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To cite this article: Dong Hae Jung , Hyun Jung Park , Ho Hee Jang , Se-Hee Kim , YunJae Jung & Won-Suk Lee (2020) Clinical Impact of PD-L1 Expression for Survival in Curatively Resected Colon Cancer, *Cancer Investigation*, 38:7, 406-414, DOI: [10.1080/07357907.2020.1793349](https://doi.org/10.1080/07357907.2020.1793349)

To link to this article: <https://doi.org/10.1080/07357907.2020.1793349>



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Clinical Impact of PD-L1 Expression for Survival in Curatively Resected Colon Cancer

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ABSTRACT

Background: Programmed death 1 (PD-1) and its ligand PD-L1 play a key dysfunction of T lymphocytes. The purpose of this study was to assess and compare the prognostic role of tumor- TILs and its relationship with PD-L1 expression in stage II and III colon cancer.

Methods: Immunohistochemistry was used to assess the densities of CD8⁺, CD4⁺, and FOXP3⁺ cells, and PD-L1 expression in intraepithelial tumor site from 58 stage II and III colon cancers. These were evaluated for association with histopathologic features and overall survival.

Results: PD-L1-positive tumors contained a higher number of CD8⁺ TILs with statistical significance ($p = 0.001$). CD4⁺ TILs showed positive correlation with PD-L1 expression ($p = 0.034$). There were no associations between PD-L1 expression and FOXP3⁺ TILs. Microsatellite instability (MSI)-high status ($p = 0.001$; Odd ratio 18.0; 95% CI = 4.3–74.8) was the strongest prognostic factor along with mucinous/poor cell differentiation, CD8 and right tumor location was associated with PD-L1 expression ($p = 0.024$, 0.035 and 0.033, respectively).

Conclusion: This study demonstrated that PD-L1 expression was associated with MSI-high, increased CD8⁺ TILs, mucinous and poor cell differentiation, and right-sided tumor location.

ARTICLE HISTORY

Received 21 February 2020
Revised 11 May 2020
Accepted 5 July 2020

KEYWORDS

Cancer biomarkers;
chemotherapy; biochemical markers

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world and exhibits heterogeneous characteristics in terms of genomic alterations, expression signature and drug responsiveness (1). Currently, the most reliable prognostic factor in colon cancer is the TNM stage. Although the prognosis for individual patients is dependent upon the extent of the disease, the prognosis of patients with the same stage varies widely, especially in those with stage II and III tumors (2). Given a significantly reduced recurrence rate from adjuvant chemotherapy, postoperative chemotherapy has become the standard treatment for stage-III colon cancer

(3). However, the role of adjuvant chemotherapy is still controversial in stage-II colon cancer and is currently not a standard of care for this group of patients (4). The overall survival rate for stage-II colon cancer after surgery alone ranges from 70% to 80%, and the risk of relapse ranges from 25% to 30% (4). Although recommendations are given for consideration of adjuvant therapy in stage-II patients with high-risk features, the current guideline admittedly states that the current definition of “high-risk” stage-II colon cancer remains inadequate (2). The limitation with TNM staging suggests the need to scrutinize beyond clinicopathologic features for recurrence prediction to search for a better prognostic marker for survival and recurrence.

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 Supplemental data for this article can be accessed [here](#).

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Recently, immune checkpoint inhibitors have dramatically improved long-term prognoses in few malignancies such as malignant melanoma, lung cancer, and renal cell cancer which had insufficient prognosis with conventional treatment (5–7). Interaction of the programmed cell death 1 (PD-1) on T cells, and its ligand PD-L1, which is expressed on tumor cells, and inflammatory immune cells including B cells, dendritic cells, and macrophages plays a crucial role in immune check point inhibition (8). It is well known that the binding of PD-L1 on tumor cells to PD-1 on T cells suppresses T-cell-mediated anti-tumor immune response, which is called “immune tolerance” (8,9), and the blockade of this pathway gives a better prognosis in some malignancies. Microsatellite instability high (MSI-H) tumors have been identified as the best candidates for immunotherapy treatment, and a number of studies have evaluated the efficacy of anti-PD-L1 and anti-CTLA-4 monoclonal antibodies in this setting. Two phase-II trials (KEYNOTE-016 and KEYNOTE-164) and one phase-II trial (CheckMate-142) conducted in previously treated MSI-H metastatic CRC patients provided objective response rates (ORR) ranging from 28% to 52%, progression-free survival (PFS) rates ranging from 34% to 59%, and overall survival (OS) rates of 72% to 76% (10–12).

