# B7-H3 expression in ductal and lobular breast cancer and its association with IL-10

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Received June 6, 2012; Accepted September 20, 2012

DOI: 10.3892/mmr.2012.1158

**Abstract.** Aberrant tumor cell expression of B7-H3, a member of the B7-family that stimulates interleukin-10 (IL-10) secretion, contributes to tumor immune evasion and tumor progression. The aim of this study was to investigate the expression of B7-H3 and IL-10 in ductal and lobular breast cancer tissues. Using immunohistochemistry, B7-H3 and IL-10 protein expression in tumor specimens of primary human breast cancer was investigated. The association between B7-H3 or IL-10 expression and clinicopathological variables was analyzed. The correlation between the expression of B7-H3 and IL-10 was also evaluated. In tumor tissues, the expression of B7-H3 and IL-10 was identified on the cell membrane and in the cytoplasm. Expression of B7-H3 was observed in 90.60% (106/117) of the specimens and 80.34% (94/117) expressed IL-10. Patients with a positive B7-H3 or high IL-10 expression were more likely to have positive lymph node metastasis (N1-3; P=0.018 or 0.035, respectively) and advanced disease (stage II-IV; P=0.011 or 0.039, respectively) compared to those with a negative or low expression. Furthermore, B7-H3 expression was correlated with IL-10 in tumor cells (R=0.545, P=0.000). High B7-H3 expression in human breast cancer tissues may be important in tumor progression and invasiveness. This expression appeared to be correlated with the ability of B7-H3 to promote IL-10 secretion.

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*Key words:* B7-H3, immunohistochemistry, breast cancer, interleukin-10

## Introduction

Breast cancer is one of the most common female malignancies. Despite the use of a wide range of adjuvant treatment options, including radiotherapy, conventional chemotherapy with cytotoxic antitumor agents alone or in combination with endocrine therapy, bisphosphonates and HER-2/neu-directed therapy, over 400,000 females are predicted to succumb to breast cancer worldwide each year (1). Thus, new therapeutic strategies for breast cancer should be identified where viable treatment options are not successful.

A defect in the immune response may contribute to tumor growth. T-cell co-stimulation is essential for the initiation of an immune response. Lack of expression of CD80 and CD86 on tumor cells is considered to be one reason for immune evasion (2,3). However, inhibitory co-stimulatory factors may be involved in the pathogenesis by inducing T-cell anergy in two ways: inducing inhibitory cofactor expression in effector immune cells or expression of ligands of inhibitory cofactors on tumor cells. Blocking the interaction of inhibitory cofactors is a new strategy in tumor therapy. Therefore, manipulation of inhibitory costimulatory molecules may be critical for the control of an immune response.

The B7 protein family provides stimulatory and inhibitory regulation of T-cell responses, depending on which B7 ligand or receptor is engaged on the target (4,5). B7-H3 is a previously identified member of the B7 family. It was initially identified as a co-stimulatory molecule. However, studies suggest a role of B7-H3 in the inhibition of T-cell response (6-8). Ling *et al* (8) demonstrated that immunoglobulin-V-like and immunoglobulin-C-like (VC) and VCVC forms of human B7-H3 inhibited $CD4^+$ T-cell proliferation and downregulated cytokine production upon TCR activation *in vitro*. Using B7-H3-deficient mice, Prasad *et al* (6) demonstrated an enhanced lung inflammatory infiltration by macrophages and lymphocytes. B7-H3 mRNA, but not protein expression, was detected in a wide range of normal somatic tissues (9,10). However, expression of the cell surface B7-H3 may be induced on monocytes and dentritic cells (DCs) by interferon-γ (9).

B7-H3 protein has modest biological activities associated with T-lymphocyte proliferation and enhanced interleukin-10 (IL-10) secretion (11). IL-10 is a multifunctional cytokine and has marked immunosuppressive effects (12,13).

In breast cancer patients, IL-10 has been reported to be overexpressed in sentinel lymph nodes of breast cancer and associated with specific tumor markers of poor prognosis (14,15). However, it is currently unclear whether IL-10 production by tumor cells is relevant to the tumor-associated B7-H3 expression.

To gain insight into the mechanism of breast cancer progression, this study examined the expression of B7-H3 and IL-10 in ductal and lobular breast cancer specimens. The association between B7-H3 or IL-10 expression and clinicopathological characteristics was then analyzed. To explore the possible mechanism underlying this association, the correlation between B7-H3 and IL-10 in tumor cells was also investigated.

