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Patterns and prognostic relevance of PD-1 and PD-L1 expression in colorectal carcinoma

Lik Hang Lee[1], Marcela S Cavalcanti[1], Neil H Segal[2], Jaclyn F Hechtman[1], Martin R Weiser[3], J Joshua Smith[3], Julio Garcia-Aguilar[3], Eran Sadot[3], Peter Ntiamoah[1], Arnold J Markowitz[2], Moshe Shike[2], Zsofia K Stadler[2], Efsevia Vakiani[1], David S Klimstra[1] and Jinru Shia[1]

[1]Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, NY, USA; [2]Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY, USA and [3]Department of Surgery, Memorial Sloan Kettering Cancer Center, New York, NY, USA

Immune checkpoint blockade targeting the programmed death-1 (PD-1) pathway has shown efficacy in several types of cancers including mismatch-repair-deficient colorectal carcinoma. In some tumor types, programmed death-ligand 1 (PD-L1) expression detected by immunohistochemistry has shown utility as a predictive marker for response to anti-PD-1 therapies. This utility, however, remains to be determined in colorectal carcinoma. In addition, although tumor-infiltrating lymphocytes have been associated with better prognosis in colorectal carcinoma, the prognostic value of PD-1 expression in these lymphocytes and its interaction with PD-L1 expression still await investigation. To address these questions, we performed a pilot study to evaluate the patterns of PD-L1 and PD-1 immunohistochemical expression on colorectal carcinoma cells and their tumor-infiltrating lymphocytes, respectively. Using tissue microarray, we found that 5% (19/394) of colorectal carcinomas exhibited high tumor PD-L1 expression, and 19% (76/392) had elevated numbers of PD-1-positive tumor-infiltrating lymphocytes. PD-L1 levels correlated with PD-1 levels ($P < 0.001$), and mismatch-repair-deficient tumors had significantly higher rates of high PD-L1 and PD-1 expression when compared with mismatch-repair-proficient tumors (18% *vs* 2% and 50% *vs* 13%, respectively; $P < 0.001$ for both). Staining intensity was also stronger for both markers in mismatch-repair-deficient tumors. Furthermore, we observed that among patients with mismatch-repair-deficient colorectal carcinoma, PD-1/PD-L1 expression stratified recurrence-free survival in an inter-dependent manner: an association between high PD-1-positive tumor-infiltrating lymphocytes and improved recurrence-free survival ($P = 0.041$) was maintained only when the tumors had low-level PD-L1 expression ($P = 0.006$); patients whose tumors had both high PD-1-positive tumor-infiltrating lymphocytes and high PD-L1 expression had a significantly worse recurrence-free survival ($P < 0.001$). Thus, our results not only provide a foundation for further assessment of PD-L1 immunohistochemistry as a predictive marker for anti-PD-1 therapy in colorectal carcinoma, they also shed light on the prognostic impact of tumor-infiltrating lymphocytes in different subsets of mismatch-repair-deficient colorectal carcinomas.
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In recent years, targeted therapies using antibodies against immune checkpoints have shown promising results in the treatment of various malignancies including melanoma, lung carcinoma, renal cell carcinoma, and urothelial carcinoma.[1–7] The rationale for such an approach lies in the fact that immune checkpoints, such as programmed death-1 (PD-1) and its ligand programmed death-ligand 1 (PD-L1), are an immune-inhibitory mechanism by which carcinoma cells evade antitumor immunity. Expression of PD-L1 can negatively regulate T-cell antitumor activity by binding to the PD-1 receptor on the T cells. Thus, it is not surprising that in many tumors showing response to anti-PD-1 therapy, the tumor cells show expression of PD-L1 when tested by immunohistochemistry.[2,3,6–8]

In colorectal carcinoma, the efficacy of immune checkpoint therapy has been limited.[2,4,5,9,10] It was confirmed only recently that colorectal carcinomas with proficient mismatch-repair function, which account for up to 96% of all metastatic colorectal carcinomas, are unlikely to respond to anti-PD-1 therapy. In a phase 2 study, objective response

Correspondence: Dr J Shia, MD, Member and Attending Pathologist, Memorial Sloan Kettering Cancer Center, Professor of Pathology, Weill Cornell Medical College, 1275 York Avenue, New York, NY 10065, USA.
E-mail: shiaj@mskcc.org
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to pembrolizumab, an anti-PD-1 antibody, was achieved only in patients with mismatch-repair-deficient colorectal carcinoma (4/10, 40%) and not in patients with proficient mismatch-repair colorectal carcinoma (0/18, 0%).[10] A question that follows is whether among the mismatch-repair-deficient colorectal carcinomas, PD-L1 and/or PD-1 expression bear any informative value in predicting response to anti-PD-1 therapy. Toward addressing this question, it is important to first determine whether immunohistochemical detection of PD-L1 and PD-1 is feasible in colorectal carcinoma and what their staining patterns are. Thus far, data in these regards are sketchy and incomplete.

Tumor-infiltrating lymphocytes have long been associated with an improved outcome in patients with colorectal carcinoma.[11] Notably, most colorectal carcinomas with significantly increased lymphocytes fall into the mismatch-repair-deficient subset.[12] In this subset, it is unclear to what extent mismatch-repair deficiency *per se versus* high number of lymphocytes or both contribute to the better prognosis observed. It is intriguing to note that recent data have indicated that not all mismatch-repair-deficient colorectal carcinomas have a good prognosis.[13,14] Could such prognostic differences be related to the lymphocyte infiltration in the tumors? Do the levels of PD-1 or PD-L1 expression in lymphocytes and tumor cells affect the prognostic impact of the tumor-infiltrating lymphocytes?

With these considerations, we designed this pilot study with two objectives. The first was to obtain preliminary data that may serve as a foundation for further exploration of the utility of PD-1 and PD-L1 immunohistochemistry as clinically relevant measures of response to anti-PD-1 therapies in colorectal carcinoma. For this, we examined the PD-1 and PD-L1 immunohistochemical expression patterns in a large series of colorectal carcinomas encompassing all tumor stages. The second objective was to evaluate the prognostic association, independent of immune checkpoint blockade, of PD-1 and PD-L1 in colorectal carcinomas with and without mismatch-repair deficiency.

