

Stromal PD-1/PD-L1 Expression Predicts Outcome in Colon Cancer Patients

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Abstract

In a study that used a next-generation tissue microarray approach, stromal expression of programmed cell death 1 (PD-1)/programmed death ligand 1 (PD-L1) in patients with colon cancer correlated with less aggressive tumor features, resulting in improved outcome. Stromal PD-L1/PD-1 expression might serve as a prognostic biomarker in colon cancer patients.

Introduction: The programmed cell death 1 (PD-1)/programmed cell death ligand 1 (PD-L1) axis plays an important role in controlling immune suppression by down-regulating T effector cell activities, enabling tumor cells to escape from the host's antitumor immunosurveillance. While only a small part of colon cancer cells express PD-L1, we sought to evaluate the differential impact of stromal and epithelial PD-L1 expression of primary tumors and liver metastasis on overall survival (OS) in colon cancer patients. **Patients and Methods:** Using a next-generation tissue microarray approach, we assessed both epithelial and stromal PD-L1 expression levels in primary tumors (n = 279) and corresponding liver metastases (n = 14) of colon cancer patients. PD-L1 positivity was graded according to the percentage (0.1%-1%, > 1%, > 5%, > 50%) of tumor cells with membranous PD-L1 expression or as the percentage of positive stroma cells and associated inflammatory infiltrates. We also assessed the interplay between stromal PD-1/PD-L1 and both intratumoral and stromal CD8 count and their impact on outcome. The primary end point was OS. **Results:** Stromal PD-L1 and PD-1 expression were both associated with less aggressive tumor behavior in colon cancer patients, which translated into better OS and disease-free survival, respectively. Conversely, PD-L1 staining in the tumor cells was less frequent than stromal staining and was associated with features of aggressive tumor biology, although without impact on outcome. Interestingly, the PD-L1 staining pattern remained similar between primary tumors and corresponding liver metastases. Stromal PD-1 expression correlated significantly with stromal PD-L1 staining and both intratumoral and stromal CD8 expression. **Conclusion:** Stromal PD-1/PD-L1 expression might serve as a prognostic marker in colon cancer patients.

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Introduction

Colon cancer is the fourth leading cause of cancer-related mortality worldwide.¹ The steadily increasing incidence, with 1.36 million new patients diagnosed per year in 2012 and an expected rate of 2.2 million new colorectal cancer patients in 2030, represents a global health problem.¹ The implementation of targeted therapies such as bevacizumab and cetuximab has led to an increased life expectancy in patients with metastatic colorectal cancer (mCRC) during the last decade.² However, the addition of biologicals is neither associated with better recurrence-free survival nor longer overall survival (OS) in the adjuvant treatment of high-risk stage II and III colon cancer.^{3,4}

To further improve treatment and to prolong survival in both the adjuvant and palliative settings, new effective therapies are needed.

The introduction of immune checkpoint inhibitors has dramatically changed the therapeutic landscape of various solid tumors such as non-small cell lung cancer, urothelial and renal cell carcinoma, and head and neck cancer as well as melanoma.⁵⁻⁹ In May 2017, the anti-programmed cell death 1 (PD-1) antibody pembrolizumab was approved by the US Food and Drug Administration for the treatment of high microsatellite instability (MSI-high) mCRC after disease progression while receiving 5-fluorouracil (5-FU)-, oxaliplatin-, or irinotecan-based chemotherapy after having exhibited promising response rates.¹⁰ Recently, the CheckMate 142 phase 2 trial showed that the PD-1 checkpoint inhibitor nivolumab provided durable disease control in treatment refractory MSI-high/mismatch repair-deficient metastatic colorectal cancer.¹¹

However, there is a need for further prognostic biomarkers enabling better prediction of outcome in colon cancer patients independent of the therapy administered. This might help to better predict which stage II and III colon cancer patients will derive benefit from adjuvant chemotherapy. Moreover, immune-related biomarkers might facilitate clinical decision making and assist us in identifying patients for whom immune checkpoint therapy might be most promising, regardless of the tumor, node, metastasis classification system (TNM) stage and MSI status. The immune response of cytotoxic T cells is regulated by an equilibrium of stimulatory and inhibitory signals, the so-called immune checkpoints.¹² One checkpoint between tumor and immune system involves PD-1/PD-L1 interaction, which results in T-cell inactivation.¹³ PD-L1 is a negative costimulatory molecule mainly expressed on T cells and antigen-presenting cells such as B cells, dendritic cells, and macrophages, but it is less frequently observed in colon cancer cells.^{14,15} The PD-L1/PD-1 axis mediates immune suppression by down-regulating T-cell effector functions, enabling tumor cells to escape from the host's antitumor immunosurveillance. This immunoevasive strategy helps tumor cells to survive and thus facilitates metastatic spread.¹⁶

Previous studies showed that epithelial PD-L1 staining in gastrointestinal tumors is less frequent compared to other solid tumors such as melanoma.¹⁷ Therefore, we sought to evaluate the impact of epithelial and stromal PD-L1 expression on outcome in colon cancer patients. We also aimed to assess whether there is a difference between epithelial or stromal PD-L1 expression between primary tumors and corresponding synchronous/metachronous metastases. Finally, to better characterize the tumor microenvironment, we also assessed the stromal PD-1 expression as well as intra- and peritumoral CD8 counts and evaluated their influence on outcome.

Patients and Methods

Patients

Patients with primary colorectal cancers treated at the University Hospital of Bern, Switzerland, between 2002 and 2013 were retrospectively enrolled into this study. From an original set of 422 cases, rectal cancers were excluded ($n = 115$), as were patients with insufficient tissue material for analysis ($n = 28$), leaving 279 patients. Adjuvant treatment for high-risk stage II and III colon cancer comprised a 5-FU- or capecitabine-based chemotherapy (5-FU/capecitabine with or without oxaliplatin). Palliative first-line chemotherapy for patients with stage IV disease consisted of a FOLFOX (5-FU, leucovorin, and oxaliplatin), XELOX (capecitabine and oxaliplatin), or FOLFIRI (5-FU, leucovorin, and

irinotecan) regimen with or without bevacizumab/cetuximab, while anti-epidermal growth factor receptor therapy was provided only to patients with *RAS* or *KRAS* wild-type tumors.

Because this cohort comprised patients enrolled between 2002 and 2013, none of the MSI-high stage IV patients received immunotherapy in subsequent treatment lines.

Histopathologic repeat review was performed according to the 7th edition of the TNM and carried out by expert gastrointestinal pathologists (A.L., H.D., V.K.). Clinical and treatment data were obtained from patient records and from direct follow-up with patients or general practitioners (M.D.B., J.W., M.H., D.I.).

Data included patient age at diagnosis, gender, histologic subtype, tumor location, pT and pN, total number of lymph nodes collected, and number of positive nodes. Distant metastasis was recorded when either pM or cM showed documented metastasis. The classification cM was used on the basis of obvious radiologic findings on computed tomography, magnetic resonance imaging, or ultrasound indicating metastases. In addition, tumor grade, and lymphatic vessel and venous vessel invasion were reviewed, as was perineural invasion. Tumor budding was scored according to the latest International Tumor Budding Consensus Conference guidelines.¹⁸ In addition, the percentage of expansive tumor growth pattern and the Klintrup-Mäkinen score were provided, the latter being an indicator of inflammatory response around the tumor. Mismatch repair (MMR) status was available for all patients, as was v-Raf murine sarcoma viral oncogene homolog B (*BRAF*) mutation status, detected by immunohistochemistry using the VE1 antibody (*BRAF* mutated protein from V600E mutation in codon 15).

Next-Generation Tissue Microarray (ngTMA)

Construction

All cases were reviewed again for tissue microarray construction, and the best 1 to 3 tissue blocks were selected in order to obtain material from normal tissue at the resection margins, tumor center, and tumor front. Hematoxylin and eosin-stained slides were recut from each block and scanned. Digital scans were uploaded to a web-based platform. Each corresponding tissue block was then placed into an automated Tissue Microarrayer (3DHitech, Budapest, Hungary). The image of each donor block was aligned with the annotated digital slides; then each annotated area was cored and the core sample transferred to an empty recipient block.¹⁹ Three cores of the tumor center as well as 3 cores of the tumor front were evaluated for each case, resulting in a next-generation tissue microarray (ngTMA) for all 422 patients with 6 tumor cores each ($n = 2532$). Tissues were obtained from the Tissue Bank Bern, archived at the Institute of Pathology, Bern, Switzerland.