## Materials and methods

*Patient characteristics*. Tumor specimens were obtained from 117 patients with primary breast cancer who underwent surgery at the Clinical Hospital of Shandong University (Shandong, China) between 2008 and 2010. The mean age of patients at the time of diagnosis was 51 years (range, 21-77). There were 97 invasive ductal and 20 lobular carcinomas. Patients were classified as American Joint Committee on Cancer pathological stage I (33 cases), stage II (52 cases) or stage III (32 cases). All patients provided written informed consent for the use of specimens and the study was approved by the Institutional Review Board.

Information on estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER-2/neu) status was collected from original surgical pathological reports and records of surgery, in-patient medical records, chest X-ray films, whole-body computed tomography (CT) films and bone scanning films were also reviewed.

*Immunohistochemistry*. Immunohistochemical staining was performed using the biotin-streptavidin-peroxidase method with a Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA, USA). Resected tissue specimens were fixed in formalin, embedded in paraffin, cut into 4-$\mu$m serial sections and then mounted on glass slides. Slides were deparaffinized with xylene and dehydrated in graded alcohol. Following this procedure, the antigen was retrieved through heating in a microwave for 2 min at 900 W and the antigen was incubated with 0.3% $H_2O_2$ solution in methanol for 30 min to block endogenous peroxidase. Slides were then washed three times with PBS and incubated in 10% normal horse serum to block nonspecific background staining. Sections were incubated with the primary antibodies in a humid chamber at 4˚C overnight. Mouse anti-B7-H3 (diluted 1:60; R&D Systems, Minneapolis, MN, USA) and anti-IL-10 (diluted 1:100; BiosPacific, Emeryville, CA, USA) were used as primary antibodies. Following additional washing with PBS, sections were incubated with biotinylated-horse anti-mouse antibodies for 30 min, washed three times with PBS and then incubated with streptavidin-conjugated peroxidase for 30 min. Sections were visualized by incubation with 3,3'-diaminobenzidine solution (0.3% hydrogen peroxide and 0.05% 3,3'-diaminobenzidine) and counterstained with hematoxylin. Negative controls were carried out by substituting a normal mouse IgG for the primary antibody.

*B7-H3 and IL-10 expression*. Histological analysis was performed simultaneously by two investigators using a double-headed light microscope without knowledge of the patients' clinical records. B7-H3 or IL-10 expression was defined as the percentage of tumor cells exhibiting immunoreactivity in the cytoplasm or on the cell membrane and calculated by counting the number of B7-H3- or IL-10-stained tumor cells among 1,000 tumor cells in each section. Cell counts were performed at x400 magnification, in at least 5 fields, in randomly selected tumor areas. The intensity of the positive cells for B7-H3 and IL-10 was also graded semiquantitatively according to the positive cell percentage: 0, expression <10%; +, 10-40%; ++, 40-80%; +++, >80%. Specimens were classified into two groups based on the staining intensity consisting of a negative group (expression <10%) and a positive group (expression 10-100%).

*Statistical analysis*. Correlation between the expression of B7-H3 or IL-10 and clinicopathological variables was analyzed using Fisher's exact or the $\chi^2$ test, where appropriate. Spearman correlation coefficient was used to determine the association between B7-H3 and IL-10 expression. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analyses were performed using SPSS 16.0 (IBM).

## Results

*B7-H3 and IL-10 expression in ductal and lobular breast cancer tissue*. B7-H3 was expressed in 106 of 117 specimens and IL-10 was expressed in 94 of 117 specimens (90.60% and 80.34%, respectively). In the majority of cases the expression pattern of B7-H3 and IL-10 in tumor cells appeared to be extremely diffuse throughout the section. Microscopically, B7-H3 and IL-10 were identified in the tumor cell membrane, cytoplasm, or both (Fig. 1A, C, E and G). Expression of IL-10 was also observed in infiltrating lymphoid cells, while little staining of B7-H3 was identified in the stromal cells. Distribution of the B7-H3 and IL-10 staining intensity in all specimens is demonstrated in Figs. 2 and 3.

