Immune-Derived PD-L1 Gene Expression Defines a Subgroup of Stage II/III Colorectal Cancer Patients with Favorable Prognosis Who May Be Harmed by Adjuvant Chemotherapy

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Abstract
A recent phase II study of patients with metastatic colorectal carcinoma showed that mismatch repair gene status was predictive of clinical response to PD-1–targeting immune checkpoint blockade. Further examination revealed strong correlation between PD-L1 protein expression and microsatellite instability (MSI) in stage IV colorectal carcinoma, suggesting that the amount of PD-L1 protein expression could identify late-stage patients who might benefit from immunotherapy. To assess whether the clinical associations between PD-L1 gene expression and MSI identified in metastatic colorectal carcinoma are also present in stage II/III colorectal carcinoma, we used in silico analysis to elucidate the cell types expressing the PD-L1 gene. We found a statistically significant association of PD-L1 gene expression with MSI in early-stage colorectal carcinoma (P < 0.001) and show that, unlike in non–colorectal carcinoma tumors, PD-L1 is derived predominantly from the immune infiltrate. We demonstrate that PD-L1 gene expression has positive prognostic value in the adjuvant disease setting (PD-L1low vs. PD-L1high HR = 9.09; CI, 2.11–39.10). PD-L1 gene expression had predictive value, as patients with high PD-L1 expression appear to be harmed by standard-of-care treatment (HR = 4.95; CI, 1.10–22.35). Building on the promising results from the metastatic colorectal carcinoma PD-1–targeting trial, we provide compelling evidence that patients with PD-L1high/MSI/immunehigh stage II/III colorectal carcinoma should not receive standard chemotherapy. This conclusion supports the rationale to clinically evaluate this patient subgroup for PD-1 blockade treatment. Cancer Immunol Res; 4(7); 582–91. ©2016 AACR.

Introduction
Stroma-derived factors have long been known to influence cancer progression (1), and the importance of the microenvironment for molecular classification of colorectal cancer tumors has been confirmed (2, 3). These studies highlight the influence of the nonneoplastic component of the tumor on patient prognosis. Expression of PD-L1, the immune checkpoint inhibitor, has been primarily detected on the surface of epithelial neoplastic cells in a number of cancers; however, in colorectal carcinoma, immunohistochemistry (IHC)-based studies of small cohorts have detected high PD-L1 expression in the stromal and immune compartments (4, 5). Although upregulation of PD-L1 in the tumor microenvironment (TME) is a recognized tumor immune-defense mechanism, these findings suggest a different origin for PD-L1 protein expression in colorectal carcinoma.

The mismatch repair system (MMR) helps preserve the fidelity of the genome (6, 7). Colorectal carcinomas that harbor defects in MMR show high microsatellite instability (MSI) and account for 12% to 15% of colorectal carcinomas. MSI tumors are generally defined by their large number of somatic mutations, compared with microsatellite stable (MSS) tumors. These tumors also exhibit heavy peritumoral/intratumoral lymphocytic infiltration, most likely due to a large number of mutated antigenic epitopes at the cell surface; this observation has been previously correlated with good prognosis in early-stage disease (8).

Investigators from a number of adjuvant trials have questioned the value of chemotherapy for defined colorectal carcinoma molecular subtypes in early-stage disease, with some studies suggesting potential harm, particularly to the overall good prognosis MSI group (9). Although it could be inferred from preclinical data that MSI tumors would not respond to 5-fluorouracil (5-FU)–based treatment (10), the first large adjuvant study published using MSI status to stratify patients revealed that patients with MSI tumors did benefit from the addition of chemotherapy following surgery (11). However, 11 subsequent studies have shown no benefit from 5-FU–based treatment for patients with MSI colorectal carcinoma in the adjuvant setting (9).
Significance of PD-L1 Gene Expression in Colorectal Cancer

Recent clinical studies in melanoma, renal cell carcinoma, and non–small cell lung cancer have reported significant positive responses to PD-1 checkpoint targeting (12). In contrast, results in colorectal carcinoma have been disappointing (12). Interrogation of factors associated with response to PD-1 blockade suggested that MSI status was a predictor of response, underpinning a phase II clinical trial (13) in patients with metastatic colorectal carcinoma stratified by MSI status. The disease control rate in this study was 90% (CI, 55–100) for patients with MSI tumors and 11% for patients with MSS tumors (CI, 1–35), supporting the hypothesis of strong predictive value of MSI status for positive response to PD-1 blockade in advanced colorectal carcinoma. In addition, many CD8+ infiltrating T lymphocytes were detected at the invasive front regions of these tumors, corresponding to increased PD-L1 expression levels at the tumor margin.