It was reported that biologic behaviors of colon cancers are associated with a unique immune response of the colon cancer microenvironment, and the outcome is governed predominantly by immune responses at the primary tumor site (13). This suggests that precise evaluation of local immune response could be useful for predicting prognosis or even have prognostic value superior to and independent of the TNM classification (13).

In this study, we aimed to evaluate the relationship between PD-L1 expression in tumor cells and clinicopathological characteristics, including FOXP3⁺, CD8⁺ and CD4⁺ tumor-infiltrating lymphocytes (TILs) density, with survival.

Patients and methods

Patients and specimens

Between January 2011 and December 2014, 507 patients underwent curative resection of CRC at

the Gil Medical Center, Gachon University. Of these patients, 342 patients were diagnosed as pathologic stage II and III colon cancer according to the American Joint Committee on Cancer staging system (14). Twenty archival specimens per year were randomly selected according to their microsatellite status. The criteria for case inclusion were as follows: (1) availability of formalin-fixed, paraffin-embedded tissue; (2) a complete set of clinical and pathologic information such as age, gender, size of tumor, lymphatic invasion, vascular invasion, and perineural invasion were included; (3) no prior history of any form of colitis during the past 1 year, (4) no rectal cancer and (5) no prior history of chemotherapy or radiation therapy. Among the 80 randomly selected archival specimens, 58 archival specimens fulfilled the inclusion criteria and were available for analysis.

Ethics statement

All the clinical specimens were approved by the clinical research ethics committee of the Gachon University, Gil Medical Center. Written informed consent was obtained from all patients according to the policies of the committee (IRB# GIRBA2216). Any information that could identify the patients was not included in this article. All methods were performed in accordance with the Helsinki guidelines and regulations.

Surveillance

Surveillance for recurrence following surgery is outlined as follows: physical examination, serum CEA (carcino-embryonic antigen), chest radiography, and spiral abdominal computed tomography were performed every 6 months for the first three years and annually thereafter. Other examinations, such as colonoscopy and abdominal ultrasound, were selected and performed every six to 12 months depending on the status of the patients. The median follow-up duration was 53.5 months (20.0–83.7 months).

Immunohistochemistry

After reviewing H&E slides of each tumor, a representative paraffin block that showed well-fixed,

viable tumor was selected. Four consecutive 4 μ m thick sections were cut with a microtome and transferred to poly-L-lysine-coated slides. The sections were dewaxed in xylene and rehydrated with 100% alcohol. Immunohistochemical staining was performed using Bond III automated IHC/ISH stainer (Leika Biosystems, Newcastle, UK). Antigen retrieval was done by heating in Epitope Retrieval Solution 1/2 (ER1/ER2) (Leika) for 20 minutes. Endogenous peroxidase activity was blocked by incubating the sections in 0.3% hydrogen peroxide for 10 minutes. The sections were incubated with primary antibodies [rabbit monoclonal antibody to PD-L1 (Abcam, ab205921, clone 28-8, 1:100 dilution), mouse monoclonal antibody to CD4 (Novocastra, ORG-8756, clone 1F6, 1:50 dilution), mouse monoclonal antibody to CD8 (Novocastra, NCL-L-CD8-4B11, clone 4B11), and mouse monoclonal antibody to FOXP3 (Abcam ab20034, clone 236 A/E7, 1:100 dilution)] for 15 minutes at 37°C, followed by visualization with Bond Intesne Detection Kit (Leika). Each immunohistochemistry run contained a positive control (on-slide tonsil tissue) and a negative antibody control (buffer, no primary antibody).