A second ngTMA was constructed similarly on liver metastases from 14 mCRC patients. In order to assess tumor heterogeneity, tissues were taken from all primary tumor blocks and metastatic tissue blocks, resulting in a total of 100 cores to be assessed by ngTMA. This study was approved by the ethics committee of the Canton of Bern under approval 200/2014.

Immunohistochemistry and Immunofluorescence

The ngTMA blocks were sectioned at 3 μ m. Immunohistochemistry was performed on an automated immunostainer (BOND RX; Leica Biosystems, Newcastle, UK). Sections were deparaffinized

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and rehydrated in Dewax Solution (Leica Biosystems), and antigen retrieval was performed with Tris-based buffer ER1 Leica Biosystems for 40 minutes at 95°C. For the conventional PD-L1 immunohistochemistry, samples were incubated with the primary antibody, anti-PD-L1 (clone SP142; Spring Bioscience, Pleasanton, CA), at 1:400 dilution for 30 minutes at room temperature. The slides were incubated with the Bond Polymer Refine visualization kit (with 3-3' diaminobenzidine as chromogen; Leica Biosystems). Finally, samples were counterstained with hematoxylin and mounted in Aquatex (Merck, Kenilworth, NJ). Human placenta was used as positive control. For PD-1-CD8 double immunohistochemistry, heat-induced epitope retrieval at pH 9 was carried out in a Tris buffer-based solution (AR9640; Leica Biosystems) for 30 minutes at 95°C. The primary antibodies were incubated sequentially: first, mouse PD-1 antibody (clone NAT105, Cell Marque; MilliporeSigma, Billerica, MA) diluted 1:200, was incubated for 30 minutes at room temperature. Then all samples were incubated with horseradish peroxidase polymer for 15 minutes and subsequently visualized using 3-3' diaminobenzidine for 10 minutes. Second, mouse CD8 antibody (clone C8/144B; Dako-Agilent, Santa Clara, CA) was diluted 1:100 and incubated for 30 minutes at room temperature. The secondary antibody was incubated with alkaline phosphatase polymer for 15 minutes, then visualized using Fast Red as a red chromogen (Red Polymer Refine Detection; Leica Biosystems).

For PD-L1-CD8 double immunofluorescence, epitope retrieval was carried out at pH 6 in a citrate buffer base (AR9961; Leica Biosystems) for 30 minutes at 100°C. The blocking step was carried out using Protein Block (Leica Biosystems). Primary antibodies were incubated for 60 minutes at room temperature together: PD-L1 (clone E1L3N; Cell Signaling Technology, Danvers, MA) at 1:200 dilution and CD8 at 1:100 dilution (clone C8/144B; Dako-Agilent, Santa Clara, CA). Secondary antibodies were incubated for 60 minutes at room temperature together: goat anti-rabbit, Alexa Fluor 488, 1:1000 dilution (Invitrogen, Thermo Fisher Scientific, Waltham, MA), and goat anti-mouse, Alexa Fluor 555 (Invitrogen, Thermo Fisher), 1:1000 dilution. Finally, the samples were mounted with mounting medium plus 4',6-diamidino-2-phenylindole (Vectashield, Burlingame, CA). Digital pictures were taken on a fluorescence microscope (BX61VS; Olympus, Tokyo, Japan).

Evaluation of Immunohistochemistry

The evaluation of PD-L1 immunohistochemical stainings was performed across all TMA cores for each individual case. Tumoral PD-L1 staining in the primary tumor and liver metastases was scored as positive if epithelial cells demonstrated a membranous staining pattern of any intensity. PD-L1 positivity was then categorized according to the percentage of positive cells among all epithelial tumor cells, as follows: 0.1%-1% (score 0), > 1% (score 1), > 5% (score 2), and > 50% (score 3). PD-L1 staining in the tumor stroma and associated inflammatory infiltrate in the primary tumor and the liver metastases were scored as positive for any staining at any intensity except for nuclear or dot-like cytoplasmic staining patterns. PD-L1 positivity was then categorized according to the area of positive cells within the total area of tumor-associated stroma and immune cells (0.1%-1%, > 1%, > 5%, > 50%).

PD-L1 positivity in areas of ulceration or necrosis, detached cells, blood vessels, neutrophils, and macrophages was not evaluated. The first half of cases from the primary tumor were scored by 2 independent reviewers (B.D. and J.W.) and the second half of cases and the liver metastases by B.D. PD-1 staining in the tumor stroma and associated inflammatory infiltrate in the tumor front was scored using the same criteria as for PD-L1. PD-1 positivity was recorded as the percentage of the area of positive cells within the total area of tumor-associated stroma and immune cells. The density of CD8-positive cells was calculated by dividing the total number of peritumoral or intratumoral positive cells by the estimated percentage of the stroma and tumor surface area, respectively.

Statistical Analysis

Associations of PD-L1 expression in tumor and stroma and PD-1 staining in stroma with categorical variables were tested by the chi-square test, while the Kruskal-Wallis or Wilcoxon rank sum test was used for continuous or ordinal variables. Survival analysis was carried out with Kaplan-Meier survival curves and log-rank test, while any multivariable survival analysis was performed by Cox regression analysis, adjusting for the possible confounding effects of pT, pN, and pM stage and postoperative therapy. OS was calculated from the date of surgery to date of death or last known follow-up. Disease-free survival (DFS) was measured from date of surgery to date of local recurrence or metastasis. $P < .05$ was considered statistically significant; no adjustment for multiple comparisons was performed. To illustrate potential associations among PD-L1, PD-1, and CD8, we created a correlation matrix using the Spearman rank-based correlation analysis. Analyses were carried out by SAS 9.2 (SAS Institute, Cary, NC). Kaplan-Meier curves were plotted by SPSS (Version 21, IBM, Armonk, NY).

Results

Baseline characteristics of our study population, comprising 279 patients with colon cancer, are listed in Table 1. Median age was 72 years (range, 30-91 years), and 41% of the patients were women. A total of 7.6% (n = 20) had stage I, 31.7% (n = 83) stage II, and 29.4% (n = 77) stage III colon cancer, whereas 31.3% (n = 82) had metastatic disease. The 5-year OS rate was 62.8%, and the 3-year DFS rate was 81.1%.

Stromal Counts of PD-L1

In our cohort, 170 (60.9%) of 279 patients displayed stromal PD-L1 expression, which has been further subdivided into stromal expression in tumor front and tumor center. PD-L1 staining was categorized according to the percentage/area of positive cells.

Clinicopathologic Correlations. Increasing counts of stromal PD-L1 positivity in the center of the tumor or invasion front were predominantly associated with less aggressive tumor features, including less frequent pT3-T4 tumors ($P = .0211$, $P = .011$, front and center, respectively), less lymph node metastasis ($P = .0196$, center), less distant metastasis ($P = .0229$, center), less tumor budding ($P = .0337$, center), and stage IV tumors ($P = .0466$, center), as well as less lymphatic vessel invasion ($P = .0448$, front) and perineural invasion ($P = .0018$, front).