Effective use of PD-1–targeting checkpoint inhibitors requires reliable biomarkers/companion diagnostics. Immunohistochemical detection of PD-L1 is currently confounded by technical variation, fluctuations in detection levels, and reproducible cutoff thresholds. Most importantly, marked intratumoral staining heterogeneity greatly hinders reproducibility of immunohistochemical scoring systems. Thus, alternative approaches for assessing PD-L1 are required.

The extremely promising results in stage IV disease prompted us to evaluate the potential for immune checkpoint targeting in the adjuvant setting. We performed extensive bioinformatics analyses using well-characterized independent transcriptional profiling datasets to determine (i) whether PD-L1 gene expression is associated with specific cell lineage compartments within the colorectal carcinoma TME; (ii) ability to stratify patients in early-stage colorectal carcinoma using PD-L1 gene expression and determine its association with MSI status/immune infiltration; and (iii) clinical relevance of PD-L1 gene expression to both prognosis and potential for benefit from adjuvant chemotherapy.

Materials and Methods

Independent datasets

Gene expression profiles from independent colorectal carcinoma datasets were downloaded from the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under accession numbers GSE14333, GSE35602, GSE13294, GSE39396, and GSE39582. GSE14333 contains microarray profiles of surgically resected specimens in 290 colorectal carcinoma patients; 185 have additional treatment and survival data and are used in this study. GSE35602 contains microarray profiles separately profiled from micro-dissected stroma or epithelium regions from 13 colorectal carcinoma tissues. GSE13294 contains 155 colorectal carcinoma microarray profiles, with MSI status, from surgically resected specimens. GSE39396 contains microarray profiles from fresh colorectal specimens where FACS has been used to divide cells into specific endothelial (CD45+ EPCAM− CD31+ FAP+), epithelial (CD45− EPCAM+ CD31− FAP−), leukocyte (CD45− EPCAM− CD31− FAP−), and fibroblast (CD45− EPCAM− CD31+ FAP+) populations prior to microarray profiling. GSE39582 contains 566 stage I–IV profiles from a large colorectal carcinoma series, of which the stage II/III profiles were selected for analysis.

Transcriptional analysis

Partek Genomics Suite was used for independent dataset analysis. For the purpose of clustering, data matrices were standardized to the median value of probe-set expression. Standardization of the data allows for comparison of expression levels for different probe sets. Following standardization, two-dimensional hierarchical clustering was performed (samples × probe sets/genome). Euclidean distance was used to calculate the distance matrix, a multidimensional matrix representing the distance from each data point (probe set-sample pair) to all the other data points. The Ward linkage method was subsequently applied to join samples and genes together, with the minimum variance, to find compact clusters based on the calculated distance matrix.

Statistical analysis

Median and tertile stratification was performed on GSE13294 by calculation of mean expression values from both CD274 probe sets. These values were then classified as high and low based on 77:78 sample distributions or as high, medium, and low based on 52:51:52 sample distributions. Student t tests and Fisher exact tests were carried out using GraphPad Prism version 5 for Windows, GraphPad Software.

Survival analysis

Survival curves, comparing expression and treatment subgroups, were estimated with the Kaplan–Meier method and compared by the log-rank test, using GraphPad Prism version 5 for Windows, GraphPad Software. Cox proportional hazards analysis, using Stata version 11.2, was applied to evaluate recurrence-free survival (RFS) according to PD-L1 gene expression levels within the indicated subgroups, prior to and after adjustment for age, sex, tumor stage and location, and receipt of adjuvant treatment. Categorical and continuous variables were compared between individuals within the overall cohort and also in PD-L1 high/MSI versus PD-L1 low/MSS tumors using χ² tests and t tests, respectively.

