

TRAIL DEATH RECEPTOR-4 EXPRESSION POSITIVELY CORRELATES WITH THE TUMOR GRADE IN BREAST CANCER PATIENTS WITH INVASIVE DUCTAL CARCINOMA

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Purpose: Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) selectively induces apoptosis in cancer cells but not in normal cells, and a number of clinical trials have recently been initiated to test the safety and antitumoral potential of TRAIL in cancer patients. Four different receptors have been identified to interact with TRAIL: two are death-inducing receptors (TRAIL-R1 [DR4] and TRAIL-R2 [DR5]), whereas the other two (TRAIL-R3 [DcR1] and TRAIL-R4 [DcR2]) do not induce death upon ligation and are believed to counteract TRAIL-induced cytotoxicity. Because high levels of DcR2 expression have recently been correlated with carcinogenesis in the prostate and lung, this study investigated the importance of TRAIL and TRAIL receptor expression in breast cancer patients with invasive ductal carcinoma, taking various prognostic markers into consideration.

Methods and Materials: Immunohistochemical analyses were performed on 90 breast cancer patients with invasive ductal carcinoma using TRAIL and TRAIL receptor-specific antibodies. Age, menopausal status, tumor size, lymph node status, tumor grade, lymphovascular invasion, perineural invasion, extracapsular tumor extension, presence of an extensive intraductal component, multicentricity, estrogen and progesterone receptor status, and *CerbB2* expression levels were analyzed with respect to TRAIL/TRAIL receptor expression patterns.

Results: The highest TRAIL receptor expressed in patients with invasive ductal carcinoma was DR4. Although progesterone receptor-positive patients exhibited lower DR5 expression, *CerbB2*-positive tissues displayed higher levels of both DR5 and TRAIL expressions.

Conclusions: DR4 expression positively correlates with the tumor grade in breast cancer patients with invasive ductal carcinoma. © 2007 Elsevier Inc.

Breast cancer, TRAIL, DR4, Apoptosis.

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer in women and represents 18% of all cancers (1). Unfortunately, breast cancer is also the leading cause of death in women 40 to 50 years of age. Breast cancer typically is a disease of the postmenopausal phase, and its incidence increases with age; among older women 70% to 80% of breast cancers are invasive ductal carcinoma, and involvement of the axillary lymph nodes worsens the prognosis (2). Because breast cancer exhibits lymphogen and hematogen distribution pattern, it

is considered a systemic disease from its onset (3). Furthermore, age, grade, histopathologic and nuclear grade of the tumor, estrogen and progesterone receptor expression, and *HER2/neu* gene amplifications are important prognostic parameters and commonly determine treatment type (4). For example, when tumor size is <2 cm, axillary lymph node involvement is 33%, which increases to 60% in patients with tumors >4 cm (5). Although the 5-year survival rate of a patient with one affected lymph node is ~63%, the rate decreases to 47% if the patient has five affected lymph nodes. Moreover, an increase in the number of positive lymph nodes

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increases the local recurrence rate from 33% to 54% (6); even if no lymph node involvement is detected, the mortality rate is still ~20% to 30% and increases to 70% to 90% with lymph node involvement. In addition to mastectomy or breast-conserving surgery, radiotherapy, chemotherapy, and hormonal therapies are used to prevent local recurrence and increase overall survival. Despite these treatment options, patients often experience serious adverse effects such as nausea, vomiting, skin reactions, and cardiac and pulmonary complications. Thus, novel treatment methods, such as gene therapy, are needed to treat patients with breast cancer.

One novel treatment method currently under clinical investigation is a recombinant adenovirus encoding human tumor necrosis factor (TNF)-related apoptosis-inducing ligand (Ad5hTRAIL) (7). Although this vector induces apoptosis in a variety of cancer cell lines (8–10) and synoviocytes from patients with rheumatoid arthritis (11), there remain a number of cancer types tested, including breast (MCF7), that are TRAIL resistant (12). Because one of the possible mechanisms for TRAIL resistance is high DcR2 surface expression, it is important to assess TRAIL receptor expression on breast cancer tissue before initiating TRAIL gene therapy. Moreover, the contribution of TRAIL and TRAIL receptor expression on breast cancer development is unknown. Thus, this study examined the expression of TRAIL and all four TRAIL receptors in 90 patients with invasive ductal carcinoma using immunohistochemistry to assess their relationship to other prognostic factors and potentially predict the feasibility of TRAIL gene therapy as a treatment.