Table 1 Characteristics of 279 Patients	
Characteristic	Value
Age (years), median (min-max)	72 (30-91)
Gender	
Male	165 (59.1)
Female	114 (40.9)
Histologic Subtype	
Adenocarcinoma	229 (86.7)
Mucinous	29 (11.0)
Other	6 (2.3)
Tumor Location	
Left-sided	140 (54.5)
Right-sided	117 (45.5)
pT	
pT1-2	37 (13.7)
pT3-4	233 (86.3)
pN	
pN0	130 (47.4)
pN1-2	144 (52.6)
Total LN collected, median (min-max)	32 (4-111)
Positive LN collected, median (min-max)	0 (0-37)
Distant Metastasis (pM or cM)	
M0	183 (67.5)
M1	88 (32.5)
TNM Stage	
I	20 (7.6)
II	83 (31.7)
III	77 (29.4)
IV	82 (31.3)
Tumor Grade	
G1-2	205 (75.9)
G3	65 (24.1)
Lymphatic Vessel Invasion	
L0	80 (32.0)
L1	170 (68.0)
Venous Vessel Invasion	
V0	114 (47.5)
V1	126 (52.5)
Perineural Invasion	
Pn0	200 (80.6)
Pn1	48 (19.4)
Tumor budding (ITBCC), median (min-max)	6 (0-78)
Expansive tumor growth pattern (%), median (min-max)	50 (0-100)
Klintrup-Mäkinen Score	
0	21 (8.5)
1	96 (38.9)
2	103 (41.7)
3	27 (10.9)
BRAF Mutation (VE1)	
Negative (wild-type)	169 (89.4)
Positive (mutated)	20 (10.6)

Table 1 Continued	
Characteristic	Value
MMR Status	
MMR deficient	26 (9.6)
MMR proficient	244 (90.4)
Postoperative Therapy	
None	163 (71.5)
Treated	65 (28.5)
Five-year overall survival rate	62.8%
Three-year disease-free survival rate	81.1%

Data are presented as n (%) unless otherwise indicated. Abbreviations: BRAF = v-Raf murine sarcoma viral oncogene homolog B; ITBCC = International Tumor Budding Consensus Conference; LN = lymph node; MMR = mismatch repair; TNM = tumor, node, metastasis classification system.

A higher PD-L1 stromal expression in both front and center correlated with a more expansive tumor growth pattern ($P = .0032$, $P = .0446$, respectively) as well as with a more pronounced inflammatory state of the tumor represented by the Klintrup-Mäkinen score ($P = .0021$, $P < .0001$) and more frequent MSI-high status ($P < .0001$, center) (Tables 2 and 3).

OS and DFS. Higher PD-L1 stromal cell counts at the invasion front were statistically associated with better OS in the entire cohort (5-year OS 75% vs. 57%, 56.6% and 57%, $P = .0361$, Figure 1) and in subgroup analysis of patients with stage II disease (5-year OS 86% vs. 81%, 100% and 61%, $P = .025$), while a trend toward improved prognosis could be seen when analyzing patients with stage III disease ($P = .0927$). The impact of stromal PD-L1 expression on OS did not show any difference between MMR-deficient and MMR-proficient tumors in the overall population (5-year OS 63% vs. 67%, $P = .9453$). Additionally, DFS did not correlate with PD-L1 expression in either the entire cohort or in patients with stage II and III disease. Here again, among patients with high stromal PD-L1 expression, no difference in DFS could be observed among patients with high stromal PD-L1 when stratified by MMR status (MMR deficient vs. MMR proficient, $P = .910$).

Tumor Cell Expression of PD-L1

Clinicopathologic Correlations. In contrast to stromal cell counts, tumor cell expression was predominantly associated with more aggressive tumor features, such as a larger number of lymph node metastases ($P = .0319$), higher tumor grade ($P = .0006$, $P < .0001$, front and center, respectively), and more frequent (albeit not significantly associated) lymphatic vessel and venous vessel positivity ($P = .0707$ and $P = .0633$, center).

PD-L1 was correlated with more right-sided tumor location ($P = .0051$, center), female gender ($P = .0004$, front), and older age ($P = .0103$, front). Expression of PD-L1 was significantly higher in MSI-high cancers ($P = .0171$, $P < .0001$, front and center, respectively), and in particular those with BRAF mutation ($P < .0001$, both) (Table 4).

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Table 2 Association of PD-L1 in Tumor-Associated Stroma (Invasion Front) and Clinicopathologic Features

Feature	Stromal Expression in Tumor Front				P
	0	1	2	3	
Age (years)					
Rank score [#]	118.6	127.2	109.1	107.3	.5046
Gender					
Male	69 (67.6)	16 (59.3)	19 (48.7)	32 (52.5)	.1146
Female	33 (32.4)	11 (40.7)	20 (51.3)	29 (47.5)	
Histologic Subtype					
Adenocarcinoma	84 (88.4)	24 (96.0)	30 (81.1)	51 (86.4)	.7235
Mucinous	9 (9.5)	1 (4.0)	5 (13.5)	6 (10.2)	
Other	2 (2.1)	0	2 (5.4)	2 (3.4)	
Tumor Location					
Left-sided	53 (58.9)	20 (74.1)	17 (47.2)	28 (48.3)	.0932
Right-sided	37 (41.1)	7 (25.9)	19 (52.8)	30 (51.7)	
pT					
pT1-2	6 (6.1)	2 (7.7)	8 (20.5)	12 (20.3)	.0211*
pT3-4	92 (93.9)	24 (92.3)	31 (79.5)	47 (79.7)	
pN					
pN0	44 (44.4)	10 (37.0)	21 (55.3)	33 (54.1)	.319
pN1-2	55 (55.6)	17 (63.0)	17 (44.7)	28 (45.9)	
Total LN Collected					
Rank score [#]	108.3	107.9	114.0	122.2	.5937
Positive LN Collected					
Rank score [#]	118.8	128.0	96.8	107.0	.1135
Distant Metastasis (pM/cm)					
M0	59 (59.6)	15 (62.5)	27 (69.2)	48 (78.7)	.0893
M1	40 (40.4)	9 (37.5)	12 (30.8)	13 (21.3)	
TNM Stage					
I	3 (3.2)	1 (4.2)	5 (13.2)	7 (11.9)	.1809
II	31 (32.6)	6 (25.0)	14 (36.8)	19 (32.2)	
III	25 (26.3)	8 (33.3)	8 (21.1)	21 (35.6)	
IV	36 (37.9)	9 (37.5)	11 (28.9)	12 (20.3)	
Tumor Grade					
G1-2	73 (74.5)	19 (73.1)	34 (87.2)	47 (79.7)	.3805
G3	25 (25.5)	7 (26.9)	5 (12.8)	12 (20.3)	
Lymphatic Vessel Invasion					
L0	24 (26.4)	6 (25.0)	10 (29.4)	27 (47.4)	.0448*
L1	67 (73.6)	18 (75.0)	24 (70.6)	30 (52.6)	
Venous Vessel Invasion					
V0	39 (45.9)	4 (18.2)	16 (48.5)	33 (56.9)	.0215*
V1	46 (54.1)	18 (81.8)	17 (51.5)	25 (43.1)	
Perineural Invasion					
Pn0	66 (72.5)	16 (66.7)	30 (88.2)	53 (94.6)	.0018*
Pn1	25 (27.5)	8 (33.3)	4 (11.8)	3 (5.4)	
Tumor Budding (ITBCC)					
Rank score [#]	36.3	41.8	28.9	22.4	.0718
Expansive Tumor Growth Pattern					
Rank score [#]	90.3	87.8	101.6	125.1	.0032*

Table 2 Continued

Feature	Stromal Expression in Tumor Front				P
	0	1	2	3	
Klintrup-Mäkinen Score					
0	12 (13.2)	2 (8.3)	3 (9.4)	1 (1.8)	.0021*
1	41 (45.1)	13 (54.2)	11 (34.4)	13 (23.2)	
2	34 (37.4)	7 (29.2)	13 (40.6)	28 (50.0)	
3	4 (4.4)	2 (8.3)	5 (15.6)	14 (25.0)	
BRAF Mutation (VE1)					
Negative (wild-type)	70 (93.3)	13 (76.5)	23 (92.0)	38 (88.4)	.2017
Positive (mutated)	5 (6.7)	4 (23.5)	2 (8.0)	5 (11.6)	
MMR Status					
MMR deficient	6 (5.9)	2 (7.4)	4 (10.8)	10 (16.7)	.1625
MMR proficient	95 (94.1)	25 (92.6)	33 (89.2)	50 (83.3)	

Data are presented as n (%) unless otherwise indicated.

Abbreviations: BRAF = v-Raf murine sarcoma viral oncogene homolog B; ITBCC = International Tumor Budding Consensus Conference; LN = lymph node; MMR = mismatch repair; TNM = tumor, node, metastasis classification system.

*Statistically significant.

[#]Rank score from Wilcoxon Rank Sum test.

OS and DFS. There was no effect of tumoral PD-L1 expression on OS ($P = .7895$, front and $P = .3482$, center). However, a trend toward a longer DFS could be observed in the overall population ($P = .1246$). Patients with positive tumors showed a 100% 5-year DFS compared to 77% for patients with no PD-L1 staining in the tumor front (Supplemental Figure 1 in the online version).