PDL-1 expression

PD-L1 staining was defined as complete circumferential or partial linear plasma membrane staining at any intensity. Cytoplasmic staining was not considered positive. The percentage of viable tumor cells exhibiting positive-membrane staining in the entire specimen was measured and categorized as one of “less than 1%”, “1% to less than 5%”, “5% to less than 10%”, and “10% or greater” groups. Cases of 1% or greater of staining percentage were regarded as PD-L1-positive. The expression of PD-L1 in tumor cells were estimated using IHC method mentioned above.

Quantitative analysis of TILs

The number of TILs were counted manually using a DP71 Digital camera (Olympus, Japan), installed on an Olympus light microscope (Olympus BX51, Japan) and attached to a personal computer. Three sets of independent 200x

microscopic fields (0.366 mm²/field) containing viable tumor and modest to dense lymphocytic infiltration were selected for each patient sample and for each of immunoantibody (CD4, CD8 and FOXP3). Each set was made to include identical microscopic field. After taking a microphotography, the number of the immunostained lymphocytes were counted and three sets of the ratios of the TIL numbers of CD4, CD8 and FOXP3 (CD4-positive lymphocytes/CD8-positive lymphocytes, CD4-positive lymphocytes/FOXP3-positive lymphocytes, CD8-positive lymphocytes/FOXP3-positive lymphocytes) were calculated. The evaluation of TILs was performed by one pathologist without knowledge of the clinicopathologic data. For data analysis, mean values were used.

Statistical analysis

Comparisons between groups were performed using the Pearson's *chi*-square test. For all immunohistochemical markers, the cutoff for definition of subgroup was the mean value. The mean cut off values for TILs are shown in [Supplementary Table 1](#). Survival curves were estimated using the Kaplan–Meier product-limit method, and the significances of differences between survival curves were determined using the log-rank test. For multivariate analysis the Cox regression model was used to identify independent prognostic factors for overall survival and recurrence. Differences with *p* value less than 0.05 were considered statistically significant. The overall survival was calculated from the date of colon resection and the date of death or the last follow-up, and disease-free survival was measured from the date of colon resection to the date of tumor recurrence or the last follow-up.

Results

Correlation of immunohistochemical variables with clinicopathologic features

All cases were classified according to their mean numbers into either low- and high-density groups for each marker: CD8⁺, CD4⁺, and FOXP3⁺. In a total of 58 colon cancer patients, the median age was 64 years (range, 35–84) and 58.6% were

Table 1. Clinicopathologic features.

	PD-L1 positive, <i>n</i> = 18 (%)	PD-L1 negative <i>n</i> = 40 (%)	<i>p</i> -value
Age, median (range)	65 (37–84)	63 (35–84)	0.401
Sex			0.796
Male	11 (61.1)	23 (57.5)	
Female	7 (38.9)	17 (42.5)	
Tumor size			0.044
>5 cm	13 (72.2)	17 (42.5)	
≤5 cm	5 (27.8)	23 (57.5)	
Tumor location			0.0001
Right	13 (72.2)	6 (15.0)	
Left	5 (27.8)	34 (85.0)	
TNM stage			0.947
T1 and T2	2 (11.1)	6 (15.0)	
T3 and T4	16 (88.9)	34 (85.0)	
N positive	1 (5.6)	1 (2.5)	0.555
N negative	17 (94.4)	39 (97.5)	
M0	18 (100.0)	40 (100.0)	0.133
Tumor Differentiation			0.009
Well + Moderate	7 (38.9)	36 (90.0)	
Mucinous and Poor	11 (61.1)	4 (10.0)	
Lymphovascular inv.			0.950
Yes	12 (66.7)	27 (67.5)	
No	6 (33.3)	13 (32.5)	
Perineural Inv.			0.156
Yes	3 (16.7)	14 (35.0)	
No	15 (83.3)	26 (65.0)	
MSI* status			0.0001
High	12 (66.7)	4 (10.0)	
Stable	6 (33.3)	36 (90.0)	
KRAS			0.471
Wild	13 (72.2)	15 (50.0)	
Mutant	5 (27.8)	15 (50.0)	
EGFR			0.829
Wild	10 (55.6)	21 (52.5)	
Mutant	8 (44.4)	19 (47.5)	
P53			0.511
Wild	13 (72.2)	32 (80.0)	
Mutant	5 (27.8)	8 (20.0)	
CEA,			0.540
>5 ng/dL	4 (22.2)	12 (30.0)	
≤5 ng/dL	14 (77.8)	28 (70.0)	

