Validation of Intratumoral T-bet\(^+\) Lymphoid Cells as Predictors of Disease-Free Survival in Breast Cancer

Anna Marie Mulligan\(^1,2\), Dushanthi Pinnaduwage\(^3\), Sandrine Tchatchou\(^3\), Shelley B. Bull\(^3,4\), and Irene L. Andrulis\(^1,5,6\)

Abstract

We previously observed T-bet\(^+\) lymphocytes to be associated with a good prognosis in a cohort of women with familial breast cancer. To validate this finding, we evaluated lymphocytic T-bet expression in an independent unselected prospectively accrued series of women with lymph node–negative breast carcinoma. T-bet and clinicopathologic data were available for 614 women. Hormone receptors, HER2, Ki-67, CK5, EGFR, and p53 status were determined using IHC and/or biochemical methods. Tumors were assigned to luminal A, luminal B, HER2, and basal subtypes based on the expression of IHC markers. Multiple cutpoints were examined in a univariate penalized Cox model to stratify tumors into T-bet\(^+\)/high and T-bet\(^+\)/low. Fisher exact test was used to analyze T-bet associations with clinicopathologic variables, IHC markers, and molecular subtype. Survival analyses were by the Cox proportional hazards model. All tests were two sided. A test with a P value < 0.05 was considered statistically significant. T-bet\(^+\)/high tumor status was significantly associated with large tumor size, high grade, hormone receptor negativity, CK5, EGFR and p53 positivity, high Ki-67, and basal subtype. With a median follow-up of 96.5 months, T-bet\(^+\)/low tumor status was associated with a reduced disease-free survival compared with T-bet\(^+\)/high tumor status in multivariate analysis (P = 0.0027; relative risk = 5.62; 95% confidence intervals, 1.48–50.19). Despite being associated with adverse clinicopathologic characteristics, T-bet\(^+\) tumor-infiltrating lymphoid cells are associated with a favorable outcome. This supports their role in Th1-mediated antitumor activity and may provide insight for the development of new therapeutic strategies.

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Introduction

Breast cancer is a complex and heterogeneous disease with significant disparity in clinical outcomes still being seen, despite improvements in disease classification using tumor-related prognostic markers. More recently, attention has been placed on the components of the tumor microenvironment, including lymphocytic infiltration, whose interaction with the tumor can strongly influence the patient’s long-term outcome. The interplay between the immune system and cancer is not straightforward: tumor cells that can suppress antitumor immunity and play an essential role in both innate and adaptive immunity and are involved in tumor immune surveillance (2, 3). Early studies that examined the relationship between lymphocytic infiltration in tumors and outcome in breast cancer showed conflicting results, with some reporting a favorable association with outcome and a dense inflammatory infiltrate (4) and others identifying an adverse association (5). More recent results from large cohorts have demonstrated a good prognosis in patients whose breast cancers showed a marked lymphocytic infiltrate as well as an association with high response rates by such tumors to neoadjuvant therapy (4, 6–12). The apparently contradictory findings may reflect the diverse functional roles of the individual components of lymphocytic subtypes (13). While recognition of the individual immune cell subsets that consistently mediate favorable effects remains elusive, the current consensus is that CD4\(^+\) Th1 and CD8\(^+\) T cells are among the players that can generate effective although potentially attenuated antitumor responses while CD4\(^+\) Th2 cells and CD4\(^+\) Tregs are among the cells that can suppress antitumor immunity and can promote tumor progression (14–16).

T-bet (T-box transcription factor 21), an immune cell–specific member of the T-box family of transcription factors, is expressed in multiple cells of the innate and adaptive immune system [including dendritic cells, natural killer (NK) cells, CD4\(^+\) and CD8\(^+\) effector cells, B cells, and a subset of regulatory T cells], and its expression is required for the survival, development, and proper functioning of immune cells (17–23). In disease states, T-bet plays an important role in infectious and inflammatory...
conditions as well as in tumor progression: in the absence of T-bet, susceptibility to metastases from melanoma was shown to be increased because of impaired NK-cell function and survival in vivo (24). Similarly, in a murine model of lung adenocarcinoma, T-bet deficiency led to a marked induction of tumor load (25). More recently, T-bet has been shown to be required for promoting blockade-induced CD8+ T-cell effector responses sufficient to eradicate disseminated leukemia in an animal model (26). Furthermore, high numbers of T-bet+ intratumoral lymphoid cells have been found to correlate with improved outcome in gastric cancer (27) and in high-grade cervical intraepithelial neoplasia (28).

