treatment.[4,5] Immune checkpoints are regulators of the immune system and are important for self-tolerance and antitumor immunity. Therefore, immune checkpoint inhibitors can be targets for cancer immunotherapy.[5] B7-H3 and B7-H4, coinhibitory molecules of the B7 family, are attractive candidate targets that can be used along with existing therapies to overcome immunosuppression.[12,13] Several studies, including our previous study have explored the potential role of B7-H3 and B7-H4 in breast cancer including TNBC.[14–20,25] Expression levels of B7-H3 and B7-H4 mRNA and protein, as measured by various methods, are higher in breast cancer tissues than in normal breast tissues.[14,16,18] However, reports on the compartmentalization of B7-H3 and B7-H4 expression in breast cancers are inconsistent and often contradictory. Most studies have shown selective increase in B7-H3 or B7-H4 expression level in tumor cells only.[14–16,18,19] In contrast, B7-H4 protein expression has been reported in both tumor and stromal cells of breast cancer and the expression in the stroma cells did not

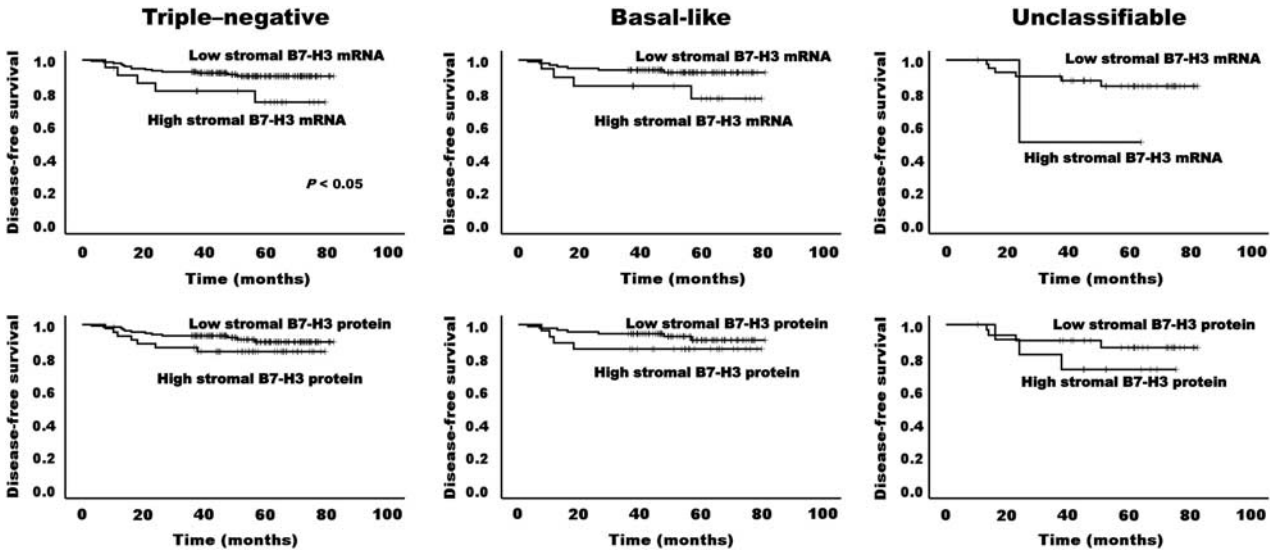

FIGURE 5. Disease-free survival of triple-negative breast cancer and its basal-like and unclassifiable subtypes grouped according to stromal B7-H3 mRNA and protein expression. The high stromal mRNA expression level shows a trend for decreased disease-free survival in patients with triple-negative breast cancer ($P < 0.05$).

appear specific for particular cell type.[17] Lee et al[20] found B7-H3 and B7-H4 expression in stromal TILs. The reason for this inconsistency is unclear but can be explained by differences in methodology, scoring systems, and protein expression cutoff levels.