Materials and methods

Patient and Tumor Characteristics

Study cases consisted of colorectal carcinomas surgically resected at a single cancer center. Only cases with accessible tissue blocks and clinical follow-up were included. Hematoxylin and eosin (H&E) sections of all cases were systematically reviewed for pathologic features including tumor histologic type, differentiation, tumor-infiltrating lymphocytes (scored 0–3 with 0 being <1 tumor-infiltrating lymphocyte per 10 high power fields, 1 being 1–15 per 10 high power fields, 2 being >15 but <215 per 10 high power fields, and 3 being 215 or more per 10 high power fields), and peritumoral lymphocyte aggregates (scored 0–2 with 0 being none, 1 being a few often <5, and 2 being >5). Tumor location was defined as being left-sided if the tumor was located at or distal to the splenic flexure. Chart review was performed to obtain clinical data including age, sex, neoadjuvant therapy, follow-up intervals, and time to recurrence. The study was approved by the institutional review board.

Tissue Microarray

The ATA-27 automated arrayer (Beecher Instruments, Sun Prairie, WI, USA) was used to create the tissue microarrays with 0.6 mm punch size. Tissue microarrays were constructed from representative tumor blocks for each case. Three cores of different areas of the tumor were sampled from each tumor specimen. Cases with no analyzable tumor in the resulting tissue microarrays were excluded from final analysis. The minimal requirement for a case to be regarded as analyzable was the presence of at least 1 intact core.

Immunohistochemical Analyses

Immunohistochemistry for PD-1 and PD-L1 was performed on tissue microarray slides. For PD-1, we used a monoclonal antibody against PD-1 (clone NAT105, ready to use, Catalog # 760–4895, Cell Marque) on the Ventana Benchmark Ultra (Ventana Medical Systems, Tucson, AZ, USA). After optimizing the immunohistochemical conditions, the slides were deparaffinized. Target retrieval was performed for 24 min using 'Cell Condition 1'. The incubation time for the primary antibody was 16 min. The slides were washed, followed by incubation with the secondary antibody and final development with the 3,3′-Diaminobenzidine Map Kit (Ventana, Catalog # 760–700) for 10 min, and detected with Ventana Optiview (Ventana, Catalog # 760–700).

For PD-L1 immunohistochemistry, we used a monoclonal antibody against PD-L1 (clone E1L3N, dilution 1:250; Cell Signaling Technology, Danvers, MA, USA) on the Leica's Bond III platform (Leica Biosystems, Buffalo Grove, IL, USA). The dilution factor of this antibody was optimized by using liver parenchyma as negative control and tonsil tissue as positive control as recommended. In addition to antibody concentration, we also optimized target retrieval buffer. After optimizing the immunohistochemical conditions, the slides were deparaffinized. Target retrieval was performed in standard Epitope Retrieval Solution 2 (Catalog # AR 9640, pH 9.0) slightly basic buffer. The primary antibody was diluted by 1:100 with a final concentration of 0.2 μg/ml. The incubation time for the primary antibody was 30 min. The slides were washed, followed by incubation with the secondary antibody and final development with the 3,3′-Diaminobenzidine Map Kit (Leica Biosystems, Catalog # DS9800) for 7 min, and detected with Leica's Bond Polymer Refine (Leica Biosystems, Catalog # DS9800).

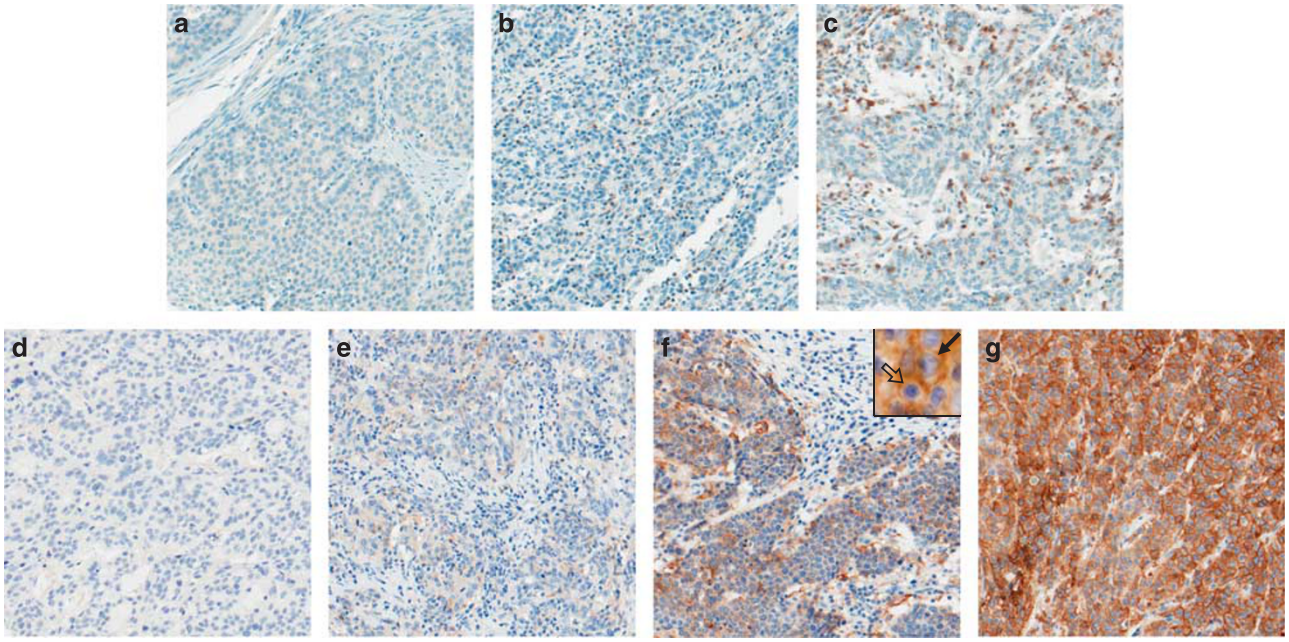

Figure 1 Immunohistochemical staining for PD-1 and PD-L1. The intensity of PD-1 expression on intraepithelial lymphocytes was scored as: 0 for no staining (**a**), 1+ for faint staining (**b**), and 2+ for moderate or strong staining (**c**). The intensity of PD-L1 expression on tumor cells was scored on a similar scale: 0 for no staining (**d**), 1+ for faint staining (**e**), and 2+ for moderate (**f**) or strong (**g**) staining. Staining of PD-L1 was seen in two patterns: staining primarily at the tumor-stroma interface and in both tumor cells and lymphocytes or immune cells (**f**), or diffuse labeling in the tumor cells (**g**). Inset in figure **f** highlights positive staining in both tumor cells (solid arrow) and lymphocytes (open arrow).

