Association of B7-H4, PD-L1, and tumor infiltrating lymphocytes with outcomes in breast cancer

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INTRODUCTION
T cell co-inhibitory molecules belonging to the B7 family in the tumor microenvironment, such as PD-L1 provide critical inhibitory signals and have been recognized as a major immune inhibitory mechanism in diverse solid tumors.1-4 Antibodies that inhibit the PD-1/PD-L1 pathway produce durable clinical responses in various solid malignancies, including breast carcinomas, yet it appears to benefit only a subset of breast tumors.5-11

B7-H4 (VTCN1) is also a T-cell co-inhibitory molecule and a member of the B7 family. It has limited expression in normal peripheral tissues, such as lung epithelium, whereas it is expressed at higher levels in several human cancers, including breast carcinomas5,12,13 (Supplementary Table 1). B7-H4 can function as a co-inhibitory factor inhibiting CD4+ and CD8+ T-cell proliferation, cytokine production, and generation of alloreactive cytotoxic T-lymphocytes (CTLs) by arresting the cell cycle.14,15 In preclinical models, IL-6 and IL-10 can stimulate B7-H4 expression by monocytes, macrophages, and myeloid dendritic cells.16-18 Efforts are ongoing to develop new therapies that target B7-H4.19

We hypothesized that B7-H4 expression may inversely correlate with PD-L1 levels in human breast cancers. We also queried whether B7-H4, PD-L1, and tumor infiltrating lymphocyte (TIL) levels differ among intrinsic subgroups of breast cancer, defined by ER, PR, and HER2 expression. Further, we investigated whether expression of these two molecules differed across cell type (tumor vs. stroma), and whether B7-H4 and/or PD-L1 expression are prognostic in early stage breast cancer.

RESULTS
B7-H4 expression in breast cancer
B7-H4 expression was analyzed in 561 and 444 tumors in the Yale and UM cohorts, respectively. B7-H4 protein expression had a predominant cytoplasmic/membranous distribution in tumor and was seen both in tumor and stroma (Fig. 1). The expression in the stroma did not appear to be specific to any specific cell type. Although we measure a continuous score for B7-H4 expression, the visually defined threshold was used to split the population into high/positive vs. low/negative expression.

B7-H4 tumor protein expression in the tumor cell component was high in 257/561 (45.8%) of the Yale cohort patients and 198/444 (44.6%) of the UM cohort patients (Table 1; Supplementary Fig. 1a, b). Stromal B7-H4 protein expression was high in 164/561 (29.2%) and 116/444 (26.1%) in the Yale and UM cohorts, respectively. B7-H4 protein expression of the tumor and stroma was highly correlated in both cohorts (Supplementary Fig. 1c, d). B7-H4 stromal and tumor protein expression was independent of the age, ER/HER2 status, and stage in both the Yale and UM cohorts (Supplementary Table 2).
PD-L1 expression in breast cancer

PD-L1 protein expression in the tumor cell component was present in 43/561 (7.7%) and 77/452 (17%) of the Yale and UM cohorts and stromal PD-L1 protein expression was detectable in 55/561 (9.8%) and 61/449 (13.6%) of the Yale and UM cohorts, respectively. Total PD-L1 expression tumor and/or stroma was detected in 12.8% and 21.8% of the Yale and UM cohorts, respectively (Table 1). PD-L1 expression in tumor and in stroma was higher in TNBC and this difference across other breast cancer subtypes was statistically significant in both Yale and UM cohorts (Table 2 and Supplementary Table 3). In combined analysis of the two cohorts, PD-L1 tumor expression was observed in 53/617 (9%) ER-positive cases, compared to 64/341 (19%) ER-negative cases. Likewise, PD-L1 stromal expression was observed in 7% vs. 21% in ER positive vs. negative cases, respectively. Otherwise there was no other clinicopathological associations found in both of the Yale and UM series for PD-L1 protein expression (Supplementary Table 4).