*Correlation between B7-H3 or IL-10 expression and clinicopathological factor*. Table I shows a comparison of the clinical pathological factors with B7-H3 or IL-10 positive and negative expression groups. Patients with positive B7-H3 or high IL-10 expression (focal expression of the staining ≥40%) were more likely to have lymph node metastasis ($P=0.018$ for B7-H3 and $P=0.035$ for IL-10) and advanced disease (stage II-IV; $P=0.011$ for B7-H3 and $P=0.039$ for IL-10) compared to those with negative B7-H3 or low IL-10 expression (focal expression of the staining <40%). IL-10 expression was higher in larger tumors (>2 cm) and HER-2/neu-positive groups, but did not approach significance. No significant correlation was identified between age, histological types, differentiation, ER or PR status and the expression of B7-H3 or IL-10.

*Correlation between the expression of B7-H3 and IL-10*. To explore the possible mechanism underlying the association between B7-H3 and tumor progression, we analyzed whether B7-H3 expression was correlated with IL-10 levels. The results indicated a positive correlation between B7-H3 and IL-10 expression ($R=0.545$, $P=0.000$) (Fig. 4).

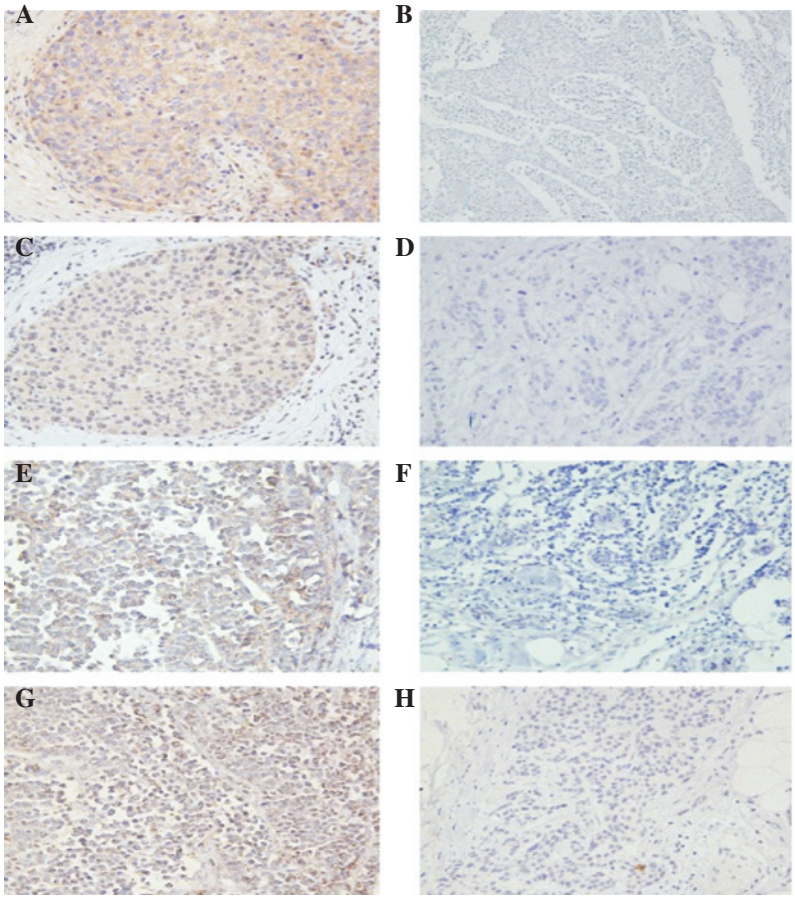


Figure 1. Sections from ductal and lobular breast cancer stained immunohistochemically for B7-H3 and interleukin-10 (IL-10). (A) B7-H3- and (C) IL-10-positive cancer cells with grade +++ (original magnification, x200) in ductal breast cancer. (B) B7-H3- and (D) IL-10-negative cancer cells (original magnification, x200) in ductal breast cancer. (E) B7-H3- and (G) IL-10-positive cancer cells with grade +++ (original magnification, x200) in lobular breast cancer. (F) B7-H3- and (H) IL-10-negative cancer cells (original magnification, x200) in lobular breast cancer. IL-10, interleukin-10.

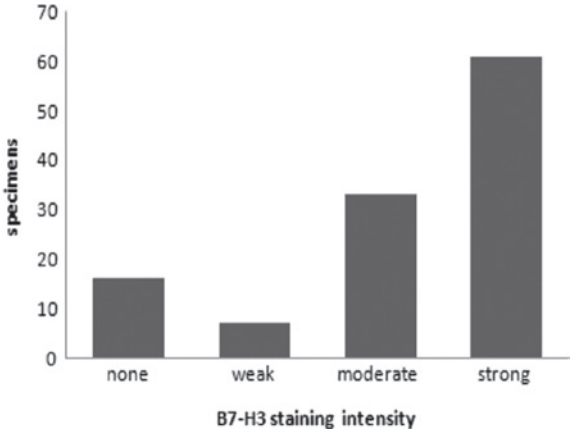


Figure 2. Distribution of B7-H3 staining intensity in ductal and lobular breast cancer tissues.