The stains for PD-1 and PD-L1 were scored as follows: (1) the amount of cells staining (percent of tumor cells staining for PD-L1, and the number of tumor-infiltrating lymphocytes per square millimeter of tumor staining for PD-1); and (2) the staining intensity using a scale of 0 to 2+: 0 for no staining, 1+ for faint staining, and 2+ for moderate or strong staining (Figure 1).

Immunohistochemical staining for MLH1, PMS2, MSH2, and MSH6 was performed on the tissue microarrays using standard streptavidin-biotin-peroxidase procedure. Primary monoclonal antibodies used included MLH1 (clone G168-728, diluted 1:250, BD PharMingen, San Diego, CA, USA), MSH2 (clone FE11, diluted 1:50, Oncogene Research Products, Cambridge, MA, USA), MSH6 (clone GRBP. P1/2.D4, diluted 1:200; Serotec, Raleigh, NC, USA), and PMS2 (clone A16-4, diluted 1:200, BD PharMingen, San Diego, CA, USA). Non-neoplastic colonic mucosa and colorectal carcinoma known to be deficient of MLH1, MSH2, MSH6 and PMS2 were used as external positive and negative controls, respectively. Negative protein expression was defined as complete absence of nuclear staining within tumor cells in the presence of concurrent positive labeling in internal non-neoplastic tissues. Negative or equivocal protein expression detected on tissue microarray was followed up with immunohistochemistry performed on corresponding whole-tumor sections of the resection specimen following the same protocol. For the cases where repeated immunohistochemical staining was performed on whole sections, the results of the whole section were used for final analysis.

Statistical Analysis

Descriptive and comparative statistics were performed using IBM Statistical Software for the Social Sciences version 22 (IBM Corporation, Armonk, NY, USA). Continuous variables were compared using the Student's *t*-test. Categorical variables were compared using χ^2 or the Fisher exact test depending on the number of observations. A *P*-value < 0.05 was considered significant. Survival distributions were estimated using the Kaplan–Meier method and differences were assessed using the log-rank statistic. Time to event was calculated from date of colonic resection to date of event. An event for recurrence-free survival was defined as recurrence or death. Patients without the event of interest at last follow-up were censored. A Cox regression model, employing the backwards conditional method, was used to adjust for confounders. Parameters with $P < 0.05$ on univariate analysis were included in the regression model.

Results

Patient Demographics

A total of 395 patients were included in the study. The average age of the patients at surgical resection

Table 1 Patient demographics ($N = 395$)

	n[a] *(%)*
Age (years, mean ± s.d. (range))	55 ± 15 (18–90)
Sex	
Male	201 (51)
Female	194 (50)
Tumor differentiation	
Well	11 (3)
Moderate	326 (83)
Poor	54 (14)
Medullary features	
Yes	16 (7)
No	230 (93)
Signet ring cell features	
Yes	5 (1)
No	371 (99)
Mucinous features	
Yes	69 (18)
No	307 (82)
Location	
Right colon	155 (39)
Left colon	239 (61)
Neoadjuvant treatment	
Yes	39 (10)
No	343 (90)
T-stage	
Tis	2 (1)
T1	28 (7)
T2	64 (17)
T3	220 (57)
T4	70 (18)
N-stage	
N0	188 (50)
N1	118 (32)
N2	67 (18)
AJCC stage	
I	60 (15)
II	114 (29)
III	132 (34)
IV	83 (21)
Tumor-infiltrating lymphocyte score	
0 – < 1 TIL per 10 high power fields	74 (19)
1–1 to 15 TILs per 10 high power fields	175 (46)
2 – > 15 but < 215 TILs per 10 high power fields	109 (28)
3 – > 215 TILs per 10 high power fields	35 (9)
Peritumoral lymphocyte aggregates	
0 – None	230 (59)
1 – Few	160 (41)
2 – Many	1 (0)
Mismatch-repair protein status	
Mismatch-repair proficient	321 (83)
Mismatch-repair deficient	68 (17)
PD-1 expression (on TILs)	
Low	316 (81)
High	76 (19)
PD-L1 expression	
Low	375 (95)
High	19 (5)
Follow-up (months, mean ± s.d. (range))	55 ± 40 (< 1–393)

Abbreviations: AJCC, American Joint Committee on Cancer; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; TILs, tumor-infiltrating lymphocytes.
[a]Discrepancy in the number of cases is due to lack of data on several cases.

was 55 ± 15 years. Of the patients assessed, 55% were high stage (AJCC stage III or IV) and 10% received neoadjuvant chemotherapy. The complete patient demographics are shown in Table 1.

Immunohistochemical Expression of PD-1, PD-L1, and Mismatch-Repair Proteins

Data on PD-1 and PD-L1 were obtained on 389 tumors. PD-1 positivity was observed in both tumor-infiltrating lymphocytes and stromal lymphocytes. PD-L1 staining was observed in tumor cells as well as lymphocytes or immune cells; tumor cell staining for PD-L1 occurred in two patterns: primarily along the tumor-stromal interface (84%, Figure 1f) or diffuse (16%, Figure 1g). In the formal scenario, the stained interfaces were not limited to the invasive front or the periphery of large groups of tumor cells; they could be intra-tumoral as well (wherever tumor cells were juxtaposed with stroma cells). For both markers, both membranous and cytoplasmic staining were regarded as positive.