Co-expression of the two checkpoint proteins in the tumor was quite low in both cohorts (illustration of mutually exclusive expression of PD-L1 (Fig. 2a, c); B7-H4 (Fig. 2b, d)). Co-expression was observed in only 6/561 (1%) cases in the Yale cohorts and 17/424 (4%) cases in the UM cohort, with similar results in the stromal components (Table 3; Supplementary Fig. 2a, b).

Association of B7-H4 with PD-L1 and TIL infiltration

More than 10% T-cell infiltration was observed in 58.2% and 49.5% of the Yale and UM cohorts, respectively. PD-L1 and B7-H4 were inversely correlated. Furthermore, TILs were weakly, positively correlated with PD-L1 but weakly, inversely correlated with B7-H4 expression in the UM cohort and weakly positively correlated with PD-L1 and B7-H4 expression in the Yale cohorts. Both cohorts, however, had a high incidence of low staining for both markers and low levels of TILs (Supplementary Table 5, Supplementary Fig. 3). Nonetheless, B7-H4 was positive and TILs were high in only 10/407 (2%) and 7/421 (2%) whereas in cases with high TILs, PD-L1 over expression was (37/44) 84% and (38/59) 64% of the Yale and UM cohorts, respectively (Table 3).

**Table 1.** Distribution of B7-H4, PD-L1 tumor, and stroma QIF and TILs quantification by H&E in Yale and University of Michigan (UM) cohorts

| Variable | Yale Cohorts | UM Cohorts |
|----------|--------------|------------|
| B7-H4 tumor | N = 561 | N = 473 |
| Positive | 257 | 198 |
| Positive | 164 | 116 |
| PD-L1 tumor | 43 | 77 |
| Positive | 55 | 61 |
| PD-L1 stroma | 72 | 98 |
| Positive | 170 | 212 |
| 0–10 | 41.8 | 21.6 |
| 11–20 | 18.4 | 18.4 |
| 21–30 | 17 | 22 |
| 31–40 | 13 | 14 |
| >40 | 44 | 60 |
| TILs Low | 363 | 360 |
| Low | 89.2 | 85.7 |
| High | 44 | 60 |
| 10.8 | 14.3 |

*The actual number of cases for analysis for each marker is different due to loss of tissue or the absence of tumor cells in some spots.

PD-L1 expression in breast cancer

Neither B7-H4 nor PD-L1 expression in either tumor or stroma was associated with survival outcome in either cohort (Fig. 3). In both cohorts, overall survival (OS) was nearly identical for those with or without elevated B7-H4 expression. Although there was a trend...
towards worse OS in one of the Yale cohorts and in the UM cohort, neither of these met statistical significance.

**DISCUSSION**

In this study, we have observed that the immune checkpoint B7-H4 is expressed in the epithelial tumor component of ~50% of breast tumors, and in the stromal element in ~25–30% of cases. Furthermore, it was unusual to have both B7-H4 and PD-L1 expressed in the same tumor, suggesting nearly total mutual exclusion. PD-L1 was weakly correlated with TILs in both cohorts, whereas B7-H4 was weakly positively correlated with TILs in one cohort and weakly negatively correlated in the other.

Taken together, these results suggest that breast cancers may employ one or the other, but rarely simultaneous, immune checkpoint pathways to evade immunity. Moreover, B7-H4 expression was not associated with any of the particular intrinsic subtypes of breast cancer, defined by expression of ER or HER2. In contrast, as previously reported, PD-L1 was more commonly expressed in TNBC cases.\(^{20,21}\)

These data may have clinical implications. Immune checkpoint inhibitors have been found to have striking activity in a variety of solid malignancies,\(^{3–8}\) including breast cancer, however, they are effective in fraction of patients.\(^{9,22–24}\) Our data suggest that cases lacking PD-L1 might escape immune surveillance by virtue of upregulation of B7-H4 or other non-redundant alternative targets.