PD-L1 Expression in Primary Colon Tumors and Matched Liver Metastases. We assessed both epithelial and stromal PD-L1 expression levels in 14 primary tumors and corresponding liver metastases. We demonstrated that both epithelial and stromal PD-L1 staining in the primary tumor strongly correlated with matched liver metastases (Supplemental Figure 2 in the online version).

Stromal Counts of PD-1

In our study, 45 (45.5%) of 99 of the patients had disease that exhibited high stromal PD-1 expression (tumor front). PD-1 staining in the stroma was subdivided according to the percentage of positive stroma surface area and was classified as high (> 10% of stroma surface area) or low (\leq 10% of stroma surface area).

Clinicopathologic Correlations. High stromal PD-1 expression in the invasion front correlated with less aggressive tumor characteristics, including less frequent vascular invasion ($P = .0173$), less nodal involvement ($P = .0491$), and less tumor budding ($P = .0032$). No association with microsatellite stable (MSS)/MSI-high status could be observed ($P = .655$) (Supplemental Table 1 in the online version).

OS and DFS. Higher PD-1 stromal cell expression at the invasion front was associated with better DFS in the overall population (3-year DFS 80% vs. 60%, $P = .0379$) (Supplemental Figure 3 in

the online version). However, OS did not correlate with stromal PD-1 expression ($P = .434$).

Intratumoral and Stromal Counts of CD8

OS and DFS. Intratumoral (i) and stromal (s) CD8 counts were significantly associated with both OS (5-year OS 71.8% iCD8 high vs. 41.3% iCD8 low, $P = .0004$) and DFS (3-year DFS 79.5% iCD8 high vs. 60.9% iCD8 low, $P = .0461$, and 78.1% sCD8 high vs. 61.0% sCD8 low, $P = .0342$ respectively).

Stromal PD-L1/CD8 Double Staining

We analyzed the cases with the strongest PD-L1 positivity (> 5% of the total stroma area) of the second half of our cohort (13/99, 13%) using double immunofluorescence against PD-L1 and CD8. Of the 13 cases evaluated, 7 showed CD8⁺ T cells that were predominantly PD-L1 negative (category 1), 1 case with predominantly PD-L1 positive CD8⁺ T cells (category 2), and 5 cases displaying a mixed pattern of PD-L1 positive and negative CD8⁺ T cells (category 3) (Supplemental Figure 4 in the online version).

Correlation Between PD-L1 and Other Immunohistochemical Markers

The associations between PD-1, CD8, and PD-L1 are depicted in a correlation matrix in Supplemental Table 2 in the online version. Stromal PD-1 expression correlated significantly with stromal PD-L1 staining and both intratumoral and stromal CD8 expression.

Discussion

We demonstrated that stromal but not epithelial PD-L1 immunostaining is associated with less aggressive tumor behavior in colon cancer, which translated into better OS.

Stromal PD-1/PD-L1 Expression

Table 3 Association of PD-L1 in Tumor-Associated Stroma (Tumor Center) and Clinicopathologic Features

Feature	Stroma Expression in Tumor Center				P
	0	1	2	3	
Age (years)					
Rank score [#]	129.6	123.8	142.9	127.6	.7513
Gender					
Male	87 (61.7)	12 (50.0)	21 (52.5)	31 (55.4)	.5566
Female	54 (38.3)	12 (50.0)	19 (47.5)	25 (44.6)	
Histologic Subtype					
Adenocarcinoma	116 (87.9)	20 (95.2)	34 (87.2)	47 (83.9)	.5963
Mucinous	13 (9.8)	1 (4.8)	5 (12.8)	6 (10.7)	
Other	3 (2.3)	0	0	3 (5.4)	
Tumor Location					
Left-sided	77 (60.2)	15 (62.5)	19 (52.8)	24 (45.3)	.269
Right-sided	51 (39.8)	9 (37.5)	17 (47.2)	29 (54.7)	
pT					
pT1-2	13 (9.6)	1 (4.5)	11 (27.5)	11 (19.6)	.011*
pT3-4	122 (90.4)	21 (95.5)	29 (72.5)	45 (80.4)	
pN					
pN0	61 (44.5)	5 (20.8)	22 (56.4)	31 (55.4)	.0196*
pN1-2	76 (55.5)	19 (79.2)	17 (43.6)	25 (44.6)	
Total LN Collected					
Rank score [#]	123.3	116.8	126.6	147.6	.1687
Positive LN Collected					
Rank score [#]	128.6	160.3	124.5	117.4	.0805
Distant Metastasis (pM or cM)					
M0	85 (62.0)	14 (60.9)	33 (84.6)	42 (76.4)	.0229*
M1	52 (38.0)	9 (39.1)	6 (15.4)	13 (23.6)	
TNM Stage					
I	7 (5.3)	0	5 (13.2)	8 (14.5)	.0466*
II	38 (29.0)	4 (19.0)	14 (36.8)	19 (34.5)	
III	38 (29.0)	9 (42.9)	14 (36.8)	15 (27.3)	
IV	48 (36.6)	8 (38.1)	5 (13.2)	13 (23.6)	
Tumor Grade					
G1-2	107 (79.3)	18 (81.8)	31 (77.5)	36 (64.3)	.1424
G3	28 (20.7)	4 (18.2)	9 (22.5)	20 (35.7)	
Lymphatic Vessel Invasion					
L0	36 (29.0)	3 (15.0)	14 (36.8)	21 (38.9)	.1909
L1	88 (71.0)	17 (85.0)	24 (63.2)	33 (61.1)	
Venous Vessel Invasion					
V0	48 (41.4)	6 (30.0)	20 (55.6)	33 (61.1)	.0274*
V1	68 (58.6)	14 (70.0)	16 (44.4)	21 (38.9)	
Perineural Invasion					
Pn0	97 (78.2)	15 (75.0)	32 (84.2)	48 (88.9)	.3133
Pn1	27 (21.8)	5 (25.0)	6 (15.8)	6 (11.1)	
Tumor Budding (ITBCC)					
Rank score [#]	43.4	30.5	31.3	25.9	.0337*
Expansive Tumor Growth Pattern					
Rank score [#]	107.2	113.2	124.8	137.3	.0446*
Klintrup-Mäkinen Score					
0	13 (10.5)	0	4 (10.5)	0	<.0001*
1	62 (50.0)	10 (50.0)	9 (23.7)	11 (20.8)	

Table 3 Continued

Feature	Stroma Expression in Tumor Center				P
	0	1	2	3	
2	43 (34.7)	8 (40.0)	24 (63.2)	24 (45.3)	
3	6 (4.8)	2 (10.0)	1 (2.6)	18 (34.0)	
BRAF Mutation (VE1)					
Negative (wild-type)	89 (91.8)	14 (93.3)	24 (88.9)	30 (78.9)	.1847
Positive (mutated)	8 (8.2)	1 (6.7)	3 (11.1)	8 (21.1)	
MMR Status					
MMR deficient	5 (3.7)	0	5 (13.2)	16 (29.1)	<.0001*
MMR proficient	130 (96.3)	24 (100.0)	33 (86.8)	39 (70.9)	

Data are presented as n (%) unless otherwise indicated.

Abbreviations: *BRAF* = v-Raf murine sarcoma viral oncogene homolog B; ITBCC = International Tumor Budding Consensus Conference; LN = lymph node; MMR = mismatch repair; TNM = tumor, node, metastasis classification system.

*Statistically significant.

#Rank score from Wilcoxon Rank Sum test.