Of the 389 tumors, 68 were found to be mismatch-repair deficient by immunohistochemistry. Mismatch-repair deficiency correlated significantly with PD-1 and PD-L1 expression by both staining intensity and the percentage of cell staining ($P < 0.001$ for both). By maximizing the sensitivity and specificity of PD-L1 or PD-1 expression in predicting mismatch-repair status through receiver-operating characteristic curve analyses,[15] we derived the following criteria to separate tumors into high and low PD-L1 and PD-1 expression categories. 'High-level PD-L1' corresponded to > 1% of tumor cells staining with 2+ intensity; 'high PD-1-positive tumor-infiltrating lymphocytes' corresponded to a measure of > 1.43 tumor-infiltrating lymphocytes of 1+ or 2+ intensity per square millimeter. With these criteria, 18 and 50% of mismatch-repair deficient tumors exhibited high-level PD-L1 and high PD-1-positive tumor-infiltrating lymphocytes, respectively; on the other hand, these rates for mismatch-repair-proficient tumors were only 2% and 13%, respectively ($P < 0.001$ for both).

For the entire group, 5% had high-level PD-L1 (19/394) and 19% had high PD-1-positive tumor-infiltrating lymphocytes (76/392). The PD-L1 staining was diffuse in 16% of the high-level positive cases. Expression of PD-L1 and that of PD-1 were positively correlated: 68% of the cases with high-level PD-L1 also displayed high PD-1 positive tumor-infiltrating lymphocytes, and 98% of the cases with low PD-1 positive tumor-infiltrating lymphocytes also had low-level PD-L1 ($r = 0.28$, $P < 0.001$). PD-1 levels maintained a positive correlation with PD-L1 levels when assessing only the mismatch-repair deficient or mismatch-repair proficient tumors ($P = 0.017$ and $P = 0.013$, respectively).

Association Between PD-L1/PD-1 and Clinicopathologic Parameters

The association between PD-1 expression, PD-L1 expression, and clinicopathologic parameters is shown in Table 2. High PD-1-positive tumor-infiltrating lymphocytes and high-level PD-L1 correlated with the various clinicopathologic parameters that were more often associated with mismatch-repair deficiency including medullary morphology, right sided location, high H&E tumor-infiltrating lymphocyte score, high H&E peritumoral lymphocyte aggregate score, and younger age (all $P < 0.05$). Many of these associations remained significant even when controlling for mismatch-repair status, including medullary morphology, high H&E tumor-infiltrating lymphocyte score, and high H&E peritumoral lymphocyte aggregate score (all $P < 0.05$). Overall, high-level PD-L1 expression was also associated with female sex ($P = 0.029$), whereas high PD-1-positive tumor-infiltrating lymphocytes was associated with lower N-stage ($P = 0.001$) and lower overall AJCC stage group ($P < 0.001$).

Survival Analysis

The follow-up time for all patients was 55 ± 40 months (range, 0.4–393 months). Univariate analysis of the entire patient cohort identified the following parameters as significant predictors for recurrence-free survival: age ($P = 0.041$), sex ($P = 0.025$), T-stage ($P < 0.001$), N-stage ($P < 0.001$), AJCC stage group ($P < 0.001$), H&E tumor-infiltrating lymphocyte score ($P = 0.002$), H&E peritumoral lymphocyte aggregate score ($P = 0.007$), and PD-1 expression ($P = 0.011$).

When the patients were stratified by mismatch-repair status, many factors remained significant. However, the significance for PD-1 expression was only observed in the mismatch-repair-deficient group (Figure 2). High-level PD-1-positive tumor-infiltrating lymphocytes correlated with a significantly better recurrence-free survival among mismatch-repair-deficient patients (Figures 2c, $P = 0.041$). In addition, as shown in Figure 2, tumor PD-L1 expression also emerged as a significant factor in the mismatch-repair-deficient group; high-level PD-L1 was associated with a significantly worse recurrence-free survival (Figures 2f, $P = 0.026$). On the other hand, in the mismatch-repair-proficient group, neither PD-1 nor PD-L1 showed a statistically significant association with recurrence-free survival.

On multivariate analysis, both PD-1 and PD-L1 expression were shown to be independent factors associated with recurrence-free survival within the mismatch-repair-deficient group (PD-1: hazard ratio 0.03, 95% confidence interval, 0.0–0.42, $P = 0.01$; PD-L1: hazard ratio 22.86, 95% confidence interval, 1.99–263.21, $P = 0.012$), along with AJCC staging (hazard ratio 5.36, 95% confidence interval, 1.81–15.84, $P = 0.002$) (Table 3). PD-1 and PD-L1 were not independent factors associated with recurrence-free survival in the mismatch-repair-proficient group or the entire patient cohort.

Further assessment revealed that within the mismatch-repair deficient group, PD-1 and PD-L1 were associated with recurrence-free survival in a co-dependent manner with combined high PD-L1 expression and high PD-1 positive tumor-infiltrating lymphocytes associating with a worse recurrence-free survival. As shown in Table 4, the association of high PD-1-positive tumor-infiltrating lymphocytes with a better recurrence-free survival was observed only among the group that had low-level PD-L1 ($P = 0.006$), and not among the group with high-level PD-L1 ($P = 0.061$). Conversely, the association of high PD-L1 levels with a worse recurrence-free survival was observed only among the group that had high PD-1-positive tumor-infiltrating lymphocytes ($P < 0.001$) and not in the group with low PD-1-positive tumor-infiltrating lymphocytes ($P = 0.082$).

Discussion

This study represents one of the first studies to systematically evaluate the frequency and pattern of immunohistochemical expression of PD-1 and PD-L1 in colorectal carcinoma. Our results showed that immunohistochemical detection of these markers was feasible and technically achievable. Of the 394 colorectal carcinomas that we evaluated, the rate of high PD-1-positive tumor-infiltrating lymphocytes was 19%, and high-level PD-L1 expression in the tumor cells was 5%. Both high PD-1-ositive tumor-infiltrating lymphocytes and high-level PD-L1 were significantly more frequent in mismatch-repair-deficient colorectal carcinomas than in mismatch-repair-proficient colorectal carcinomas. In addition, our results showed for the first time that the positive prognostic impact of tumor lymphocytes in patients with mismatch-repair-deficient colorectal carcinoma was negated by the presence of high-level PD-L1 in the tumors.

