PD-L1 expression in colorectal cancer is associated with microsatellite instability, *BRAF* mutation, medullary morphology and cytotoxic tumor-infiltrating lymphocytes

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Programmed cell death 1 (PD-1) and its ligand (PD-L1) are key suppressors of the cytotoxic immune response. PD-L1 expression on tumor cells may be induced by the immune microenvironment, resulting in immune escape (adaptive immune resistance), and an adverse prognosis in many malignancies. In colorectal carcinoma the response to PD-1/PD-L1 inhibition is correlated with microsatellite instability. However, little is known about the clinicopathologic, molecular, and prognostic characteristics of colorectal carcinoma with PD-L1 expression. We performed immunohistochemistry for PD-L1 on 181 cases of colorectal carcinoma with known microsatellite instability and mutational status, and correlated PD-L1 expression with clinicopathologic features including tumor-infiltrating lymphocyte burden/immunophenotype, tumor mutational profile, and disease-specific survival. PD-L1 was expressed in tumors from 16 patients (9%) who were more often older ($P = 0.006$) and female ($P = 0.035$), with tumors exhibiting a larger size ($P = 0.013$), but lower stage ($P < 0.001$). PD-L1 expression was associated with increased CD8 and TBET-positive tumor-infiltrating lymphocytes, medullary phenotype, poor differentiation, microsatellite instability, *BRAF* mutation ($P < 0.001$ for each), and a lower frequency of *KRAS* mutation ($P = 0.012$). On multivariate analysis, PD-L1 expression was associated with medullary morphology and frequent CD8-positive tumor-infiltrating lymphocytes, suggesting adaptive immune resistance. PD-L1 positivity was not predictive of survival in the entire cohort, but it was associated with a lower disease-specific survival within the microsatellite-instability high cohort. PD-L1 expression in colorectal carcinoma is associated with clinicopathologic and molecular features of the serrated pathway of colorectal carcinogenesis, and is associated with a worse outcome within microsatellite-unstable tumors. These findings support the role of PD-L1 expression in providing normally immunogenic colorectal carcinoma a means of immune evasion and a more aggressive biology, provide a potential mechanistic explanation for the favorable response of microsatellite-unstable colorectal carcinoma to PD-1/PD-L1 pathway blockade, and suggest potential predictive and prognostic roles of PD-L1 immunohistochemistry in colorectal carcinoma.
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Programmed cell death ligand 1 (PD-L1) is a key immunoregulatory molecule that, upon interacting with its receptor, PD-1, suppresses the CD8 cytotoxic immune response in both physiologic and pathologic pathways.[1] PD-L1 and PD-1 have come under close scrutiny in recent years as potential targets for cancer therapy, and there are multiple ongoing clinical trials with anti-PD-1 or PD-L1 agents. PD-L1 expression on tumor cells has been linked to a weakened host immune response and consequent poor prognosis in several malignancies, including melanoma and esophageal, gastric, hepatocellular and urothelial carcinomas, among others.[2,3] The role of PD-L1 expression in colorectal carcinoma is less clear, with some published studies reporting conflicting results as to whether PD-L1 expression indicates a better or worse prognosis.[4,5]

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In contrast to some malignancies such as melanoma, renal, and lung cancers, colorectal carcinoma generally demonstrates a very low response rate to PD-1 or PD-L1 blockade.[6–8] However, a subset of colorectal carcinomas harboring microsatellite instability and increased tumor-infiltrating lymphocytes has been shown to respond to an anti-PD-1 agent, although microsatellite-stable tumors did not.[9] However, there are conflicting results regarding the association of microsatellite instability with PD-L1 expression in colorectal carcinoma. Some studies have shown PD-L1 expression on colorectal carcinoma tumor cells to be rare in both microsatellite-unstable and microsatellite-stable tumors,[9,10] whereas others have reported strong PD-L1 expression in up to one third of both microsatellite-unstable and microsatellite-stable tumors.[4] In addition, the clinicopathologic features of colorectal carcinoma with PD-L1 expression are not well defined.

In this study we sought to evaluate clinicopathologic and molecular characteristics, including microsatellite instability, and prognosis of tumors harboring PD-L1 expression in a large cohort of resected colorectal carcinomas.

Materials and methods

This study was approved by the institutional review board of the Massachusetts General Hospital, Boston, MA, USA. Patients with colorectal carcinoma who underwent surgical resection of primary or metastatic tumors and clinical molecular testing between the years of 1997 and 2012 were identified from the pathology database. We selected 181 cases with available tissue blocks, including resections of primary tumors ($n=138$) and metastatic foci ($n=43$), including both pre- ($n=124$) and post- ($n=57$) chemotherapy specimens. For the purpose of this study, the tissue microarrays were enriched for cases with microsatellite instability through the inclusion of 31 cases from 2011 through 2012 that were known to be microsatellite unstable.