METHODS AND MATERIALS

Evaluation of patients with breast cancer

Patients were selected from a cohort of 133 consecutive patients with nonmetastatic invasive ductal carcinoma treated at the Department of Radiation Oncology at the Akdeniz University Faculty of Medicine, Antalya, Turkey, between 2000 and 2005; written informed consent was obtained from all patients before treatment. All patients were uniformly staged using physical examination, complete blood count, blood chemistry, chest radiography, abdominal ultrasonography, and bone scan. Of these 133 patients, 90 (67.6%) had paraffin-embedded breast cancer tissue samples available for examination and immunohistochemical staining, as well as information on various characteristics such as age, menopausal status, tumor size, axillary lymph node involvement, histologic grade, lymphovascular invasion (LVI), perineural invasion, extracapsular extension (ECE), presence of an extensive intraductal component (EIC), multicentricity, estrogen and progesterone receptor status, *CerbB2* (*HER2/neu*) oncogene status, and adjuvant radiotherapy and/or systemic treatment.

All tumor sections were stained initially with hematoxylin/eosin and examined for the histologic typing based on the World Health Organization system. Based on the tumor histology, all patients displayed invasive ductal cell carcinoma. Histopathologic grading was performed using a semi-quantitative method described by Elston and Ellis (13). The extent of the intraductal component was assessed as a percent area of the whole tumor (invasive + intraductal) (14). The LVI was identified microscopically and confirmed by occasional immunohistochemical staining for CD34 (15–18). Extracapsular

tumor extension was analyzed pathologically (19–22). Perineural invasion was spotted by the pathologic examination of the tissue samples. Hormonal receptor status was revealed using antibodies specific for the estrogen receptor (RM9101 SP1, 1:100 dilution, Labvision) or the progesterone receptor (RM9102 SP2, 1:100 dilution, Labvision) (23). *CerbB2* expression was detected using immunohistochemistry (MS730 e-24001-3B5, 1:400 dilution, Labvision). Hormone receptors were evaluated based on nuclear staining, whereas *CerbB2* expression was determined by membranous staining pattern.

Local relapse was defined as a relapse within the breast or chest wall. Regional relapse was defined as a relapse in the ipsilateral axillary, supraclavicular, and internal mammary nodal areas. Distant metastasis was defined as the occurrence of tumors beyond the local or regional area. All patients were followed for relapse recurrence, distant metastasis, and survival status by clinical, biochemical, and radiologic studies. Patient follow-up consisted of physical examination, routine laboratory studies, chest radiographs, mammograms, and bone scans. Ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI) were not routinely ordered during the follow-up period but were performed when indicated to further evaluate the clinical findings consistent with the possible recurrence.

TRAIL/TRAIL receptor immunohistochemistry on breast cancer tissues

Immunohistochemistry for TRAIL and each of the TRAIL receptors was performed as recently described (24, 25). All primary antibodies were purchased from Alexis Biochemicals (Switzerland). Breast cancer tissues were stained using the following primary antibodies at 1/300 dilution: anti-human TRAIL mAb (III6F; ALX-804-326-C100), anti-human TRAIL-R1 mAb (HS101; ALX-804-297A-C100), anti-human TRAIL-R2 pAb (ALX-210-743-C200), anti-human TRAIL-R3 pAb (ALX-210-744-C200), and anti-human TRAIL-R4 mAb (HS402; ALX-804-299A-C100). Negative controls included samples that were stained only with the appropriate secondary Ab.