### Table 2. Comparison of B7-H4 and PD-L1 protein expression in tumor and stroma for breast cancer intrinsic subtypes (merged data for Yale and UM Cohorts)

|                      | Yale and UM cohorts merged\(^a\) | B7-H4 positive | PD-L1 positive |
|----------------------|----------------------------------|----------------|---------------|
|                      | Tumor                            | Stroma         | Tumor         | Stroma        |
| Triple negative      | 65/124 (52%)                     | 43/124 (35%)   | 34/123 (28%)  | 38/123 (31%)  |
| ER or PR positive/HER2 positive | 23/50 (46%)                     | 17/50 (34%)   | 4/47 (9%)     | 3/47 (6%)     |
| ER or PR positive/HER2 negative | 236/476 (49%)                | 137/476 (29%) | 48/481 (10%)  | 37/478 (8%)   |
| ER or PR negative/HER2 positive | 23/51 (45%)                   | 8/51 (16%)    | 6/50 (12%)    | 10/50 (20%)   |
| \(p\)-value\(^b\)    | 0.79                             | 0.08           | <0.0001       | <0.0001       |

\(^a\)Triple negative only Yale cohort is excluded from the pooled analysis

\(^b\)Difference in the proportion of positive B7-H4 or PD-L1 between subgroups using chi-square test. \(p\)-values are considered statistically significant if <0.05 and are indicated in bold

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**Fig. 2** Representative fluorescence image illustrating inverse correlation of tumor PD-L1 and B7-H4 protein expression. Serial sections of two different cases (A, B = case 1; C, D = Case 2). Cytokeratin (green), DAPI (Blue). a, c: PD-L1 staining (red), b, d: B7-H4 staining (red). PD-L1 is high in tissue from case 1 in (a), and low in tissue from case 2 in (c). B7-H4 protein expression is low in tissue from case 1 in (b) and high in tissue from case 2 in (d).
Table 3. Association of B7-H4, PD-L1 expression by QIF and TILs count in tumor and stromal components of primary breast cancer

### Yale cohorts

| PDL1   | N^a | p-Value^b |
|--------|-----|-----------|
|        |  N  |          |
|        | Neg | Pos      |
| **Tumor B7H4** |     |          |
| Negative | 267 (47%) | 37 (7%) | 304 | <0.0001 |
| Positive | 251 (45%) | 6 (1%)  | 257 |
|          | 518  | 43       | 561 |
| **Stromal B7H4** |     |          |
| Negative | 292 (60%) | 37 (7%) | 329 | 0.0018 |
| Positive | 156 (32%) | 5 (1%)  | 161 |
|          | 448  | 42       | 490 |
| **TIL** |     |          |
|       | N   |          |
|       | Low | High     |
| **Tumor B7H4** |     |          |
| Negative | 147 (36%) | 34 (8%) | 181 | <0.0001 |
| Positive | 216 (53%) | 10 (3%) | 226 |
|          | 363  | 44       | 407 |
| **Stromal B7H4** |     |          |
| Negative | 242 (59%) | 39 (10%) | 281 | 0.003 |
| Positive | 121 (30%) | 5 (1%)  | 126 |
|          | 363  | 44       | 407 |
| **Tumor PD-L1** |     |          |
| Negative | 359 (88%) | 7 (2%)  | 366 | <0.0001 |
| Positive | 4 (1%)  | 37 (9%)  | 41  |
|          | 363  | 44       | 407 |
| **Stromal B7H4** |     |          |
| Negative | 331 (81%) | 25 (6%) | 356 | <0.0001 |
| Positive | 32 (8%)  | 19 (5%)  | 51  |
|          | 363  | 44       | 407 |