Defining the patterns of immunohistochemical expression of PD-1 and PD-L1 in colorectal carcinoma is clinically relevant as it provides a foundation for further exploration of the test's utility in predicting treatment response to immune checkpoint inhibition targeting the PD-1 pathway. In our analysis, we found immunohistochemical assessment of PD-1 to be a reliable and objective measure to evaluate the extent of PD-1 expression. For PD-L1 staining, we used a monoclonal antibody against PD-L1 from Cell Signaling (clone E1L3N), and observed two readily recognizable positive staining patterns in colorectal carcinoma tumor cells: diffuse and focal. The former is rare, constituting 16% of the positive cases. When the staining is focal, it is characteristically present along the tumor-stromal interface. In the positive cases, positive staining in background lymphocytes and mononuclear stromal cells frequently exists.

Table 2 Comparison between PD-L1 high and low cases, and between PD-1 high and low cases[a]

	PD-L1			*PD-1 (on TILs)*		
	Low (N = 375)	*High (N = 19)*		*Low (N = 316)*	*High (N = 76)*	
	N (%)	*N (%)*	P-*value*	*N (%)*	*N (%)*	P-*value*
Age (years, mean ± s.d.)	55 ± 15	65 ± 15	**0.002**	55 ± 14	59 ± 16	**0.031**
Sex			**0.029**			0.650
M	195 (52)	5 (26)		163 (52)	37 (49)	
F	180 (48)	14 (74)		153 (48)	39 (51)	
Tumor differentiation			**0.008**			0.098
Well	10 (3)	1 (5)		9 (3)	2 (3)	
Moderate	314 (85)	11 (58)		267 (85)	57 (76)	
Poor	47 (13)	7 (37)		16 (21)	37 (12)	
Medullary features			< **0.001**			< **0.001**
Yes	10 (4)	6 (46)		5 (3)	11 (21)	
No	223 (96)	7 (54)		118 (97)	41 (79)	
Signet ring cell features			1.000			0.588
Yes	5 (1)	0 (0)		0 (0)	0 (0)	
No	353 (99)	17 (100)		296 (98)	73 (100)	
Mucinous features			0.958			0.442
Yes	65 (18)	3 (18)		57 (19)	11 (15)	
No	293 (82)	14 (82)		244 (81)	62 (85)	
Location			**0.028**			< **0.001**
Right colon	142 (38)	12 (63)		106 (34)	49 (64)	
Left colon	232 (62)	7 (37)		209 (66)	27 (36)	
Neoadjuvant treatment			0.142			0.050
Yes	39 (11)	0 (0)		36 (12)	3 (4)	
No	324 (89)	18 (100)		270 (88)	71 (96)	
T-Stage			0.323			0.238
Tis	2 (1)	0 (0)		2 (1)	0 (0)	
T1	26 (7)	2 (11)		19 (6)	9 (12)	
T2	59 (16)	5 (28)		50 (16)	14 (19)	
T3	213 (58)	6 (33)		176 (57)	43 (57)	
T4	65 (18)	5 (28)		61 (20)	9 (12)	
N-stage			0.067			**0.001**
N0	178 (51)	9 (50)		136 (45)	51 (71)	
N1	109 (31)	9 (50)		105 (35)	13 (18)	
N2	67 (19)	0 (0)		59 (20)	8 (11)	
AJCC stage			0.397			< **0.001**
I	56 (15)	4 (21)		41 (13)	19 (25)	
II	110 (30)	3 (16)		82 (26)	31 (41)	
III	123 (33)	9 (47)		113 (36)	19 (25)	
IV	80 (22)	3 (16)		75 (24)	7 (9)	
Tumor-infiltrating lymphocyte score			< **0.001**			< **0.001**
0 – < 1 TIL per 10 high power fields	71 (19)	3 (16)		69 (22)	3 (4)	
1–1 to 15 TILs per 10 high power fields	175 (47)	0 (0)		161 (51)	13 (17)	
2 – > 15 but < 215 TILs per 10 high power fields	101 (27)	7 (37)		76 (24)	33 (43)	
3 – > 215 TILs per 10 high power fields	26 (7)	9 (47)		8 (3)	27 (36)	
Peritumoral lymphocyte aggregates			**0.003**			< **0.001**
0 – None	225 (61)	4 (21)		203 (65)	24 (32)	
1 – Few	145 (39)	15 (79)		109 (35)	51 (68)	
2 – Many	1 (0)	0 (0)		1 (0)	0 (0)	
Mismatch-repair protein status			< **0.001**			< **0.0001**
Mismatch-repair proficient	314 (85)	7 (37)		277 (89)	41 (55)	
Mismatch-repair deficient	55 (15)	12 (63)		34 (11)	34 (45)	
PD-1 expression on TILs			< **0.001**			
Low	309 (83)	6 (32)				
High	63 (17)	13 (68)				

Abbreviations: AJCC, American Joint Committee on Cancer; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; TILs, tumor-infiltrating lymphocytes.
[a]Number of cases for each category may be less than the total due to lack of data on several cases. Statistically significant *P*-values ($P < 0.05$) are highlighted in bold.