Immunohistochemical scoring of TRAIL/TRAIL receptor expression in patients with invasive ductal carcinoma of the breast

Three independent pathologists with no prior knowledge of the clinical data performed the specimen analysis. Both intensity and marker distribution (percentage of the positively stained epithelial cells) were used for the calculation of the scores in the breast cancer tissues. The intensity of the staining was scored as follows: 0 = negative; 1 = weak; 2 = moderate; and 3 = strong. Similarly, the marker distribution was scored as follows: 0 = less than 10% of the epithelial cells stained on the sections; 1 = 10% to 40%; 2 = 40% to 70%; and 3 = more than 70%. The final immune-staining score was given by adding the scores of both the intensity and the marker distribution for each patient.

Statistical analysis

The SPSS 13.0 software for Windows package (SPSS Inc., Chicago, IL) was used to perform the statistical analysis, as specified either in Results or in the figure legends. The following variables were considered for this analysis: age, menopausal status, tumor size, axillary lymph node involvement, histologic grade, lymphovascular invasion, presence of an extensive intraductal component, perineural invasion, extra capsular extension, multicentricity,

presence of *in situ* disease, estrogen, progesterone receptor status, HER-2 oncogene status, and adjuvant radiotherapy and/or systemic therapy. Statistical significance was considered at the 5% probability level ($p < 0.05$). Error bars for all data points in all figures represent the standard error of the mean (\pm SEM).

RESULTS

Clinical assessment of the breast cancer patients with invasive ductal carcinoma

At diagnosis, the median patient age was 43 (range, 34–81 years), and 50% (45 of 90) of women were premenopausal. All patients were pathologically staged according to the guidelines of 2002 American Joint Committee on Cancer (13, 26, 27), resulting in the following breakdown: 29 cases (32.2%) were T1, 49 cases (54.4%) were T2, 11 cases (12.2%) were T3, and 1 case (1.1%) was T4. In all, 65 patients (72.2%) had histopathologically confirmed lymph node metastasis, and the number of involved lymph nodes was less than four in 34 cases (37.7%), four to nine in 23 cases (25.5%), and more than nine in eight cases (8.8%). Lastly, 14 patients had stage I disease (15.5%), 19 had stage IIA disease (21.1%), 24 had stage IIB disease (26.6%), 23 had stage IIIA (25.5%) disease, 2 had stage IIIB disease (2.2%), and 8 had stage IIIC disease (8.8%).

Of these patients, 25 patients (27.7%) were treated with breast-conserving surgery (BCS), and 65 patients (72.2%) were treated with modified radical mastectomy and level I to II axillary dissection. Adjuvant chemotherapy was given to 79 patients (87.7%) with axillary lymph node metastasis or a tumor >1 cm. Overall, 79 patients (87.7%) received adjuvant chemotherapy, 11 patients (12.2%) received adjuvant hormonal therapy alone, and 79 patients (87.7%) received both types of systemic therapy sequentially. Indications for chemotherapy varied, depending on the protocols at the time of the treatment as well as patient and physician preferences. All patients underwent postoperative external beam radiation therapy (RT) as a component of their treatment. Radiotherapy was indicated mainly for one of the following criteria: more than four positive axillary nodes, extracapsular extension, and T3 tumor. The internal mammary region was not routinely irradiated, but it was irradiated in the group of patients with mediocentral quadrant tumor. For radiation treatment planning, a virtual computed tomographic simulation was performed for all patients, and the dose distribution was calculated using a computerized planning system. The whole breast or chest wall was irradiated by two tangential fields using 6-MV x-rays at a dose of 50.4 Gy in 5.5 weeks with a dose per fraction of 1.8 Gy. Additional boost doses of 10 Gy were administered to all BCS patients. In patients with >4 axillary lymph nodes involved, 50.4 Gy in 5.5 weeks was given to the regional lymph nodes. The median follow-up period was 39 months, and the patient mortality rate was 4.4% ($n = 4$) as revealed during the patient follow-up stage. During the follow-up, 4 patients developed bone metastasis and 1 patient had brain metastasis.

