Characterization of type III TGF- β receptor expression in invasive breast carcinomas: a potential new marker and target for triple negative breast cancer

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Abstract Invasive breast carcinomas are heterogeneous and exhibit distinct molecular features and biological behavior. Understanding the underlying molecular events that promote breast cancer progression is necessary to improve treatment and prognostication. TGF- β receptor III (TBR3) is a member of the TGF- β signaling pathway, with functions in cell proliferation and migration in malignancies, including breast cancer. Recent studies propose that TBR3 may function as a tumor suppressor and that its loss may correlate with disease progression. However, there are limited data on the expression of TBR3 in breast cancer in relationship to tumor type, hormonal receptor status and HER-2/neu, and patient outcome. In this study, we investigated the expression of TBR3 in a cohort of 205 primary invasive breast carcinomas in tissue microarrays (TMAs), with comprehensive clinical, pathological and follow-up information. Sections were stained for TBR3 and evaluated for intensity of reactivity based on a 4-tiered scoring system (1 to 4; TBR3 low=scores 1–2; TBR3 high=scores 3–4). Of the 205 invasive carcinomas, 123 were luminal type (95 type A, 28 type B), 8 were HER-2 type, and 62 were triple negative (TN). TBR3 was high in 112 (55 %) and low in 93 (45 %) cases. Low TBR3 was associated with higher

histological grade and worse disease free and overall survival, all features of biologically aggressive breast carcinomas. TBR3 was significantly associated with the subtype of breast cancer, as low TBR3 was detected in 95 % of TN compared to 22 % of luminal tumors ($p<0.0001$). We discovered a significant association between low TBR3 protein expression, TN breast cancer phenotype, and disease progression. These data suggest that TBR3 loss might be linked to the development of TN breast cancers and pave the way to investigating whether restoring TBR3 function may be a therapeutic strategy against TN breast carcinomas.

Keywords TGF- β · TGF- β receptor III · TBR3 · TGFBR3 · Breast cancer · Luminal · Triple negative · Prognosis

Introduction

Invasive carcinomas of the breast constitute a group of heterogeneous tumors with distinct molecular features and different abilities to invade and metastasize. Expression profiling studies have allowed the categorization of breast carcinomas into different subtypes including luminal, HER2, and basal-like (Kittaneh et al. 2013; Rivenbark et al. 2013). The luminal tumors harbor the expression of estrogen receptor (ER) and progesterone receptor (PR) and are responsive to anti-hormonal treatment. HER2 tumors overexpress HER-2/neu and respond to anti-HER2 therapies. In contrast, basal-like tumors are often triple negative (ER-, PR-, HER2-) and are not candidates for effective targeted treatments (Diaz et al. 2007; Kim et al. 2006; Rakha et al. 2008a). Understanding the molecular pathways associated with basal-like triple negative breast carcinomas is thus necessary for developing more effective therapies.

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Table 1 Clinico-pathological characteristics of the 205 patients with invasive carcinoma

Characteristics	N (%)
Race	
White	178 (86.8)
Black	18 (8.8)
Other/Unknown	9 (4.4)
Menopausal Status	
Pre	59 (28.8)
Peri	16 (7.8)
Post	118 (57.6)
Unknown	12 (5.8)
Tumor Size (cm)	
≤ 2	63 (30.7)
> 2	131 (63.9)
Unknown	11 (5.4)
Tumor Grade	
I	26 (12.7)
II	80 (39.0)
III	75 (36.6)
Unknown	24 (11.7)
Lymph Nodes	
Negative	87 (42.4)
Positive	99 (48.3)
Unknown	19 (9.3)
Tumor Stage	
I	64 (31.2)
II	92 (44.9)
III	40 (19.5)
IV	1 (0.5)
Unknown	8 (3.9)
Lymphovascular Invasion	
Present	60 (29.3)
Absent	135 (65.8)
Unknown	10 (4.9)
Estrogen Receptor	
Positive	130 (63.4)
Negative	71 (34.6)
Unknown	4 (2.0)
Progesterone Receptor	
Positive	112 (54.6)
Negative	89 (43.4)
Unknown	4 (2.0)
HER2/neu Status	
Overexpressed	37 (18.0)
Not overexpressed	157 (76.6)
Unknown	11 (5.4)
TBR3 Expression	
1	45 (21.9)
2	48 (23.4)
3	66 (32.2)
4	46 (22.4)

