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ORIGINAL ARTICLE

Retrospective Study

Clinical significance of programmed cell death-ligand expression in small bowel adenocarcinoma is determined by the tumor microenvironment

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Abstract

BACKGROUND
Comprehensive genomic analysis has shown that small bowel adenocarcinoma (SBA) has different genomic profiles from gastric and colorectal cancers. Hence, it is essential to establish chemotherapeutic regimens based on SBA characteristics. The expression of programmed cell death-ligand 1 (PD-L1) and programmed cell death-ligand 2 (PD-L2) in SBA is not fully understood. Anti-PD-L1/PD-1 therapy uses tumor-infiltrating lymphocytes (TILs); therefore, the status of TILs in the tumor microenvironment (TME) may influence their efficacy. The ratio of FoxP3+ to CD8+ T cells has been reported to be useful in predicting the prognosis of digestive system cancers.

AIM
To investigate the clinicopathological significance of PD-L1/2 expression according to the status of TILs in SBA tissues.

METHODS
We performed immunohistochemical analysis for PD-L1, PD-L2, CD8, FoxP3, and DNA mismatch repair (MMR) proteins using formalin-fixed, paraffin-embedded tissues from 50 patients diagnosed with primary SBA. The immunoreactivities of PD-L1 and PD-L2 were determined separately in tumor cells and tumor-infilt-

rating immune cells throughout the tumor center and invasive margins, and finally evaluated using the combined positive score (CPS). We assessed CD8+ and FoxP3+ T cells in the intratumoral and tumor-surrounding stroma. Subsequently, we calculated and summed the ratio of FoxP3 to CD8+ T cell counts. Immune-related cell densities were graded as low or high. Immunohistochemical results were compared with clinicopathological factors and patient prognosis. The distribution of cancer-specific survival (CSS) was estimated using the Kaplan–Meier method, and the log-rank test was used to test for significant differences in CSS. A Cox proportional hazard model was also used to assess the effect of tumor variables on CSS.

RESULTS
PD-L1 expression was positive in 34% in tumor cells (T-PD-L1) and 54% in tumor-infiltrating immune cells (I-PD-L1) of the cases examined. T-PD-L2 was positive in 34% and I-PD-L2 was positive in 42% of the cases. PD-L1 CPS ≥ 10 and PD-L2 CPS ≥ 10 were observed in 50% and 56% of the cases, respectively. Deficient MMR (dMMR) was 14% of the cases. T-PD-L1, I-PD-L1 and PD-L1 CPS ≥ 10 were all significantly associated with dMMR ($P = 0.037$, $P = 0.009$, and $P = 0.005$, respectively). T-PD-L1, I-PD-L1, and PD-L1 CPS ≥ 10 were all associated with deeper depth of invasion ($P = 0.001$, $P = 0.024$, and $P = 0.002$, respectively). I-PD-L2 expression and PD-L2 CPS ≥ 10 were significantly higher in the differentiated histological type ($P = 0.015$ and $P = 0.030$, respectively). The I-PD-L1 and I-PD-L2 levels were significantly associated with better CSS ($P = 0.037$ and $P = 0.015$, respectively). CD8-high was significantly associated with less lymph node metastasis ($P = 0.047$), less distant metastasis ($P = 0.024$), less peritoneal dissemination ($P = 0.034$), and earlier TNM stage ($P = 0.047$). The CD8-high group had better prognosis than the CD8-low group ($P = 0.018$). FoxP3 expression was not associated with any clinicopathological factors or prognosis. We found that patients with PD-L2 CPS ≥ 10 tended to have worse prognosis in the FoxP3/CD8-low group ($P = 0.088$).

CONCLUSION
The clinicopathological significance of PD-L1/2 expression may differ depending on the TME status. Immune checkpoint inhibitors may improve the prognosis of SBA patients with low FoxP3/CD8 ratio and PD-L2 expression.

Core Tip: We investigated the clinicopathological significance of programmed cell death-ligand 1 (PD-L1) and programmed cell death-ligand 2 (PD-L2) expression in association with the infiltration of FoxP3+ and CD8+ T cells into the tumor microenvironment (TME) to identify PD-L/PD-1 immunotherapy candidates among patients with small bowel adenocarcinoma (SBA). We demonstrated that the status of the TME affects the clinical significance of PD-L1 and PD-L2. PD-L2 may be associated with poor prognosis of SBA patients with a high immune cell infiltration.