### UM cohort

| PDL1   | N^a | p-Value^b |
|--------|-----|-----------|
|        |  N  |          |
|        | Neg | Pos      |
| **Tumor B7H4** |     |          |
| Negative | 175 (41%) | 59 (14%) | 234 | <0.0001 |
| Positive | 173 (41%) | 17 (4%)  | 190 |
|          | 348  | 76       | 424 |
| **Stromal B7H4** |     |          |
| Negative | 265 (63%) | 45 (11%) | 310 | 0.82 |
| Positive | 95 (23%)  | 15 (3%)  | 110 |
|          | 360  | 60       | 420 |
| **TIL** |     |          |
|       | N   |          |
|       | Low | High     |
| **Tumor B7H4** |     |          |
| Negative | 174 (41%) | 52 (12%) | 226 | <0.0001 |
| Positive | 188 (45%) | 7 (2%)   | 195 |
|          | 362  | 59       | 421 |
| **Stromal B7H4** |     |          |
| Negative | 249 (59%) | 57 (13%) | 306 | <0.0001 |
Most clinical trials of immune checkpoint inhibition have been focused on TNBC, since PD-L1 is more likely, although not exclusively, expressed in this sub-type, and there are few other treatment options except chemotherapy. Our data demonstrate that B7-H4 is not only inversely related to PD-L1 expression but also its expression appears to be independent of breast cancer intrinsic subtype.

In our study, neither B7-H4 nor PD-L1 expression was associated with patient outcome, although we detected a statistically insignificant trend towards worse survival for over-expression of the latter. However, our datasets were serially collected retrospective studies, with mixes of intrinsic subtypes and a variety of treatments. It is possible that either or both of the immune checkpoints might be prognostic in subgroups of patients according to hormone receptor or HER2 expression, or according to specific treatments. However, our power to conduct such subgroup analyses was limited. Nonetheless, we speculate that these molecules may serve as general mechanisms of tolerance to the immune system employed by epithelial tissue, and identification of mechanism of tolerance and breaking tolerance with checkpoint specific or with use of combined immune checkpoint inhibitors may result in reduction in tumor burden and patient benefit.

The presence of increased TILs is associated with increased PD-L1 levels in diverse tumors suggesting that these factors are biologically linked. It has been postulated that B7-H4 over-expression in immune cells may impair the immune response against tumors by decreasing the TILs via various mechanisms. A study revealed that T cells isolated from cervical cancer patients co-cultured with recombinant B7-H4 for 48 h, which resulted activated T cell arrest at the G1/G2 phase, another experiment with co-cultured cytotoxic lymphocytes with Lewis lung carcinoma cell line showed that membrane bound B7-H4-induced CTL apoptosis which was partially reversed when B7-H4 blocked with a neutralizing monoclonal antibody. Decreased TILs in the tumor microenvironment decreases production of pro-inflammatory cytokines such as IL-6 and IL-10. Decreased TILs and microenvironmental cytokines, such as IL-6 and IL-10, may alter the immune balance towards tumor-associated FOXP3+ T-reg cells, which can trigger APCs including macrophages to produce inhibitory cytokines, such as IL-6 and IL-10.

Number of preclinical studies support the hypothesis of B7-H4 generating an inhibitory tumor microenvironment, for example, in one study it has been shown that the quantity of B7-H4 on the surface of pancreatic islets cells positively correlates with their resistance to T cell attack in murine models of type 1 diabetes. In another study when 4T1 metastatic breast cancer cells were transferred into both wildtype and B7-H4−/− mice. In B7-H4−/− mice, the percentage and overall number of CD4+Foxp3+ Tregs was reduced and a significantly higher ratio of effector CD4+ and CD8+ T cells was observed. Indeed, our results, showing an inverse relationship of B7-H4 and TILs are consistent with this hypothesis. In this regard, our group recently reported similar findings in non-small cell lung cancer, further supporting the biologic balance of PD-L1 and B7-H4 to control immune response across different tumor types.

A scenario can be envisioned in which a B7-H4 drug is only effective in combination with a PD-L1 inhibitor. B7-H4 inhibition might trigger lymphocyte infiltration and activate an immune response that could in turn be complemented by a PD-1 axis checkpoint inhibitor. However, with the existing data, we can only state that the tumors that express B7-H4 appear to be a different subset than those that are more likely to respond to current immune therapies, as indicated by TILs or PD-L1 expression.

The lack of concordance of these markers, especially PD-L1 in the tumor and stroma compartments (Supplementary Fig. 5) could be explained by the expression of PD-L1 in macrophages and morphologically fibroblast-like cells in addition to TILs in breast cancer stroma, as previously described. Future studies focused on phenotype of these cells would provide further evidence.

