In Situ Tumor PD-L1 mRNA Expression Is Associated with Increased TILs and Better Outcome in Breast Carcinomas

Kurt A. Schalper1, Vamsidhar Velcheti3, Daniel Carvajal1, Hallie Wimberly1, Jason Brown1, Lajos Pusztai2, and David L. Rimm1

Abstract

**Purpose:** Blockade of the PD-1/PD-L1 axis emerged as a promising new therapeutic option for cancer that has resulted in lasting responses in metastatic renal, lung carcinomas, and melanomas. Tumor PD-L1 protein expression may predict response to drugs targeting this pathway. Measurement of PD-L1 protein is limited by the lack of standardized immunohistochemical methods and variable performance of antibodies. Our goal was to correlate PD-L1 mRNA expression with clinical variables in primary breast carcinomas.

**Experimental Design:** The fluorescent RNAscope paired-primer assay was used to quantify *in situ* PD-L1 mRNA levels in 636 stage I–III breast carcinomas on two sets of tissue microarrays [YTMA128 (*n* = 238) and YTMA201 (*n* = 398)]. Tumor-infiltrating lymphocytes (TIL) were assessed by hematoxylin/eosin stain and quantitative fluorescence.

**Results:** On YTMA128 and YTMA201, 55.7% and 59.5% of cases showed PD-L1 mRNA expression, respectively. Higher PD-L1 mRNA expression was significantly associated with increased TILs (*P* = 0.04) but not with other clinical variables. Elevated TILs (scores 2 and 3+) occurred in 16.5% on YTMA128 and 14.8% on YTMA201 and was associated with estrogen receptor–negative status (*P* = 0.01 on YTMA128 and 0.0001 on YTMA201). PD-L1 mRNA expression was associated with longer recurrence-free survival (log-rank *P* = 0.01), which remained significant in multivariate analysis including age, tumor size, histologic grade, nodal metastasis, hormone receptor, HER2 status, and the extent of TILs (HR, 0.268; CI, 0.099–0.721; *P* = 0.009).

**Conclusions:** PD-L1 mRNA expression is identified in nearly 60% of breast tumors and it is associated with increased TILs and improved recurrence-free survival. These observations support the evaluation of PD-1/PD-L1–targeted therapies in breast cancer. Clin Cancer Res; 20(10); 1–10. © 2014 AACR.

Introduction

Breast cancers harbor a large number of genomic alterations that can result in mutated proteins. These mutations not only contribute to the malignant transformation but also lead to neoantigens that may serve as targets for a local immune response, which could exert some control on tumor growth (1). Indeed, the presence of lymphocytes in the tumor microenvironment (TILs) and gene expression signatures representative of these cells have long been associated with (slightly but significantly) better prognosis, particularly among high-grade and estrogen receptor (ER)–negative tumors (2–5). Numerous attempts have been made in the past to exploit adoptive immunotherapy in breast cancer (e.g., vaccines, cytokines) that have met with limited success (6). It is increasingly recognized that inhibition of TIL activity in the tumor microenvironment limits the extent of antitumor immune response (7).

Recent evidence highlights the pivotal role of the PD-1 (programmed cell death-1) receptor pathway in maintaining an immunosuppressive tumor microenvironment. PD-1 is a member of the B7-CD28 family of T-cell coregulatory receptors and contributes inhibitory signals that mediate the physiologic immune tolerance and limit the inflammatory response to infections (8, 9). PD-1 is expressed in various immune cell types and its activation attenuates T-cell function, survival, and expansion (10, 11). The PD-1 ligand, PD-L1 is expressed on activated T cells, B cells, dendritic cells, and macrophages, in addition to some immune-privileged non-hematopoietic tissues (e.g., retina and placenta; refs. 12, 13). Tumors from diverse locations can express PD-L1, including breast, ovarian, gastric, pancreatic, lung, and renal cell carcinomas (14–18). PD-L1 expression by tumor cells is believed to mediate the inhibition of local immune responses, thus shielding the tumor from T-cell–mediated killing. In support of this notion, early-phase trials using monoclonal antibodies targeting PD-1 or PD-L1 have shown substantive and durable clinical responses in
Translational Relevance