Distinctive expression profile of TRAIL and TRAIL receptors in breast cancer patients

Immunohistochemical staining of 90 breast cancer patients with invasive ductal carcinoma were analyzed in terms of TRAIL and TRAIL receptor expression, as described in Methods and Materials. Expression of DR4 was significantly higher compared with TRAIL, DR5, DcR1, and DcR2 expression (Fig. 1). Multiple statistical analyses were performed to determine the comparative expression pattern of TRAIL and TRAIL receptors in the patient samples. The Kalmogorov-Smirnov test demonstrated that expression patterns of TRAIL and TRAIL receptors in patients with invasive ductal carcinoma did not exhibit a Gaussian distribution; thus the Friedman test was used to document the statistical significance among the markers. The Wilcoxon signed rank test with Bonferroni's correction was also used to compare the groups in pairs. Based on these analyses, DR4 expression was the highest in breast cancer tissues (Fig. 2), which was followed by DcR2 expression. In contrast, DcR1 was expressed the lowest. Thus, we concluded that DR4 expression is the prominent TRAIL receptor type expressed in breast cancer patients with local invasive ductal carcinoma.

DR4 expression positively correlates with tumor grade but not with tumor stage

Breast cancer tumor staging and grading were performed, resulting in 9 cases (10%) having histopathologic low grade tumor, 38 cases (42%) were intermediate grade tumor, and 43 cases (47.7%) were high grade tumor. Spearman's rho correlation test was used to reveal any possible correlation between the expression of TRAIL and TRAIL receptors and tumor stage or grade. Based on this analysis, DR4 expression positively correlated only with the tumor grade but not with the tumor stage (Table 1; tumor-stage correlation data not shown). Interestingly, DR4 expression also correlated with DcR2, DR5, and TRAIL expression, but not with DcR1 expression.

Patients were next categorized into two groups based on the presence of an *in situ* component, multicentricity, perineural invasion, lymphovascular, and extracapsular involvement. Breast cancer tissue of 37 patients (41%) displayed an extensive intraductal component, and 18 patients (20%) had multicentric lesions. Perineural invasion was observed in 8 patients (8%), and lymphovascular invasion was present in 40 (44.4%). Of the 65 node-positive patients, extracapsular tumor extension was present in 28 (31.1%). Despite these results, the Mann-Whitney *U* test did not show any significant differences between TRAIL expression, expression of the TRAIL receptors, and the above-mentioned parameters valuable in the prognosis of the breast cancer (data not shown).

*Significance of TRAIL and TRAIL receptor expression with respect to expression of the hormone receptors or *CerbB2**

Patients were categorized into two groups based on the presence or absence of estrogen receptor (ER), progesterone receptor (PR), and *CerbB2* expression. The Mann-Whitney *U* test was administered to determine any statistical divergence