This study has number of limitations. The protein expression and TILs assessments were performed in archival tissue without serial biopsies, thus, we cannot assess the temporal dynamics of protein expression and lymphocytic infiltration. Further, the work was entirely done on TMAs. The use of TMAs may not provide the same information as if the expression patterns had been performed on whole sections. It is reassuring that the TMA data were comparable in cohorts from two different institutions. Also defining the cut points are difficult to determine due to the nature of the continuous data generated in these studies. In this work, visual cut off is selected after reviewing many images and after the specific staining pattern, which has been perimembranous/cytoplasmic for the B7-H4, subjectively visualized. Once a cut off is established the same cut off is used for all cohorts to be consistent throughout the study.

In summary, we report that a second immune checkpoint protein, B7-H4, is commonly expressed in breast cancer, that its expression is independent of intrinsic subtypes, and that it is inversely related to PD-L1 and TILs. Although not related to prognosis in standardly treated patients with early stage disease, we speculate that these findings could have implications for...
further development and studies of immune checkpoint inhibitors in this disease.

METHODS

Patients, cohorts, and tissue microarrays (TMAs)

Samples from four retrospective collections of breast cancer, three from Yale University (cohorts A–C) and one from University of Michigan (UM) represented in TMAs were used. The major clinicopathological characteristics of the cohorts are presented in Supplementary Table 6. Two of the Yale University cohorts (cohorts A and B) have been previously described38,39 and a third cohort (cohort C) consists of tissues collected from patients with stages I–III triple negative breast cancer (TNBC). The fourth, cohort D consists of a set of 473 breast cases treated at the University of Michigan Comprehensive Cancer Center (UMCCC).

Yale University cohorts were retrospectively derived from stages I–III breast cancer collections of patients who underwent surgical tumor resection and were followed at Yale University from 1976 to 2010. Cases in cohort D were derived from all patients who had definitive surgery for invasive breast cancer at UMCCC between 2004 and 2005, and for whom breast cancer tissue blocks were therefore available, and who underwent subsequent therapy and follow-up at UMCCC.

Tissue and associated clinicopathological information was used after approval from the Yale Human Investigation Committee (protocol #9505008219) or from the University of Michigan Institutional Review Board (IRBMED#2001-0788; HUM00042180). Both review boards provided waiver of consent, since these were otherwise discarded tissues collected during routine medical care.

Two different built TMA blocks from each cohort using 0.6 mm tissue cores were included. These blocks were constructed with the same TMA map with cores obtained by nonadjacent sampling of the same tumor blocks to examine tumor heterogeneity.

Antibody validation—cell line transfection and assessment for B7-H4 and PD-L1

Open-access databases (Expression Atlas European Bioinformatics Institute EMBL-EBI, https://www.ebi.ac.uk/gxa/home and Cell Line Atlas-The Human Protein Atlas, www.proteinatlas.org/cell) were reviewed for cell line mRNA and protein expression of B7-H4 (VTCN1). The cultured human MCF7 breast cancer cell line (American Type Culture Collection) was selected for negative control due to low mRNA expression.

MCF7 cells were seeded in 24-well plates in duplicates in Gibco™ RPMI Medium (ThermoFisher Scientific, USA), supplemented with 10% FBS, Penicillin–Streptomycin (10,000 U/ml) and incubated at 37°C, 5% CO₂.
When they reached 70% confluency, they were transferred to Optimem-
Low Serum Medium (ThermoFisher Scientific, USA), transfected with
500 ng of B7-H4 plasmid (provided by Cell Signaling Technologies (CST, MA) with different Lipofectamine 2000 concentrations ranging from 2 to 5 μl (ThermoFisher, USA), following manufacturer’s instructions. Empty vector and untreated cells (no plasmid, no lipofectamine) were included as negative controls. After 72 h incubation, cells were collected and replated in eight-chamber polystyrene vessel tissue culture-treated glass slides (Falcon, Cat. no. 354108) in duplicates. Glass slides were washed twice in PBS (Life Technologies) and fixed in 4% paraformaldehyde for 10 min. Then, following double wash in PBS they were permeabilized in 0.2% Triton x100-PBS for 3 min.