The presence of tumor-infiltrating lymphocytes in breast cancer is associated with better prognosis and higher response to preoperative chemotherapy. Unfortunately, the prognostic and predictive value of tumor immune infiltrates is significant but modest. In most instances, the immune system mounts only a partially effective antitumor response. Expression of Programmed death ligand-1 (PD-L1) is a key mechanism of tumor immune evasion. PD-L1 is expressed in various human cancers and tumor PD-L1 protein positivity using immunohistochemistry predicted response to anti-PD1 monoclonal antibody therapy. The literature suggests the different immunohistochemical methods yield discordant results that hinder progress in this field. Herein, we describe a reproducible, antibody-independent, and compartment-specific method of measuring PD-L1 mRNA in formalin-fixed, paraffin-embedded tissue samples and demonstrate the prognostic value of this marker in breast cancer. Further evaluation of PD-L1 mRNA in a prospective clinical trial may help select patients for immunostimulatory drugs targeting the PD-1/PD-L1 pathway.

patients with refractory solid tumors, including melanoma, renal, and non–small cell lung carcinomas (19, 20).

PD-L1 protein has been reported not to be expressed in normal breast but to be increased in nearly half of breast cancers, particularly in hormone receptor–negative and high-grade, proliferative tumors (14, 21). In addition, high PD-L1 protein expression in TILs from breast cancer specimens was observed in large, high-grade HER2-positive tumors. The presence of regulatory T cells (Tregs), tumor PD-L1 expression, and PD-L1–positive TILs was associated with high histologic grade, ER negativity, and prominent lymphocytic infiltrates (22). Preliminary data show that tumor PD-L1 protein expression using immunohistochemistry (IHC) in formalin-fixed, paraffin-embedded tissue samples and demonstrate the prognostic value of this marker in breast cancer. Further evaluation of PD-L1 mRNA in a prospective clinical trial may help select patients for immunostimulatory drugs targeting the PD-1/PD-L1 pathway.

In situ mRNA hybridization

In situ detection of PD-L1 transcripts in FFPE TMA samples was performed using the RNAscope assay with custom-designed in situ hybridization probes (Advanced Cell Diagnostics) coupled to automated quantitative fluorescence (QIF) detection as described (33–35). Briefly, 5 μm sections were deparaffinized, boiled with preamplification reagent for 15 minutes, and submitted to protease digestion followed by hybridization for 2 hours with target probes to human PD-L1 mRNA, Ubiquitin C (UbC) as a positive control, or the bacterial gene DapB mRNA as a negative control. Hybridization signals were detected with Cy5-tyramide. Preparations were then incubated with a wide-spectrum rabbit anti-cow cytokeratin antibody (clone Z0622 1:100, DAKO Corp) in bovine serum albumin/Tris-buffered saline for 1 hour followed by detection with a secondary Alexa-546 conjugated goat-anti-rabbit antibody (1:100, Molecular Probes). Slides were mounted using ProlongGold plus 4',6-diamidino-2-phenylindole (DAPI) to highlight nuclei. Assay specificity was assessed measuring the signal in positive and negative control samples. Reproducibility was assessed by staining control preparations on different days using the same protocol and determining the linear regression coefficients ($R^2$) between such runs.
Automated quantitative fluorescence

QIF using the AQUA method enables objective and sensitive measurement of targets within user-defined tissue compartments (33–35, 37, 38). Briefly, the QIF score of mRNA signal in the tumor was calculated by dividing the target mRNA pixel intensities with the area of the tumor compartment defined by the cytokeratin positivity. Scores were normalized to the exposure time and bit depth at which the images were captured, allowing scores collected at different exposure times to be comparable. The experimenters visually evaluated all acquired histospots and cases with staining artifacts and/or presence of less than 2% tumor were excluded. As shown in Supplementary Fig. S3, PD-L1 protein was measured using QIF with the validated mouse monoclonal antibody clone 5h1 as recently reported (35). Detailed description of PD-L1 protein staining is provided in the Supplementary Methods.