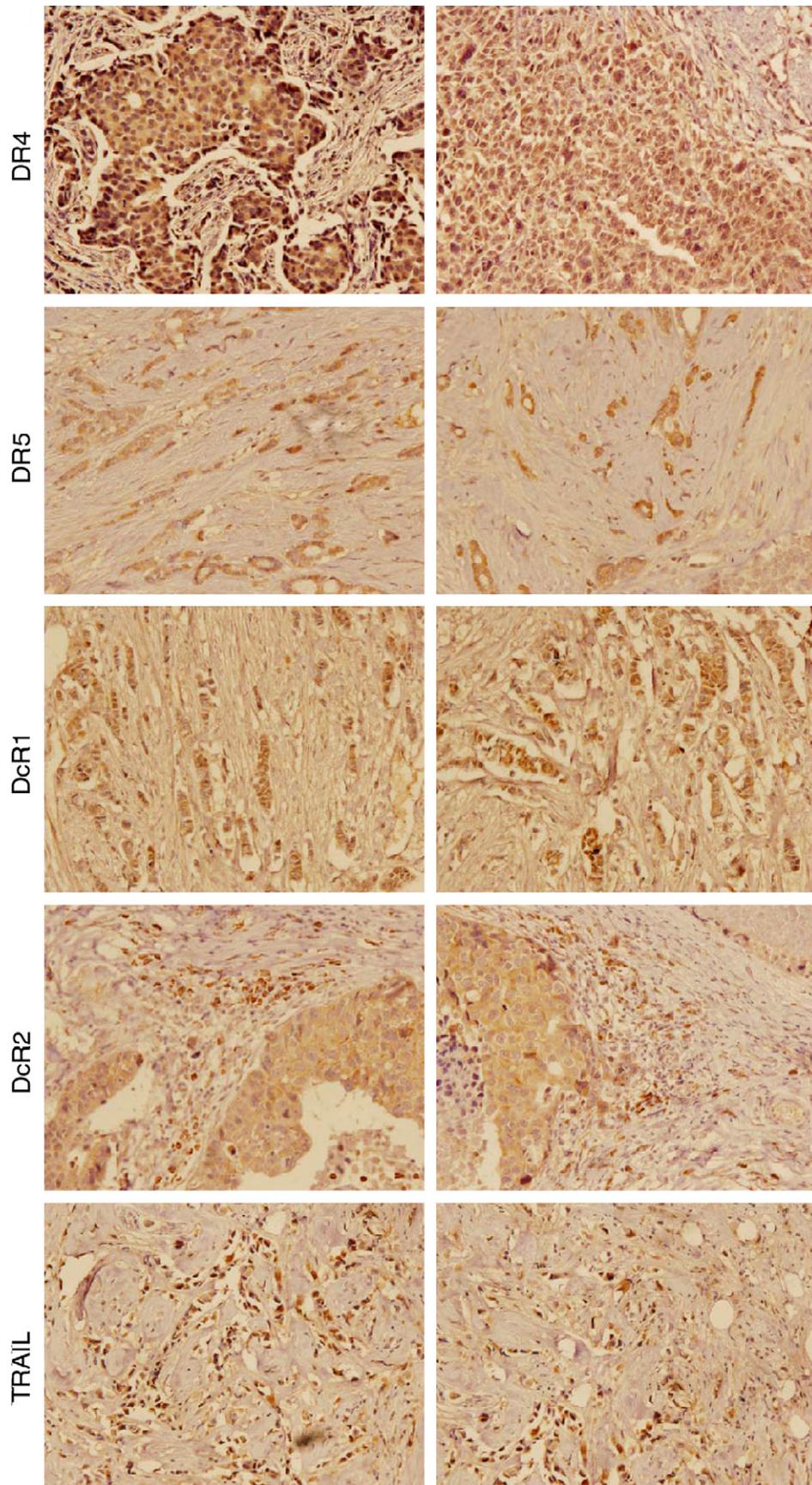


Fig. 1. Immunohistochemical analysis of invasive ductal breast carcinoma samples. Brown precipitate indicates positive staining on the sections. Duplicate samples from two different patients (left and right panels) are shown. Antibodies used for the staining are specific for the proteins listed to the left of each panel. Magnification = $\times 200$.

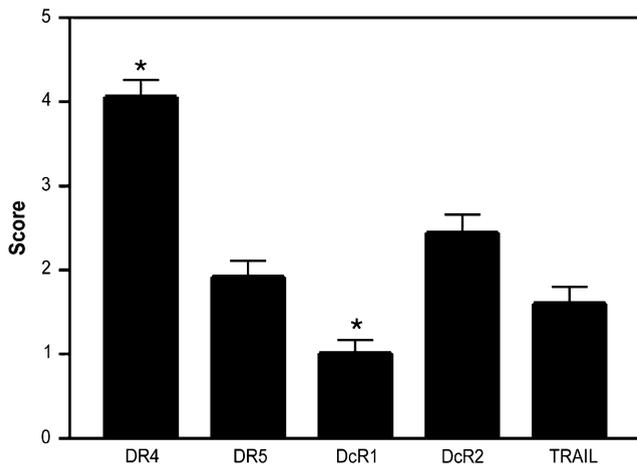


Fig. 2. Comparative analysis of tumor necrosis factor–related apoptosis inducing ligand (TRAIL) and TRAIL death receptor (DR4, DR5, DcR1, and DcR2) expression in breast cancer patients with invasive ductal carcinoma. Each bar represents the mean (\pm SEM) of 90 different breast cancer patients examined. Statistically significant differences (*) are based on Friedman test followed by the Wilcoxon signed rank test with Bonferroni's correction.