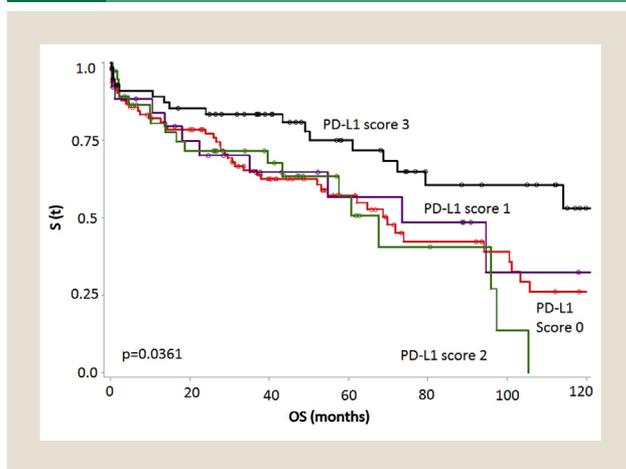
Conversely, PD-L1 staining in the tumor epithelium was less frequent than stromal staining and was predominantly associated with features of aggressive tumor biology such as higher tumor grade, lymph node infiltration, and *BRAF* mutation. However, no association of epithelial PD-L1 staining with OS could be observed. Interestingly, despite the association with negative prognostic factors, patients with positive tumoral PD-L1 expression showed a trend toward an improved DFS, which might be at first sight mainly explained by the higher percentage of MSI-high cancers in tumors exhibiting PD-L1-positive cancer cells. The small percentage of epithelial PD-L1 staining in the tumors did not allow us to perform further analyses stratified by MMR status. However, after adjusting for MMR status in the multivariate analysis, this favorable trend of tumoral PD-L1 staining on DFS was maintained. Contrary to other studies, we intentionally excluded rectal cancer patients from our analysis; rectal cancer differs from colon cancer in many ways, such as therapeutic approach, tumor biology, and prognosis.^{20,21}

Our findings suggest that stromal PD-L1 expression might serve as a prognostic marker in colon cancer patients. Contrary to our findings, Thompson et al²² showed that in patients with locally advanced gastric cancer, both epithelial and stromal PD-L1 expression were associated with worse outcome. These opposite findings regarding the impact of stromal and tumoral PD-L1 expression on outcome in our colon cancer and their gastric cancer cohorts suggest that the interaction between tumor and tumor-associated stroma might be different among various tumor types. The prognostic value of PD-L1 expression in colorectal cancer is highly debated. While some studies demonstrated that epithelial PD-L1 staining is associated with better outcome,²³ others have shown a deleterious effect on survival.^{24,25}

Whereas Masugi et al²⁶ found that stromal PD-L1 expression was only present in 5%, our study found that stromal PD-L1 was expressed in 60.9%. Additionally, the correlation between MMR status and tumoral PD-L1 expression observed in our study could not be shown by Masugi et al. However, there are several possible reasons for these contradictory findings. First, there is no established consensus regarding PD-L1 scoring. While in some studies the cutoff level determining PD-L1 positivity was set at > 5%, other groups used different cutoffs.^{24,27} In our study, we used a finely tuned grading system indicating the percentage/area of positively stained cells at different levels (0.1%-1%, > 1%, > 5%, > 50%),²⁸ whereas in Masugi et al the PD-L1 expression level was scored as 0 (absent), 1 (weak), 2 (moderate), and 3 (strong) without indicating any percentages/area of positively stained cells according to their grading system.

Second, there is a considerable heterogeneity in PD-L1 antibodies used for immunohistochemical staining across studies.^{24,25} We used in our study an established and validated monoclonal rabbit antibody for PD-L1 staining (clone SP142; Spring Bioscience, Pleasanton, CA; 1:400 dilution). Masugi et al²⁶ used a different antibody clone that is targeted to a different epitope (mouse monoclonal anti-CD274 antibody, clone MIH1; eBioscience, San Diego, CA; dilution, 1:50), which most likely differs in sensitivity and specificity toward the PD-L1 protein. Third, inter-assay variations, differences in formalin fixation of surgical specimens, and the use of different immunohistochemistry platforms

Figure 1 Stromal PD-L1 Expression at Invasion Front and Effect on Overall Survival in Colon Cancer Patients (All Stages)



Abbreviation: PD-L1 = programmed cell death ligand 1.

Stromal PD-1/PD-L1 Expression

Table 4 Association of PD-L1 in Tumor and Clinicopathologic Features

Feature	Tumor Front			Tumor Center		
	Negative	Positive	P	Negative	Positive	P
Age (years)						
Rank score [#]	110.9	149.7	.0103*	129.5	151.3	.2363
Gender						
Male	131 (63.3)	5 (23.8)	.0004*	143 (58.8)	8 (44.4)	.2324
Female	76 (36.7)	16 (76.2)		100 (41.2)	10 (55.6)	
Histologic Subtype						
Adenocarcinoma	172 (87.8)	16 (84.2)	.0851	201 (87.4)	16 (88.9)	.5522
Mucinous	20 (10.2)	1 (5.3)		24 (10.4)	1 (5.6)	
Other	4 (2.0)	2 (10.5)		5 (2.2)	1 (5.6)	
Tumor Location						
Left-sided	110 (58.2)	8 (38.1)	.0781	131 (58.5)	4 (23.5)	.0051*
Right-sided	79 (41.8)	13 (61.9)		93 (41.5)	13 (76.5)	
pT						
pT1-2	26 (12.9)	2 (10.5)	.7689	34 (14.5)	2 (11.1)	.6944
pT3-4	176 (87.1)	17 (89.5)		201 (85.5)	16 (88.9)	
pN						
pN0	102 (50.2)	6 (28.6)	.0584	114 (47.9)	5 (27.8)	.0989
pN1-2	101 (49.8)	15 (71.4)		124 (52.1)	13 (72.2)	
Total LN Collected						
Rank score [#]	111.5	121.7	.4918	125.6	167.3	.0211*
Positive LN Collected						
Rank score [#]	110.0	136.4	.055	125.2	172.2	.0051*
Distant Metastasis (pM or cM)						
M0	135 (66.8)	13 (65.0)	.8683	161 (68.2)	13 (72.2)	.7246
M1	67 (33.2)	7 (35.0)		75 (31.8)	5 (27.8)	
TNM Stage						
I	14 (7.1)	2 (10.5)	.6502	19 (8.4)	1 (5.6)	.6204
II	66 (33.7)	4 (21.1)		71 (31.3)	4 (22.2)	
III	54 (27.6)	7 (36.8)		68 (29.9)	8 (44.4)	
IV	62 (31.6)	6 (31.6)		69 (30.4)	5 (27.8)	
Tumor Grade						
G1-2	164 (81.2)	9 (47.4)	.0006*	186 (79.1)	6 (33.3)	<.0001*
G3	38 (18.8)	10 (52.6)		49 (20.9)	12 (66.7)	
Lymphatic Vessel Invasion						
L0	63 (33.9)	4 (21.1)	.2565	72 (32.9)	2 (11.8)	.0707
L1	123 (66.1)	15 (78.9)		147 (67.1)	15 (88.2)	
Venous Vessel Invasion						
V0	82 (45.8)	10 (55.6)	.4295	103 (49.0)	4 (25.0)	.0633
V1	97 (54.2)	8 (44.4)		107 (51.0)	12 (75.0)	
Perineural Invasion						
Pn0	148 (80.0)	17 (89.5)	.3173	179 (81.7)	13 (76.5)	.5913
Pn1	37 (20.0)	2 (10.5)		40 (18.3)	4 (23.5)	
Expansive Tumor Growth Pattern						
Rank score [#]	102.7	84.4	.1879	117.8	113.2	.7847
Tumor Budding (ITBCC)						
Rank score [#]	32.9	39.3	.4396	37.0	43.8	.4951
Klintrup-Mäkinen Score						
0	18 (9.8)	0	.3668	17 (7.8)	0	.0008*
1	70 (38.3)	7 (36.8)		88 (40.4)	4 (23.5)	

Table 4 Continued

Feature	Tumor Front			Tumor Center		
	Negative	Positive	P	Negative	Positive	P
2	74 (40.4)	8 (42.1)		93 (42.7)	6 (35.3)	
3	21 (11.5)	4 (21.1)		20 (9.2)	7 (41.2)	
BRAF Mutation (VE1)						
Negative (wild-type)	137 (94.5)	7 (50.0)	<.0001*	152 (91.6)	5 (45.5)	<.0001*
Positive (mutated)	8 (5.5)	7 (50.0)		14 (8.4)	6 (54.5)	
MMR Status						
MMR deficient	16 (7.9)	5 (23.8)	.0171*	19 (8.1)	7 (38.9)	<.0001*
MMR proficient	187 (92.1)	16 (76.2)		215 (91.9)	11 (61.1)	

Data are presented as n (%) unless otherwise indicated.

Abbreviations: BRAF = v-Raf murine sarcoma viral oncogene homolog B; ITBCC = International Tumor Budding Consensus Conference; LN = lymph node; MMR = mismatch repair; TNM = tumor, node, metastasis classification system.