INTRODUCTION

Small bowel adenocarcinoma (SBA) is an uncommon condition, accounting for less than 3% of all gastrointestinal neoplasms; however, the small intestine accounts for 95% of the surface area of the entire gastrointestinal tract[1]. According to a recent report from the United States, the incidence of small bowel cancer has been increasing, with an annual increase of 1.8% between 2006 and 2015[2]. Although there are no definitive risk factors for SBA, diets containing high volumes of animal fat and protein have been reported to increase the risk, which may be related to an increase in the number of SBA[3]. SBA accounts for 30%–50% of all small bowel malignancies, and limited data exist on its molecular and clinicopathological features[4]. SBA has a high probability of being discovered at an advanced stage owing to a delay in diagnosis. No effective chemotherapy has been established for unresectable SBAs, and the regimens for colorectal cancer are palliatively administered. However, comprehensive genomic analyses have revealed that SBA and colorectal cancer have different genomic profiles, and it is assumed that the molecular pathways leading to carcinogenesis may also be different[3,5,6]. Therefore, it is essential to establish chemotherapeutic regimens based on the specific characteristics of SBA.

The small bowel is the largest organ of the human immune system. Lymphoid tissues in the lamina propria and various immune-related cells are prevalent in the small intestine and contribute to immune surveillance. Programmed cell death-ligand 1 (PD-L1) is the primary PD-1 Ligand that is upregulated in various solid tumors, and plays a pivotal role in modulating the tumor microenvironment (TME) to inhibit cytokine production and the cytolytic activity of PD-1+ tumor-infiltrating CD4+ and CD8+ T cells[7]. Several studies have demonstrated the efficacy of blocking the PD-1/PD-L1 signaling pathway in gastrointestinal cancers with high microsatellite instability (MSI-high)[8-11], and recent studies have demonstrated a significant association between MSI-high and PD-L1 expression in SBA[12,13]. PD-L1 expression has been reported to be associated with gastrointestinal cancer prognosis, despite several conflicting reports. While some studies have reported that PD-L1 expression is associated with favorable prognosis in gastric and colorectal cancers[14-18], others have reported that PD-L1 expression is associated with poorer prognosis in these cancers[19-22]. Considering the role of PD-1/PD-L1 in the TME, PD-L1 expression is predicted to be associated with worse prognosis in cancer patients.

Programmed cell death-ligand 2 (PD-L2) was discovered as a second ligand for PD-1 and has been reported to inhibit T cell proliferation by blocking cell cycle progression, similar to PD-L1[23,24]. PD-L2 suppresses the proliferation and cytokine production of CD4+ T cells through T cell receptors[23]. Its expression is significantly associated with PD-L1 expression in melanoma, non-small cell lung cancer, head and neck squamous cell carcinoma, renal cell carcinoma, bladder cancer, gastric cancer, and triple-negative breast cancer[25]. Although the function and expression pattern of PD-L2 are considered to be similar to those of PD-L1, PD-L2 has not received much attention, and its role in modulating tumor immunity remains undetermined.

Anti-PD-L1/PD-1 therapy utilizes tumor-infiltrating lymphocytes (TILs). Therefore, the status of TILs in the TME may influence their efficacy. Although TILs are mainly composed of CD8+ T cells, they are heterogeneous cell populations that also contain regulatory T cells (Tregs), which are believed to inhibit CD8+ T cells[26]. Tregs are an immunosuppressive subset of CD4+ T cells that orchestrate cellular and molecular networks to induce an immunosuppressive environment favorable to tumorigenesis[27]. Tregs are characterized by the expression of the master regulatory transcription factor FoxP3[28]. A high ratio of FoxP3+ to CD8+ T cells is associated with poor clinical outcomes in digestive system cancers [29-31].

This study investigated the clinicopathological significance of PD-L1 and PD-L2 expression in association with the infiltration of FoxP3+ and CD8+ T cells into the TME to identify PD-L/PD-1 immunotherapy candidates among SBA patients.