In a cohort of women with familial breast cancer from the Ontario site of the Breast Cancer Family Registry (29), studied for expression of chemokine CXCL10 and tumoral lymphocytic infiltration, we observed that T-bet+ lymphocytes were associated with the basal molecular subtype as well as with morphologic features characteristic of such tumors, including high-grade, p53 expression, ER negativity, CK5 positivity, and EGFR positivity (30). Despite the association with an aggressive phenotype, T-bet positivity was associated with a good prognosis. To confirm this finding in an independent unselected hospital-based cohort, we assessed the presence of T-bet+ immune cells in a large cohort of axillary node negative (ANN) breast cancer patients and determined the relationship between T-bet positivity and IHC biomarkers, clinicopathologic characteristics, molecular subtypes (luminal A, luminal B, HER2, and basal), and patient outcome. We hypothesized that intratumoral T-bet+ lymphoid cells would correlate with the basal subtype and would be associated with a more favorable outcome.

Materials and Methods
Patient cohort and clinical follow-up

The patient cohort comprised a prospectively accrued consecutive series of 1,561 women with lymph node–negative, invasive breast carcinoma enrolled at eight Toronto hospitals from September 1987 to October 1996, as previously described (31, 32). This included 887 women on whom paraffin-embedded tissue blocks were available for use in the construction of tissue microarrays (TMA). The characteristics of the whole cohort and TMA cohort have been reported previously (33). Written informed consent was obtained from all study participants.

We followed women in the cohort for recurrence and death. Disease-free survival (DFS) was taken as the time between diagnosis and the confirmation of non-breast recurrence. All patients were monitored for death whether or not they experienced disease recurrence. Using clinical follow-up data, patient status on January 10, 2002, determined DFS time and censoring status. Follow-up data were monitored for an additional 6 months to confirm patient status at the termination date. Excluding the patients lost to follow-up or with distant recurrence, the minimum follow-up time was 56 months after surgery and the median follow-up time was 100 months. Approval of the study protocol was obtained from the Research Ethics Boards of Mount Sinai Hospital (#01-0313-U; Toronto, Ontario, Canada) and the University Health Network (#02-0881-C; Toronto, Ontario, Canada).

Hormone receptor status, tissue microarray construction, and IHC staining

Estrogen receptor (ER) and progesterone receptor (PgR) status were determined biochemically at the time of surgery by ligand-binding assays of frozen tissue, which was the standard approach used at the time, and by IHC detection on the TMA. Formalin-fixed paraffin-embedded tumor blocks were available for 887 patients. Areas of invasive carcinoma were selected from an H&E-stained section of each tumor, and two 0.6-mm cores of tissue were taken from the corresponding areas of the paraffin block. The selected donor cores were embedded in a recipient paraffin block, and 4-µm sections were cut and immunohistochemically stained for ER, PgR, HER2, Ki-67, CK5, EGFR, p53, and T-bet, under the conditions described in Table 1. Microwave antigen retrieval was carried out in a Micromed T/T Mega Microwave Processing Lab Station (ESBE Scientific). Sections were developed with diaminobenzidine tetrahydrochloride and counterstained in Mayer’s hematoxylin.

Except for Ki-67 and T-bet, each of the IHC-stained sections was scored using the Allred scoring method (34). Nuclear staining was assessed for ER, PgR, and p53. Strong complete membrane staining was assessed for HER2. Membranous and/or cytoplasmic membrane staining was scored for CK5 and EGFR. The Ki-67 labeling index was determined based on the percentage of positive tumor nuclei, regardless of intensity of staining. In all, 50 nuclei were counted and therefore tissue microarray core samples with <50 tumor cells were deemed unsatisfactory, resulting in exclusion of 220 cases. Absolute counts of T-bet+ lymphoid cells were conducted, and these were categorized as intratumoral [when within the epithelial nests or within close proximity (the distance between positive lymphocyte and tumor nest is equal to or less than the size of one tumor cell)] or peritumoral (at a distance from the epithelial nests).