Hematoxylin and eosin-stained sections of all formalin-fixed paraffin-embedded cases were reviewed by two pathologists (MM-K and JRB) to confirm the presence of adequate lesional tissue for tissue microarray construction and to evaluate pathological features including tumor histology, grade, abundance of tumor-infiltrating lymphocytes, lymphatic, vascular, and perineural invasion, and pattern of advancing border (pushing or infiltrating). Tissue microarrays were constructed using two to three (median three) 2-mm cores of representative tumor tissue from each case, along with multiple cores of non-neoplastic colonic tissue as controls.

The tumor mutation status had been determined before the study as part of the clinical work-up by a multiplex PCR-based assay (SNaPshot platform; Applied Biosystems) to detect a panel of commonly mutated genes implicated in oncogenesis, including *BRAF*, *KRAS*, *APC*, and *TP53,* among others, as previously described.[11] Microsatellite-instability testing by PCR and/or by immunohistochemistry for mismatch-repair proteins (MLH1, MSH2, PMS2, and MSH6) was also conducted.

Immunohistochemistry was performed on 5-μm sections cut from tissue microarray blocks using an automated stainer (Bond Rx, Leica Microsystems, Bannockburn, IL) and the following antibodies in accordance with the manufacturer's recommendations for each antibody: PD-L1 (clone:E1L3N #13684, 1:200, Cell Signaling Technology, MA, USA), PTEN (clone:138G6 # 9559S 1:100, Cell Signaling Technology), CD8 (clone: IgG2b 4B11, RTU, Leica, IL), GATA3 (clone: L50-823# CM405A 1:250, Biocare, Concord, CA), and TBET (clone: D6N8B XP Rabbit mAb, 1:100, Cell Signaling Technology).

PD-L1 expression on tumor cells was evaluated using a three-tiered grading system: $0 = <5\%$ of the tumor cells; $1 = 5–49\%$ of tumor cells; and $2 = \geq 50\%$ tumor cells with membranous staining of any intensity. Cytoplasmic staining was not considered in this study. Scores of 1 and 2 were considered to be positive for PD-L1 expression (Figure 1a–c). Cytoplasmic and/or nuclear PTEN staining was semi-quantitatively evaluated as either lost (0: $<10\%$ of tumor cells), reduced (1: 10–90% of tumor cells), or preserved (2: $>90\%$ of the tumor cells with positive staining; Figure 1d–f). PTEN expression was then dichotomized as lost ($<10\%$ of tumor cells with expression of PTEN) or retained ($\geq 10\%$ of tumor cells with expression).

Expression of CD8, TBET (a Th1 pathway transcription factor), and GATA3 (a Th2 pathway transcription factor) in tumor-infiltrating lymphocytes was semi-quantitatively evaluated on a scale of 0–3 based on the number of positive lymphocytes infiltrating tumor cells. The score was defined based on the percentage of tumor cells with overlying positively-staining T cells: 0 = none or rare; 1+ = $<5\%$; 2+ = $\geq 5\%$ and $<25\%$; 3+ = $\geq 25\%$ (Figure 1g–j). Subsequently, the grades were dichotomized into high (scores 2–3) and low/negative (scores 0–1) for tumor-infiltrating lymphocytes.

Three of the authors (MWR, JRB, and MM-K), blinded to the clinical and pathological data, independently evaluated the immunohistochemical stains. In cases with discrepant scores between cores of an individual case or between observers, a final score was recorded based on a consensus assessment at a multi-head microscope.

Statistical Methods

Statistical analysis was performed using Stata (Version 14.0, StataCorp, College Station, Texas). Univariate analysis of categorical variables was done using a Pearson χ^2-test or a Fisher exact test, where appropriate; continuous variables were evaluated using a Wilcoxon rank-sum test. Multivariate