Table 1 (continued)

Characteristics	N (%)
Breast Cancer Subtype	
Luminal A	95 (49.2)
Luminal B	28 (14.5)
HER2	8 (4.2)
Triple Negative	62 (32.1)
Adjuvant Hormonal Therapy	
Yes	101 (49.3)
No	86 (41.9)
Unknown	17 (8.3)
Radiation Therapy	
Yes	121 (59.0)
No	66 (32.2)
Unknown	17 (8.3)
Death of any cause	
Yes	74 (36.1)
No	131 (63.9)
Death from breast cancer	
Yes	29 (14.1)
No	176 (85.9)

Transforming Growth Factor- β (TGF- β) has an important role in both normal mammary tissue development and early mammary carcinogenesis. During breast development, TGF- β is a potent inhibitor of mammary epithelial proliferation and regulator of mammary ductal and alveolar development. In breast cancer, the TGF- β signaling pathway functions in a cell type and context specific manner. In early stages of cancer, it functions as a tumor suppressor. However, at later stages, TGF- β has tumor promoting effects by suppressing immune surveillance and enhancing angiogenesis, and by inducing cancer cell migration and invasion. The overall effect is increased tumor invasiveness and metastases and poorer prognosis for breast cancer patients (Walker and Dearing 1992; Dalal et al. 1993; Gorsch et al. 1992; Ghellal et al. 2000).

TGF- β receptor III (TBR3), or betaglycan, is the most abundant TGF- β superfamily coreceptor, and has been shown to modulate TGF- β signaling through mechanisms that are not fully elucidated (Dong et al. 2007; Gatza et al. 2010; Mythreye and Blobel 2009). Loss of TBR3 expression has been reported in multiple cancers, with loss of expression correlating with progression and worse prognosis (Dong et al. 2007; Gatza et al. 2010; Mythreye and Blobel 2009; Hanks et al. 2013; Gordon et al. 2008). In breast cancer, TBR3 inhibits cell migration and invasion, supporting a tumor suppressor function (Dong et al. 2007). TBR3 is located on chromosome 1p32 and loss of heterozygosity in this region in breast cancer has been associated with a poorer prognosis

Fig. 1 Expression levels of TBR3 protein in invasive carcinomas of the breast. Shown are representative pictures of four different invasive carcinomas stained with H&E and with anti-TBR3 antibody. Invasive carcinomas with scores 1 and 2 were designated as TBR3 Low, and those with scores 3 and 4 were TBR3 High

(Ragnarsson et al. 1999; Borg et al. 1992; Bieche et al. 1999). Although several studies have shown a role of TBR3 in breast carcinogenesis, there are limited studies evaluating the expression of TBR3 protein in light of clinical and pathological features of breast cancer. We undertook this study to define the expression patterns of TBR3 within breast cancer subtypes and its associations with clinical and pathological features of invasive carcinomas of the breast.

Materials and methods

Study population

Breast tissues from the Surgical Pathology files at the University of Michigan arranged in high density tissue microarrays (TMAs) were employed. Our cohort comprised 205 cases ($n=615$ tissue microarray elements). Clinical and outcome information on the 205 patients was obtained by chart review, with institutional review board approval. Patient characteristics including age, race, menopausal status, treatment, and clinical follow up (i.e. recurrence, death) as well as tumor characteristics including, size, nodal involvement,

hormone receptor status, and HER-2/neu expression were recorded.

Immunohistochemical studies

Formalin fixed, paraffin embedded tissue blocks of the TMAs were sectioned at 5 μ and placed on charged slides. Slides were deparaffinized in xylene and rehydrated through graded alcohols. Heat Induced Epitope Retrieval (HIER) was performed in the Decloaking Chamber (Biocare Medical) with Target Retrieval, (Dakocytomation). Slides were incubated in Peroxidazed (Biocare Medical) for 5 min to quench endogenous peroxidases. Slides were incubated for 1.5 h at room temperature with rabbit polyclonal anti-TGFbR3 (Novus Biologicals, Cat#27030002) diluted 1:1400. This antibody has been validated by Western blot and immunohistochemistry in our laboratory (Pal et al. 2012). The antibody was detected with anti-rabbit Envision⁺ HRP Labelled Polymer (DakoCytomation) for 30 min at room temperature. HRP staining was visualized with the DAB⁺ Kit (DakoCytomation). Negative control slides were run. Slides were counterstained in hematoxylin, blued in running tap

water, dehydrated through graded alcohols, cleared in xylene and then mounted with Permount.