Evaluation of tumor-infiltrating lymphocytes

The scoring of TILs was performed in hematoxylin/eosin–stained TMA preparations independently by two pathologists (K.A. Schalper and D. Carvajal) using a four-tiered scale based on the visual estimation of the amount of lymphocytes in each histospot, as described (39). A score of 0 indicated virtual absence of TILs; 1+, low TILs (<30%); 2+, moderate (30%–60%); and 3+, marked increase in the lymphocytic infiltrate (>60%). Cases that could not be appropriately evaluated for technical reasons (e.g., bad staining, low tumor area, etc) were designated as not evaluable. Spots with discordance in TIL category between pathologists were reviewed jointly and a single consensus category was established. In addition, the signals of different TIL subtypes were simultaneously measured using QIF with a multiplexed immunofluorescence protocol (Supplementary Fig. S4). Detailed description of the protocol and reagents is provided in the Supplementary Methods.

Determination of PD-L1 mRNA positivity

The cutoff for PD-L1 mRNA positivity was defined as the noise threshold of the system. This was determined by using the average QIF score of DapB (negative control bacterial gene) in situ hybridization in a serial section slide.
stained in the same batch as the experimental samples. PD-L1 mRNA scores between experiments were mean-normalized using the DapB scores from each independent run as reference. Cases with PD-L1 mRNA signal above the DapB were considered as positive and cases with scores equal to or lower were considered as negative. Cases with very low UbC (positive control) QIF scores (<60) were excluded from further analysis to rule out the possibility of false negative technical results.

**Statistical analysis**

Patient characteristics were compared using a $t$ test for continuous variables and a $\chi^2$ test for categorical variables. Recurrence-free survival (RFS) and disease-specific survival (DSS) functions were compared using Kaplan–Meier estimates and statistical significance was determined using the log-rank test. A multivariate Cox proportional hazards model including age, tumor size, lymph node status, estrogen receptor, and HER2 status as covariates was built. All statistical analyses were performed using JMP Pro software (version 9.0.0, 2010, SAS Institute Inc.).

**Results**

**PD-L1 mRNA assay validation**

As shown in Supplementary Fig. S1A, PD-L1 mRNA signal was higher in PD-L1–transfected Mel624 cells than in parental cells and was recognized as multiple relatively small dots with predominant perinuclear cellular distribution (Supplementary Fig. S1A, red fluorescence channel). The PD-L1 mRNA QIF score was significantly higher than DapB (negative control) in FFPE preparations from PD-L1 Mel624 transfectants, but not in parental Mel624 cells (P < 0.001; Supplementary Fig. S1A). In samples from human placenta, PD-L1 mRNA signal was located predominantly in the trophoblastic cell compartment characterized by intense cytokeratin positivity (Supplementary Fig. S1B, left, green fluorescence channel). In contrast, the UbC mRNA signal was evenly distributed within the epithelial and mesenchymal areas of the chorionic villi (Supplementary Fig. S1B, central panels). As expected, the DapB mRNA signal was faint throughout the placental tissue, indicating a low background signal (Supplementary Fig. S1B, right).

**In situ PD-L1 mRNA expression in breast cancer**

In breast cancer samples, PD-L1 mRNA signal showed a similar dotted hybridization pattern and was predominantly located within the tumor (cytokeratin-positive) compartment (Fig. 1A, left). As expected, consecutive serial section samples hybridized with UbC and DapB mRNA probes showed high and low signal, respectively (Fig. 1A, middle and right). The cytokeratin signal and staining pattern were comparable between the serial section specimens.

In YTMA128, 178 spots (75%) were informative for all three mRNA targets and only 2 cases (0.8%) showed extremely low UbC mRNA scores and were excluded from the analysis. Ninety-eight cases (55.7%) showed positive PD-L1 mRNA signal (Fig. 1B). In YTMA201, 358 cases (89.5%) were informative and 22 cases (5.5%) were excluded for low UbC scores. In this cohort, 201 cases (59.5%) showed PD-L1 mRNA levels above the detection threshold (Fig. 1C). Although the serial section reproducibility of PD-L1 mRNA assay on YTMA245 was high ($R^2 = 0.73$, Supplementary Fig. S2A), the regression between measurements in 2 cores from the same tumor block (intercore regression) on YTMA201 was considerably lower ($R^2 = 0.2$, Supplemental Fig. S2B), suggesting that PD-L1 mRNA is a rather heterogeneous marker in breast cancer. Serial section regression of UbC was comparable with that of PD-L1 mRNA ($R^2 = 0.81$, Supplementary Fig. S2C), but the intercore regression was higher using the same experimental conditions ($R^2 = 0.48$, Supplementary Fig. S2D).