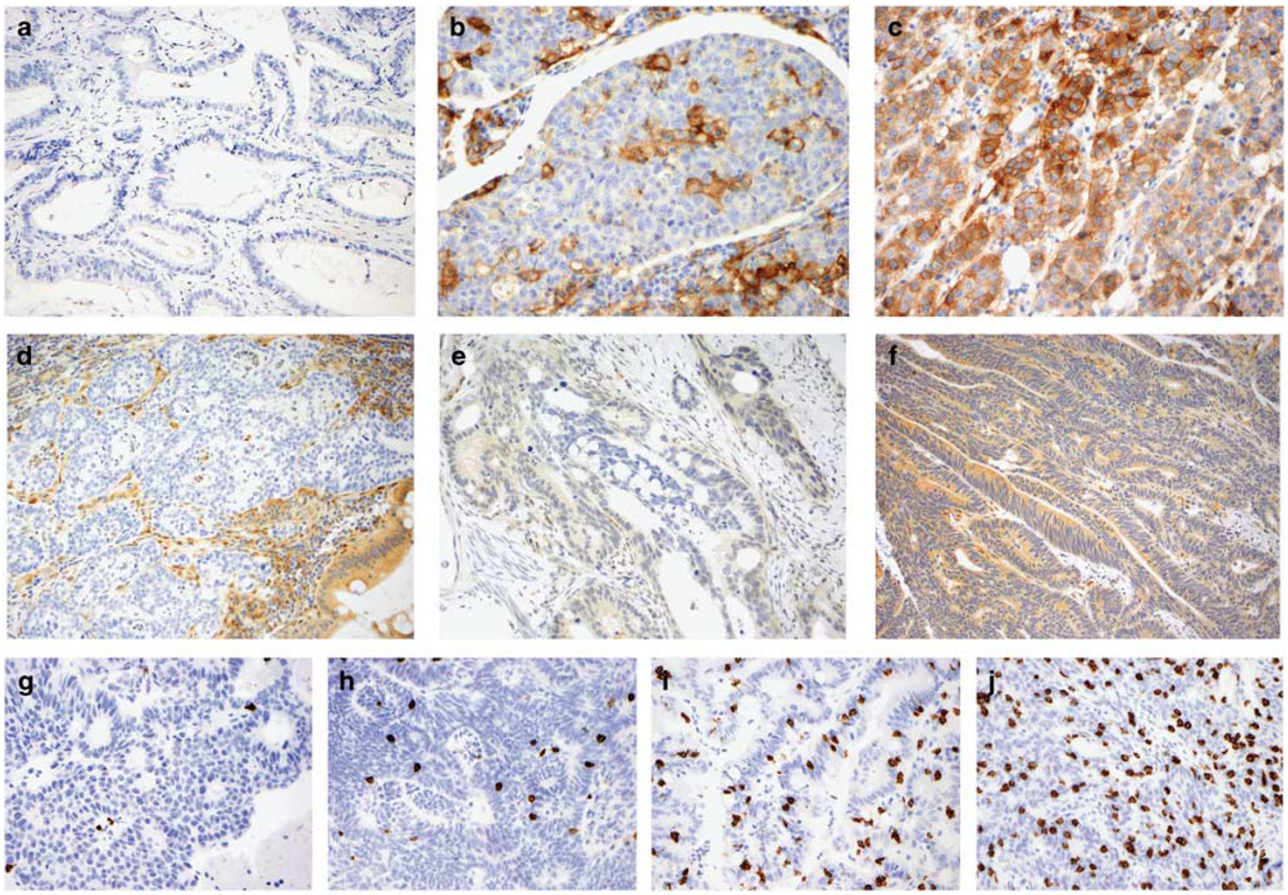

Figure 1 Immunohistochemical scoring of PD-L1 (**a–c**), PTEN (**d–f**) and CD8 (**g–j**). PD-L1 expression was scored on a scale of 0–2: 0 = < 5% (**a**), 1+ = 5–49% (**b**), and 2+ = ≥ 50% (**c**) of the tumor cells exhibiting membranous staining of any intensity. Cytoplasmic and/or nuclear PTEN staining was semi-quantitatively evaluated as either lost (0) = < 10% of tumor cells with positive staining ((**d**) negative tumor cells in the background of positive normal epithelium and stromal cells), reduced (1) = 10–90% of tumor cells with positive staining (**e**) or preserved (2) = > 90% of the tumor cells with positive staining (**f**). PTEN expression was then dichotomized as lost (< 10% of tumor cells with expression of PTEN) or retained (≥10% of tumor cells with expression). Tumor-infiltrating CD8+, TBET+, or GATA3+ lymphocytes were scored on a scale of 0–3 based on the percentage of tumor cells with overlying positive cells: 0 = none or rare (**g**), 1 + = < 5% (**h**), 2+ = ≥ 5% and < 25% (**i**), and 3+ = ≥ 25% (**j**). CD8, TBET, and GATA3 scores were subsequently dichotomized into cases with high (scores 2+ to 3+) and low (scores 0 and 1+) expression. PD-L1, programmed cell death ligand 1.

analysis was performed to identify independent predictors of PD-L1 positivity using exact logistic regression. Variables with *P*-value < 0.1 on univariate analysis were considered in the multivariate model, and the most parsimonious model including the covariates with the highest biological and clinical relevance was selected. The covariates included in the final model were age, sex, CD8+ tumor-infiltrating lymphocytes, microsatellite instability, *BRAF* mutation, and medullary histology. To limit confounding by prior treatment, survival analysis was performed on a subset of the cohort in which PD-L1 expression was examined on untreated, primary tumors using the Kaplan–Meier method, log-rank tests, and the Cox proportional hazards model. Odds ratios (OR) and 95% confidence intervals (CI) were obtained. Statistical significance was set at $\alpha = 0.05$.