Results

PD-L1 gene expression associated with immune component of TME

Unlike its expression in other cancers, PD-L1 expression in the colorectal TME may not be exclusive to epithelial tumor cells (4). To further investigate this preliminary finding, we used two colorectal carcinoma gene expression transcriptomic datasets, derived from samples either laser-microdissected to purify stromal and epithelial regions (GSE35602), or separated into epithelial, leukocyte, endothelial, or fibroblast components using FACS (GSE39396) prior to microarray profiling of the separated cells. Scatterplot and boxplot assessment of gene expression levels of PD-L1 (CD274), according to region of origin, revealed significantly increased gene expression in the stroma compared with neoplastic epithelial cells in the laser-microdissected dataset (two-tailed Student t test P < 0.0001; Fig. 1A and C). In the FACS-derived microarray dataset, significantly higher levels of PD-L1 gene expression were observed in tumor-associated leukocytes compared with epithelial cells (two-tailed Student t test P = 0.0071; Fig. 1B and D).

To assess the purity of the microdissection of the samples from the GSE35602 dataset, we used a previously
published gene expression list derived exclusively from nonepithelial cells. These 213 genes were defined from three signatures specifically expressed by cancer-associated fibroblasts (n = 131), leukocytes (n = 47), and endothelial cells (n = 35; ref. 3). Using hierarchical clustering and principal component analyses, we robustly identified two distinct groups in the GSE35602 dataset that corresponded to each "region of origin" with 100% accuracy (Supplementary Fig. S1A and S1C). When the same approach was applied to GSE39396 (FACS-sorted dataset), region of origin was again identified with 100% accuracy (Supplementary Fig. S1B and S1D).

In addition to PD-L1, expression of other immune therapy targets (CTLA4, LAG3, and IDO1) has also been reported to be upregulated in immune-infiltrating cells of MSI tumors compared with MSS tumors (4), although their gene expression levels in individual cell compartments have not been assessed. Using region-specific and cell-specific gene expression profiles, we found that CTLA4 (6.3-fold, P < 0.001), LAG3 (4.5-fold, P < 0.001), PD-L1 (4.2-fold, P < 0.001), and IDO1 (9.2-fold, P < 0.001) are all

**Figure 1.**

PD-L1 gene expression is higher in the stromal immune compartment of the TME. A, scatterplot indicating higher PD-L1 gene expression in stromal cells compared with the epithelium of 13 microdissected primary tumor samples (GSE35602). B, PD-L1 gene expression was higher in the leukocyte population compared with fibroblast, endothelial, and epithelial populations isolated by FACS (GSE39396). C and D, dot plot with associated box and whisker plots representing the mean expression values for PD-L1 in each sample set; 10% lower and 90% upper values are indicated. E and F, Euclidean clustering for gene expression profiles of CTLA4, LAG3, PD-L1, and IDO1 in each sample set. Overlay bar indicates the region or cell of origin as used in A–D.
elevated in stroma compared with epithelium in colorectal carcinoma tumor samples (Fig. 1D). Whereas IDO1 expression was elevated in all stromal compartments compared with epithelial cells, the elevated expression of CTLA4, LAG3, and PD-L1 was confined to the leukocyte-specific compartment (Fig. 1E and F).

We also confirm that expression of IFNγ (IFNG) is specific to the immune-derived compartment (Supplementary Fig. S1E), consistent with previous findings (14).

Collectively, these analyses provide compelling evidence that PD-L1 in colorectal tumors is predominantly derived from infiltrating immune cells rather than neoplastic epithelial cells.

Association between MSI, immune infiltration, and PD-L1 gene expression

To assess whether the clinical associations between PD-L1 gene expression and MSI identified in metastatic colorectal carcinoma are also present in stage II/III colorectal carcinoma, we evaluated transcriptomic data derived from a cohort of 155 patients (GSE13294), enriched to contain approximately

Table 1. Characteristics of colon cancer patients and tumors according to PD-L1 gene expression status