The raw score data were processed using a TMA deconvoluter software program into a format suitable for statistical analysis (35). As two cores from each tumor were assessed, the larger of the two values was chosen for use in statistical analysis to minimize the effect of false negatives on the array. For ER, PgR, HER2, and p53, cutpoints to define positivity were based on previous

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Dilution</th>
<th>Source</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td>6F11</td>
<td>1/75</td>
<td>Vector</td>
<td>Tris buffer (pH 9.0)</td>
</tr>
<tr>
<td>PgR</td>
<td>PgR 1294</td>
<td>1/1000</td>
<td>DAKO</td>
<td>Tris buffer (pH 9.0)</td>
</tr>
<tr>
<td>p53</td>
<td>D.07</td>
<td>1/400</td>
<td>ID Lab</td>
<td>Tris buffer (pH 9.0)</td>
</tr>
<tr>
<td>CK5</td>
<td>XM26</td>
<td>1/400</td>
<td>Vector</td>
<td>Tris buffer (pH 9.0)</td>
</tr>
<tr>
<td>HER2</td>
<td>CB11 TAB250 (cocktail)</td>
<td>1/300</td>
<td>Novocastra</td>
<td>Pepsin 10 minutes at 37°C</td>
</tr>
<tr>
<td>EGFR</td>
<td>31G7</td>
<td>1/25</td>
<td>Zymed</td>
<td>Pepsin 10 minutes at 37°C</td>
</tr>
<tr>
<td>Ki67</td>
<td>MiB1</td>
<td>1/300</td>
<td>DAKO</td>
<td>Citrate buffer (pH 6.0)</td>
</tr>
<tr>
<td>T-bet</td>
<td>4B10</td>
<td>1/100</td>
<td>Santa Cruz Biotechnology</td>
<td>Citrate buffer (pH 6.0)</td>
</tr>
</tbody>
</table>
validation studies (34, 36–39). For CK5 and EGFR, the cutpoint for positivity was arbitrarily specified as $\geq 4$. Ki-67 was dichotomized into Ki-67 high (Ki-67 labeling index $\geq 14\%$) or Ki-67 low (Ki-67 labeling index $< 14\%$; ref. 40). For T-bet, following the examination of multiple cutpoints, an absolute count of 30 positive intratumoral lymphoid cells (within or within close proximity of the epithelial cell nests) was used as the cutoff for positivity (T-bet $^{+/+}$). Tumors with lower levels or absence of T-bet $^{+/+}$ intratumoral lymphoid cells were considered as T-bet $^{-/-}$. Interpretable scores were obtained in 618 tumors. In four cases, clinicopathologic characteristics were unavailable, resulting in the inclusion of 614 patients in the final statistical analyses.

Definitions of intrinsic subtypes

Tumors from each group were assigned to molecular subtypes based on previous publications (41–44). Tumors that were positive for HER2 protein overexpression were assigned to the HER2 subtype. Tumors that were negative for HER2 but positive for ER were assigned to the luminal subtype. Tumors that were negative for HER2 and ER, and positive for CK5 or EGFR, were assigned to the basal subtype. The luminal subtype was subsequently subdivided into luminal A and luminal B based on PgR, p53, and Ki-67 labeling index. Tumors that had a Ki-67 labeling index $\geq 14\%$ and were negative for PgR or positive for p53 were assigned to the luminal B subtype (44).

Statistical analysis

Fisher exact test was used to analyze the T-bet marker associations with clinicopathologic variables, IHC markers (markers used to define molecular subtype), and molecular subtype. Clinicopathologic variables used in analyses represent traditional and/or known prognostic factors for node-negative breast cancer and were chosen based on the literature and on previous prognostic modeling we conducted in this cohort (31–33, 44). Analyses of the association of DFS with T-bet marker status were conducted by the univariate Cox proportional hazards model. Adjustment of the minimum $P$ value for multiple testing of five correlated tests (47) yielded a strict T-bet significance test criterion of $P$ value $< 0.016$ for strong family-wise type I error control. A further evaluation of the association between DFS and a three-level categorization (absent, low count, high count) was performed and reported in Supplementary Tables S1 and S2, with RRs and $P$ values for the five cutpoints examined. It is evident in the three-level KM plots (Supplementary Fig. S1A and S1B) that the absent and low count groups are indistinguishable no matter which of the cutpoints examined used to define the high-versus the low-count group, and the RRs comparing absent versus high and low versus high are remarkably similar (Supplementary Tables S1 and S2). Further division of the low-count group according to present/absent did not improve discrimination further. A $P$ value $< 0.05$ was applied for tests of all other factors. Statistical analyses of associations were performed using SAS 9.1 software (SAS Institute, Inc.). Survival curves were plotted using R statistical software, version 2.15.0 (http://www.r-project.org/).