Results

The study cohort ($n = 181$) had a mean age of 62 years (range: 26–91), and 53% were female. PD-L1 expression was scored as 0, 1, and 2 in 165 (91%), 4 (2%), and 12 (7%) cases, respectively. Using the 5% cut-off (PD-L1 score 1–2), 16 cases (9%) were deemed positive for PD-L1 expression. Clinicopathologic and molecular characteristics of the study cohort stratified by PD-L1 status are shown in Table 1. PD-L1 expression was significantly associated with older age at the time of resection ($P = 0.006$) and female gender ($P = 0.035$). Interestingly, PD-L1 expression was only seen in resections of the primary tumor ($P = 0.014$), mostly in cases without neoadjuvant therapy ($P = 0.023$). Although the median tumor size was larger in the PD-L1 positive tumors ($P = 0.013$), this did not reach significance in terms of T-staging. Histologically, the PD-L1 positive

Table 1 Clinicopathological characteristics

	PD-L1 positive, n *(%)*	*PD-L1 negative,* n *(%)*	P-*value*
Age, years, median (range)	74.5 (50–92)	61 (24–91)	0.006
Sex			
Male	3 (19)	78 (47)	0.035
Female	13 (81)	87 (53)	
Primary or recurrence/metastasis			
Primary	16 (100)	122 (74)	0.014
Recurrence/ metastasis	0	43 (26)	
Prior treatment			
No	15 (94)	109 (66)	0.023
Yes	1 (6)	56 (34)	
Tumor size, cm, median (range)	6.4 (2.9–13)	4.5 (0.8–13.9)	0.013
pT			
1	0	8 (5)	0.97
2	2 (12.5)	18 (12)	
3	10 (62.5)	83 (53)	
4	4 (25)	46 (30)	
pN			
0	7 (44)	70 (45)	0.32
1	6 (37)	35 (23)	
2	3 (19)	49 (32)	
Stage			
I	1 (6)	14 (9)	0.001
II	6 (38)	37 (22)	
III	8 (50)	33 (20)	
IV	1 (6)	81 (49)	
Tumor differentiation			
Well/moderate	2 (13)	112 (72)	< 0.001
Poor	14 (87)	43 (28)	
Mucinous histology			
Yes	3 (19)	33 (20)	1
No	13 (81)	128 (80)	
Medullary histology			
Yes	12 (75)	23 (7)	< 0.001
No	4 (25)	149 (93)	
Tumor-infiltrating lymphocytes			
Yes	12 (80)	34 (26)	< 0.001
No	3 (20)	98 (74)	
Small vessel invasion			
Yes	9 (56)	77 (54)	1
No	7 (44)	65 (46)	
Large vessel invasion			
Yes	6 (40)	66 (46)	0.79
No	9 (60)	77 (54)	
Perineural invasion			
Yes	6 (37)	58 (42)	0.71
No	10 (63)	79 (58)	
Tumor border			
Pushing	7 (47)	35 (26)	0.13
Infiltrating	8 (53)	100 (74)	

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

tumors were more likely to be poorly differentiated ($P < 0.001$) with a medullary phenotype ($P < 0.001$), and were more likely to exhibit prominent tumor-infiltrating lymphocytes ($P < 0.001$) (Figure 2), although angiolymphatic and perineural invasion had no bearing on PD-L1 expression.

Regarding the subsets of tumor-infiltrating lymphocytes (Table 2), 10 (62%) PD-L1-positive tumors contained a high number of CD8-positive tumor-infiltrating lymphocytes (five each of scores 2+ and 3+) and the remaining six (38%) PD-L1-positive tumors had score 1+ CD8-positive tumor-infiltrating lymphocytes. Only five (3%) of 164 PD-L1-negative tumors in which immunohistochemistry for tumor-infiltrating lymphocytes could be examined had a high number of CD8-positive tumor-infiltrating lymphocytes (all five with a score of 2+) and tumor-infiltrating lymphocytes were significantly more frequent in PD-L1-positive tumors ($P < 0.001$). Although no cases were scored 3+ for TBET-positive tumor-infiltrating lymphocytes, PD-L1-positive tumors had a significantly higher proportion of score 2+ TBET-positive tumor-infiltrating lymphocytes (62% *vs* 3% of PD-L1-negative tumors, $P < 0.001$). There was no association between PD-L1 expression and GATA3-postitive tumor-infiltrating lymphocytes.

Of 181 cases, 54 (30%) were determined to be microsatellite-instability high by immunohistochemistry combined with PCR ($n = 39$), or immunohistochemistry alone ($n = 15$). Of those, four (7%) patients were considered to have hereditary nonpolyposis colorectal cancer by clinical history and genetic testing. As for molecular testing, 118 patients exhibited one or more mutations with 83 harboring one mutation, 30 with two, and 5 with three. These included 44 (24%) with *BRAF* mutations, 44 (24%) with *KRAS* mutations and 37 (20%) with *TP53* mutations (Supplementary Table 1). In 11 microsatellite-unstable tumors *BRAF* mutation status was not known. Loss of PTEN expression by immunohistochemistry (< 10% of tumor cells staining) was seen in 35 (20%) of the 178 evaluated cases. PD-L1 expression was present in 12 (22%) of the 54 microsatellite-instability high tumors, 14 (32%) of the 44 BRAF-mutated tumors, three (8.1%) of the 37 *TP53* mutated tumors, the one *IDH* mutated tumor, as well as six (17%) of the 35 tumors with loss of PTEN protein expression on immunohistochemistry. Interestingly, the four hereditary nonpolyposis colorectal cancer cases did not exhibit PD-L1 expression. Of note, nine (75%) of the 12 microsatellite-unstable tumors with PD-L1 expression and nine (64%) of the 14 *BRAF*-mutated tumors with PD-L1 expression also showed increased CD8+ tumor-infiltrating lymphocytes (Figure 2). Overall, PD-L1 positive tumors were more likely to harbor microsatellite-instability ($P < 0.001$) and *BRAF* mutations ($P < 0.001$), but were less likely to exhibit *KRAS* mutations ($P = 0.012$). Furthermore, there was a trend towards tumors with PD-L1 expression exhibiting loss of PTEN expression