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All colon cancers</th>
<th>PD-L1high only</th>
<th>PD-L1low only</th>
<th>PD-L1high only</th>
<th>PD-L1low only</th>
<th>Pn</th>
<th>Pn</th>
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<tbody>
<tr>
<td>Age, years, mean (SD)</td>
<td>67.6 (12.7)</td>
<td>69.5 (13.9)</td>
<td>0.23</td>
<td>70.8 (17.0)</td>
<td>68.6 (11.2)</td>
<td>0.47</td>
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<td>Sex, n (%)</td>
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<td></td>
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</tr>
<tr>
<td>Male</td>
<td>193 (56.8)</td>
<td>49 (58.3)</td>
<td>0.80</td>
<td>19 (52.8)</td>
<td>30 (62.5)</td>
<td>0.37</td>
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</tr>
<tr>
<td>Female</td>
<td>147 (43.2)</td>
<td>35 (41.7)</td>
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<td>17 (47.2)</td>
<td>18 (37.5)</td>
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<tr>
<td>Tumor stage, n (%)</td>
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<tr>
<td>II</td>
<td>181 (53.2)</td>
<td>41 (48.8)</td>
<td></td>
<td>19 (52.8)</td>
<td>22 (45.8)</td>
<td></td>
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<tr>
<td>III</td>
<td>159 (46.8)</td>
<td>43 (51.2)</td>
<td>0.47</td>
<td>17 (47.3)</td>
<td>26 (54.2)</td>
<td>0.53</td>
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<tr>
<td>Tumor location, n (%)</td>
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<tr>
<td>Proximal</td>
<td>125 (36.8)</td>
<td>54 (64.5)</td>
<td>&lt;0.001</td>
<td>32 (88.9)</td>
<td>22 (45.8)</td>
<td>&lt;0.001</td>
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<td>Distal</td>
<td>215 (63.2)</td>
<td>30 (35.7)</td>
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<td>4 (11.1)</td>
<td>26 (54.2)</td>
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<td>Adjuvant treatment receipt, n (%)</td>
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<td>No</td>
<td>178 (52.4)</td>
<td>54 (64.3)</td>
<td>0.07</td>
<td>8 (22.2)</td>
<td>21 (43.8)</td>
<td>0.07</td>
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<tr>
<td>Yes</td>
<td>161 (47.4)</td>
<td>29 (34.5)</td>
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<td>MSI status, n (%)</td>
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<td>MSI</td>
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<td>36 (42.9)</td>
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<td>MSS</td>
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<td>48 (57.1)</td>
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<td>NA</td>
<td>NA</td>
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<td>CIMP, n (%)</td>
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<td></td>
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<tr>
<td>Negative</td>
<td>246 (72.4)</td>
<td>41 (48.8)</td>
<td>0.07</td>
<td>22 (47.3)</td>
<td>8 (16.7)</td>
<td>&lt;0.001</td>
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<tr>
<td>Positive</td>
<td>101 (27.6)</td>
<td>30 (35.7)</td>
<td></td>
<td>52 (76.7)</td>
<td>22 (47.3)</td>
<td>&lt;0.001</td>
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<td>45 (13.1)</td>
<td>10 (11.9)</td>
<td></td>
<td>3 (8.3)</td>
<td>10 (20.8)</td>
<td>&lt;0.001</td>
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<tr>
<td>CIN, n (%)</td>
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<tr>
<td>Negative</td>
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<td>27 (32.1)</td>
<td>&lt;0.001</td>
<td>22 (61.1)</td>
<td>5 (10.4)</td>
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<tr>
<td>Positive</td>
<td>215 (64.1)</td>
<td>41 (48.8)</td>
<td></td>
<td>17 (47.3)</td>
<td>26 (54.2)</td>
<td>&lt;0.001</td>
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</tr>
<tr>
<td>Unknown</td>
<td>54 (15.9)</td>
<td>10 (11.9)</td>
<td></td>
<td>3 (8.3)</td>
<td>10 (20.8)</td>
<td>&lt;0.001</td>
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<tr>
<td>p53, n (%)</td>
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<tr>
<td>WT</td>
<td>100 (29.4)</td>
<td>33 (39.3)</td>
<td>&lt;0.001</td>
<td>15 (41.7)</td>
<td>18 (37.5)</td>
<td>&lt;0.001</td>
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<tr>
<td>MT</td>
<td>131 (38.5)</td>
<td>26 (31.0)</td>
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<td>3 (8.3)</td>
<td>23 (47.9)</td>
<td>&lt;0.001</td>
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<td>109 (31.1)</td>
<td>25 (29.8)</td>
<td>0.20</td>
<td>18 (50.0)</td>
<td>7 (14.6)</td>
<td>&lt;0.001</td>
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<td>KRAS, n (%)</td>
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<tr>
<td>WT</td>
<td>194 (57.1)</td>
<td>53 (63.1)</td>
<td>&lt;0.001</td>
<td>25 (69.4)</td>
<td>28 (58.3)</td>
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<tr>
<td>MT</td>
<td>128 (37.7)</td>
<td>28 (33.3)</td>
<td></td>
<td>10 (27.8)</td>
<td>18 (37.5)</td>
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<tr>
<td>Unknown</td>
<td>18 (5.3)</td>
<td>3 (3.6)</td>
<td>0.56</td>
<td>1 (2.8)</td>
<td>2 (4.2)</td>
<td>0.58</td>
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<tr>
<td>BRAF, n (%)</td>
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<tr>
<td>WT</td>
<td>278 (81.8)</td>
<td>55 (65.5)</td>
<td>&lt;0.001</td>
<td>18 (50.0)</td>
<td>37 (77.1)</td>
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<tr>
<td>MT</td>
<td>18 (5.3)</td>
<td>19 (22.6)</td>
<td></td>
<td>17 (47.2)</td>
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<tr>
<td>Unknown</td>
<td>44 (12.9)</td>
<td>10 (11.9)</td>
<td>&lt;0.001</td>
<td>1 (2.8)</td>
<td>9 (18.8)</td>
<td>&lt;0.001</td>
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</tr>
</tbody>
</table>