After a double wash in PBS followed by blocking in 2% BSA-PBS for 1 h at room temperature, primary antibodies against cytokeratin (monoclonal mouse antihuman pan-cytokeratin (clone AE1/AE3, M3515; Dako Corp., Carpinteria, CA, USA)) B7-H4 (monoclonal rabbit antibody, Cell Signaling Technologies, clone D1M8I) at a working concentration of 1.305 μg/ml were added overnight at 4 °C, in a light protected chamber. After two washes of 1% tween-PBS and one in PBS 5 min each, secondary antibodies were added using GAM/Alexa Fluor 546-conjugated goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR, USA) diluted 1:100 in rabbit EnVision reagent (Dako) for 1 h at room temperature. After PBS-tween/PBS wash Cyanine 5 (Cy5) directly conjugated to tyramide (Perkin-Elmer, Waltham, MA, USA) at 1:50 dilution in amplification buffer was added for 10 min for target antibody detection. Finally, after a last PBS-tween/PBS wash, mounting was performed with Prolong Gold-DAPI (Life Technologies). Quantitative immunofluorescence (QIF) microscopy was used for assessment of protein expression of both positive and negative controls in MCF7 for B7-H4 transfection (Supplementary Fig. 4).

For PD-L1 QIF we used a validated antibody (clone SP142, rabbit or mouse EnVision reagent) for 1 h in rabbit EnVision reagent (Dako) for 1 h at room temperature. After PBS-tween/PBS wash Cyanine 5 (Cy5) directly conjugated to tyramide (Perkin-Elmer, Waltham, MA, USA) at 1:50 dilution in amplification buffer was added for 10 min for target antibody detection. Finally, after a last PBS-tween/PBS wash, mounting was performed with Prolong Gold-DAPI (Life Technologies). Quantitative immunofluorescence (QIF) microscopy was used for assessment of protein expression of both positive and negative controls in MCF7 for B7-H4 transfection (Supplementary Fig. 4).

Quantitative immunofluorescence
We measured the levels of B7-H4 (clone D1M8I, CST), PD-L1 (clone SP142, Spring Bioscience) combined with pancytokeratin to determine the tumor and stromal compartment by QIF in TMA slides containing the cohort cases. Serial section slides were used for analyzing the staining and protein expression patterns for the two markers. TMA slides were baked overnight at 60 °C and then soaked in xylene twice for 20 min each. Slides were rehydrated in two 1 min washes in 100% ethanol followed by one wash in 70% ethanol and finally rinsed in streaming tap water for 5 min. Antigen retrieval was performed in sodium citrate buffer, pH 6 for B7-H4 antibody and with EDTA buffer, pH 8 for PD-L1 antibody in the PT module-LabVision for 20 min at 97 °C in a pressure-boiling container. Blocking was performed with 0.3% bovine serum albumin in 0.05% tween solution for 30 min after antigen retrieval. Each of the B7 family antibodies (B7-H4 and PD-L1) was combined with 1:100 pan-cytokeratin antibody (Dako) in 0.5% BSA in TBST and incubated overnight at 4 °C. Primary antibodies were followed by incubation with Alexa 546-conjugated goat anti-mouse or anti-rabbit secondary antibody (Molecular Probes, Eugene, OR, USA) diluted 1:100 in rabbit or mouse EnVision reagent (Dako) for 1 h. Signal was amplified with Cyanine 5 (Cy5) directly conjugated to tyramide (Perkin-Elmer, Waltham, MA, USA) at 1:50 dilution. ProLong mounting medium (ProLong Gold; Molecular Probes) with 4,6-diamidino-2-phenylindole (DAPI) was used to stain nuclei.