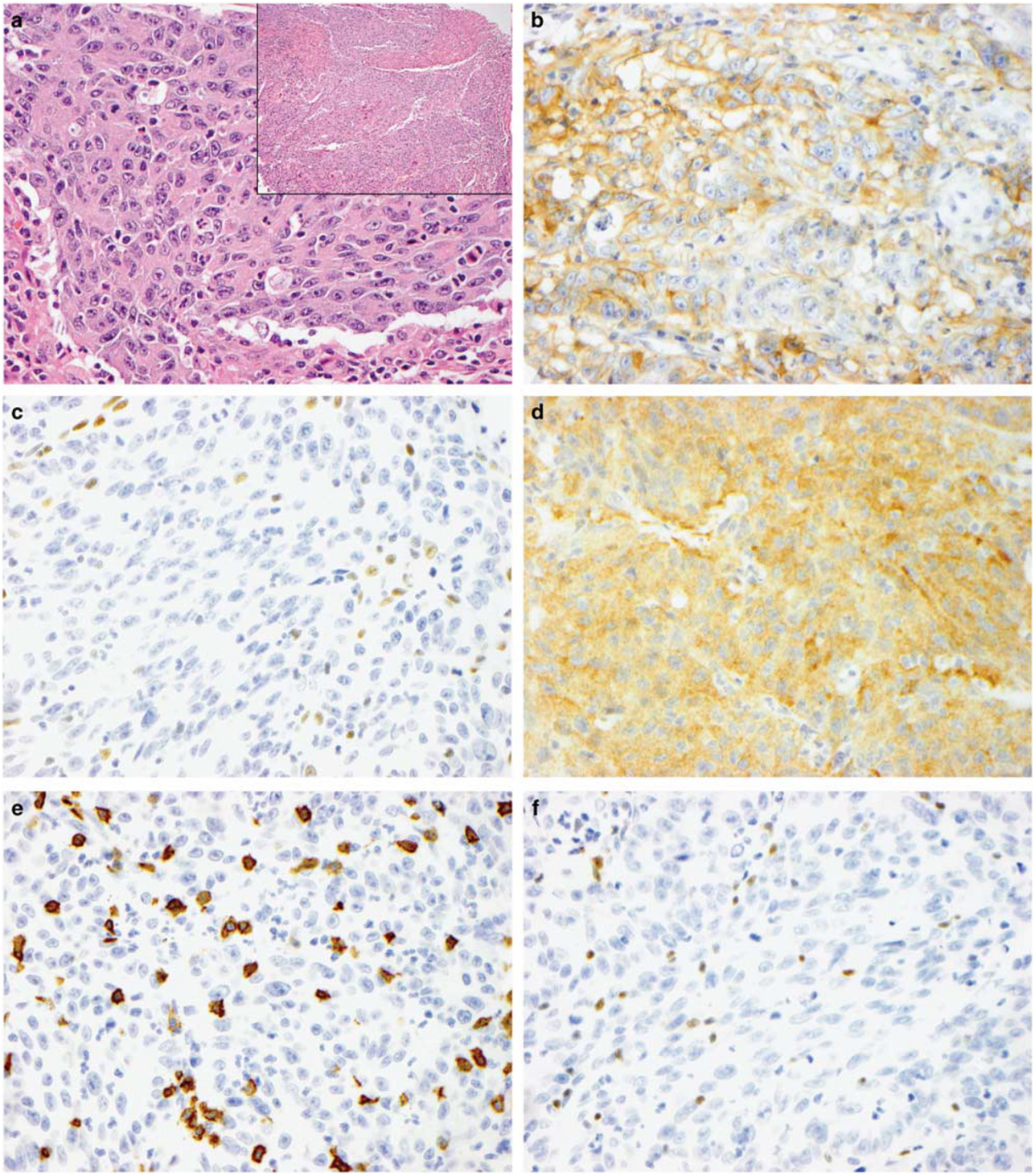

Figure 2 (**a–f**) Colorectal adenocarcinoma with PD-L1 expression. Poorly differentiated adenocarcinomas (**a**) with high PD-L1 expression (**b**) often demonstrated features of a medullary phenotype including syncytial growth and tumor-infiltrating lymphocytes. PD-L1 expression was associated with microsatellite instability ((**c**) loss of MLH1 nuclear expression in the tumor cells with preserved nuclear expression in the stromal cells) and *BRAF* mutation ((**d**) positive staining with BRAF V600E immunohistochemistry). These tumors also demonstrated high numbers of intraepithelial CD8+ (**e**) and TBET+ (**f**) small lymphocytes.

by immunohistochemistry ($P=0.092$), but not with *PTEN* mutation (Table 3).