TBR3 staining was evaluated at least three times for every tissue microarray element and at least nine times for each tumor by two pathologists (NV and CGK), blinded to tumor stage and clinical information. As observed previously (Pal et al. 2012), TBR3 protein was strongly expressed in the cytoplasm and cytoplasmic membrane of breast cancer cells. TBR3 expression was scored from 1 to 4 as follows: score 1 no staining, score 2 weak staining, score 3 moderate staining, and score 4 strong staining. Scores 3 and 4 were considered high and scores 1 and 2 were considered low TBR3 respectively.

Statistical analysis

We tabulated the association between TBR3 status and clinical and pathological characteristics and investigated the differences between TBR3 status groups using a *t*-test for age and chi-square test or Fisher's exact test for categorical data excluding the unknown category. The primary outcome was overall survival defined as the time from initial treatment to death from any cause. The secondary endpoint was disease-free survival defined as the time from initial treatment to the date of first progressive disease or death. Five and 10 year survival was estimated using the Kaplan-Meier method of various strata defined by clinical and pathological characteristics. The difference in survival was compared using the log-rank test. Cox proportional hazards models investigated the association between TBR3 status controlling for pathological and clinical characteristics. All analyses were conducted in SAS v9.3.

Results

Expression of TBR3 and associations between TBR3 expression with clinical and pathological features of invasive carcinomas

We identified a total of 205 primary invasive carcinomas. The median follow up was 7.83 years (range 3 months to 25.3 years). Clinical and pathological characteristics are summarized in Table 1. In our cohort of 205 invasive breast carcinomas TBR3 expression was high (scores 3 and 4) in 112 (55 %) and low (scores 1 and 2) in 93 (45 %) tumors. Figure 1 shows representative cases of TBR3 immunohistochemical staining. TBR3 was expressed in the cytoplasm and cytoplasmic membrane of breast cancer cells.

The association between TBR3 expression with clinical and pathological characteristics is shown in Table 2. We found that TBR3 expression is significantly associated with tumor size, tumor grade, ER, PR, HER2, and subtype of breast

Table 2 Association of TBR3 expression with clinical and pathologic characteristics

Characteristic	TBR3		<i>p</i> -value
	LOW (<i>n</i> =93)	HIGH (<i>n</i> =112)	
Age	53.5±11.5	56.4±13.3	0.10
Race			0.21
White	81 (87.1)	97 (86.6)	
Black	11 (11.8)	7 (6.3)	
Other/Unknown	1 (1.1)	8 (7.1)	
Menopausal Status			0.842
Pre	28 (30.1)	31 (27.7)	
Peri	7 (7.5)	9 (8.0)	
Post	54 (58.1)	64 (57.1)	
Unknown	4 (4.3)	8 (7.1)	
Tumor Size (cm)			0.0001
≤2	41 (44.1)	22 (19.6)	
>2	45 (48.4)	86 (76.8)	
Unknown	7 (7.5)	4 (3.6)	
Tumor Grade			<0.0001
I	1 (1.1)	25 (22.3)	
II	25 (26.9)	55 (49.1)	
III	60 (64.5)	15 (13.4)	
Unknown	7 (7.5)	17 (15.2)	
Lymphovascular Invasion			0.675
Present	30 (32.3)	30 (26.8)	
Absent	59 (63.4)	76 (67.9)	
Unknown	4 (4.3)	6 (5.4)	
Lymph Nodes			0.138
Negative	46 (49.5)	41 (36.6)	
Positive	38 (40.9)	61 (54.5)	
Unknown	9 (9.7)	10 (8.9)	
Tumor Stage			0.611
I	31 (3.3)	33 (29.5)	
II	39 (41.9)	53 (47.3)	
III	17 (18.3)	23 (20.5)	
IV	1 (1.1)	0 (0)	
Unknown	5 (5.4)	3 (2.7)	
Estrogen Receptor			<0.0001
Positive	27 (29.0)	103 (92.0)	
Negative	64 (68.8)	7 (6.2)	
Unknown	2 (2.2)	2 (1.8)	
Progesterone Receptor			<0.0001
Positive	20 (21.5)	92 (82.1)	
Negative	71 (76.3)	18 (16.1)	
Unknown	2 (2.2)	2 (1.8)	
HER2/neu Status			0.0003
Overexpressed	9 (9.7)	28 (25.0)	
Not overexpressed	83 (89.2)	74 (66.1)	
Unknown	1 (1.1)	10 (8.9)	
Adjuvant Hormonal Therapy			<0.0001
Yes	24 (25.8)	77 (68.8)	
No	63 (67.7)	23 (20.5)	
Unknown	6 (6.5)	12 (10.7)	
Radiation Therapy			0.48
Yes	57 (61.3)	64 (57.1)	
No	27 (29.0)	39 (34.8)	
Unknown	9 (9.7)	9 (8.0)	