These patterns are similar to that observed in other cancer types where the utility of immunohistochemistry as predictive markers is better defined, including melanoma[16] and lung carcinoma.[17,18]

In our study, we further demonstrated that, in colorectal carcinoma, the high expressers of both PD-1 and PD-L1 were mostly mismatch-repair-deficient tumors. Recently, molecular analyses have indicated that only a small subset of colorectal carcinomas has upregulated mRNA expression of the major immune checkpoint genes (including *PDCD1*, *CD274*, *CTLA4*, *LAG3*, and *TIGIT*) and this subset falls within the category of mismatch-repair-deficient tumors.[19,20] Clinically, as reported by Le *et al.*,[10] in a phase II clinical trial, colorectal carcinoma patients that showed immune-related objective response to anti-PD-1 treatment all had mismatch-repair-deficient tumors. Thus, all evidence suggests the existence of a particular subset of colorectal carcinomas that are mismatch-repair-deficient, have activated PD-L1 function, and respond to immune checkpoint inhibition. However, as yet, data directly indicating that the positive cases by immunohistochemistry are indeed the cases with upregulated immune checkpoint genes and/or the cases respond to anti-PD-1 therapy are still lacking. Thus, further studies are needed. The PD-1 and PD-L1 immunohistochemical staining patterns observed in our study should serve as a foundation for such studies, and incorporation of immunohistochemistry in clinical trials is warranted.

Tumor-infiltrating lymphocytes are in general regarded as an indicator of host immune response to the tumor,[11,21] and have long been recognized as a favorable prognostic marker in colorectal carcinoma.[22–25] Prognostic studies thus far, however, have mostly evaluated tumor-infiltrating lymphocytes outside the context of immune checkpoints. In this study, we demonstrated that the expression of immune checkpoint receptor PD-1 and its ligand PD-L1 can indeed affect the impact of tumor-infiltrating lymphocytes on recurrence-free survival. Specifically, we demonstrated that overall, high PD-1-positive tumor-infiltrating lymphocytes were associated with a favorable outcome in mismatch-repair deficient colorectal carcinoma patients, but high-level PD-L1 expression was associated with a worse recurrence-free survival. More interestingly, we showed that when patients were further stratified by PD-L1 or PD-1 levels, the favorable association with outcomes of PD-1-positive tumor-infiltrating lymphocytes was only observed in group of tumors that did not have high-level PD-L1 expression. Tumors with high-level PD-L1 expression had poor prognosis regardless of PD-1 expression. Conversely, the association of high-level PD-L1 expression with worse recurrence-free survival was observed only in the group of tumors that had high PD-1-positive tumor-infiltrating lymphocytes. Tumors with low levels of PD-1 tumor-infiltrating lymphocytes had poor prognosis, regardless of PD-L1 expression.

Immune checkpoints represent the major defense system of the tumor against the host's antitumor immunity.[26] Efficient checkpoint function of the tumor can lead to immune evasion and consequently tumor progression. As PD-1 is a major component of the PD-1 checkpoint pathway, our observation that high-level PD-1-positive tumor-infiltrating lymphocytes correlated with favorable recurrence-free survival in mismatch-repair-deficient colorectal carcinomas seems unexpected. However, the interpretation of our observation has to be made in conjunction with the fact that the frequency of high-level PD-L1 expression is very low in colorectal carcinoma. High tumor-infiltrating lymphocytes are common in mismatch-repair-deficient colorectal carcinomas (presumably secondary to neo-antigens generated by a high burden of frameshift mutations),[27,28] and as an inherent component of this adaptive immunity that elicits the tumor-infiltrating lymphocytes, PD-1-positive tumor-infiltrating lymphocytes develop as well. However, for PD-1 to exert its immune-inhibitory effect, there also needs to be PD-L1 expression by the tumor cells or other immune cells. Thus, given the low frequency of high PD-L1 expression in colorectal carcinoma, the high PD-1-positive tumor-infiltrating lymphocytes we observed in most cases may merely reflect an elevation of effector T cells in general. This assumption is indeed supported by the fact that, when we sub-stratified our cases, the favorable survival associated with high PD-1-positive tumor-infiltrating lymphocytes is only maintained in tumors with no or low PD-L1, and not in tumors with high PD-L1, and the negative association with recurrence-free survival of PD-L1 was no longer significant when the tumors did not have high levels PD-1-positive tumor-infiltrating lymphocytes.

Thus, mismatch-repair-deficient colorectal carcinomas with 'high PD-1 and no or low PD-L1' are likely to have an anti-tumoral immune microenvironment in which the cytotoxic tumor-infiltrating lymphocytes prevails and consequently they are likely to have a better prognosis, whereas those with both 'high PD-1 and high PD-L1' are likely to harbor ongoing immune checkpoint activity and are consequently more prone to progression and poor prognosis. It can be further inferred that in colorectal carcinoma, particularly in those that are mismatch-repair-deficient, tumor-infiltrating lymphocytes are necessary for both anti-tumoral and pro-tumoral immunity. The balance between the two opposing immune functions is largely dependent on the level of PD-L1 expression. Further exploration in this area may shed light not only on the understanding of PD-1-positive tumor-infiltrating lymphocytes as a prognostic indicator, but also on the utility of PD-1 and PD-L1 expression as predictors for treatment response to immune therapy. Colorectal carcinomas with both 'high PD-1 and PD-L1' may have an active immune checkpoint activity and may therefore represent the subset that benefit from anti-PD-1 therapy.