By multivariate analysis using exact logistic regression, a high number of CD8-positive tumor-infiltrating lymphocytes (odds ratio (OR) 8.67, 95% CI 1.29, 77.93; $P=0.022$) and medullary histology (OR 10.33, 95% CI 1.18, 155.17; $P=0.032$) were independent predictors of PD-L1 expression after adjusting for other covariates in the model (Table 4). Age, microsatellite instability, and female gender

Table 2 Tumor-infiltrating lymphocyte immunophenotype

	PD-L1 positive, n *(%)*	*PD-L1 negative,* n *(%)*	P-*value*
CD8			
Low	6 (37.5)	159 (97)	< 0.001
High	10 (62.5)	5 (3)	
TBET			
Low	6 (37.5)	157 (97)	< 0.001
High	10 (62.5)	5 (3)	
GATA3			
Low	16 (100)	161 (100)	1
High	0	0	

Abbreviation: PD-L1, programmed cell death ligand 1.
Low: scores 0–1. High: scores 2–3.

Table 3 Microsatellite instability and mutational status

	PD-L1 positive, n *(%)*	*PD-L1 negative,* n *(%)*	P-*value*
MSI status			
Stable	4 (25)	120 (74)	< 0.001
Instable	12 (75)	42 (26)	
BRAF			
WT	2 (13)	121 (80)	< 0.001
Mutant	14 (87)	30 (20)	
KRAS			
WT	15 (100)	102 (70)	0.012
Mutant	0	44 (30)	
PIK3CA			
WT	15 (100)	130 (91)	0.62
Mutant	0	13 (9)	
PTEN			
WT	15 (100)	139 (97)	1
Mutant	0	4 (3)	
PTEN IHC			
Retained	10 (63)	133 (82)	0.09
Lost	6 (37)	29 (18)	
APC			
WT	15 (100)	133 (93)	0.60
Mutant	0	10 (7)	
IDH			
WT	14 (93)	143 (100)	0.10
Mutant	1 (7)	0	
NRAS			
WT	15 (100)	137 (96)	1
Mutant	0	6 (4)	
TP53			
WT	12 (80)	109 (76)	1
Mutant	3 (20)	34 (24)	

Abbreviations: IHC, immunohistochemistry; MSI, microsatellite instability; PD-L1, programmed cell death ligand 1.

were not associated with PD-L1 expression in this analysis. Although it did not reach significance, there was a strong trend towards BRAF-mutated cancers having an independent predictive value for PD-L1 expression (OR 11.74, 95% CI 0.90, 203.93; $P = 0.064$).

Table 4 Multivariate analysis, predictors of PD-L1 positivity

	OR (95% CI)	P-*value*
Sex (female)	1.97 (0.21, 26.8)	0.83
Age (≥62 y/o)	0.14 (0.004, 2.07)	0.23
CD8 TIL	8.67 (1.29, 77.93)	0.022
BRAF mutation	11.74 (0.90, 203.93)	0.064
Medullary histology	10.33 (1.18, 155.17)	0.032
MSI	0.91 (0.03, 17.51)	1

Abbreviations: CI, confidence interval; MSI, microsatellite instability; OR, odds ratio.

Table 5 Multivariate survival analysis (Cox proportional hazards model)

	HR (95% CI)	P-*value*
Sex (male *vs* female)	1.6 (0.6–4.2)	0.38
Age	1 (0.96–1.04)	0.92
pT	1.9 (0.9–4.0)	0.12
pN	1.9 (1.0–3.5)	0.038
PD-L1 score 1 (*vs* 0)	1.3 (0.1–13.6)	0.84
PD-L1 score 2 (*vs* 0)	16.9 (1.0–297.7)	0.053
CD8 TIL	0.14 (0.12–1.8)	0.13
BRAF mutation	1.8 (0.5–6.8)	0.39
KRAS mutation	2.4 (0.8–7.1)	0.11
Medullary histology	0.13 (0.01–3.7)	0.23
MSI	4.8 (0.8–29.6)	0.094

Abbreviations: CI, confidence interval; HR, hazard ratio; MSI, microsatellite instability; PD-L1, programmed cell death ligand 1.

Among the patients who had undergone resection of the primary tumor without neoadjuvant therapy, the 3-year disease-specific survival rate for the PD-L1-positive ($n = 15$) and -negative ($n = 98$) groups were 76% and 72%, respectively ($P = 0.1896$). Interestingly, among the microsatellite-instability-positive patients, PD-L1 expression was associated with reduced disease-specific survival (3-year survival rate of 72% *vs* 97% of the PD-L1-negative group, $P = 0.0319$). By multivariate survival analysis using Cox proportional hazards model (Table 5), when individual PD-L1 expression scores (0, 1, and 2) were considered, nodal status was significantly associated with decreased survival (hazard ratio 1.9, 95% CI 1.04, 3.51, $P = 0.038$), and tumors with the highest PD-L1 expression (score 2) had marginally significant but markedly reduced disease-specific survival (hazard ratio 16.9, 95% CI 0.96, 297.67, $P = 0.053$) compared with those with negative PD-L1 expression (score 0). The significance of this association was reduced when PD-L1 expression was dichotomized into high (scores 1–2) *vs* low (score 0; $P = 0.096$). CD8-positve tumor-infiltrating lymphocytes, medullary histology, mutation status, and microsatellite-instability status were not independently prognostic.