cancer; this is the same as when TBR3 is kept as a 4-level variable. Invasive carcinomas with High TBR3 tend to be larger than those with low TBR3. However, tumors with low TBR3 are more likely to exhibit more aggressive pathological features including higher histological grade, a measure of the degree of tumor differentiation and poor prognostic indicator in breast cancer (Parker et al. 2009; Rakha et al. 2008b), and triple negative phenotype.

Based on these data, we hypothesized that low TBR3 protein expression may be associated with worse patient survival. At the univariate level histological grade, tumor stage and lymphovascular invasion were associated with disease-free and overall survival (Tables 3 and 4). Hormone receptor status was inversely associated with outcome. We found a significant association between TBR3 expression and survival. Specifically, low TBR3 was associated with a shorter

disease-free interval after initial surgical treatment as well as lower overall survival (Tables 3 and 4 and Fig. 2). The 10 year disease-free survival for patients with low TBR3 expressing tumors was 53.2 % compared to 65.2 % for high TBR3 expressing tumors ($p=0.038$, Fig. 2). Similarly, the 10 year overall survival for patients with low TBR3 expressing tumors was 57.5 % compared to 73.4 % for high TBR3 expressing tumors ($p=0.046$, Fig. 2). The best multivariate model of disease-free and overall survival only included tumor grade and stage.

TBR3 emerges as a biomarker of triple negative invasive carcinomas

We found that TBR3 expression was strongly associated with the expression of ER, PR, and HER-2/neu overexpression

Table 3 Univariate analysis of overall survival

Characteristic	5 year		10 year		Log-rank <i>p</i> -value
	Estimate	95 % CI	Estimate	95 % CI	
Menopausal Status					
Pre	77.7	64.0–86.7	65.7	50.3–77.4	0.938
Peri	87.1	57.3–96.6	79.1	47.9–92.8	
Post	83.7	75.3–89.4	64.9	54.2–73.7	
Tumor Size (cm)					
≤2	78.9	65.7–87.4	61.9	46.2–74.2	0.021
>2	85.5	78.0–90.6	72.5	63.0–79.9	
Tumor Grade					
I/II	87.3	79.2–92.4	69.6	58.9–78.1	0.002
III	70.0	57.4–79.6	54.7	40.5–66.9	
Lymphovascular Invasion					
Present	69.5	55.6–79.8	46.6	32.5–59.6	0.010
Absent	86.7	79.5–91.5	75.8	66.8–82.8	
N Stage					
N0	82.7	72.8–89.2	71.6	59.8–80.5	0.080
N1	87.6	76.8–93.6	72.6	59.0–82.4	
N2/N3	74.0	54.6–86.0	59.3	39.3–74.7	
Tumor Stage					
I	83.2	71.0–90.6	76.0	61.7–85.5	0.008
II	87.2	78.1–92.7	72.9	61.3–81.6	
III or IV	70.8	53.4–82.6	52.1	34.3–67.2	
Estrogen Receptor					
Positive	84.7	77.1–90.0	68.6	59.2–76.2	0.130
Negative	74.8	62.1–83.8	63.0	47.2–75.3	
Progesterone Receptor					
Positive	87.8	79.9–92.7	71.7	61.6–79.6	0.018
Negative	72.8	61.6–81.2	59.5	46.3–70.4	
HER2/neu Status					
Overexpressed	80.6	63.5–90.2	68.0	49.5–80.9	0.874
Not Overexpressed	81.5	74.2–86.9	65.9	56.8–73.6	
TBR3 Expression					
Low (1,2)	73.9	63.1–82.0	57.5	45.0–68.1	0.046
High (3, 4)	87.8	79.9–92.7	73.4	63.3–81.1	