*Microsatellite instability.

male. PD-L1 expression was significantly associated with right colon ($p < 0.0001$), tumor size ($p = 0.044$), mucinous differentiation ($p = 0.009$) and MSI-high cancers ($p < 0.0001$) (Table 1). Gender, lymphovascular invasion, KRAS mutation and EGFR expression were not statistically associated with PD-L1 expression. The representative TILs for MSI-high and microsatellite stable (MSS) cancer is shown in Figures 1 and 2.

Correlation between PD-L1 expression and TILs

The correlations between PD-L1 expression in tumor cells and TILs are shown in Table 2. PD-L1 expression was evaluated in 58 tumors. PD-L1-positive tumors contained a higher number of CD8 positive TILs with statistical significance ($p = 0.010$). CD4 TILs showed positive correlation

with PD-L1 expression ($p = 0.034$). A significant association between PD-L1 expression and FOXP3⁺ TILs was also shown (p value = 0.029) (Table 2). The ratios of CD4⁺/CD8⁺ TILs, CD4⁺/FOXP3⁺ TILs, and CD8⁺/FOXP3⁺ TILs were not significantly different between PD-L1-positive and -negative tumors (Supplementary Table S1 and Supplementary Figure S1).

Independent predictors of PD-L1 expression

A multivariate model was developed to test the independent prognostic factors and TIL densities, as proposed by Salama *et al.* (15). The variables included this features were the densities of each T-cell marker with cell differentiation, MSI status, and tumor location. This analysis revealed that MSI-high status ($p = 0.001$; Odd ration 18.0; 95% CI = 4.3–34.8) was the strongest prognostic factor. It is interesting to note that mucinous histology, CD8 expression, and right tumor location were associated with PD-L1 expression ($p = 0.024$, 0.035, and 0.033 respectively). Expression of CD4 ($p = 0.248$) and FOXP3 ($p = 1.555$) was not significantly associated with PD-L1 expression (Table 3).

Overall survival

The overall survival 82.3% at 5- years for all patients. The forward conditional Cox regression model was used to delineate significant prognostic factors for survival. MSI-high status showed tendency toward better survival but did not showed significance ($p = 0.059$). Lymph node positivity was the only independent prognostic factor for overall survival. No significant advantage for overall survival was found for CD4 high or CD8 high groups (high-density groups). PD-L1 expression was not associated with increased overall survival ($p = 0.175$, Figure 3, Table 4) or disease free survival ($p = 0.834$, Supplementary Fig. S2).

Discussion

PD-L1 expression by tumor cells has been shown to provide a mechanism of immune evasion through downregulation of the active T-cell-