*Statistically significant.

#Rank score from Wilcoxon Rank Sum test.

might have contributed to the discrepancies in results among our studies. Therefore, because of the lack of standardization, drawing cross-comparisons between studies is still challenging. As we move forward in the field of immunotherapy, it will be of utmost importance to develop a uniform consensus guideline regarding PD-L1 scoring that will enable us to better interpret the findings from different studies.

Contrary to Masugi et al,²⁶ who reported a stromal PD-L1 expression of 5%, the study by Taube et al²⁹ displayed a stromal PD-L1 expression rate of 50%, which is almost within the same range as the stromal PD-L1 expression rate found in our study (60.9%).

The reasons mentioned above might explain why we found an association between stromal PD-L1 expression and OS whereas the study by Masugi et al²⁶ did not. Given the higher percentage of stromal PD-L1 expression in our study (n = 170, 60.9%) compared to the study of Masugi et al (n = 44, 5%), we might have sufficient statistical power to detect a statistically and clinically meaningful association between OS and stromal PD-L1 expression. Moreover, our study population and that of Masugi et al are significantly different. In order to have a homogenous study population, we restricted our analysis to patients with colon cancer; we intentionally excluded rectal cancer patients because of that disease's different tumor biology, treatment, and prognosis.²⁰ Furthermore, our study population was enrolled between 2002 and 2013. During this time, a considerable improvement in outcome could be achieved not only as a result of the introduction of oxaliplatin in both the adjuvant and palliative treatment settings but also as a result of the implementation of biological therapies such as anti-VEGF- and anti-EGFR-targeted agents in the palliative setting.³⁰ Conversely, 50% of the patients in the study of Masugi et al were diagnosed before 1999, when adjuvant and palliative chemotherapy consisted mainly of 5-FU and outcome was significantly worse compared to the last 15 years. All these points might provide an additional potential explanation as to why we found an association of stromal PD-L1 expression and OS, whereas this correlation was not found by Masugi et al.

Furthermore, we used a multicore digital TMA (ngTMA®) approach to reliably investigate tumor heterogeneity. Notably, no

difference in DFS or OS could be observed between patients with MMR-deficient tumors and high stromal PD-L1 expression, and those with MMR-proficient tumors and high stromal PD-L1 staining. Thus, we can conclude that the beneficial impact of stromal PD-L1 expression on outcome is independent of MMR status.

As opposed to other solid tumors, such as head and neck cancer, lung cancer, renal cell carcinoma, and urothelial carcinoma, where tumoral PD-L1 expression is more frequent, PD-L1 staining in colorectal cancers is almost exclusively restricted to tumor-infiltrating immune cells and is rarely observed on tumor cells.^{17,31-34} The stromal predominance of PD-L1 staining compared to the epithelial expression in colon cancers and its prognostic significance will serve as a basis to further explore the colon cancer microenvironment and to reveal its pro- and anticancer properties.

According to the different effects of PD-L1 expression in tumor epithelium versus stroma on OS, one might speculate that treatment with anti-PD-L1 antibodies not only stimulates PD-1-positive effector T cells targeting PD-L1-positive tumor cells but also eliminates the beneficial, antitumorigenic effect of PD-L1 in the stroma within the tumor microenvironment. To date, the exact mechanism on how PD-L1 expression in stromal cells exerts its antitumoral effects within the tumor microenvironment remains elusive.³⁵

At first sight, it might seem counterintuitive that the stromal expression of an immunosuppressive ligand such as PD-L1 correlates with improved survival. However, this association might be explained if PD-L1 expression within the tumor microenvironment is not solely considered as a result of an increased immune inhibiting PD-1/PD-L1 interplay but rather is viewed as a reflection of adaptive antitumor immunity, where tumor-infiltrating lymphocytes are activated in response to tumor antigens. This is consistent with our findings that stromal PD-L1 expression correlates with an inflammatory state of the tumor represented by the Klintrup-Mäkinen score and more frequent MSI-high status. In this context, stromal PD-L1 overexpression might represent an immune status whose equilibrium is shifted more toward tumor suppression than tumor promotion. Our assumptions

Stromal PD-1/PD-L1 Expression

are further supported by several previous reports showing that PD-L1, besides its inhibitory properties, also exerts costimulatory effects on T-cell activation.³⁶⁻³⁹ Moreover, Ni et al⁴⁰ demonstrated that PD-L1 overexpression in CD8⁺ donor T cells and their interaction with CD80 resulted in enhanced CD8⁺ donor T-cell proliferation in the spleen, which in turn led to enhanced graft-versus-leukemia effect early after hematopoietic stem cell transplantation. Previous reports showed increased PD-L1 expression in activated T cells and also showed that PD-L1 deficiency impairs proliferation and development of CD8⁺ T effector cells,^{41,42} resulting in decreased protective immunity.

Additionally, Mlecnik and colleagues showed that PD-L1 expression significantly correlates with the immunoscore in colorectal cancer independent of microsatellite status, and that a higher immunoscore is associated with improved outcome in colorectal cancer.^{43,44} Another group demonstrated a correlation between PD-L1 expression and CD8⁺ tumor-infiltrating lymphocytes.¹⁵ Similarly, our group showed that CD8⁺ T-cell infiltration in endoscopic biopsy samples of colorectal cancer patients predicts longer OS independent of TNM stage.⁴⁵

It is widely established that overexpression of the ligand PD-L1 in cancer cells results in tumor evasion from the host immune system by providing binding sites for the coinhibitory receptor PD-1 mainly located on the surface of T cells.⁴⁶ The resulting PD-L1/PD-1 interplay suppresses the antitumor immune response by inhibiting T-cell activation.⁴⁷

Contrary to other solid tumors such as urothelial carcinoma, hepatocellular carcinoma, and gastric cancer, in which PD-L1 staining in tumor cells was identified as a poor prognostic indicator, PD-L1 staining in our colon cancer study cohort did not correlate with worse outcome even though more aggressive tumor features were observed.^{22,34,48,49} Tumoral PD-L1 staining in our cohort even showed a trend toward a longer DFS. Because of the small number of patients with PD-L1-positive tumor cells, the findings did not reach statistical significance. Remarkably, no event could be observed in patients with tumoral PD-L1 independent of the MMR status (2 of 9 patients with tumoral PD-L1 expression were MMR deficient, whereas 7 patients were MMR proficient).

While there is an inequality of PD-L1 expression between primary tumors and metastases among melanoma patients, we demonstrated that PD-L1 expression in primary colon tumors largely corresponds to matched liver metastases.⁵⁰ Thus, PD-L1 analysis in metastatic lesions might not be required when planning anti-PD-L1 treatment for metastatic colon cancer.

Moreover, stromal PD-L1 expression correlated significantly with stromal PD-1 staining and both intratumoral and stromal CD8 expression. Similar to PD-L1, stromal PD-1 expression was also associated with less aggressive tumor characteristics, resulting in improved outcome. These findings suggest that a finely tuned interplay between stromal PD-L1 and PD-1 in combination with intratumoral and stromal CD8 is a prerequisite for an effective antitumoral immune response.

Limitations of our study are the small number of patients and its retrospective, single-center design. Strengths are that we restricted our analysis to colon cancer patients and performed subgroup analyses of patients with stage II and III colon cancer. By using a

multicore tissue microarray procedure (6 cores per case) we were able to take tumor heterogeneity into account. Additionally, we evaluated the correlation between various clinicopathologic features (such as tumor budding, MMR, and *BRAF* mutational status as well as tumor growth pattern and Klintrup-Mäkinen score) and PD-L1/PD-1 expression. Furthermore, we are one of the first to describe that stromal PD-L1 expression in colon cancer patients might have a predictive value for OS independent of MMR deficiency/MSI status, and that both epithelial and stromal PD-L1 expression in the primary tumor is largely consistent with those observed in the corresponding metastases. Future studies are mandatory to evaluate the predictive impact of stromal PD-L1 expression in both MSI-high and MSS colon cancer patients treated with anti-PD-L1 antibodies. This might help us identify those patients who will derive the most benefit from PD-L1-directed therapy and who will not. Our findings suggest that the distinction of expression patterns of PD-L1 (epithelial vs. stromal) is important for prognostic purposes because stromal expression is associated with less aggressive tumor characteristics and better OS, whereas epithelial staining correlates with predominantly adverse tumor features.