Consistent with previous findings in non–small cell lung cancer (35), breast tumor cells showed a predominant presence of inflammatory infiltrates in breast carcinomas. A, representative microphotographs of hematoxylin/eosin preparations from breast cancer samples showing different TILs categories (TILs scores 0–3+). B and C, graphs showing the number and proportion of cases in each TILs category on YTMA128 (B) and YTMA201 (C). NE, not evaluable (see Materials and Methods). D and E, PD-L1 mRNA scores in cases with low TILs (score 0 and 1+) and high TILs (score 2 and 3+) from YTMA128 (D) and YTMA201 (E). The chart shows mean ± SEM of QIF scores, and the number within each bar indicates the amount of cases in each group.
membranous-like PD-L1 protein staining pattern (Supplementary Fig. S3A) and the levels of PD-L1 mRNA showed a positive nonlinear relationship with PD-L1 protein in both breast cancer collections [regression coefficient (R) of 0.2 on YTMA128 (P = 0.01) and 0.16 on YTMA201 (P = 0.003), Supplementary Fig. S3C and S3D).

**Characterization of lymphocytic infiltrates in breast cancer**

Representative pictures of breast tumors cases showing different levels of TILs (scores 0–3+; see Materials and Methods) are depicted in Fig. 2A. Both YTMA128 and YTMA201 showed a high proportion of cases with low TILs (scores 0 and 1+) of 73.6% and 77.7%, respectively (Fig. 2B and C). The number of cases with more prominent lymphocytic infiltrates (scores 2 and 3+) was comparably low in both cohorts (16.5% on YTMA128 and 14.8% on YTMA201; Fig. 2B and C). Cases with elevated TILs showed significantly higher PD-L1 mRNA levels in both YTMA128 and YTMA201 (P < 0.01; Fig. 2D and E). The overall concordance for categorical TIL status determination between pathologists was 93% in YTMA128 (κ = 0.77) and 95% in YTMA201 (κ = 0.83).

Characterization of TIL subpopulations using multiplexed QIF revealed that cases from YTMA128 showing PD-L1 mRNA expression had 14.3% more CD3 signal, 21% more CD20 signal, and 8.8% higher CD8 signal than PD-L1 mRNA-negative tumors (Supplemental Fig. S4C). Similarly, PD-L1 mRNA expressing samples from YTMA201 showed 18.3% higher CD3 signal, 19.2% more CD20, and only 2.6% change in CD8 fluorescence (Supplementary Fig. S4D).

**Clinicopathological associations of PD-L1 mRNA status and TILs breast carcinomas**

In both YTMA128 and YTMA201, PD-L1 mRNA expression was significantly associated with the presence of elevated TILs (scores 2 and 3+, P = 0.04; Table 1), but not with age, tumor size, lymph node positivity, histologic grade, estrogen receptor positivity, and HER2 status. The presence of elevated TILs was significantly associated with ER-negative status in both YTMA128 and YTMA201 (P = 0.01 and 0.001, respectively; Table 2). The presence of prominent lymphocytic infiltrates was also significantly associated with younger age at diagnosis in YTMA128 (P = 0.006; Table 2). The latter association was also apparent in YTMA201, but did not reach statistical significance (P = 0.08; Table 2).

**Association of PD-L1 mRNA status and TILs with survival in breast cancer patients**

In patients from YTMA201 with a relatively prolonged follow-up (median follow-up, 139 months; Supplementary Table S1), expression of PD-L1 mRNA in the tumor compartment was significantly associated with longer RFS (log-rank P = 0.01; Fig. 3A). The presence of elevated TILs in the TMA spot was also associated with longer RFS, although

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**Table 1. PD-L1 mRNA associations on YTMA128 and YTMA201**