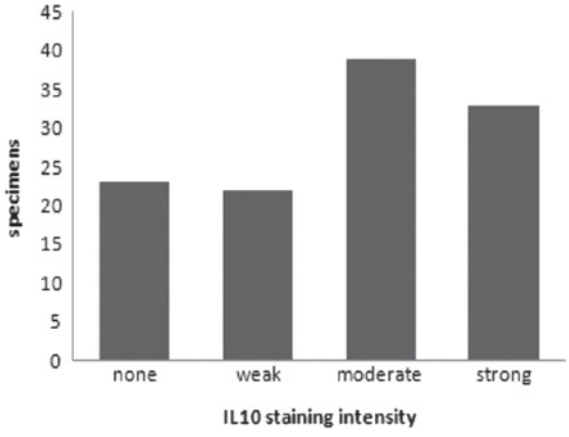


Figure 3. Distribution of interleukin-10 (IL-10) staining intensity in ductal and lobular breast cancer tissues.

## Discussion

Numerous therapeutic modalities are available for adjuvant treatment of advanced breast cancer, including radiotherapy, conventional chemotherapy with cytotoxic antitumor agents, hormone therapy and signal-transduction inhibitors (16). However, a number of patients respond poorly to existing therapeutic modalities. Thus, new molecular targets are required for the development of novel therapeutic strategies for the treatment of breast cancer.

B7-H3 is a transmembrane glycoprotein and a member of the B7 family of proteins, previously known as an immunoregulatory molecule, demonstrated to be inducible in macrophages and DCs (6). More recently, it was identified to

Table I. Correlation between B7-H3 or IL-10 expression in tumor cells and clinicopathological factors.

| | | B7-H3 expression | | | IL-10 expression | | |
|---|---|---|---|---|---|---|---|
| Clinicopathological factors | No. | Positive | Negative | P-value | Positive | Negative | P-value |
| Age (years) | | | | | | | |
| ≤50 | 63 | 56 | 7 | 0.543 | 34 | 29 | 0.854 |
| >50 | 54 | 50 | 4 | | 28 | 26 | |
| Histological cancer types | | | | | | | |
| Ductal | 97 | 89 | 8 | 0.397 | 53 | 44 | 0.432 |
| Lobular | 20 | 17 | 3 | | 9 | 11 | |
| Differentiation | | | | | | | |
| Well | 22 | 19 | 3 | 0.431 | 10 | 12 | 0.483 |
| Moderate and poorly | 95 | 87 | 8 | | 52 | 43 | |
| Tumor size (cm) | | | | | | | |
| ≤2 | 50 | 42 | 8 | 0.053 | 21 | 29 | 0.061 |
| >2 | 67 | 64 | 3 | | 41 | 26 | |
| Lymph nodes | | | | | | | |
| N0 | 43 | 35 | 8 | 0.018[a] | 17 | 26 | 0.035[a] |
| N1-3 | 74 | 71 | 3 | | 45 | 29 | |
| Pathological stage | | | | | | | |
| I | 33 | 26 | 7 | 0.011[a] | 12 | 21 | 0.039[a] |
| II-III | 84 | 80 | 4 | | 50 | 34 | |
| Biological markers | | | | | | | |
| ER | | | | | | | |
| Positive | 82 | 73 | 9 | 0.502 | 40 | 42 | 0.225 |
| Negative | 35 | 33 | 2 | | 22 | 13 | |
| PR | | | | | | | |
| Positive | 78 | 69 | 9 | 0.332 | 38 | 40 | 0.239 |
| Negative | 39 | 37 | 2 | | 24 | 15 | |
| HER-2/neu | | | | | | | |
| Positive | 30 | 25 | 5 | 0.147 | 11 | 19 | 0.055 |
| Negative | 87 | 81 | 6 | | 51 | 36 | |

ER, estrogen receptor; PR, progesterone receptor; HER-2/neu, human epidermal growth factor receptor 2; IL-10, interleukin-10. [a]P<0.05.