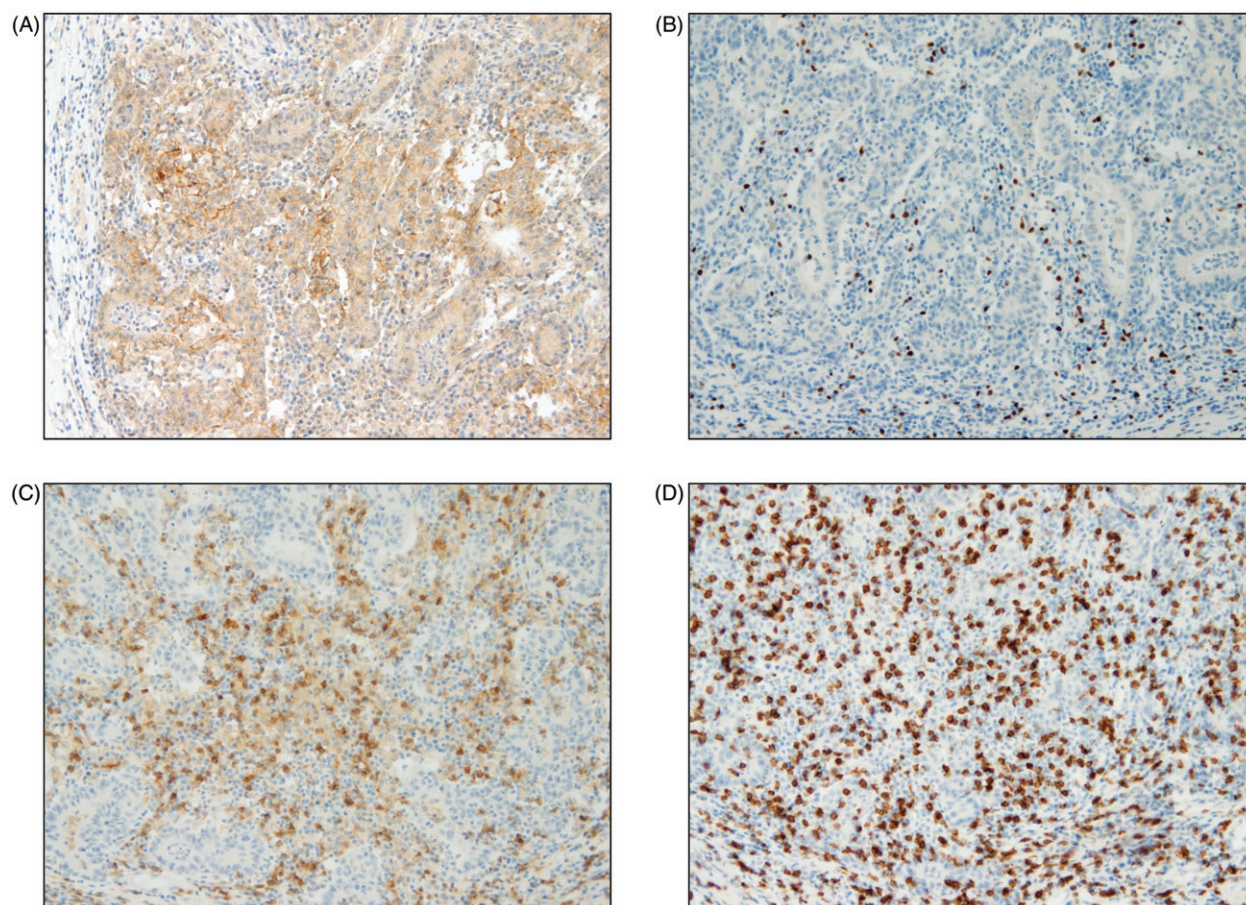


Figure 1. Representative examples of immunohistochemical findings of intraepithelial infiltrating lymphocytes. Microsatellite instability high PD-L1 expression (A) CD4⁺ T cells (B), CD8⁺ T cells (C), FOXP3⁺ T cells (D) (magnification $\times 400$).

mediated immune response (16,17). In this study, we demonstrate that PD-L1 expression is scarcely seen in sporadic colon cancer; however, it is associated with signatures MSI, mucinous/poor cell differentiation, right-sided colon, and frequent TILs. PD-L1 was not associated with overall survival in curatively resected stage II and III colon cancer.

To date, two different mechanisms of PD-L1 upregulation on tumor cells have been reported: innate immune resistance and adaptive immune resistance (17,18). 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 (17,19,20). By contrast, adaptive immune resistance refers to the induction of PD-L1 expression on tumor cells in response to local inflammatory signals produced by active anti-tumor immune response (cytotoxic T cell and/or Th1 pathway activation) (17,21). This in turn

induces PD-1 expression on T cells (22). 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 (17,18,23). Our findings suggest that upregulation of PD-L1 in colon cancer is probably due to an adaptive immune response with regards to frequent TILs expressing CD8, CD4, and FOXP3.