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<tr>
<th>Parameter</th>
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<th>YTMA201</th>
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<td>PD-L1 mRNA (+)</td>
<td>P</td>
<td>PD-L1 mRNA(−)</td>
<td>PD-L1 mRNA (+)</td>
<td>P</td>
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<tr>
<td>&lt;50</td>
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<td>29 (58)</td>
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<td>82 (37.6)</td>
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<td>Tumor size</td>
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<td>Number (%)</td>
<td></td>
<td>Number (%)</td>
<td>Number (%)</td>
<td></td>
</tr>
<tr>
<td>&lt;2 cm</td>
<td>39 (39)</td>
<td>61 (61)</td>
<td>0.297</td>
<td>61 (33.9)</td>
<td>119 (66.1)</td>
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<td>2–5 cm</td>
<td>28 (47.5)</td>
<td>31 (52.5)</td>
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<td>22 (33.8)</td>
<td>43 (66.2)</td>
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</tr>
<tr>
<td>Nodal status</td>
<td>Number (%)</td>
<td>Number (%)</td>
<td></td>
<td>Number (%)</td>
<td>Number (%)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>45 (44.6)</td>
<td>56 (55.4)</td>
<td>0.367</td>
<td>57 (32.6)</td>
<td>118 (67.4)</td>
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<td>36 (61)</td>
<td></td>
<td>27 (37.5)</td>
<td>45 (62.5)</td>
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<tr>
<td>Histologic grade</td>
<td>Number (%)</td>
<td>Number (%)</td>
<td></td>
<td>Number (%)</td>
<td>Number (%)</td>
<td></td>
</tr>
<tr>
<td>GI–II</td>
<td>10 (41.7)</td>
<td>14 (58.3)</td>
<td>0.472</td>
<td>21 (24.1)</td>
<td>66 (75.9)</td>
<td>0.676</td>
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<td>GIlll</td>
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<td>10 (47.6)</td>
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<td>25 (26.9)</td>
<td>68 (73.1)</td>
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</tr>
<tr>
<td>ER</td>
<td>Number (%)</td>
<td>Number (%)</td>
<td></td>
<td>Number (%)</td>
<td>Number (%)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>16 (64)</td>
<td>11 (44)</td>
<td>0.081</td>
<td>36 (47.3)</td>
<td>40 (52.6)</td>
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<td>Positive</td>
<td>46 (40.4)</td>
<td>68 (59.6)</td>
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<td>91 (37.8)</td>
<td>150 (62.2)</td>
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<tr>
<td>HER2</td>
<td>Number (%)</td>
<td>Number (%)</td>
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<td>Number (%)</td>
<td>Number (%)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>51 (41.1)</td>
<td>73 (58.9)</td>
<td>0.098</td>
<td>91 (36.5)</td>
<td>158 (63.5)</td>
<td>0.204</td>
</tr>
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<td>Positive</td>
<td>9 (64.3)</td>
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<td>4 (22.2)</td>
<td>14 (77.8)</td>
<td></td>
</tr>
<tr>
<td>TILs</td>
<td>Number (%)</td>
<td>Number (%)</td>
<td></td>
<td>Number (%)</td>
<td>Number (%)</td>
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</tr>
<tr>
<td>Low (0 and 1)</td>
<td>64 (46.4)</td>
<td>74 (53.6)</td>
<td>0.043</td>
<td>117 (43)</td>
<td>155 (57)</td>
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<tr>
<td>High (2 and 3)</td>
<td>7 (25.9)</td>
<td>20 (74.1)</td>
<td></td>
<td>16 (28.6)</td>
<td>40 (71.4)</td>
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**NOTE:** Bold values indicate statistical significance with P < 0.05.
without reaching statistical significance (log-rank, $P = 0.07$; Fig. 3B). Concordant with PD-L1 mRNA, elevated levels of PD-L1 protein were also significantly associated with longer RFS in YTMA201 (Wimberly and colleagues, 2013, SABCS December 2013, manuscript in preparation).

The presence of increased TILs was not significantly associated with the risk of recurrence in multivariate analysis (HR, 0.765; CI, 0.188–2.548; $P = 0.67$). In the multivariate model, PD-L1 mRNA expression in breast tumors from YTMA201 was significantly associated with lower risk of recurrence (HR, 0.268; CI, 0.099–0.721; $P = 0.009$; Table 3) and this association was independent from age, tumor size, lymph node status, histologic grade, ER status, HER2 status, and the presence of low or high TILs. As expected, larger tumor size (>2 cm) and tumor involvement of lymph nodes were significantly associated with higher recurrence risk (HR, 2.872; CI, 0.865–6.919; $P = 0.02$ and HR, 5.305; CI, 1.961–14.878; $P = 0.001$, respectively; Table 3). In addition, PD-L1 mRNA expression or the presence of elevated TILs showed a mild, nonstatistically significant association with longer DSS (log-rank $P = 0.16$ and 0.06, respectively; Fig. 3C and D).