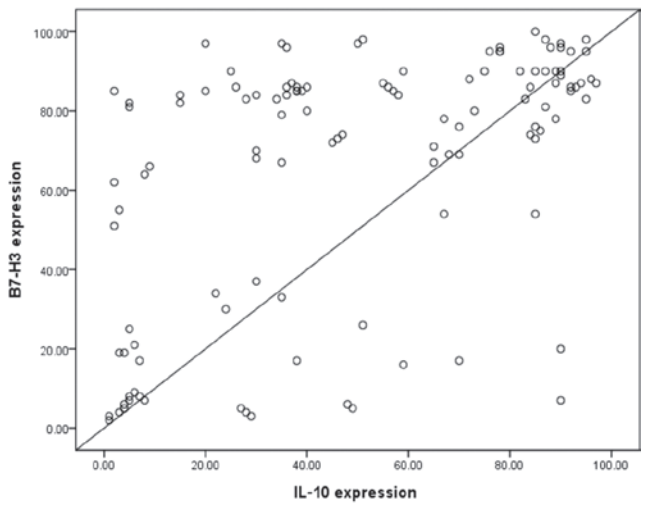


Figure 4. Correlation between B7-H3 and interleukin-10 (IL-10) expression. IL-10, interleukin-10.

be of clinical significance in various types of cancer. In certain tumor types, high expression of B7-H3 has been linked to poor prognosis (17-19), whereas in other cancer types the opposite effect has been observed (20,21). IL-10 is a pleiotropic cytokine produced by Th2 cells. Several studies have indicated that IL-10 is present in the tumor site and suggest that it mediates immunosuppression (13). A significant correlation between the expression of IL-10 and specific poor prognostic factors in human breast carcinomas has been observed (14,15). The present study has examined B7-H3 and IL-10 expression in ductal and lobular breast cancer tissues. Immunohistochemical staining of B7-H3 and IL-10 in tumor cells revealed elevated cell surface and cytoplasmic staining. Our results have demonstrated that B7-H3 and IL-10 expression in breast cancer was associated with important prognostic factors associated with lymph node metastasis and tumor stage. B7-H3 expression was correlated with IL-10 in the tumor cells, which may be responsible for the clinical significance of B7-H3 expression.

The mechanism accounting for the expression of B7-H3 in tumor cells remains unknown. However, expression of other inhibitory costimulatory factors has previously been described in various tumor cells. For example, a high expression of B7-H1, B7-H4 and ILT4 has been documented in certain malignant carcinomas (17,22,23).

A common mechanism may exist in regulating the over-expression of the inhibitory factors in tumor cells. Studies have indicated that expression of some inhibitory costimulatory factors in tumor-related macrophages and DCs are upregulated by environmental IL-10 of cancer (24,25). These observations suggest that the cytokine microenvironment induces expression of those inhibitors in tumor environmental cells. Expression of B7-H3 and IL-10 on breast cancer cells in the present study expands the information on immune inhibition.

The role of B7-H3 expression in malignant transformation or tumor progression has not been determined. B7 family members and their receptors are known to regulate antigen-specific immune response through inhibition of T-cell activation, cytokine secretion and the development of cytotoxicity (4-6). Extensive laboratory and histopathological data indicate that T-cell immune reactivity is a favorable prognostic indicator in non-metastatic breast cancer and the suppression of cell-mediated immunity may be critically involved in breast cancer progress (12-14). B7-H3 protein has modest biological activities associated with the proliferation of T lymphocytes and enhanced IL-10 secretion (11). Expression of IL-10 in tumors has been associated with immunosuppression of a Th1 response and increased tumorigenicity (15). In addition, animals receiving xenotransplants of B16 melanoma cells demonstrate increased tumor growth following intralesional injection of IL-10 (26). These immunosuppressions may allow tumor cells to lodge and facilitate the growth of metastasis. Thus, it is likely that tumor B7-H3 contributes to breast carcinoma aggressiveness by promoting excessive IL-10 secretion, leading to a strong immunosuppressive effect.

In summary, our findings suggest for the first time that B7-H3 expression is correlated with IL-10 in tumor cells. This suggests a new mechanism by which breast carcinoma escapes immune defenses. Recognition of this new mechanism of tumor evasion is likely to necessitate a new approach to the design of B7-H3-based immunotherapy.

## Acknowledgements

This study was partially sponsored by the Department of Science and Technology of Shandong Province.

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