Recently, a trial found a significantly higher response rate and survival in colon cancer with MSI as compared with MSS (24). The significance of MSI in identifying molecular profile-driven therapies in mCRC was recently elaborated in a phase-II trial enrolling 41 patients with treatment-refractory metastatic cancers stratified into three cohorts: MSI-high colon cancer (cohort A), MSS colon cancer (cohort B), and MSI-high non-CRC (cohort C) (24). Treatment with the anti-PD-1 immune checkpoint inhibitor

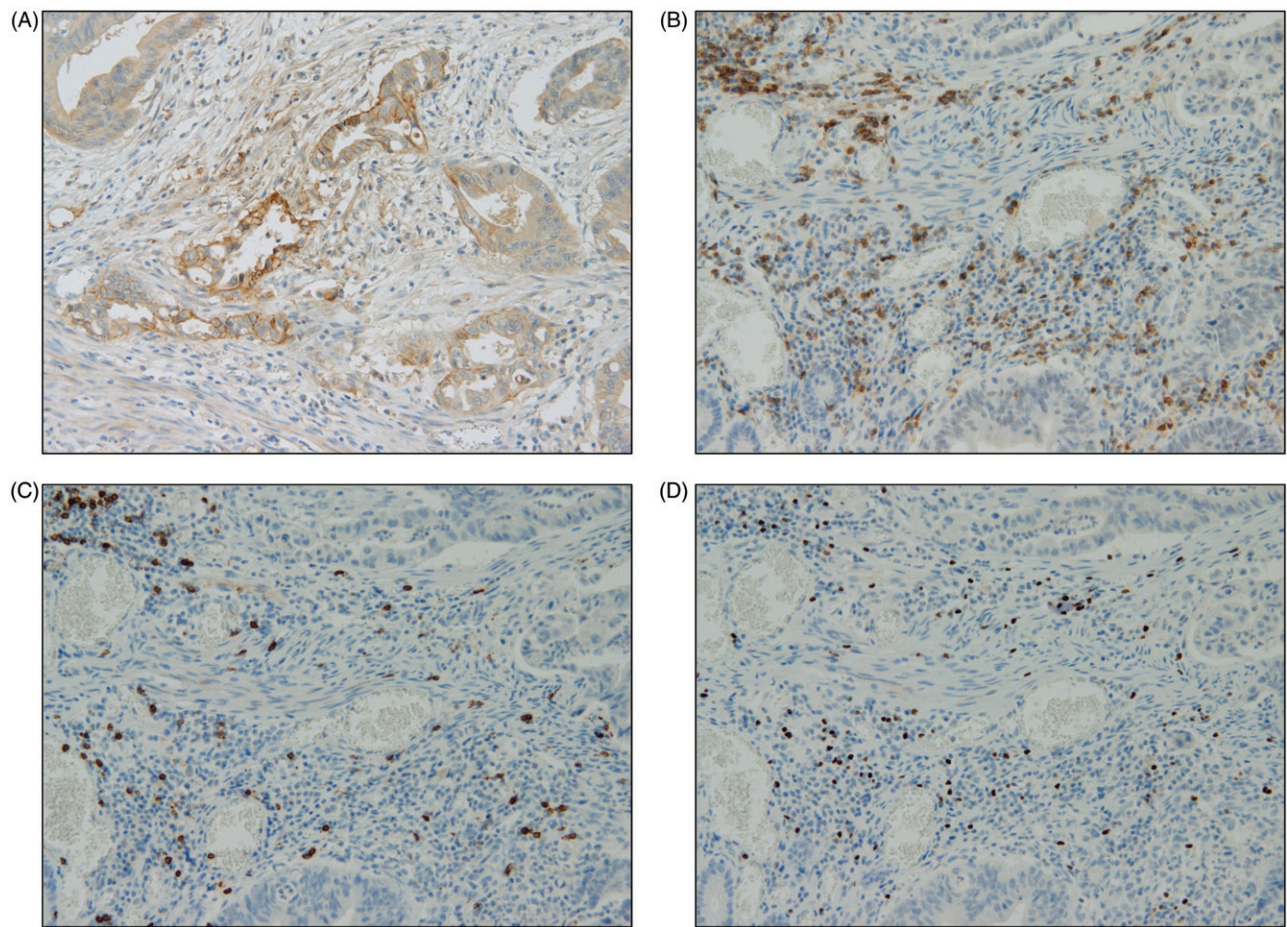


Figure 2. Representative examples of immunohistochemical findings of intraepithelial infiltrating lymphocytes. Microsatellite stable PD-L1 expression (A) CD4⁺ T cells (B), CD8⁺ T cells (C), FOXP3⁺ T cells (D) (magnification $\times 400$).