between TRAIL and TRAIL receptor expression versus ER, PR, or CerbB2. Breast cancer tissues of 61 patients (67.7%) were positive for ER expression and 62 patients (68.8%) were positive for PR expression. Although the presence of ER did not seem to influence the level of TRAIL and TRAIL receptor expression (data not shown), PR⁺ breast cancer tissue did manifest statistically lower amounts of DR5 expression than PR⁻ breast cancer tissue (Fig. 3). Furthermore, breast cancer tissues from 56 patients (62.2%) were positive for CerbB2 expression. Interestingly, CerbB2⁺ tissues manifested statistically higher levels of both DR5 and TRAIL expression compared with CerbB2⁻ tissues (Fig. 4).

Menopausal status differentially influences TRAIL and TRAIL receptor expression profiles in breast cancer patients with invasive ductal carcinoma

Interestingly, although half (45 of 90) of the breast cancer patients were classified as being premenopausal, the postmenopausal patients exhibited significantly higher TRAIL expression compared with premenopausal women, as demonstrated by the Mann-Whitney *U* test (Fig. 5). No such correlation was observed among the TRAIL receptors.

DISCUSSION

As malignant breast tumors exhibited altered levels of the death ligand expression (FasL and TRAIL) (28), death ligand, and cognate receptor expression profiles in patients with breast cancer have recently been assessed for their potential use as prognostic markers. For example, although Fas expression correlated with lymph node involvement and the number of recurrences, FasL expression was linked to the histologic grade of the tumor (29). In addition, several studies have found that the Fas/FasL status of breast cancer patients had a significant impact on clinical outcome (30–32). Despite these results, there have been few studies examining the expression of TRAIL and TRAIL receptors in cancer patients. Our group have recently reported the examination of the *in vivo* expression profiles of TRAIL and TRAIL receptors in prostate carcinoma (24, 33) or lung carcinoma (25) patients. Both studies demonstrated that DcR2 expression was the prominent TRAIL receptor expressed in these patient groups. Similarly, high DcR2 expression was detected in the MCF7 breast cancer cell line (12). In another study, the entire coding regions of the TRAIL receptors were analyzed to evaluate their involvement in breast carcinogenesis (34). Although no correlation

Table 1. Correlations of tumor necrosis factor–related apoptosis inducing ligand (TRAIL) death receptor (DR) expression with tumor grade in breast cancer patients (determined by Spearman's rho)

		Grade	DR4	DR5	DcR1	DcR2	TRAIL
Spearman's rho	Grade	1.000	0.208*	0.085	0.098	-0.006	0.123
	Significance (2-tailed)		0.049	0.423	0.357	0.955	0.248
	<i>N</i>	90	90	90	90	90	90
DR4	Correlation coefficient	0.208*	1.000	0.236*	0.171	0.283 [†]	0.291 [†]
	Significance (2-tailed)	0.049		0.025	0.106	0.007	0.005
	<i>N</i>	90	90	90	90	90	90
DR5	Correlation coefficient	0.085	0.236*	1.000	0.312 [†]	0.200	0.027
	Significance (2-tailed)	0.423	0.025		0.003	0.059	0.800
	<i>N</i>	90	90	90	90	90	90
DcR1	Correlation coefficient	0.098	0.171	0.312 [†]	1.000	0.098	0.240*
	Significance (2-tailed)	0.357	0.106	0.003		0.359	0.022
	<i>N</i>	90	90	90	90	90	90
DcR2	Correlation coefficient	-0.006	0.283 [†]	0.200	0.098	1.000	0.154
	Significance (2-tailed)	0.955	0.007	0.059	0.359		0.146
	<i>N</i>	90	90	90	90	90	90
TRAIL	Correlation coefficient	0.123	0.291 [†]	0.207	0.240*	0.154	1.000
	Significance (2-tailed)	0.248	0.005	0.800	0.022	0.146	
	<i>N</i>	90	90	90	90	90	90

* Correlation is significant at the 0.05 level (2-tailed).

[†] Correlation is significant at the 0.01 level (2-tailed).