Abbreviations: MT, mutant; NA, not applicable; WT, wild-type.

*Adjuvant chemotherapy treatment receipt, unknown for two individuals (one PD-L1high, one PD-L1low).

bp53 results confounded by lack of information for mutational status in 50% of PD-L1high MSI cases.

Unadjusted and adjusted analyses of RFS

Table 2. Unadjusted and adjusted analyses of RFS

| Entire cohort | PD-L1low vs. high | HR 1.64 (95% CI, 0.91–2.97) | HR 1.32 (95% CI, 0.69–2.54) |
|---------------|------------------|----------------|----------------| | | | | |
| Untreated only | PD-L1low vs. high | HR 9.09 (95% CI, 2.31–39.10) | HR 4.81 (95% CI, 0.79–29.19) |
| Treated only | PD-L1low vs. high | HR 0.86 (95% CI, 0.45–1.66) | HR 0.80 (95% CI, 0.40–1.60) |
| PD-L1high only | Treated vs. untreated | HR 4.95 (95% CI, 1.10–22.35) | HR 1.62 (95% CI, 0.25–10.36) |
| PD-L1low only | Treated vs. untreated | HR 0.49 (95% CI, 0.29–0.83) | HR 0.44 (95% CI, 0.25–0.80) |

*Adjusted for all confounders in Table 1, with those that were significant at the P < 0.25 level kept in the model as covariates. PD-L1 results adjusted for age, sex, CIMP, CIN, and KRAS. Tested for tumor location, BRAF, MSI, and p53.

RFS analysis was performed using the Cox proportional hazards method stratified by PD-L1 levels or treatment expression levels. Analysis was performed both before and following adjustment.
equal numbers of MSI (n = 78) and MSS (n = 77) tumors. Using a median- and tertile-stratification approach based on mean PD-L1 gene expression, we differentiated samples based on high or low PD-L1 gene expression (Fig. 2A) and high, medium, or low gene expression (Supplementary Fig. S2A). Using a median-stratified approach, PD-L1 gene expression was...
Significantly associated with MSI status (Fisher exact two-tailed test $P < 0.0001$; Fig. 2B). In addition, this PD-L1$^{high}$/MSI-rich subgroup was significantly associated with the 47 leukocyte-specific nonepithelial gene signature (Fisher exact two-tailed test $P < 0.0001$; Fig. 2C). When the intermediate group ($n = 51$) was removed from the tertile analysis, we again found a