In our previous and present study, we performed ISH and IHC to investigate the expression and location of B7-H3 and B7-H4 mRNA and protein in breast cancer including TNBCs. RNAscope ISH technology maintains morphologic features and can detect and quantify mRNA expression at a single cell level with high sensitivity and specificity.[25,30] B7-H3 mRNA and protein were expressed in the tumor and stromal cells. In stromal region, B7-H3 mRNA was mainly observed in the endothelial cells. Conversely, B7-H4 mRNA and protein were expressed only in the tumor cells. Although B7-H3 in the stromal region is mainly expressed in immune cells or fibroblasts,[20,33,34] vascular endothelial cells, which are stromal cells, can also highly express B7-H3,[35–38] as shown in our study. Expression of B7-H3 and B7-H4 proteins was closely correlated with that of their mRNAs according to the tumor compartment. Our ISH and IHC data

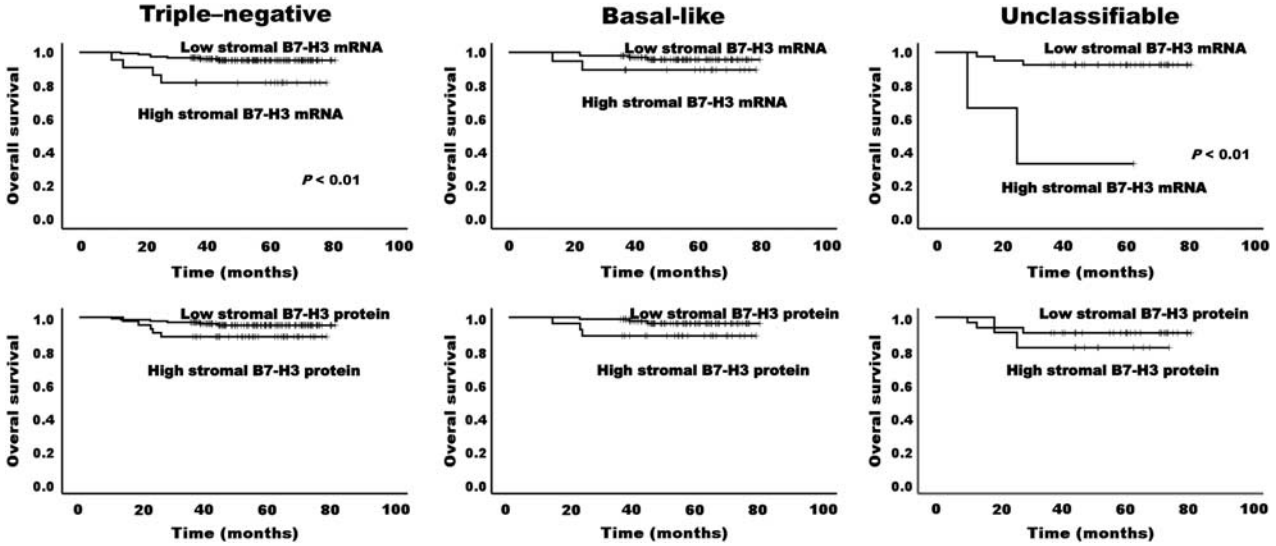

FIGURE 6. Overall survival of triple-negative breast cancer and its basal-like and unclassifiable subtypes grouped according to stromal B7-H3 mRNA and protein expressions. When Kaplan-Meier survival curves for overall survival are plotted, poor prognoses are observed for patients with high stromal mRNA expression level in triple-negative breast cancer and the unclassifiable subtype ($P < 0.01$ and <0.001, respectively).

TABLE 3. Multivariate Analysis With Cox's Proportional Hazard Model for Prognostic Factors in Patients With Basal-like Subtype

	Disease-free Survival			Overall Survival		
	HR	95% CI	*P*	HR	95% CI	*P*
Age ($\leq$52 vs. >52 y)	1.626	0.622-9.417	0.202	1.649	0.475-35.541	0.199
Tumor size ($\leq$2 vs. >2 cm)	0.000	0.112-8.585	0.987	0.718	0.255-31.405	0.397
Lymph node status (negative vs. positive)	2.962	0.033-1.249	0.085	0.349	0.031-6.476	0.554
Stage (I/II vs. III)	0.003	0.063-18.712	0.955	0.580	0.007-8.681	0.446
Stromal B7-H3 mRNA expression (low vs. high)	4.595	0.036-0.862	0.032	1.396	0.020-2.644	0.237
Tumor $CD3^+$ T-cell infiltrate (low vs. high)	0.004	0.000-6.415E+106	0.948	0.003	0.000-3.866E+108	0.957
Tumor $CD8^+$ T-cell infiltrate (low vs. high)	0.002	0.000-1.168E+107	0.961	0.002	0.000-1.087E+108	0.966
Stromal $CD3^+$ T-cell infiltrate (low vs. high)	0.008	0.000-2.898E+107	0.928	0.005	0.000-4.361E+131	0.944
Stromal $CD8^+$ T-cell infiltrate (low vs. high)	0.007	0.000-2.970E+98	0.935	0.003	0.000-2.057E+123	0.953

CI indicates confidence intervals; HR, hazard ratios.

confirmed that B7-H3 and B7-H4 expression in TNBCs is primarily regulated at the transcriptional level, although the mechanisms underlying their expression remain unclear.