Table 2. Correlation between tumor-infiltrating lymphocyte and PD-L1 expression.

	PD-L1 positive, n (%)	PD-L1 negative, n (%)	p-value
CD8			0.010
Low	3 (16.7)	21 (52.5)	
High	15 (83.3)	19 (47.5)	
CD4			0.034
Low	5 (27.8)	23 (57.5)	
High	13 (72.2)	17 (42.5)	
FOXP3			0.029
Low	7 (38.9)	35 (87.5)	
High	11 (61.1)	5 (12.5)	

Table 3. Multivariate analysis on predictors of PD-L1 expression.

	Odd Ratio (95% CI)	p-value
MSI-high	18.0 (4.3–34.8)	0.001
Tumor location (right)	6.2 (1.2–23.1)	0.033
CD8	4.4 (1.1–28.9)	0.035
CD4	0.8 (0.17–4.1)	0.248
FOXP3	0.5 (0.7–3.3)	0.974
Mucinous/Poor cell differentiation	9.1 (1.17–124.7)	0.024

pembrolizumab (10 mg/kg intravenously every 14 days) showed significantly improved immune-related response rates and survival for cohorts A

and C compared with cohort B. This study validated the concept that MSI high tumor and high tumor mutational burden can predict response to pembrolizumab in metastatic CRC (24,25). Predictive markers of anti-EGFR mAbs resistance has been extensively studied. Cetuximab or panitumumab are effective in 10–20% of unselected metastatic colorectal patients. KRAS mutations account for approximately 30–50% of patients who are not responsive. The oncogenic activation of BRAF affects the response to anti-EGFR mAbs in approximately 10% of mCRC. Other biomarkers, such as NRAS, PTEN and PI3K, may allow further selection of the appropriate patient group (26).

Somatic mutations found in tumors can be recognized by the patient's own immune system, and mismatch repair-deficient CRCs have ~100 times as many somatic mutations as mismatch repair-proficient CRCs (27). Two possible hypothesis explaining such event; (1) higher the

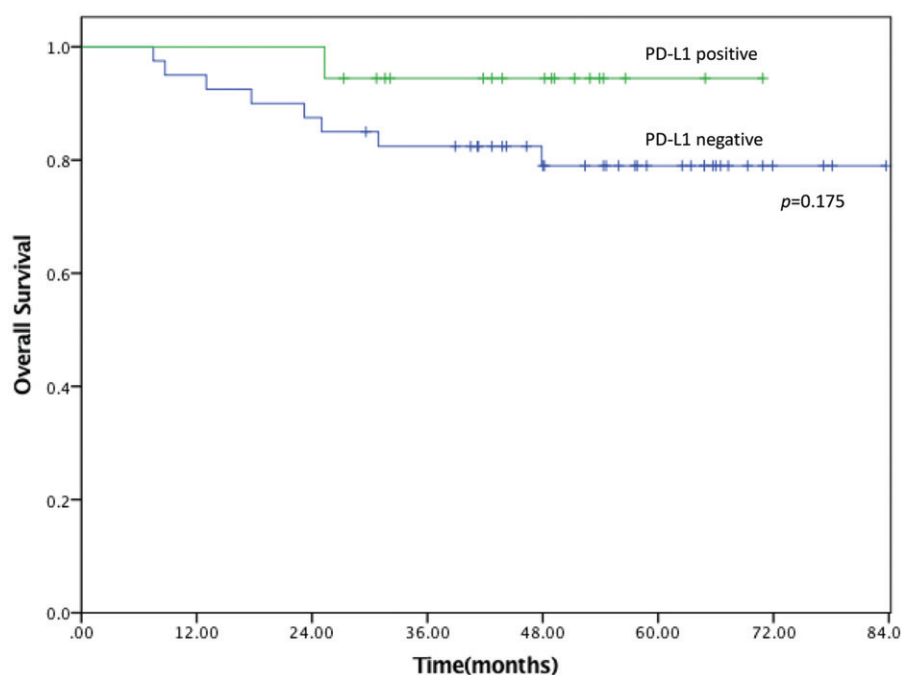


Figure 3. Kaplan–Meier analysis of overall survival according to PD-L1 expression.