The QIF measurements were performed using the AQUA® method of QIF (Genoptix Medical Laboratory) as previously described.44 The QIF score of B7-H4 antibody in the tumor and in the stroma was calculated by dividing the target B7-H4 pixel intensity in the area of the tumor and stroma compartment defined by the cytokeratin positivity in tumor cells. In order to better represent tumor heterogeneity in the quantitative analysis of B7-H4 and PD-L1 protein expression, we performed QIF staining in two different TMA builds. To address run-to-run variability, slides were stained and analyzed on the same day using the same protocol. Serial cut index slides were used for quality control and regression coefficients (R²) between independent runs for these index slides were found to be high (>0.9). Two TMA histospots were evaluated for each case and the average score was determined. Cases which had <2% tumor tissue, extensive necrosis or non-invasive tumor tissue in a histospot were excluded from the analysis.

TILs assessment
Stromal TILs were assessed in H&E-stained TMA sections by two experienced Yale pathologists (V.P., D.C.H.) and expressed as the percentage of total cells using 10% increments as suggested by International TILs Working Group.45 Sections with 50% or greater TIL infiltrate were denoted as lymphocyte predominant breast cancer.46

ER, PR, and HER2 determination
ER, PR, and HER2 were collected from the clinical charts at both institutions. All of the specimens from both institutions were collected from cases that preceded the 2010 ASCO/CAP guidelines for ER/PR.46 Any case with more than 10% of tumor cells showing reactivity in the nucleus was called positive. For HER2, the conventional scoring system was used including a four-point subjective scale (0–3). HER2 IHC staining of 3+ (uniform, intense membrane staining of >30% of invasive tumor cells) and HER2 IHC staining of +2 (were considered equivocal) that were re-assayed for gene amplification using FISH as suggested in ASCO/CAP 2007 guidelines.46

Statistical analysis
All results are presented using REMARK criteria.46 The three breast cancer cohorts from Yale University were pooled for the analysis. The fourth cohort from UM was analyzed separately. A visual cut point was determined for QIF positivity and same cut off was used for all cohorts. Pearson’s correlation coefficient (R) was used to assess the reproducibility of the assay between near-serial sections of the index array. Differences between QIF signals between groups were analyzed using Fisher’s exact test. Two-sided p-value < 0.05 was considered statistically significant. Linear regression was used to determine the association between continuous scores after a natural log transformation of the data to satisfy linearity assumptions and the coefficient of determination (R²) was calculated. Chi-square tests were performed to compare characteristics between groups. Spearman’s correlation was used to assess the correlation between TILs and PD-L1 and B7-H4 expressions.

Survival analysis of continuous marker scores for the Yale combined cohorts was performed using the X-tile software (Yale University, New Haven, CT, USA) for disease-specific survival differences.47 This analysis exported to GraphPad Prism 7.01 software for Kaplan–Meier OS curve presentations. Statistical analysis system (SAS) v 9.4 was used for analysis of the Michigan cohort.

DATA AVAILABILITY
The data analyzed during this study are described in the following metadata record: https://doi.org/10.6084/m9.figshare.c.4304642.46 Expression data are available from the EMBL-EBI Expression Protein Atlas and Human Protein Atlas as outlined in the following metadata record: https://doi.org/10.6084/m9.figshare.7352912. Quantitative immunofluorescence image data are available on request as outlined in the following metadata record: https://doi.org/10.6084/m9.figshare.c.41523-018-0095-1. Additional analyzed data files are available in the Supplementary Information of this article.

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AUTHOR CONTRIBUTIONS
M.A., D.L.R., D.F.H., and K.M.K participated in the concept design, coordination of the study and drafted the manuscript. M.A., K.M.K., V.P., D.E.C, M.T., D.G.T., K.A.S., and M.S. involved in the execution, acquisition, analysis of all data. D.L.R. and D.F.H. provide patient cohorts, supervised, and funded the project. All the authors made substantial contributions to the discussion of content, and reviewed and edited the manuscript before submission.

ADDITIONAL INFORMATION
Supplementary information accompanies the paper on the npj Breast Cancer website (https://doi.org/10.1038/s41523-018-0095-1).
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