Figure 3.
PD-L1 gene expression profile in stage II/III colorectal carcinoma. A (left), hierarchical clustering of stage II/III colorectal carcinoma patient cohort (GSE39582) based on expression profiles of PD-L1 (CD274) identifies a strong positive subgroup accounting for 20% of the overall population. Overlay bar indicates assigned PD-L1 subgroup (PD-L1$^{high}$, red; PD-L1$^{low}$, blue) and MSI/MSS status (MSI, black; MSS, gray; NA, white). Right, scatterplot of PD-L1 gene expression values highlights the positive and negative groups identified in the heatmap. B (left), scatterplot of PD-L1 gene expression values according to MSI/MSS status. Right, Fisher exact test confirms a statistically significant correlation between high PD-L1 transcript amounts and MSI status. C, hierarchical clustering of patient cohort based on leukocyte-specific gene signature stratifies population into 3 groups based on their immune infiltrate content. Overlay bar indicates that the high immune infiltrate subgroup overlaps strongly with the high PD-L1 gene expression subgroup.
significant correlation between PD-L1 gene expression and both MSI and the leukocyte-specific signature (Supplementary Fig. S2B–S2D). These results reveal the relationship between high PD-L1 gene expression, MSI, and immune infiltration in stage II/III disease.

Identification of a subgroup of patients with high PD-L1 gene expression

Using hierarchical clustering of microarray gene expression profiles from a large stage II/III colorectal carcinoma dataset (GSE39582), and using Euclidean and Ward metrics, we identified a distinct subgroup of patients with high PD-L1 gene expression relative to the remaining population (Fig. 3A). This PD-L1\text{high} subgroup accounted for 20% of the overall cohort, which we used as our threshold for all subsequent analyses; further investigation highlighted a strong correlation between elevated PD-L1 gene expression and MSI genotype (Fisher exact two-tailed test $P < 0.0001$; Fig. 3A and B; Supplementary Fig. S3A), further validating our earlier findings (Fig. 2).

Stratification of the data was performed to facilitate an evaluation of the available clinicopathologic factors using two different comparisons: PD-L1\text{high} gene expression subgroup (PD-L1\text{high}) versus PD-L1\text{low} gene expression subgroup (PD-L1\text{low}) in the entire cohort and MSI versus MSS in the PD-L1\text{high} subgroup only. Within the entire cohort, individuals with PD-L1\text{high} tumors did not differ from those with PD-L1\text{low} tumors in terms of age, sex, or stage distribution. PD-L1\text{high} tumors were less likely to be treated with adjuvant chemotherapy, although this finding did not reach statistical significance ($P = 0.07$). PD-L1\text{high} tumors were more likely to be right-sided, MSI, CpG island methylator phenotype (CIMP) positive, chromosome instability (CIN) negative, and protein kinase BRAF mutant than PD-L1\text{low} tumors, but not p53 or KRAS mutant (Table 1).

Findings from the PD-L1\text{high} subgroup stratified by MSI or MSS confirm that MSS/PD-L1\text{high} tumors were less likely to be right-sided, CIMP, CIN, and BRAF mutant than MSI/PD-L1\text{high} tumors. This analysis again highlighted that whereas MSI status was significantly associated with high PD-L1 gene expression, a subgroup of 13% of MSS tumors were also classified as PD-L1\text{high}. Using the previously described leukocyte-specific signature (3) that is solely attributed to the leukocyte compartment of the TME, we found strong overlap between those patients in the PD-L1\text{high} subgroup and those with a gene expression profile indicative of an increased immune infiltrate (Fisher exact two-tailed test $P < 0.001$; Fig. 3C). This finding further confirmed that the PD-L1\text{high} subgroup, which is significantly enriched for MSI ($P < 0.001$), was also associated with more tumor-infiltrating immune cells and highlights PD-L1 expression as a robust transcriptional marker for this subgroup. Although we do find significant clinicopathologic differences between MSI and MSS in the PD-L1\text{high} subgroup, in agreement with our data presented in Figs. 1 and 2, it is the biological signature indicative of a large immune infiltration that appears to dictate the level of PD-L1 gene expression.

Further analysis confirmed coexpression and elevated expression of CTLA4, LAG3, and IDO1 in stage II/III tumor samples that have high expression of PD-L1 (Supplementary Fig. S3B), in addition to significant upregulation of IFNγ (two-tailed Student t test $P < 0.001$; Supplementary Fig. S3C).