mutational burden may result in the formation of more “neo-antigens” in microsatellite-unstable tumors, leading to a higher anti-tumor immune response (17); (2) Mismatch repair-deficient cancers contain prominent lymphocyte infiltrates, a finding consistent with an immune response (28). Upregulation of PD-L1 expression in tumor cells within this environment may lead to cascade of the active immune responses and more aggressive tumor biology (17,29). Although this study did not show an overall survival difference between the PD-L1-positive and PD-L1-negative groups, there was tendency toward worse survival outcome in PD-L1-negative group. This may be due to the small sample size. The correlation between mismatch-repair deficiency and PD-L1 expression in colon cancer supports the idea that immunogenic mismatch-repair deficiency cancers may acquire PD-L1 expression on the cancer cells to escape from the cytotoxic T-cell/Th1 immune response. Thus, leading to poor adaptive immune response and survival.

It is worth noting multivariate analysis revealed that increased CD8-positive TILs, and mucinous and poorly differentiated cell morphology, MSI, and right-sided tumor location were significant independent predictors of PD-L1 expression (Table 3). These results may insinuate

Table 4. Multivariate analysis on predictors of survival.

	Odd Ratio (95% CI)	p- value
MSI-high	1.4(0.1–4.7)	0.059
Tumor location(right)	1.1(0.4–3.4)	0.114
Mucinous histology	0.9(0.1–8.6)	0.190
CD8	0.4(0.9–1.7)	0.089
PD-L1	0.8(0.9–6.8)	0.175
Node positive	4.7(0.8–29.4)	0.032

the possibility that unique subgroups of colon cancer may arise within the DNA mismatch repair pathway of carcinogenesis, including a PD-L1-positive immune activated subtype, associated with increased TILs and adaptive immune resistance, and a PD-L1-negative immune-tolerant subtype (17).

The prognostic role of nodal status was the only independent predictor of overall survival. PD-L1 expression was not correlated with significantly worse survival; however, there was a tendency toward prolonged survival and this finding should be interpreted with caution as our cohort had limited sample size (Figure 3). In a recent report, PD-L1 expression was not correlated with prolonged survival in left-sided colon cancer and rectal cancer. However, right-sided colon cancer was associated with prolonged survival (30). Although we did not statistical significance in overall survival due to small sample size, we are

agreement with previous study that PD-L1 expression is associated with better survival (30,31).

We demonstrated that PD-L1 expression is associated with less aggressive cancer biology in colon cancer, which may result in better overall survival. Due to the small sample size our study did not reach statistical significance regarding overall survival. However, we demonstrated tendency toward better outcome ($p=0.175$; Figure 3). This is in line with previous finding in colon cancer (32,33). The prognostic value of PD-L1 expression in colon cancer is highly debated. While some studies demonstrated with better outcome (32), others have shown a worse outcome (9,31). Although our study is in agreement with Drosser et al. (32), survival data should be interpreted with caution. In order to have a uniform study samples, we only included colon cancer and excluded rectal cancer. We believe rectal cancer is a different disease with different tumor biology, treatment plan and prognosis. The discrepancy regarding overall survival with Masugi et al. (9) may be also due to the time of patient recruitment. This study population was enrolled between 2011 and 2014. During this time, a considerable improvement in outcome was possible with addition of oxaliplatin. This contrast with Masugi et al. (9) where more than 50% of patient population was diagnosed before 1999 and 5-FU was the main chemotherapeutic regimen at the time.

In conclusion, we demonstrate that PD-L1 expression is characteristic of MSI-high, with increased CD8-positive TILs, mucinous and poor cell differentiation, and right-sided tumor location, which being the independent predictors of high PD-L1 status.

Disclosure statement

All of the listed authors claim no conflict of interest and financial disclosure.

Funding

This study was supported by funding from the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of

Education NRF-2017R1D1A1B03035453 and GAIIB2019103 to W.S. Lee and 2019R1F1A1052209 to Y Jung.

Data availability statement

All data are available for disclosure.

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