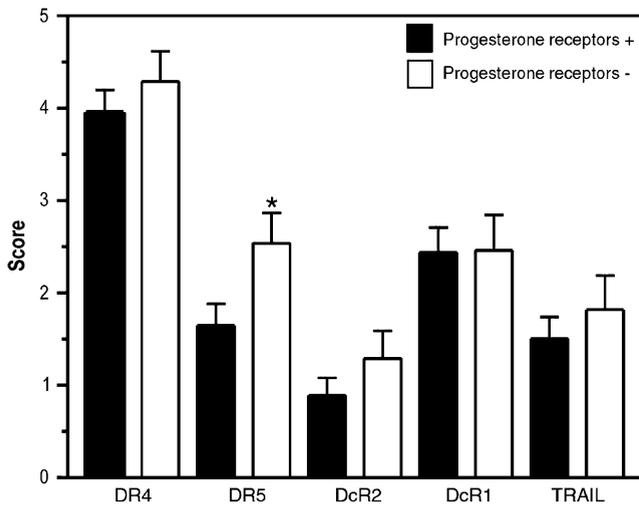


Fig. 3. Expression profile of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and TRAIL death receptor (DR4, DR5, DcR1, and DcR2) expression in breast cancer patients based on progesterone receptor status. Each column represents the mean (\pm SEM) of 62 progesterone receptor positive (PR⁺) patients or 28 progesterone receptor negative (PR⁻) patients with breast cancer. Statistical significance (*) was determined using the Mann-Whitney *U* test.

was found between TRAIL expression compared with clinical and histopathologic variables, this study did not exclude the involvement of TRAIL receptor expression in breast cancer pathogenesis. However, inactivating mutations of DR4 and DR5 were linked to breast cancer metastasis (35). Furthermore, 70% of the primary breast cancer samples exhibited aberrant methylation patterns in the TRAIL decoy receptor genes leading to gene silencing (36). Therefore, the goal of this study was to investigate the importance of TRAIL/TRAIL receptor expression profiles in breast cancer patients with locally invasive ductal carcinoma.

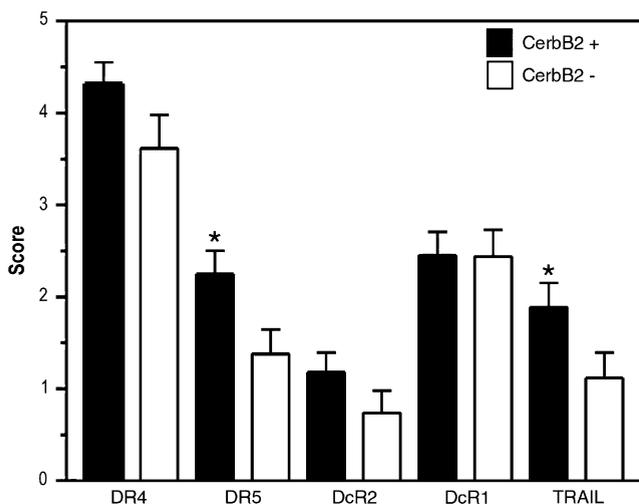


Fig. 4. Differential regulation of TRAIL and TRAIL death receptor (DR4, DR5, DcR1, and DcR2) expression in patients with breast cancer based on CerbB2 expression. Each column displays the mean (\pm SEM) of 56 CerbB2⁺ patients or 34 CerbB2⁻ patients with breast cancer. Statistical significance (*) was determined using the Mann-Whitney *U* test.