PD-L1 is a significant positive prognostic marker in early-stage disease

To investigate the clinical relevance of PD-L1 gene expression, we used relapse follow-up data associated with the well-characterized GSE39582 dataset. Patients ($n = 201$) were stratified based on PD-L1 subgroup, stage, and treatment. In the untreated stage III population, we found a clear difference in RFS between low and high PD-L1 subgroups, with the PD-L1\text{low} subgroup having a significantly worse outcome ($P = 0.0003$; HR = 9.09; 95% CI, 2.11–39.10; Fig. 4A and B; Table 2). However, in the treated cohort, the correlation between survival and high PD-L1 expression was lost ($P = 0.6514$; HR = 0.86; 95% CI, 0.45–1.66), suggesting that PD-L1 gene expression also has value for predicting benefit from standard adjuvant chemotherapy (Fig. 4B, Table 2).

To address this question, we performed treatment interaction analyses and found that, whereas patients with low expression of the PD-L1 gene significantly benefit from adjuvant chemotherapy ($P = 0.0062$; HR = 0.49; 95% CI, 0.29–0.83), patients in the PD-L1\text{high} subgroup have poorer RFS following treatment ($P = 0.0208$; HR = 4.95; 95% CI, 1.10–22.35; Fig. 4A and B; Table 2). An adjusted analysis for the known confounders and covariates of PD-L1 gene expression (Table 1) again confirmed that PD-L1 gene expression could be considered as an independent biomarker for patient stratification, as although the prognostic and predictive trend remained the same, the adjusted multivariate significance was lost (Table 2).

To confirm these findings in an independent patient cohort, we interrogated a further early-stage colorectal carcinoma dataset (GSE14333). Patients ($n = 185$) were again stratified into high and low PD-L1 subgroups in similar proportions as identified using our initial dataset. In Dukes B patients within this cohort, high PD-L1 gene expression was significantly associated with better disease-free survival (DFS) in the untreated population compared with those who received adjuvant treatment ($P = 0.0371$; HR = 10.18; CI, 1.15–90.14). This trend was also observed in the combined Dukes B/C cohort, but failed to reach significance, most likely due to the small number of patients in this combined cohort compared with the original dataset (Fig. 4C).

These data indicate that TME-derived PD-L1 transcription levels are both a positive prognostic marker for improved RFS/DFS in early-stage disease, but importantly are also a negative predictive marker for chemotherapy in the adjuvant setting.

Discussion

Since the FDA’s approval of the first immune checkpoint therapy (the CTLA4-specific antibody ipilimumab), a number of clinical trials have demonstrated the potential for targeting this pathway in a variety of cancers. However, immune therapy has had surprisingly little impact in colorectal carcinoma. A recent phase II study gave the first indication that PD-1 targeting of colorectal carcinoma in metastatic disease significantly benefited patients with MSI tumors when compared with those with MSS disease (13). This study also indicated that PD-L1 expression (assessed by IHC) was strongly associated with MSI, suggesting that expression of PD-L1 may be a useful predictive biomarker of response to PD-1 immune checkpoint targeting in this setting.

Using an in silico approach, we assessed whether PD-L1 gene expression was associated with MSI in early-stage colorectal carcinoma, which would provide a rationale for pursuing PD-1 checkpoint targeting in the adjuvant disease setting. We
found that PD-L1 transcription levels are significantly elevated in the immune cells present in the TME, in agreement with an earlier study in a small patient cohort (4). Given that high immune infiltration can occur in colorectal carcinoma, these findings may explain the difference in response rates to immune-checkpoint targeting in colorectal carcinoma compared with other tumors, e.g., lung cancer and melanoma, where PD-L1 expression has been detected in the membrane of epithelial neoplastic cells. Using a transcriptomic dataset from a cohort of 155 colorectal carcinoma patients, enriched to include ~50% MSI/MSS, we found that PD-L1^{high} tumors are significantly associated with the MSI genotype. Additionally, a statistically significant correlation between high PD-L1 gene expression and substantial immune cell infiltration was found, further supporting the hypothesis that these patients would benefit from PD-1–targeting agents. This significant association with MSI was also evident in a large well-characterized stage II/III clinical cohort, which also confirms that PD-L1 gene expression is significantly associated with right-sided, CIMP, and BRAF-mutant tumors.
A recent clinical trial in metastatic colorectal carcinoma uncovered a subgroup of patients with MSI genotype and high PD-L1 levels using IHC; we identified a distinct subgroup of stage II/III colorectal carcinomas, this time identifiable by high PD-L1 transcription levels. While this subgroup was significantly associated with MSI, it was not exclusive to this genotype, with a small number of MSS tumors also being PD-L1high. Conversely, a small proportion of MSI patients were classified as PD-L1low. These results suggest that while the MSI genotype results in high mutation rates which promote high levels of immune infiltration, the expression of PD-L1, and indeed immune infiltration levels, can also be upregulated by MSI-independent mechanisms. Thus, PD-L1 gene expression, rather than MSI status, may be a more powerful predictive biomarker for response to PD-1/PD-L1 checkpoint inhibition in colorectal carcinoma. Previous studies have shown that patients with MSI colorectal carcinoma generally have a good overall prognosis in early-stage disease; however, there is still debate as to the benefit of adjuvant chemotherapy in this group (9). Recently, a large meta-analysis concluded that there was no effect of adjuvant treatment for MSI patients, whereas there was a significant benefit in MSS patients (15). Data presented here now show that high PD-L1 transcription levels, which are significantly associated with the MSI genotype, identify a subgroup of patients with a significantly better prognosis in early-stage disease. In addition, we show that this PD-L1high subgroup derives no clinical benefit and indeed may be harmed by adjuvant 5-FU-based chemotherapy using an unadjusted analysis.