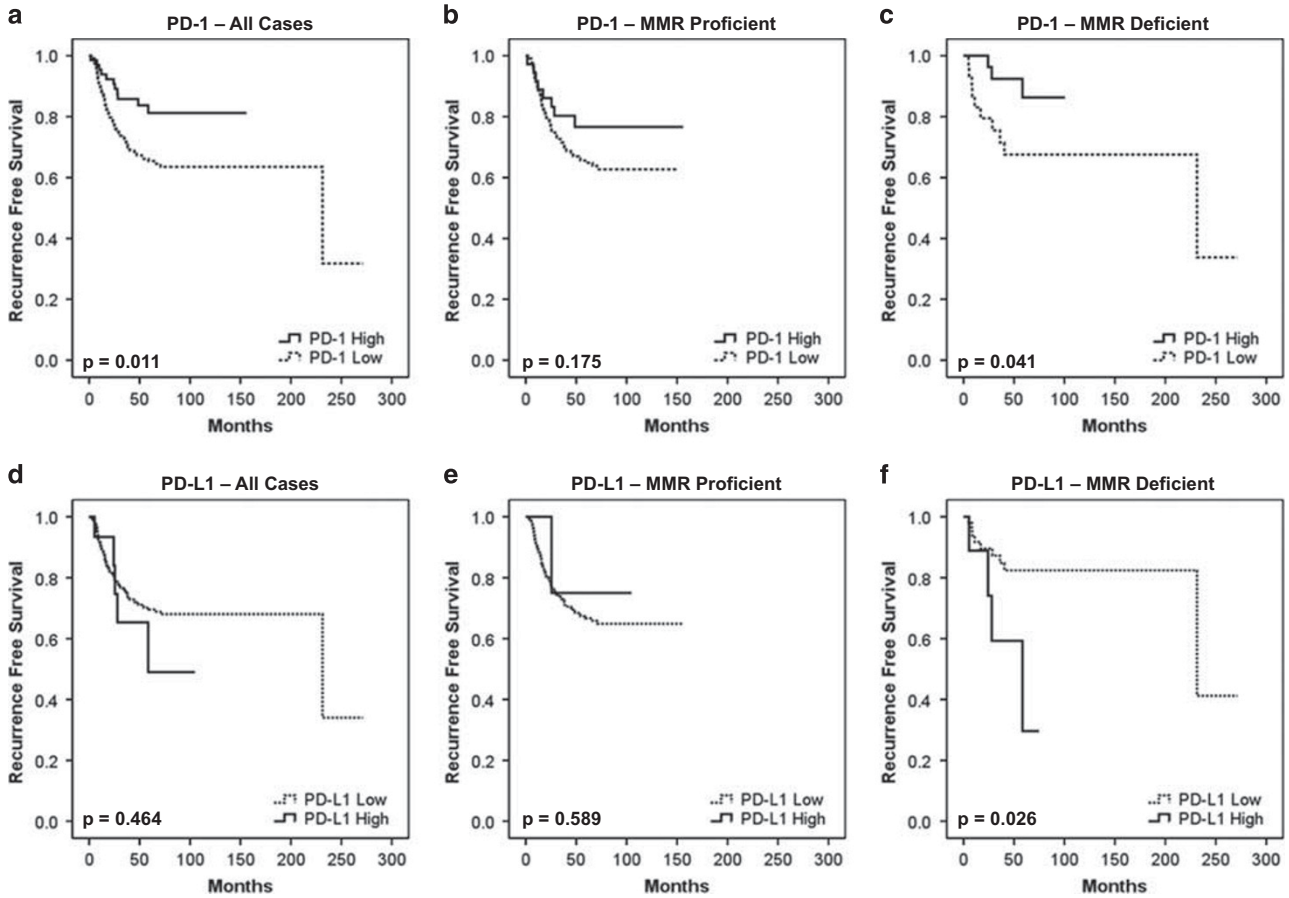

Figure 2 Kaplan–Meier survival curves depicting recurrence-free survival of patients with colorectal carcinoma stratified by tumor-infiltrating lymphocyte PD-1 expression and tumor cell PD-L1 expression. PD-1 expression stratified survival when all patients were assessed (**a**). It was not a significant prognostic factor in patients with mismatch-repair proficient carcinomas (**b**), but was significant among mismatch-repair-deficient carcinomas (**c**). PD-L1 expression did not stratify survival when assessing all patients (**d**) or in patients with mismatch-repair proficient carcinomas (**e**), but did significantly stratify patients with mismatch-repair deficient carcinomas (**f**). *P*-values were calculated by log-rank test.

Table 3 Relationship between clinicopathological characteristics and RFS among mismatch-repair-deficient tumors

	Univariate analysis			*Multivariate analysis*	
	Hazard ratio	P-*value (Cox)*	P-*value (log-rank)*	*Hazard ratio*	P-*value*
Age	0.98 (0.95–1.01)	0.256		–	–
Sex	1.16 (0.37–3.59)	0.801	0.801	–	–
Tumor differentiation	1.32 (0.48–3.63)	0.587	0.525	–	–
Medullary features	1.67 (0.36–7.72)	0.514	0.510	–	–
Signet ring cell features	No events			–	–
Mucinous features	1.18 (0.35–3.91)	0.793	0.793	–	–
Tumor location	0.26 (0.07–0.91)	**0.034**	**0.026**	NS	NS
Neoadjuvant treatment	No events			–	–
T-Stage	3.42 (1.29–9.07)	**0.014**	0.073	NS	NS
N-Stage	2.49 (1.23–5.07)	**0.012**	**0.023**	NS	NS
AJCC Stage	5.01 (2.43–10.35)	< **0.001**	< **0.001**	5.36 (1.81–15.84)	**0.002**
Tumor-infiltrating lymphocyte score	0.49 (0.27–0.90)	**0.021**	0.077	NS	NS
Peritumoral lymphocyte aggregates	0.87 (0.26–2.88)	0.813	0.812	–	–
PD-1 expression on tumor-infiltrating lymphocytes	0.28 (0.08–1.03)	0.056	**0.041**	0.03 (0.00–0.42)	**0.010**
PD-L1 expression on tumor	3.60 (1.08–12.0)	**0.037**	**0.026**	22.86 (1.99–263.21)	**0.012**

Abbreviations: AJCC, American Joint Committee on Cancer; N, node; NS, not significant; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; T, tumor.
Statistically significant *P*-values ($P < 0.05$) are highlighted in bold.

Table 4 Interaction between PD-L1 and PD-1 on RFS among mismatch-repair-deficient tumors

		5-year RFS (95% CI)	P-*value*
Low-level PD-L1	Low PD-1-positive TILs	68% (50%–87%)	**0.006**
	High PD-1-positive TILs	All cases censored	
High-level PD-L1	Low PD-1-positive TILs	50% (0%–100%)	0.061
	High PD-1-positive TILs	33% (0%–83%)	
Low PD-1-positive TILs	Low-level PD-L1	68% (50%–87%)	0.082
	High-level PD-L1	50% (0%–100%)	
High PD-1-positive TILs	Low-level PD-L1	All cases censored	< **0.001**
	low-level PD-L1	33% (0%–83%)	

Abbreviations: CI, confidence interval; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; RFS, recurrence-free survival; TILs, tumor-infiltrating lymphocytes.
P-value calculated by log-rank test, with statistically significant differences ($P < 0.05$) in bold.