Using the online biomarker validation tool SurvExpress (ref. 40; see Supplementary Fig. S5 and Supplementary Methods) containing information from 30 published breast cancer mRNA datasets, we identified 6 cohorts including PD-L1 mRNA records. Of them, only 1 had recurrence annotations and increased PD-L1 mRNA was significantly associated with longer RFS (Wang-Richardson GSE19615, $P = 0.02$; Supplementary Fig. S5A and S5B). The other five datasets had only overall survival data and high PD-L1 mRNA was marginally significantly associated with longer overall survival in one of them (Kao-Huang GSE20685, $P = 0.05$; Supplementary Fig. S5C and S5D). In the other 4 cohorts, elevated PD-L1 mRNA trended, but was not significantly associated with overall survival (TCGA, Ma-Sgroi GSE1378, Miller-Bergh GSE3494, and Enerly-Yakhini GSE19536; data not shown).

### Discussion

Recent studies suggest that determination of PD-L1 status in tumor samples could help select patients for novel anti-PD-1/PD-L1 monoclonal antibody therapies (19). However, accurate determination of PD-L1 protein levels in FFPE tumor samples is limited by the absence of validated assays, reliable antibodies, and interpretative uncertainties (e.g., cutoff for positivity, marker heterogeneity). We describe herein a reproducible, antibody-independent assay for in situ PD-L1 mRNA measurement. A major strength of this method relies in the simultaneous measurement of a negative control indicator (DapB) that helps identifying the assay detection threshold and a positive control probe (UbC) used to exclude inadequate samples. Our data demonstrate that in situ PD-L1 mRNA expression can be measured in FFPE breast tumor samples and in TMAs. We also observed substantial variability between cores in PD-L1 expression, which will need to be taken into consideration when interpreting results from a single core biopsy of a large tumor. A limitation of our results is the lack of mRNA assessment by reverse transcription-PCR or array-based methods to establish concordance and quantification across mRNA measurement platforms. An advantage of the

### Table 2. TILs associations on YTMA128 and YTMA201

<table>
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<th>YTMA201</th>
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<td>Number (%)</td>
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<td>53 (73.6)</td>
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<td>&gt;50</td>
<td>118 (88.7)</td>
<td>15 (11.3)</td>
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<tr>
<td>Tumor size</td>
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<tr>
<td>&lt;2 cm</td>
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<td>19 (14.6)</td>
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<td>2–5 cm</td>
<td>62 (79.5)</td>
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<tr>
<td>Positive</td>
<td>14 (82.3)</td>
<td>3 (16.6)</td>
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**NOTE:** Bold values indicate statistical significance with $P < 0.05.$
The RNAscope technique is that it provides in situ measurement and can quantify mRNA in the epithelial cells within the TMA spot. As all other mRNA measurement methods grind the sample to produce the mRNA, from these methods the cellular source of the mRNA cannot be determined. However, we could validate the prognostic effect of PD-L1 mRNA expression in publicly available breast cancer mRNA datasets (Supplementary Fig. S5). Overall and despite the methodologic differences, the results are consistent with our findings measuring PD-L1 mRNA in situ.

A number of smaller studies have been published that assess expression levels of PD-L1 in breast cancer using IHC. A study by Ghebeh and colleagues (14), including 44 specimens with available frozen tissue and using the mouse monoclonal MIH1 clone for PD-L1 IHC, showed absence of signal in normal breast and expression of PD-L1 in 22 cases (50%). Fifteen of these cases showed signal in tumor cells with membranous/cytoplasmic pattern and were associated with higher histologic grade and hormone-receptor negativity. PD-L1–positive TILs were found in 18 of these cases (41%) and were associated with larger tumors, histologic grade III, HER2 positivity, and increased inflammatory infiltrates (14). In a subsequent study from the same group and using an expanded version of the cohort (N = 68), similar associations were found (22). Our results using mRNA measurements show that nearly 60% of breast carcinomas express PD-L1 transcripts and this was similar in two independent cohorts. However, no significant association with high grade/hormone receptor negativity was found. This difference might be due, at least in part, to the distinct properties and independent information provided by PD-L1 protein and mRNA molecules, as reported (33). Methodologic differences might also account for this apparent inconsistency. In fact, the MIH1 clone used in some studies failed validation using Western blot analysis and IHC in our laboratory together with two other commercially available antibodies (35). The same antibody clone was reported by others not to be suitable for FFPE samples (23).