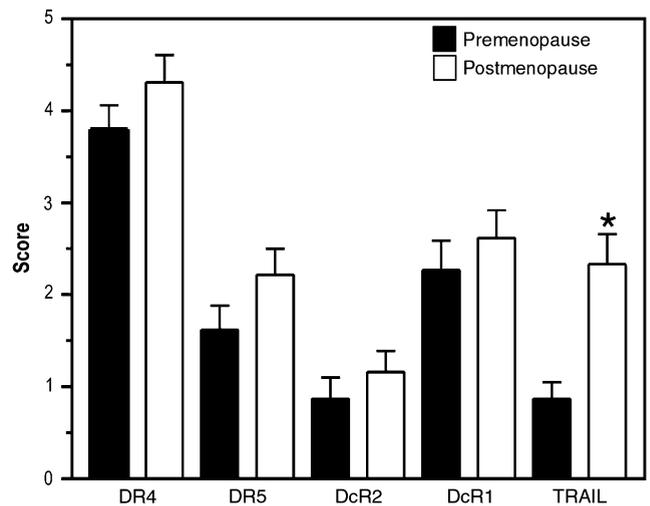


Fig. 5. Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and TRAIL receptor (DR4, DR5, DcR1, and DcR2) expression in premenopausal breast cancer patients versus postmenopausal women. Half of the breast cancer patients enrolled in the study ($n = 45$) were premenopausal, whereas the other half ($n = 45$) were postmenopausal. Statistical significance (*) was determined using the Mann-Whitney *U* test.

Contrary to our previous findings in patients with prostate or lung carcinoma, DR4 expression was the prominent TRAIL receptor expressed in 90 breast cancer patients. Although considerable levels of DcR2 expression were detectable, the level of expression was not statistically higher than DR5 or TRAIL expression levels. DcR1 expression, in contrast, was expressed to the lowest extent—a finding similar to that detected in prostate or lung carcinoma patients. Even though one of our previous studies investigating breast cancer cells (MDA-MB231) showed higher DR5 expression level compared with the other TRAIL receptors using flow cytometry (12), the current immunohistochemistry study demonstrated that DR4 was the prominent TRAIL receptor expressed in breast cancer patients. Moreover, tumor levels of the steroid hormone receptors (estrogen and progesterone) are very useful in the selection of an adjuvant treatment for early breast cancer stages (37). Despite the presence of ER, half of the patients will still not respond to the hormonal therapy. For example, patients with ER⁺/CerbB2⁺ metastatic breast cancer are less likely to respond to hormonal therapy and have shorter survival times than those patients with ER⁺/CerbB2⁻ status (38). Therefore, CerbB2 expression has been linked to a poor response to endocrine therapy in patients with recurrent or metastatic breast cancer (39, 40), making CerbB2 expression another parameter influencing the outcome of the breast cancer patients (41–43). In our study, both TRAIL and DR5 expression were significantly higher in CerbB2⁺ patients compared with CerbB2⁻ patients. Knowing that TRAIL-transduced dendritic cells protect mice from acute graft-versus-host disease and leukemia relapse through the suppression of antigen-specific T-cell activity (44), it is reasonable to predict that CerbB2⁺ breast cancer cells with high TRAIL expression might escape the cytotoxic

effects of the antitumoral immune response leading to a poor outcome.

Despite the fact that the ER status of patients is useful in the decision-making process of hormone therapy, PR status appears independently associated with the disease-free and overall survival among the endocrine-treated patients (45). Therefore, the presence of PR is considered to be a good prognostic marker in patients receiving hormonal therapy, whereas its absence is correlated with a poor response to therapy. In our study, the PR⁻ breast cancer patients exhibited significantly higher levels of DR5 expression compared with the other TRAIL receptors. Separately, the menopausal status of women is another prognostic factor affecting the outcome of breast cancer patients. Interestingly, postmenopausal breast cancer patients exhibited higher TRAIL expression compared with premenopausal breast cancer patients.

The biologic significance of this finding is not clear at this time and remains to be elucidated.

Histology-based tumor grading is one of the important parameters affecting the prognosis of the breast cancer patient (46–48). Compared with other TRAIL receptors, DR4 is the only marker that correlated with the tumor grade. Our analysis demonstrated that DR4 is the prominent TRAIL receptor expressed in breast cancer patients with invasive ductal carcinoma, and as DR4 expression increased, the tumor grade increased. This is a novel finding linking a TRAIL death receptor to the tumor grade in breast cancer patients. Given this correlation, DR4 expression might be important for the transition from a low-grade to a high grade tumor. In addition, because DR4 overexpression kills breast cancer cells irrespective of their TRAIL sensitivity (49, 50), DR4 might play an important role during breast carcinogenesis.

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