Discussion

PD-L1 expression by tumor cells has been shown to provide a mechanism of immune evasion through downregulation of the active T-cell-mediated immune response.[12,13] In this study, we demonstrate that although PD-L1 expression is infrequent in sporadic colorectal carcinomas, it is associated with signatures of the serrated pathway of colorectal carcinogenesis, including *BRAF* mutation, microsatellite instability, poor differentiation (with medullary morphology), and frequent tumor-infiltrating lymphocytes.[14] By multivariate analysis, PD-L1 expression is independently associated with medullary morphology and frequent tumor-infiltrating lymphocytes, and shows a trend towards association with *BRAF* mutation. In addition, the majority of PD-L1 positive tumors contained an increased number of TBET-positive tumor-infiltrating lymphocytes, indicative of activated Th1 pathway but not GATA3-positive tumor-infiltrating lymphocytes, which are characteristic of Th2 pathway activation.

To date, two different mechanisms of PD-L1 upregulation on tumor cells have been reported: innate immune resistance and adaptive immune resistance.[1] The former refers to the upregulation of PD-L1 expression as a result of constitutive oncogenic signaling within tumor cells, as seen in *ALK*-rearranged and *EGFR*-mutant lung cancer.[15,16] By contrast, adaptive immune resistance refers to the induction of PD-L1 expression on tumor cells in response to local inflammatory signals (eg, interferons) produced by active anti-tumor immune response (cytotoxic T cell and/or Th1 pathway activation).[13] This in turn induces PD-1 expression on T cells.[17] When engaged by PD-L1 or other ligands, PD-1 inhibits kinases that are involved in T-cell activation through the phosphatase SHP250, which leads to the apoptosis of T cells, although additional signaling pathways are likely also induced.[1,18,19] Our findings suggest that upregulation of PD-L1 in colorectal carcinoma is due to an adaptive immune response based on the association of PD-L1 expression and frequent tumor-infiltrating lymphocytes including CD8-positive and TBET-positive tumor-infiltrating lymphocytes.

In contrast to other tumor types, colorectal carcinoma in general shows a low response rate to PD-1 or PD-L1 blockade.[6–8] However, two clinical trials of anti-PD-1 antibodies for patients with colorectal carcinoma selected for microsatellite-instability high status have been initiated (NCT01876511 and NCT02060188), and a recent report from one of these trials found a significantly higher response rate and survival in colorectal carcinomas with microsatellite instability compared with their microsatellite-stable counterparts.[20] That study also revealed a greater than tenfold higher rate of somatic mutations in microsatellite-instability high tumors compared with microsatellite-stable colorectal carcinomas, as might be expected in tumors with global DNA-repair defects.[10] It has been hypothesized that this higher mutational burden may result in the formation of more neo-antigens in microsatellite-unstable tumors, leading to a robust anti-tumor immune response. Upregulation of PD-L1 expression in tumor cells within this environment may lead to abrogation of the active immune response and more aggressive tumor biology. This hypothesis is supported by a recent study with a small cohort of colorectal carcinomas ($n = 25$).[9] That study showed highly upregulated expression of multiple immune checkpoints, including PD-1, PD-L1, CTLA-4, LAG-3, and IDO in tumor-infiltrating inflammatory cells in tumors with activated CD8+ cytotoxic T cells as well as an activated Th1 pathway in the context of microsatellite instability, although the PD-L1 expression was identified in immune cells, not on the tumor cells.[9] Our study also confirms the link between microsatellite instability and PD-L1 expression in colorectal carcinoma, and supports the idea that immunogenic microsatellite-instability high tumors may acquire PD-L1 expression on the tumor cells under selective pressure to escape from the cytotoxic T-cell/Th1 immune response.

We also identified a positive association of PD-L1 expression with *BRAF* mutation and an inverse association with *KRAS* mutation in this colorectal carcinoma cohort. These findings are most likely explained by the frequent co-occurrence of *BRAF* mutation and microsatellite instability in the serrated pathway of colorectal carcinogenesis,[14] as well as the mutual exclusivity of *BRAF* and *KRAS* mutations in colorectal carcinoma.[20] Interestingly though, in lung adenocarcinoma *KRAS* mutations are positively correlated with PD-L1 expression.[21] The link between *KRAS* mutation and PD-L1 expression in non-small cell lung cancer is attributed in part to smoking, which increases the risk of developing *KRAS* mutations along with other passenger mutations.[21] As is hypothesized to occur in microsatellite-unstable colorectal carcinoma, the high mutational burden in such non-small cell lung cancer appears to lead to neo-antigen formation and increased immunogenicity.[22] In addition, as in microsatellite-unstable colorectal carcinoma, lung cancers with a high mutational burden and neo-antigen formation appear to be associated not only with induction of PD-L1 expression on tumor cells, but also with an improved objective response to anti-PD-1 agents with durable clinical benefit and better progression-free survival.[10,22] These findings suggest a common mechanism for induction of PD-L1 expression in these tumors despite different site of origin and driver mutations.