Reports on the prognostic significance of B7-H3 and B7-H4 expression in breast cancer are conflicting.[15–18,20,25] High or over expressed levels of B7-H3 and B7-H4 in tumor cells were associated with poor prognosis.[15,16,18] However, report by Altan et al[17] showed no association between B7-H4 expression in either tumor or stromal cells and survival. Recently, Lee et al[20] reported that the expression of B7-H3 in stromal TILs was associated with poor prognosis. In contrast, the expression of B7-H4 in stromal TILS was associated with a favorable prognosis.[20] In our previous study, neither B7-H3 nor B7-H4 protein expression in tumor cells was associated with disease-free and overall survival.[25] The prognostic effect based on the expression of B7-H3/B7-H4 in stromal cells was not investigated.

Little is known regarding the clinical impact and prognostic or predictive value of B7-H3 and B7-H4 in TNBC subtypes. Here, we evaluated the prognostic significance of B7-H3/B7-H4 mRNA and protein expression in tumor cells and stroma. The patients with TNBC having high stromal B7-H3 mRNA or protein expression levels showed worse disease-free and overall survival than those having low stromal B7-H3 mRNA or protein expression levels. These trends were also found in the BL and UN subtypes. However, only the stromal B7-H3 mRNA expression showed statistical significance. The high stromal B7-H3 mRNA expression level was significantly associated with poor disease-free and overall survival in the TNBCs and overall survival in the UN subtype. Stromal B7-H3 mRNA expression was independently associated with overall survival in the TNBCs and with disease-free survival in the BL subtype. The absolute numbers of LAR and UN subtypes included in this study were relatively small compared with the BL subtype. Considering the potential prognostic value of stromal B7-H3 mRNA expression in patients with LAR and UN subtype, further studies in larger cohorts of LAR and UN cases with a longer follow-up period are needed.

Similar to our findings, B7-H3 expression in stromal endothelial cells was associated with aggressive clinical behavior in ovarian and renal cell carcinomas.[35,38] In renal cell carcinoma, B7-H3 expression in either tumor or endothelial cells was significantly associated with an increased risk of death.[38] Although B7-H3 expression in ovarian carcinoma cells did not correlate with prognosis, B7-H3 expression in the endothelium correlated with a high-grade serous histologic subtype, increased recurrence, and significantly decreased survival.[35]

These results indicate that the expression of B7-H3 in stromal cells, especially in endothelial cells, plays an important role in TNBC progression. Angiogenesis is an essential process for tumor growth and metastasis. It is not currently known which signals trigger B7-H3 expression in tumor endothelial cells; however, mounting evidence suggests that B7-H3 significantly influences the process of

TABLE 4. Multivariate Analysis With Cox's Proportional Hazard Model for Prognostic Factors in Patients With Unclassifiable Subtype

	Disease-free Survival			Overall Survival		
	HR	95% CI	*P*	HR	95% CI	*P*
Age ($\leq$ 52 vs. >52 y)	0.011	0.154-8.088	0.915	1.263	0.006-5416235.963	0.261
Tumor size ($\leq$ 2 vs. > 2 cm)	0.000	0.000-9.836E+148	0.999	0.000	0.000-1.119E+137	0.999
Lymph node status (negative vs. positive)	0.062	0.169-9.894	0.803	0.916	0.000-150.396	0.339
Stage (I/II vs. III)	0.004	0.000-1.2636E+143	0.952	0.001	0.000-1.173E+135	0.976
Stromal B7-H3 mRNA expression (low vs. high)	0.020	0.000-2.413E+54	0.886	1.642	0.000-253.171	0.200
T $CD3^+$ T-cell infiltrate (low vs. high)	0.004	0.000-3.503E+117	0.950	0.000	0.000-1.794E+194	0.996
T $CD8^+$ T-cell infiltrate (low vs. high)	0.004	0.000-1.113E+110	0.947	0.000	0.000-2.240E+176	0.986
Stromal $CD3^+$ T-cell infiltrate (low vs. high)	0.009	0.000-1.113E+101	0.924	0.011	0.000-1.306E+125	0.915
Stromal $CD8^+$ T-cell infiltrate (low vs. high)	0.006	0.000-2.358E+92	0.936	0.000	0.000-3.483E+166	0.996

CI indicates confidence intervals; HR, hazard ratios.

tumor angiogenesis. B7-H3 promotes tumor angiogenesis by recruiting vascular endothelial growth factor and matrix metalloproteinases to tumor lesions.[39,40] These properties allow B7-H3 to be a highly selective target for destroying tumor vasculature.