Table 4 Univariate analysis of disease-free survival

Characteristic	5 year		10 year		Log-rank <i>p</i> -value
	Estimate	95 % CI	Estimate	95 % CI	
Menopausal Status					
Pre	72.6	58.7–82.5	58.6	43.3–71.0	0.98
Peri	80.8	51.4–93.3	64.6	34.1–83.8	
Post	77.6	68.6–84.3	58.8	48.0–68.0	
Tumor Size (cm)					
≤2	72.3	58.7–82.1	55.2	39.9–68.1	0.059
>2	80.0	71.8–86.0	67.0	57.3–74.9	
Tumor Grade					
I/II	83.5	74.8–89.4	64.6	53.7–73.5	0.0006
III	58.7	46.0–69.5	46.9	33.2–59.5	
Lymphovascular Invasion					
Present	64.6	50.7–75.5	41.7	28.1–54.8	0.019
Absent	79.8	71.7–85.8	67.4	57.8–75.3	
N Stage					
N0	73.6	63.0–81.7	64.5	52.8–74.1	0.13
N1	84.6	73.3–91.4	64.7	50.3–76.0	
N2/N3	67.8	48.4–81.2	47.0	27.0–64.7	
Tumor Stage					
I	76.8	64.0–85.6	68.3	54.1–78.9	0.033
II	80.4	70.3–87.3	65.2	53.1–74.9	
III or IV	65.8	48.5–78.5	42.4	25.0–58.8	
Estrogen Receptor					
Positive	80.1	72.0–86.1	62.5	52.9–70.7	0.045
Negative	66.0	53.0–76.2	55.3	40.5–67.8	
Progesterone Receptor					
Positive	82.3	73.7–88.4	64.7	54.2–73.4	0.015
Negative	65.9	54.5–75.1	52.9	40.1–64.1	
HER2/neu Status					
Positive	72.2	54.5–84.0	54.7	35.7–70.3	0.54
Negative	76.3	68.6–82.4	61.1	52.0–69.1	
TBR3 Expression					
Low (1, 2)	66.1	54.9–75.1	53.2	41.1–63.8	0.038
High (3, 4)	83.3	74.8–89.1	65.2	54.6–73.9	

status in invasive carcinomas. Low TBR3 was significantly associated with negative ER and PR ($p < 0.0001$ for both receptors), and absence of HER-2/neu overexpression ($p = 0.0003$, Table 2). Our cohort of 205 invasive carcinomas included 95 luminal A, 28 luminal B, 8 HER2, and 62 triple negative tumors. Expression of TBR3 was significantly associated with the breast cancer subtype ($p < 0.0001$, Table 5). Of the 95 luminal A tumors, TBR3 was high in 71 (75 %) and low in 24 (25 %). Conversely, TBR3 protein was low in 59 of 62 (95 %) of TN carcinomas.

The association between low TBR3 and survival, as well as with hormone receptor status, prompted us to test whether low TBR3 may identify luminal tumors with aggressive behavior. We found that low TBR3 expression in luminal A and B tumors ($n = 123$) was associated with high histological grade ($p = 0.013$). Survival analysis of patients with ER positive

(luminal) tumors receiving adjuvant hormonal therapy suggested that patients with tumors expressing low TBR3 ($n = 12$) had worse overall survival than those expressing high TBR3 protein ($n = 15$; $p = 0.003$, Fig. 2).