Conclusion

Stromal PD-L1 expression might serve as a prognostic marker in colon cancer patients independent of MSS/MSI status. Especially for patients with stage II disease, assessment of stromal PD-L1 status might help identify patients with stage II colon cancer who might benefit from adjuvant chemotherapy.

Because PD-L1 expression was concordant in primary tumors and corresponding metastases, PD-L1 expression in the primary tumor might also serve as a predictor of PD-L1 staining in metastatic lesions among colon cancer patients not previously treated with PD-L1 inhibitors. Consistent with the findings obtained for PD-L1, stromal PD-1 expression correlated with less aggressive tumor features, resulting in improved outcome. Moreover, stromal PD-L1 expression was associated with stromal PD-1 staining and both intratumoral and stromal CD8 expression. These results will contribute to a better understanding of the interaction between the tumor and its microenvironment, enabling us to identify those patients who will benefit most from immune-checkpoint therapy regardless of the tumor's MMR status.

Clinical Practice Points

- PD-L1 is an inhibitory immune-checkpoint molecule mainly expressed on T cells and antigen-presenting cells such as B cells, dendritic cells, and macrophages, but less frequently observed in colon cancer cells.
- Stromal but not epithelial PD-L1 immunostaining was associated with less aggressive tumor behavior in colon cancer patients, which translated into better OS.
- PD-L1 staining in the tumor epithelium was less frequent than stromal staining and was associated with features of aggressive tumor biology, such as higher tumor grade, lymph node infiltration, and *BRAF* mutation. However, no association of epithelial PD-L1 staining with OS could be observed.
- Stromal PD-L1 expression might serve as a prognostic marker in colon cancer patients independent of MSS/MSI status.

- Because stromal PD-L1 expression in stage II colon cancer patients was associated with better OS, assessment of stromal PD-L1 status might help us to identify those patients with stage II colon cancer who might benefit from adjuvant chemotherapy.
- Similar to PD-L1, stromal PD-1 expression was also associated with less aggressive tumor characteristics, resulting in improved DFS.
- Stromal PD-L1 expression correlated significantly with stromal PD-1 staining and both intratumoral and stromal CD8 expression.
- The main objective for the future is to identify patients who will benefit most from immunotherapy, regardless of the tumor's MMR status.
- A better understanding of the interplay between the tumor and its microenvironment will help us to further improve our treatment strategy against colon cancer.

Disclosure

The authors have stated that they have no conflict of interest.

Supplemental Data

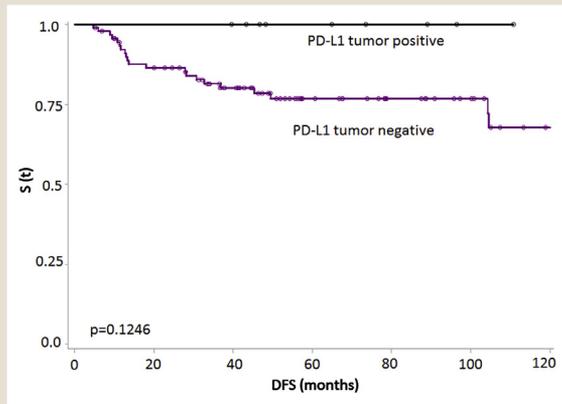
Supplemental tables and figures accompanying this article can be found in the online version at <https://doi.org/10.1016/j.clcc.2018.09.007>.

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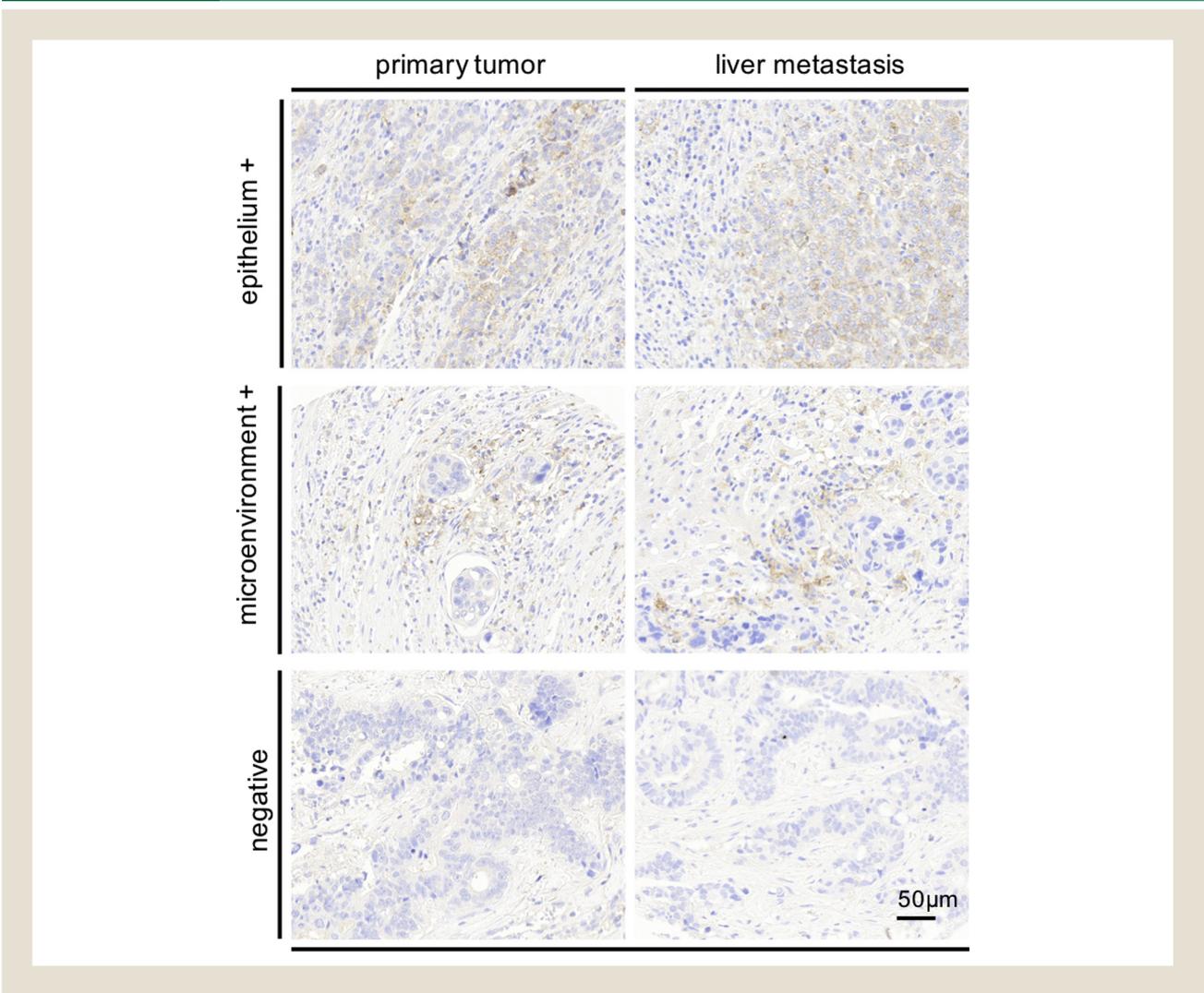
Stromal PD-1/PD-L1 Expression

Supplemental Figure 1 PD-L1 Expression in Tumor Cells at Tumor Front and Effect on Disease-Free Survival in Colon Cancer Patients (All Stages)



Abbreviation: PD-L1 = programmed cell death ligand 1.