Results

Frequency and localization of T-bet $^{+/+}$ lymphoid cells

T-bet $^{+/+}$ lymphoid cells were distributed among defined tumor compartments as intratumoral [when within the epithelial nests or within close proximity (the distance between positive lymphocyte and tumor nest is equal to or less than the size of one tumor cell)] or peritumoral [at a distance from the epithelial nests; Fig. 1]. Intratumoral T-bet $^{+/+}$ cells were more numerous than were peritumoral cells. The mean, median, and range of intratumoral T-bet $^{+/+}$ lymphoid cells were 7.4, 0.0, and
Adjuvant treatment

Histologic grade

Progesterone receptor

IHC biomarkers of intratumoral T-bet\(^+\) lymphoid cells

Molecular subtypes of intratumoral T-bet\(^+\) lymphoid cells

Prognostic relevance of intratumoral T-bet\(^+\) lymphoid cells

Discussion

In this prospectively accrued cohort of women with lymph node–negative breast cancer, we have demonstrated that intratumoral T-bet\(^+\) lymphoid cells are significantly associated with a good prognosis. This is despite being associated with adverse clinicopathologic features, including larger tumor size, higher histologic grade, hormone receptor negativity, and the basal phenotype. The data from this independent cohort of women unselected for family history add to the findings from our previous study with a cohort of women with familial breast cancer which had examined expression of the chemokine CXCL10 and tumoral lymphocytic infiltration (30). The familial cohort included tumors from patients with a strong family history of breast cancer, a significant proportion of whom carry a germline BRCA mutation. The cohort, representing incident breast cancer cases identified from population-based cancer registries, included large numbers of high-grade (54%) and basal-type (25%) tumors compared with 33% and 16%, respectively, in our current cohort. Furthermore, 46% were associated with lymph node metastases at diagnosis, in contrast with the current cohort in which patients were selected based on lymph node–negative status alone. The ANN cohort has many strengths for prognostic biomarker
T-bet is Associated with Improved Survival in Breast Cancer

validation: its multi-institutional nature allows generalizability of the study population with regard to patient ethnicity, disease severity, differences in treatment, and variations in follow-up and endpoint assessments. Furthermore, the prospective accrual of patients in this cohort has the advantage of decreasing potential biases inherent in many tissue microarray studies. Thus, this current study provides important validation data for the use of T-bet as a prognostic marker in early-stage breast cancer.

Many studies have examined the prognostic effect of inflammation in breast cancer. The methods have varied considerably, with some studies assessing lymphoid infiltrates without subtype specification and other studies examining a specific subtype of the immune cell infiltrate using IHC markers. More recently, the prognostic relevance of tumor-infiltrating lymphocytes has been examined in two large prospectively accrued cohorts. In the first, the prognostic effect of tumor-infiltrating lymphocytes was assessed according to molecular subtype and type of chemotherapy in over 2,000 women with lymph node–negative breast cancer who were enrolled in the BIG 02-98 adjuvant phase III trial (12). This study found that incremental increases in lymphocytic infiltration (whether intratumoral or stromal) resulted in improved outcome in patients who had hormone receptor and HER2–negative disease only, regardless of chemotherapeutic regimen. Benefit (stromal lymphocytic infiltration only) was also seen in women with HER2–positive disease in just one of the treatment arms (anthracycline-only). In a follow-up study from the same group (48) using tumor tissue from over 900 women enrolled in the prospective FinHER trial, a decreased distant disease recurrence rate was seen in women with increasing numbers of stromal lymphocytes in the triple-negative breast cancer group only. The strength of the effect was similar to what was seen in the earlier study.

The association of a lymphoid rich stroma with basal-like cancers is well recognized. Although this class is generally associated with a poor prognosis, outcome data suggest that it is a heterogeneous group of tumors and we and others have demonstrated that a subset of patients with basal-like breast cancer can expect to show long-term survival (33). Furthermore, medullary carcinomas cluster with the basal group, and this rare subset has been reported to be associated with a better prognosis than that of other grade 3 carcinomas (49, 50). Although one of the definitional criteria for a diagnosis of medullary carcinoma is a moderate or marked lymphoid infiltrate in the stroma between tumor nests, Rakha and colleagues (11) found no statistically significant outcome difference in patients with grade 3 medullary carcinomas and those with grade 3 ductal carcinomas with prominent inflammation, suggesting that the presence of the inflammatory infiltrate confers an improved prognosis rather than the diagnosis of medullary carcinoma per se and that this prominent inflammatory component may play an important role in determining outcome in basal-like tumors. In our study, high numbers of T-bet–lymphoid cells were associated with the basal subtype; however, we were not able to detect a survival difference within this subgroup when analyzed separately, due to small numbers in this subanalysis. However, in a similar analysis of the basal subtype performed in our previous study (30), we found the association of higher T-bet expression with improved outcome to be of borderline significance (data not shown).