Discussion

In this study, we comprehensively characterized the expression of TBR3, an understudied component of the TGF- β receptor family, in a cohort of invasive breast carcinomas with long term follow up information. Previous studies have demonstrated an important role for TBR3 in breast cancer progression (Dong et al. 2007). However, there is limited knowledge on TBR3 protein expression in human breast cancer

Fig. 2 TBR3 protein expression is associated with survival in patients with breast cancer. **a.** Tissue microarray elements containing representative invasive carcinomas with negative (1), weak (2), moderate (3), and strong (4) TBR3 staining intensities. Original magnification 100x. **b.** Low TBR3 expression in invasive carcinomas is associated with worse overall, disease-free, and with worse survival following hormonal therapy

tissues. The identification of TBR3 as a novel biomarker of the triple negative breast cancer and aggressive breast cancer phenotypes has the potential to assist in patient management.

Based on a cohort of histologically characterized 205 invasive breast carcinomas, we made several novel observations. We found that TBR3 protein expression is reduced in 45 % of invasive breast carcinomas, which is consistent with its reported tumor suppressor function during breast cancer progression (Dong et al. 2007). We discovered that low TBR3 protein expression is significantly associated with higher histological grade, a measure of tumor differentiation which takes into consideration nuclear pleomorphism, mitotic activity and glandular formation (Parker et al. 2009; Rakha et al. 2008b). Consistent with these data, we found that low TBR3 protein is significantly associated with reduced disease-free and overall survival up to 12 years after initial diagnosis of breast cancer. These results validate and further extend those of Dong et al. (Dong et al. 2007) who reported that low TBR3 messenger RNA expression was associated with a decrease in recurrence-free survival at 5 years by analyzing a publicly available microarray dataset of 286 tumors. Together, these data highlight the potential clinical relevance of detecting

TBR3 protein in breast cancer tissue samples and provide the rationale for further studies to validate its prognostic significance.

The identification of new biomarkers and potential targets for the treatment for patients with TN breast cancer are needed, and constitute a focus of intensive investigation. The present study identified that TBR3 expression is associated with the expression of ER, PR and HER-2/neu, and that low TBR3 is strongly correlated with the TN breast cancer subtype, as 95 % of TN carcinomas exhibited low TBR3 compared to 22 % of ER positive luminal tumors ($p < 0.0001$). The previously unconsidered association between low TBR3 expression and TN breast carcinomas provide solid rationale for investigating the functional and mechanistic role of TBR3 in the development of TN tumors. Mechanistic studies have demonstrated that TBR3 undergoes ectodomain shedding from the cell surface to generate a soluble form that sequesters TGF- β superfamily ligands and inhibits their downstream signaling (Dong et al. 2007; Hanks et al. 2013; Andres et al. 1989; Lopez-Casillas et al. 1994). Of note, treatment with soluble TBR3 inhibits breast cancer tumor growth, angiogenesis, and reduces metastases (Dong et al. 2007; Gatz et al. 2010; Bandyopadhyay et al. 2002). Despite important challenges, these data suggest the intriguing possibility that soluble TBR3 may be a promising agent to be used in anti-cancer therapy for patients with TN breast cancer for whom there are no effective targeted treatments (Diaz et al. 2007).

The association between low TBR3 and worse outcome, together with the discovery that low TBR3 is present in a small subset of ER positive luminal tumors led us to hypothesize that low TBR3 may identify ER positive tumors with more aggressive phenotype. Indeed, we found that in ER positive luminal tumors, low TBR3 was associated with higher histological grade ($p = 0.013$). Further, Kaplan Meier

Table 5 TBR3 expression by breast cancer subtype

Breast Cancer Subtype	TBR3 protein		<i>p</i> -value
	LOW (<i>n</i> =93)	HIGH (<i>n</i> =112)	
Luminal A	24 (25.8)	71 (63.4)	<0.0001
Luminal B	3 (3.2)	25 (22.3)	
Her2	5 (5.4)	3 (2.7)	
Triple Negative	59 (63.4)	3 (2.7)	
Unknown	2 (2.2)	10 (8.9)	

analyses revealed that patients with ER positive luminal tumors and low TBR3 expression had a worse outcome than those with high TBR3 expression after anti-hormonal therapy. We recognize that our study is limited by a small number of patients who received hormonal therapy and had available follow up information. Nonetheless, these preliminary data pave the way to investigating whether detection of TBR3 in luminal tumors may predict response to hormonal therapy in larger groups of patients.

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Conflict of interest None.

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