Interestingly, multivariate analysis revealed that only increased CD8-positive tumor-infiltrating lymphocytes, and medullary morphology, but not microsatellite instability, were significant independent predictors of PD-L1 expression. Given that only 14 (26%) of the 54 microsatellite-unstable tumors showed significant CD8-positive tumor-infiltrating

lymphocytes in our cohort, it seems that the presence of a cytotoxic T-cell (and Th1)-rich tumor microenvironment is more closely associated with PD-L1 expression than the presence of microsatellite instability in general. Interestingly, *BRAF* mutation was also not an independent predictor of PD-L1 expression, although it did show a trend toward significance. These results suggest the possibility that distinct subtypes of colorectal carcinoma may arise within the classic microsatellite-instability pathway of colorectal carcinogenesis, including a PD-L1-positive immune activated subtype with increased tumor-infiltrating lymphocytes and subsequent, adaptive immune resistance, and a PD-L1-negative immune-tolerant subtype. Of note, the four hereditary nonpolyposis colorectal cancer cases did not show PD-L1 expression in this study. Additional studies with larger numbers of hereditary nonpolyposis colorectal cancer and sporadic microsatellite-unstable colorectal carcinomas may be helpful to clarify whether sporadic microsatellite instability is more prone to adaptive immune resistance than hereditary nonpolyposis colorectal cancer.

As for the prognostic role of clinicopathological variables, nodal status was an independent predictor of decreased disease-specific survival. In a dichotomized model, high PD-L1 expression did not show a convincing trend toward decreased survival ($P=0.1896$). However, when comparing those cases with very high PD-L1 expression (2+) to those with very low or no expression (0), high PD-L1 expression was more closely associated with a lower disease-specific survival (hazard ratio 16.9; $P=0.053$). The marginal statistical significance of these associations may be due to the small number of patients with PD-L1 expression and limited follow up in a number of PD-L1-positive cases, however, the magnitude of the difference (high hazard ratio) may make this finding clinically meaningful. Further studies are needed to elucidate these findings. When restricted to microsatellite-unstable cases, PD-L1 expression was correlated with significantly decreased survival ($P=0.0319$). This finding suggests that one of reasons microsatellite-unstable colorectal carcinoma has a more favorable prognosis in general may be due to immune surveillance. Negation of that immune surveillance (such as through the expression of PD-L1) may allow for the escape and spread of cancerous cells.

Our study has several important limitations. First, there is currently no gold standard in PD-L1 testing, and a number of different antibodies and scoring protocols have been applied.[23] To account for this, we used a commercially available, anti-PD-L1 antibody that has been independently validated.[24–26] We also evaluated PD-L1 expression using cut-offs that have been associated with beneficial response to PD-1/PD-L1 inhibitors in clinical studies.[7,27] Another issue associated with the analysis is the use of tissue microarrays, in which heterogeneous staining of tumors is difficult to evaluate. A recent study on non-small cell lung cancer demonstrated significant heterogeneity of stain with PD-L1 expression.[28] To overcome this issue, we constructed tissue microarrays of multiple 2-mm cores for each of the study cases, and the final score was made based on their collective assessment. Finally, molecular testing results were limited in some (6.1%) of the study cohort, although the vast majority was successfully evaluated for microsatellite-instability status.

In conclusion, we demonstrate that PD-L1 expression is characteristic of a subset of microsatellite-unstable colorectal carcinoma, with increased CD8-positive tumor-infiltrating lymphocytes and a medullary phenotype being independent predictors of high PD-L1 status. Our findings support the hypothesis that expression of PD-L1 may be induced by a cytotoxic T-cell/Th1-mediated immune reaction to tumor cells that arises in the setting of microsatellite instability and high neo-antigen load. Interestingly, a high level of PD-L1 expression showed a trend toward markedly reduced survival on multivariate analysis, and was associated with adverse prognosis within microsatellite-unstable tumors. This suggests that colorectal carcinoma with high PD-L1 expression may acquire a survival advantage though downregulation of the immune response. Our finding of PD-L1 expression in microsatellite instability also provides a histopathologic correlate to the mechanism underlying the known response of microsatellite-unstable colorectal carcinoma to PD-1 blockade.[10] This study calls attention to the importance of studying PD-1/PD-L1 axis blockade not only in colorectal carcinoma but in other tumors with microsatellite instability, frequent tumor-infiltrating lymphocytes, and/or a medullary phenotype. Furthermore, PD-L1 expression may be useful in prognostic stratification of microsatellite-instability high colorectal carcinoma, and potentially identifying colorectal carcinoma cases that might respond to PD-1/PD-L1 blockade.

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Disclosure/conflict of interest

MM-K has served as a consultant for Merrimack Pharmaceuticals. The remaining authors declare no conflict of interest.

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