Traditionally, CD8+ cytotoxic T cells have been considered to be the key component in mounting an effective antitumor immune response and higher numbers have been associated with better patient survival (9, 51). However, CD4+ T cells have been shown to be necessary for full functioning of CD8+ cytotoxicity in vivo (52, 53). Furthermore, CD4+ T cells influence innate immunity by helping to shape the character and magnitude of the inflammatory response (54). Many breast cancer investigations before this have focused on the effects of CD4+ Tregs (55); however, increasing evidence suggests that the development of Th1 adaptive immunity is associated with improved outcome in various cancer types (56, 57), and T-bet, as the master regulator of Th1 cell differentiation, plays a pivotal role (21, 58). T-bet has been associated with better clinical outcomes in colorectal carcinoma (59) and gastric carcinoma (27); nevertheless, in breast cancer T-bet has rarely been studied. Ladoire and colleagues (60) examined T-bet expression in intratumoral lymphoid structures in women with HER2+ breast cancer who had been given prior treatment with neoadjuvant trastuzumab as well as anthracycline or taxanes. In 102 women, better recurrence-free survival was seen in those women who were treated with trastuzumab and taxanes who had T-bet+ cells in peritumoral lymphoid structures after chemotherapy. A correlation with T-bet+ cells following therapy was not seen with pathologic complete response to therapy. This study highlighted the importance, not only of the host’s immune response but also of T-bet in determining outcome. As we and others have demonstrated previously, patients with T-bet+ breast cancer who had been given prior trastuzumab and taxanes had a better outcome than those who had been given chemotherapy alone (21).

Table 3. Association between T-bet+ and IHC markers

<table>
<thead>
<tr>
<th>Marker</th>
<th>T-bet+/low (n = 556)*</th>
<th>T-bet+/high (n = 48)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number (%)</td>
<td>Number (%)</td>
</tr>
<tr>
<td>HER2</td>
<td>Negative 500 (92.1)</td>
<td>44 (93.6)</td>
</tr>
<tr>
<td></td>
<td>Positive 43 (7.9)</td>
<td>3 (6.4)</td>
</tr>
<tr>
<td>ER</td>
<td>Negative 129 (25.7)</td>
<td>30 (73.2)</td>
</tr>
<tr>
<td></td>
<td>Positive 373 (74.3)</td>
<td>11 (26.8)</td>
</tr>
<tr>
<td>PgR</td>
<td>Negative 217 (41.6)</td>
<td>34 (81.0)</td>
</tr>
<tr>
<td></td>
<td>Positive 305 (58.4)</td>
<td>8 (19.0)</td>
</tr>
<tr>
<td>pS3</td>
<td>Negative 417 (77.5)</td>
<td>24 (50.0)</td>
</tr>
<tr>
<td></td>
<td>Positive 121 (22.5)</td>
<td>24 (50.0)</td>
</tr>
<tr>
<td>EGFR</td>
<td>Negative 489 (94.6)</td>
<td>30 (65.2)</td>
</tr>
<tr>
<td></td>
<td>Positive 28 (5.4)</td>
<td>16 (34.8)</td>
</tr>
<tr>
<td>CKS</td>
<td>Negative 459 (85.8)</td>
<td>21 (44.7)</td>
</tr>
<tr>
<td></td>
<td>Positive 76 (14.2)</td>
<td>26 (55.3)</td>
</tr>
<tr>
<td>K6/67</td>
<td>&lt;14% 181 (40.9)</td>
<td>2 (4.8)</td>
</tr>
<tr>
<td></td>
<td>≥14% 262 (59.1)</td>
<td>40 (95.2)</td>
</tr>
</tbody>
</table>

*By Fisher exact test.