B7-H3 and B7-H4 are important immune molecules regulating the function of T cells in tumorigenesis.[12,13] B7-H3 appears to downregulate T-cell proliferation and activation.[12] B7-H4 is also a T-cell coinhibitory molecule and can function as a negative regulator of $CD8^+$ T-cell activation, expansion, and cytotoxicity.[13] However, studies on the relationship between TILs including $CD8^+$ T-lymphocyte and B7-H3/B7-H4 expression in breast cancer are few, and results are inconclusive. Altan et al[17] reported contradictory results on the correlation between TILs and B7-H4 according to the patient study set. TILs showed weak inverse correlation with B7-H4 expression in the University of Michigan cohort and weak positive correlation with B7-H4 expression in the Yale cohort.[17] Gruosso et al[19] integrated spatial resolution of immune cells with gene expression profiles using laser capture microdissection in TNBC. Expression levels of B7-H3 and B7-H4 were elevated in tumors with low $CD8^+$ T-cell expression level.[19]

In our previous study, B7-H3 and B7-H4 expression levels in tumor cells of breast cancer were negatively correlated with stromal $CD3^+$ and $CD8^+$ T-cell infiltration density.[25] Here, B7-H3 mRNA and protein expression levels were associated with $CD3^+$ and $CD8^+$ T-cell infiltration based on the tumor and/or stromal region. Expression levels of B7-H3 mRNA and protein in the tumor cells were negatively correlated with $CD3^+$ and $CD8^+$ T-cell infiltration density in the tumor and/or stromal region of the TNBCs and their subtypes. The high stromal B7-H3 mRNA expression level in the UN subtype was associated only with low stromal $CD3^+$ T-cell infiltration level. However, B7-H4 expression did not correlate with $CD3^+$ and $CD8^+$ T-cell infiltration. Our results indicate that B7-H3 expression in tumor cells of TNBCs can trigger tumor evasion from immunosurveillance through the suppression of T-lymphocyte infiltration, particularly that of cytotoxic $CD8^+$ T lymphocytes. Previous studies have also reported that B7-H3 overexpression in tumor cells is associated with reduced tumor infiltrating $CD8^+$ lymphocytes in various carcinomas. Guo et al[33] determined that B7-H3 expression in tumor cells of gastric neoplasia and adenocarcinoma was negatively correlated with CD8 + lymphocytes. Brustmann et al[36] also revealed that B7-H3 expression in tumor and endothelial cells of cervical squamous cell carcinoma was inversely related to TILs and $CD8^+$ TILs.

In this study, high tumor B7-H3 mRNA and protein expression in the BL subtype showed low $CD3^+$ and $CD8^+$ T infiltration. However, statistical significances were not synchronous. High tumor B7-H3 mRNA expression level was associated with low stromal $CD3^+$ and low stromal $CD8^+$ T-cell infiltration. In contrast, high tumor B7-H3 protein expression level was associated with low tumor $CD3^+$ and $CD8^+$ T-cell infiltration. The crosstalk between B7-H3 expression and various other factors could potentially affect the creation of an immunosuppressive tumor microenvironment in the BL subtype. Further studies are needed to elucidate this possibility.

Recently, B7-H3 has affected various T cells in the regulation of T-cell-mediated immune responses against cancer. Inamura et al discovered that B7-H3 expression was positively correlated with the density of tumor infiltrating $FOXP3^+$ regulatory T cells, which help tumor cells evade immunosurveillance.[38] For targeting B7-H3 in TNBC treatment, the correlation between B7-H3 expression in TNBCs and other immune cells, including $FOXP3^+$ regulatory T cells, should be examined.

In conclusion, B7-H3 mRNA and protein expression in tumor cells was negatively correlated with $CD8^+$ T-lymphocyte infiltration in TNBCs and its subtypes. Stromal B7-H3 mRNA expression was independently associated with poor overall survival in the TNBCs and with poor disease-free survival in the BL subtype. Considering the immunoinhibitory roles of B7-H3, our results indicate that this molecule is an attractive immunotherapy target for TNBCs, especially for the BL subtype.

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