A limitation of our study is that the assessment of PD-1 and PD-L1 was performed on tissue microarray only. As the staining of these markers may be focal and heterogeneous, tissue array cores may under-represent cases with focal positivity. Furthermore, even though our overall case number is relatively large, the positive events both in terms of positive PD-1/PD-L1 labeling and clinical outcome are still small.

Nonetheless, our study provided data indicating that PD-1 and PD-L1 immunohistochemistry is feasible in colorectal carcinoma, and in this tumor type, high-level expression of these markers is a feature tightly associated with mismatch-repair deficiency. Our results may serve as a foundation to further explore whether there is indeed added value of PD-1/PD-L1 immunohistochemistry to MSI in selecting colorectal carcinoma that might respond to anti-PD-1 therapy. Furthermore, our data indicated that immunohistochemical detection of PD-1 and PD-L1 has the potential to refine our understanding of the prognostic value of tumor-infiltrating lymphocytes in colorectal carcinoma; PD-L1 expression may allow the identification of the mismatch-repair-deficient colorectal cancers that have a poor prognosis despite having high-level tumor-infiltrating lymphocytes.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

References

1 Hamid O, Robert C, Daud A *et al.* Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. N Engl J Med 2013;369:134–144.
2 Herbst RS, Soria JC, Kowanetz M *et al.* Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature 2014;515:563–567.
3 Powles T, Eder JP, Fine GD *et al.* MPDL3280A (anti-PD-L1) treatment leads to clinical activity in metastatic bladder cancer. Nature 2014;515:558–562.
4 Topalian SL, Hodi FS, Brahmer JR *et al.* Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 2012;366:2443–2454.
5 Brahmer JR, Tykodi SS, Chow LQM *et al.* Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med 2012;366:2455–2465.
6 Topalian SL, Sznol M, McDermott DF *et al.* Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab. J Clin Oncol 2014;32:1020–1030.
7 Sunshine J, Taube JM. PD-1/PD-L1 inhibitors. Curr Opin Pharmacol 2015;23:32–38.
8 Taube JM, Klein A, Brahmer JR *et al.* Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy. Clin Cancer Res 2014;20:5064–5074.
9 Brahmer JR, Drake CG, Wollner I *et al.* Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. J Clin Oncol 2010;28:3167–3175.
10 Le DT, Uram JN, Wang H *et al.* PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. N Engl J Med 2015;372:2509–2520.
11 Mantovani A, Romero P, Palucka AK *et al.* Tumour immunity: effector response to tumour and role of the microenvironment. Lancet 2008;371:771–783.
12 Shia J, Ellis NA, Paty PB *et al.* Value of histopathology in predicting microsatellite instability in hereditary nonpolyposis colorectal cancer and sporadic colorectal cancer. Am J Surg Pathol 2003;27:1407–1417.
13 Goldstein J, Tran B, Ensor J *et al.* Multicenter retrospective analysis of metastatic colorectal cancer (CRC) with high-level microsatellite instability (MSI-H)Ann Oncol 2014;25:1032–1038.
14 Nordholm-Carstensen A, Krarup P-M, Morton D *et al.* Danish Colorectal Cancer Group. Mismatch repair status and synchronous metastases in colorectal cancer:

a nationwide cohort study. Int J Cancer 2015;137: 2139–2148.
15 Perkins NJ, Schisterman EF. The inconsistency of "Optimal" cut-points using two ROC based criteria. Am J Epidemiol 2006;163:670–675.
16 Madore J, Vilain RE, Menzies AM *et al.* PD-L1 expression in melanoma shows marked heterogeneity within and between patients: implications for anti-PD-1/PD-L1 clinical trials. Pigment Cell Melanoma Res 2015;28:245–253.
17 Garon EB, Rizvi NA, Hui R *et al.* Pembrolizumab for the treatment of non-small-cell lung cancer. N Engl J Med 2015;372:2018–2028.
18 Passiglia F, Bronte G, Bazan V *et al.* PD-L1 expression as predictive biomarker in patients with NSCLC: a pooled analysis. Oncotarget 2016; e-pub ahead of print.
19 Shia J, Kuk D, Schultz N *et al.* Comprehensive morphological assessment of colorectal cancers characterized by The Cancer Genome Atlas (TCGA) identifies specific morphology-molecular alteration associations [abstract]. Mod Pathol 2016;29(Suppl 2):200A.
20 Lal N, Beggs AD, Willcox BE *et al.* An immunogenomic stratification of colorectal cancer: implications for development of targeted immunotherapy. Oncoimmunology 2015;4:e976052.
21 Pagès F, Berger A, Camus M *et al.* Effector memory T cells, early metastasis, and survival in colorectal cancer. N Engl J Med 2005;353:2654–2666.
22 Klintrup K, Mäkinen JM, Kauppila S *et al.* Inflammation and prognosis in colorectal cancer. Eur J Cancer 2005;41:2645–2654.
23 Roxburgh CSD, Salmond JM, Horgan PG *et al.* Comparison of the prognostic value of inflammation-based pathologic and biochemical criteria in patients undergoing potentially curative resection for colorectal cancer. Ann Surg 2009;249:788–793.
24 Morris M, Platell C, Iacopetta B. Tumor-infiltrating lymphocytes and perforation in colon cancer predict positive response to 5-fluorouracil chemotherapy. Clin Cancer Res 2008;14:1413–1417.
25 Ropponen KM, Eskelinen MJ, Lipponen PK *et al.* Prognostic value of tumour-infiltrating lymphocytes (TILs) in colorectal cancer. J Pathol 1997;182:318–324.
26 Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer 2012;12: 252–264.
27 Llosa NJ, Cruise M, Tam A *et al.* The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints. Cancer Discov 2015;5:43–51.
28 Smyrk TC, Watson P, Kaul K *et al.* Tumor-infiltrating lymphocytes are a marker for microsatelliteinstability in colorectal carcinoma. Cancer 2001;91: 2417–2422.