Table 4. Association between T-bet+ and intrinsic subgroups defined by IHC markers

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>T-bet+/low (n = 556)*</th>
<th>T-bet+/high (n = 48)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number (%)</td>
<td>Number (%)</td>
</tr>
<tr>
<td>Basal</td>
<td>51 (12.0)</td>
<td>24 (63.2)</td>
</tr>
<tr>
<td>HER2</td>
<td>43 (10.3)</td>
<td>3 (7.9)</td>
</tr>
<tr>
<td>Luminal A</td>
<td>282 (66.4)</td>
<td>8 (21.0)</td>
</tr>
<tr>
<td>Luminal B</td>
<td>49 (11.5)</td>
<td>3 (7.9)</td>
</tr>
<tr>
<td>Unassigned</td>
<td>141</td>
<td>10</td>
</tr>
</tbody>
</table>

*Test was performed without the ‘Unassigned’ group.

By Fisher exact test.

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Table 5. Results of DFS analysis by Cox proportional hazards model

<table>
<thead>
<tr>
<th>Prognostic factor</th>
<th>RR (95% CI)</th>
<th>Univariate</th>
<th>P</th>
<th>RR (95% CI)</th>
<th>Multivariate</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T-bet</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low vs. high HER2</td>
<td>4.72 (1.30−4.15)</td>
<td>0.0133b</td>
<td></td>
<td>5.62 (1.48−50.19)</td>
<td>0.0023b</td>
<td></td>
</tr>
<tr>
<td>Positive vs. negative Menopausal status</td>
<td>2.00 (1.00−3.92)</td>
<td>0.0023</td>
<td></td>
<td>0.92 (0.40−18.6)</td>
<td>0.8209</td>
<td></td>
</tr>
<tr>
<td>Pre/peri vs. post ER</td>
<td>0.88 (0.28−2.85)</td>
<td>0.2022</td>
<td></td>
<td>0.84 (0.36−1.94)</td>
<td>0.6824</td>
<td></td>
</tr>
<tr>
<td>Negative/equivocal vs. ND/positive Tumor size</td>
<td>2.68 (0.28−2.85)</td>
<td>0.2858</td>
<td></td>
<td>1.14 (0.62−2.03)</td>
<td>0.6661</td>
<td></td>
</tr>
<tr>
<td>Grade 2−3 vs. grade 1</td>
<td>2.20 (1.45−3.20)</td>
<td>0.0001</td>
<td></td>
<td>2.01 (1.20−3.45)</td>
<td>0.0102</td>
<td></td>
</tr>
<tr>
<td>ND vs. grade 1</td>
<td>3.46 (1.68−7.11)</td>
<td>0.0007</td>
<td></td>
<td>2.64 (1.00−6.02)</td>
<td>0.0332</td>
<td></td>
</tr>
<tr>
<td>Lymphatic invasion</td>
<td>3.48 (1.94−6.24)</td>
<td>&lt;0.0001</td>
<td></td>
<td>3.78 (1.67−10.44)</td>
<td>0.0044</td>
<td></td>
</tr>
<tr>
<td>Present vs. absent Age at diagnosis, years</td>
<td>5.16 (1.94−7.38)</td>
<td>0.0010</td>
<td></td>
<td>5.08 (1.79−15.87)</td>
<td>0.0033</td>
<td></td>
</tr>
<tr>
<td>Linear Quadratic Adjuvant treatment Hormonal vs. none</td>
<td>0.88 (0.74−1.04)</td>
<td>0.1317</td>
<td></td>
<td>0.94 (0.67−1.34)</td>
<td>0.7334</td>
<td></td>
</tr>
<tr>
<td>Linear Quadratic Adjuvant treatment Chemotherapy vs. none</td>
<td>0.93 (0.81−1.06)</td>
<td>0.2615</td>
<td></td>
<td>0.99 (0.83−1.17)</td>
<td>0.9777</td>
<td></td>
</tr>
<tr>
<td>Linear Quadratic Adjuvant treatment</td>
<td>0.55 (0.36−0.82)</td>
<td>0.0038</td>
<td></td>
<td>0.51 (0.29−0.86)</td>
<td>0.0132</td>
<td></td>
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<tr>
<td>Quadratic Adjuvant treatment</td>
<td>0.94 (0.58−1.52)</td>
<td>0.8087</td>
<td></td>
<td>0.60 (0.29−1.17)</td>
<td>0.1415</td>
<td></td>
</tr>
</tbody>
</table>

aFirth penalized regression.
bFrom the likelihood ratio test; all other P values are from the Wald test.