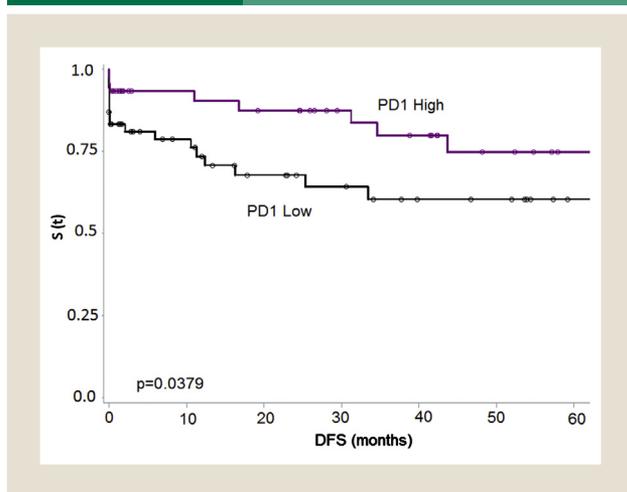
Supplemental Figure 2 Representative Images of Immunohistochemical Stainings Against PD-L1 with SP142 Antibody Clone in Colorectal Cancer and Corresponding Liver Metastases. Three Cases Illustrate Examples of PD-L1 Positivity in Tumor or Tumor Microenvironment, or Absent Positivity for PD-L1. Note Overall Weak Staining Intensity



Abbreviation: PD-L1 = programmed cell death ligand 1.

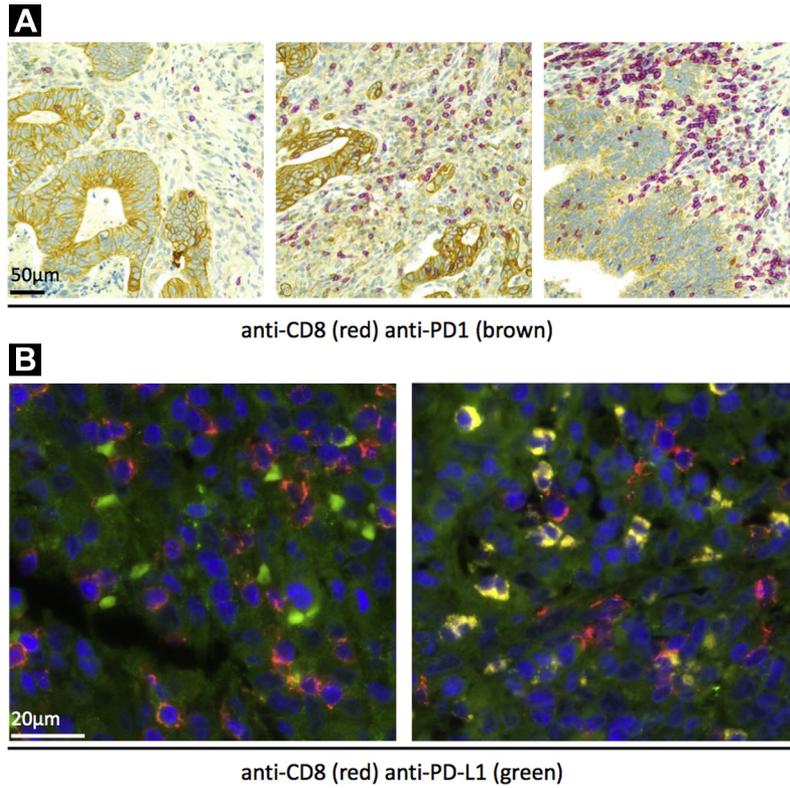
Stromal PD-1/PD-L1 Expression

Supplemental Figure 3 Stromal PD-1 Expression and Disease-Free Survival in Colon Cancer Patients (All Stages)



Abbreviation: PD-L1 = programmed cell death ligand 1.

Supplemental Figure 4 Stainings Against PD-1 and CD8. (A) Representative Images of Immunohistochemical Stainings Against PD-1 and CD8 of Tumor Front of 3 Different Cases of Colorectal Cancer. These Cases Illustrate Differences in Amount of Peri- and Intratumoral CD8 Positive Lymphocytes (Increasing From Left to Right) and Percentage of PD-1-positive Cells in Tumor Stroma (best seen on the second image). (B) Representative Images of Immunofluorescent Stainings Against PD-L1 and CD8 in Stroma at Tumor Front. (Left) Case of CD8-positive Cells (Red) in PD-L1 Positive Stroma (Green). (Right) Case of PD-L1-positive Stroma (Green) and Dual Population of CD8-positive Cells. Some CD8-positive Cells (Red) do not Express PD-L1, whereas Lymphocytes in Yellow (Merged Red and Green Signal) Coexpress CD8 and PD-L1



Abbreviation: PD-L1 = programmed cell death ligand 1.

Stromal PD-1/PD-L1 Expression

Supplemental Table 1 Association of Stromal PD-1 (Tumor Front) and Clinicopathologic Features (n = 99)

Feature	PD-1 Stroma Percentage		P
	Low (n = 54)	High (n = 45)	
Age (years)			
Rank score [#]	51.0	48.8	.6991
Gender			
Male	16 (29.6)	20 (44.4)	.1271
Female	38 (70.4)	25 (55.6)	
Histologic Subtype			
Adenocarcinoma	46 (97.9)	38 (100.0)	.3657
Mucinous	1 (2.1)	0	
Other	0	0	
Tumor Location			
Left-sided	22 (47.8)	14 (35.0)	.2606
Right-sided	11 (23.9)	16 (40.0)	
Not otherwise specified	13 (28.3)	10 (25.0)	
pT			
pT1-2	6 (12.2)	8 (19.0)	.3699
pT3-4	43 (87.8)	34 (81.0)	
pN			
PN0	22 (44.0)	27 (64.3)	.1406
pN1-2	28 (56.0)	15 (35.7)	
Total LN Collected			
Rank score [#]	49.9	46.9	.5935
Positive LN Collected			
Rank score [#]	53.0	43.1	.0491*
Distant Metastasis (pM or cM)			
M0	35 (64.8)	35 (77.8)	.1582
M1	19 (35.2)	10 (22.2)	
TNM Stage			
I	3 (5.9)	5 (11.9)	.4718
II	17 (33.3)	16 (38.1)	
III	12 (23.5)	11 (26.2)	
IV	19 (37.3)	10 (23.8)	
Tumor Grade			
G1-2	35 (83.3)	32 (94.2)	.2005
G3	7 (16.7)	2 (5.8)	
Lymphatic Vessel Invasion			
L0	19 (40.4)	21 (55.3)	.173
L1	28 (59.6)	17 (44.7)	
Venous Vessel Invasion			
V0	20 (42.6)	26 (68.4)	.0173*
V1	27 (57.4)	12 (31.6)	
Perineural Invasion			
Pn0	31 (66.0)	30 (79.0)	.1859
Pn1	16 (34.0)	8 (21.0)	
Expansive Tumor Growth Pattern			
Rank score [#]	37.4	49.9	.0201*
Tumor Budding (ITBCC)			
Rank score [#]	49.6	33.9	.0032*

Supplemental Table 1 Continued

Feature	PD-1 Stroma Percentage		P
	Low (n = 54)	High (n = 45)	
Klintrup-Mäkinen Score			
0	5 (10.6)	2 (5.3)	.288
1	29 (61.7)	18 (47.4)	
2	11 (23.4)	15 (39.5)	
3	2 (4.3)	3 (7.9)	
MMR Status			
MMR deficient	5 (9.4)	4 (9.1)	.6549
MMR proficient	48 (90.6)	40 (90.9)	

Data are presented as n (%) unless otherwise indicated.

Abbreviations: *BRAF* = v-Raf murine sarcoma viral oncogene homolog B; ITBCC = International Tumor Budding Consensus Conference; LN = lymph node; MMR = mismatch repair; TNM = tumor, node, metastasis classification system.

*Statistically significant.

#Rank score from Wilcoxon Rank Sum test.

Stromal PD-1/PD-L1 Expression

Supplemental Table 2 Correlation Matrix

	PD-1 Stroma Front	iCD8	sCD8	PD-L1 Tumor Front	PD-L1 Tumor Center	PD-L1 Stroma Front
PD-1 Stroma front	1.0					
iCD8	0.29*	1.0				
sCD8	0.6*	0.64*	1.0			
PD-L1 Tumor front	0.16	0.06	0.03	1.0		
PD-L1 Tumor center	0.1	0.01	-0.02	0.68*	1.0	
PD-L1 Stroma front	0.45*	0.51*	0.53*	0.26*	0.2*	1.0
PD-L1 Stroma center	0.23*	0.28*	0.23*	0.3*	0.35*	0.52*

Abbreviations: iCD8 = intratumoral CD8; PD-1 = programmed cell death 1; PD-L1 = programmed cell death ligand 1; sCD8 = stromal CD8.
*Statistically significant correlations ($P < .05$).