Although this dataset, and the independent validation set, were not generated from material collected from randomized controlled trials, our findings on high PD-L1 transcriptional levels have clinical implications above and beyond MSI status alone, for further stratifying patients into those likely to benefit from standard adjuvant chemotherapy and those who may potentially be harmed. Our analyses suggest that the PD-L1high subgroup should not be given adjuvant 5-FU-based chemotherapy following surgery, whereas patients with low PD-L1 gene expression significantly benefit from adjuvant treatment, in both unadjusted and adjusted models for survival analysis. Moreover, although data presented here strongly indicate that PD-L1high patients may not need any systemic therapy, their PD-L1 levels, MSI status, and immune infiltrate levels confirm that this clinical subgroup (based on the recent clinical trial in the metastatic setting) that may instead benefit from PD-1/PD-L1 immune checkpoint inhibitors, notably in stage III disease. Patients in this PD-L1high subgroup also displayed increased expression of a number of other immunotherapeutic targets, namely, CTLA4, LAG3, and IDO1. Similar to the findings presented here for PD-L1, expression of each of these targets is confined to the stroma, in particular to the immune compartment, with the exception of the metabolic regulator IDO1, which is found in all stromal compartments. These findings highlight the potential for combination immunotherapies in this immune checkpoint overexpressing subgroup. Although PD-L1 gene expression had statistically significant prognostic and predictive value in two independent cohorts, final validation requires transcriptional data, detailed treatment information, and clinical follow-up from an independent, well-balanced cohort, enriched for MSI stage II/III colorectal carcinoma patients enrolled in a prospective clinical trial. This type of patient stratification approach is ongoing in current clinical trials (16, 17) that will enable further biomarker-based validation in this setting. Ongoing debate surrounds the definition of a clinically relevant companion diagnostic threshold for assessing PD-L1 protein levels by IHC to predict benefit from PD-1 blockade. This will only be possible by retrospective outcome-supervised analysis of the tumor tissue from ongoing and completed PD-1/PD-L1 checkpoint inhibition trials in colorectal carcinoma. Matched IHC and microarray profiling from the same tissue would allow the generation of these urgently required thresholds for prospective use.

The transcriptional profiles we have analyzed are representative of primary colorectal carcinoma tumors prior to therapy, and as such provide insight into the cell populations present and their signaling activities during development of the primary tumor. It is conceivable that the majority of these tumors, which we now show have a paucity of immune cells, initiate and develop by circumventing a widespread immune response (18). It is only tumors that have escaped immunosurveillance, allowing development of invasive malignancy and subsequent metastatic spread, which have high relapse rates and poor survival. The small proportion of tumors that we have identified with an inherently large immune infiltrate (resulting from high numbers of mutated antigenic epitopes due to their MSI status; ref. 8) can now show the potential for combination immunochemotherapy in this clinically relevant and PD-L1–definable subgroup.
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Immune-Derived PD-L1 Gene Expression Defines a Subgroup of Stage II/III Colorectal Cancer Patients with Favorable Prognosis Who May Be Harmed by Adjuvant Chemotherapy

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