Our results show that tumor PD-L1 mRNA expression is significantly associated with increased local immune cell infiltrates and with longer RFS. Moreover, the survival effect of PD-L1 mRNA was independent from other well-known prognostic factors such as tumor size, lymph node involvement, and hormone receptor/HER2 status, suggesting that PD-L1 mRNA is independently associated with favorable prognosis in breast cancer. However, these results are not in agreement with previous studies showing an adverse prognostic effect of PD-L1 expression by IHC in various malignancies, including melanoma, renal, urothelial, gastric, lung, and colorectal carcinomas (24–28). In contrast, our observations are consistent with 3 recent studies in metastatic melanomas, Merkel cell carcinomas and lung non–small cell carcinomas using the validated monoclonal antibody clone 5H1 and
Our data also show that the presence of elevated TILs was significantly associated with hormone receptor-negative tumors and with a clear trend towards longer survival (Fig. 3B–D). Similar findings have been reported by others (3–5) and point to the critical role of local immunity in limiting tumor progression, particularly in more aggressive triple-negative and basal-like breast neoplasms. In addition, increased TILs have also been shown to predict response to neoadjuvant chemotherapy in breast cancer (42, 43). However, our results show that the proportion of breast tumors with prominent lymphocytic infiltration is relatively low (~15% of cases) and the prognostic information provided by TILs is limited.

Our analysis has a number of limitations. One major limitation is that it includes only retrospectively collected cases and that mature survival information was only available for one cohort. A second issue is that the use of TMAs may underestimate or overestimate the mRNA markers expression due to intratumoral heterogeneity of expression. Assays in the clinic always use whole slides and examination of high number of fields seen in a histologic section may attenuate the sampling effect of TMAs. Given these limitations, the results of this study should be considered as hypothesis generating. Although our results support the value of measuring PD-L1 in TMA samples, translation of these findings into the clinical setting could certainly benefit from using whole tissue section samples. This could better reflect the tissue distribution of PD-L1 and its topographical relationships with tumor cells and TILs. Determination of the amount of tumor tissue necessary to accurately measure PD-L1 in patient samples requires further evaluation, but should likely consider the tumor size, availability of sample material (core vs. tumor resection), clinical purpose (prognostic vs. predictive; adjuvant vs. neoadjuvant), and disease stage (one primary vs. multiple primaries vs. disseminated disease).

One key unaddressed issue is the potential of PD-L1 mRNA, alone or in combination with other markers such as PD-L1 protein, to predict response to anti-PD-1/PD-L1 therapies. Ongoing studies measuring PD-L1 mRNA and protein levels using QIF in whole tissue section specimens from patients treated with anti-PD-1 therapy might provide a better substrate to conclusively address the prognostic/predictive value of these markers.

**Disclosure of Potential Conflicts of Interest**
D.L. Rimm has a commercial research grant from Genoptix and is a consultant/advisory board member for Genoptix and Novartis. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**
Conception and design: K.A. Schalper, V. Velcheti, L. Pusztai, D.L. Rimm
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.A. Schalper, V. Velcheti, D. Carvajal, H. Wimberly, J. Brown
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.A. Schalper, V. Velcheti, D. Carvajal, L. Pusztai, D.L. Rimm
Writing, review, and/or revision of the manuscript: K.A. Schalper, V. Velchetti, D. Carvajal, J. Brown, L. Puzsztai, D.L. Rimm
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.A. Schalper, V. Velchetti, D.L. Rimm
Study supervision: K.A. Schalper, D.L. Rimm

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References


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Kurt A. Schalper, Vamsidhar Velcheti, Daniel Carvajal, et al.

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