In this study, we based analyses on intratumoral rather than peritumoral T-bet + lymphoid cells for two reasons. First, because of our prior findings of the prognostic significance of intratumoral T-bet + lymphoid cells in the Breast Cancer Family Registry, Ontario site (30), and second, because we used TMAs in this study. TMAs preferentially include areas rich in tumor cells, and peritumoral areas may not be represented consistently, thereby introducing a potential bias in peritumoral T-bet + lymphoid cell counts.

In conclusion, intratumoral T-bet + lymphoid cells in breast cancer are associated with adverse pathologic features, including the basal subtype. Nevertheless, their presence confers a favorable outcome which is independent of traditional clinicopathologic parameters and HER2 status. The potential use of T-bet as a prognostic marker in breast cancer needs to be evaluated in additional larger cohorts, particularly those rich in basal-like or HER2 + breast cancer. Understanding the mechanisms mediating these immunologic responses and biomarkers of such responses may help in better tailoring specific therapies including combinations of agents for women with breast cancer. Furthermore, modulation of T-bet expression has the potential to become a powerful therapeutic target for the treatment of cancer in the future.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A.M. Mulligan, I.L. Andrulis
Development of methodology: A.M. Mulligan, I.L. Andrulis
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.M. Mulligan, I.L. Andrulis
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.M. Mulligan, D. Pinnaduwage, S.B. Bull
Writing, review, and/or revision of the manuscript: A.M. Mulligan, D. Pinnaduwage, S. Tchatchou, S.B. Bull, I.L. Andrulis
Study supervision: I.L. Andrulis

response in breast cancer, but also of the interaction between certain chemotherapeutic regimens and the immune system. The role the immune system plays in response to trastuzumab is well described: trastuzumab activates the host’s immune system through antibody-dependent cellular toxicity (ADCC; ref. 61), which leads to Th1 activation and production of IFNγ that has been implicated in control of cancer growth (62). Evidence also suggests that taxanes could exert an immunostimulatory effect against breast cancer (63–66) by inducing a Th1 response. Paclitaxel has been shown to stimulate the secretion by macrophages of proinflammatory and Th1 cytokines such as IL1β or IL12 (67, 68). Carson and colleagues (65) showed that in phagocytes of proinflammatory cytokines, including combinations of agents for women with breast cancer. Nevertheless, their presence confers a favorable outcome which is independent of traditional clinicopathologic parameters and HER2 status. The potential use of T-bet as a prognostic marker in breast cancer needs to be evaluated in additional larger cohorts, particularly those rich in basal-like or HER2 + breast cancer. Understanding the mechanisms mediating these immunologic responses and biomarkers of such responses may help in better tailoring specific therapies including combinations of agents for women with breast cancer. Furthermore, modulation of T-bet expression has the potential to become a powerful therapeutic target for the treatment of cancer in the future.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A.M. Mulligan, I.L. Andrulis
Development of methodology: A.M. Mulligan, I.L. Andrulis
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.M. Mulligan, I.L. Andrulis
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.M. Mulligan, D. Pinnaduwage, S.B. Bull
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In this study, we based analyses on intratumoral rather than peritumoral T-bet + lymphoid cells for two reasons. First, because of our prior findings of the prognostic significance of intratumoral T-bet + lymphoid cells in the Breast Cancer Family Registry, Ontario site (30), and second, because we used TMAs in this study. TMAs preferentially include areas rich in tumor cells, and peritumoral areas may not be represented consistently, thereby introducing a potential bias in peritumoral T-bet + lymphoid cell counts.

In conclusion, intratumoral T-bet + lymphoid cells in breast cancer are associated with adverse pathologic features, including the basal subtype. Nevertheless, their presence confers a favorable outcome which is independent of traditional clinicopathologic parameters and HER2 status. The potential use of T-bet as a prognostic marker in breast cancer needs to be evaluated in additional larger cohorts, particularly those rich in basal-like or HER2 + breast cancer. Understanding the mechanisms mediating these immunologic responses and biomarkers of such responses may help in better tailoring specific therapies including combinations of agents for women with breast cancer. Furthermore, modulation of T-bet expression has the potential to become a powerful therapeutic target for the treatment of cancer in the future.

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Figure 2.
Kaplan-Meier DFS curves stratified by T-bet + status showing a longer DFS in patients with T-bet +/high tumors.
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