MATERIALS AND METHODS

Patients and tissue samples

We obtained 50 duodenal, jejunal, and ileal adenocarcinoma tissue samples from the archives of the Department of Pathology at Nippon Medical School Hospital for immunohistochemical analysis of PD-L1, PD-L2, CD8, FoxP3, and DNA mismatch repair (MMR) protein expression. Samples from patients with predisposing conditions, including Lynch syndrome, familial adenomatous polyposis, celiac disease, and Crohn's disease, were excluded to focus on sporadic SBA. Furthermore, samples from patients with ampullary adenocarcinoma or metastatic cancer were excluded. Cancer-specific survival (CSS) was defined as the interval from the date of the first surgery until death due to SBA; patients who died due to other causes were excluded. All patients provided informed written consent prior to study enrollment, and the study was approved by the Ethics Committee of Nippon Medical School (approval no. B-2020-164). All staging criteria were defined following the International Union for Cancer TNM classification.

Immunohistochemical analysis

Specimens were fixed in 10% formalin, embedded in paraffin wax, and immersed in 0.5% H_2O_2–methanol for 10 min to block endogenous peroxidase activity. Subsequently, the sections were microwaved in 0.01 mol/L citrate phosphate buffer (pH = 6.0) or EDTA (pH = 9.0) for antigen retrieval and incubated with 10% normal horse or goat serum for 10 min at 37°C to block nonspecific IgG binding. Thereafter, the sections were incubated for 18 h at 4°C with the primary antibodies listed in Supplementary Table 1. Next, they were treated with their respective biotinylated antibodies, namely anti-mouse IgG or anti-rabbit IgG (1:200; Vector) for 30 min at 25°C, followed by treatment with avidin-biotin-peroxidase complex for 30 min at 25°C. The reaction products were developed by immersing the sections in a 3,3'-diaminobenzidine tetrahydrochloride solution containing 0.03% H_2O_2.

Evaluation of immunohistochemical staining

Each patient was blindly evaluated by two independent observers (A.H. and A.T.). Any disagreements were resolved using a multi-headed microscope. The immunoreactivities of PD-L1 and PD-L2 were determined separately in tumor cells and tumor-infiltrating immune cells, such as lymphocytes and macrophages, throughout the tumor center and invasive margins. Tumor samples were defined as PD-L1 and PD-L2 positive when ≥ 1% of the tumor cells and/or tumor-infilt-rating immune cells were immunoreactive with unequivocal intensity. Subsequently, for PD-L1 and PD-L2 expression, a combined positive score (CPS) was calculated by dividing the total number of both tumor cells and immune cells above the positive threshold by the total number of viable tumor cells[32,33]. We set the PD-L1 and PD-L2 CPS cutoffs at ≥ 10% [34].

We assessed CD8+ T cells in ten randomly selected microscopic areas, including the intratumoral and tumor-surrounding stroma, no further than one high-power field from the tumor edge, by light microscopy (400 ×; BX63; Olympus, Tokyo, Japan). FoxP3+ T cells were assessed in the same ten high-power fields. Cell counts were determined

using CellSens Dimension software (Olympus, Tokyo, Japan). Immune-related cell densities were graded as low ($\leq$ median cell counts/mm^2 tumor area) or high (> median cell counts/mm^2 tumor area).

MMR status was defined by immunostaining for MLH1, MSH2, MLH6, and PMS2. Tumors were considered negative when there was a complete absence of nuclear staining of neoplastic cells in the presence of an internal positive control assessed on a whole slide. Tumors with negative staining for one of the MMR proteins were considered deficient MMR (dMMR), and all others were considered proficient MMR (pMMR). Each section was evaluated microscopically (400 ×; BX63; Olympus, Tokyo, Japan).

Statistical analysis

The immunostaining results for each protein were compared with the clinicopathological factors using the chi-square test or Fisher's exact test. Association between protein immunostaining was assessed using the chi-square test or Fisher's exact test, as appropriate. The distribution of CSS was estimated using the Kaplan–Meier method and the log-rank test was used to test for significant differences in CSS. A Cox proportional hazards model was used to assess the effects of tumor variables on CSS. In the multivariate analysis, variables with $P < 0.05$ in the univariate analysis were included. Statistical significance was set at $P < 0.05$.

RESULTS

Demographic data of patients

Demographic and baseline clinicopathological characteristics are listed in Table 1. The study included 35 men and 15 women ranging in age from 32 to 84 years (mean age, 65 years; median age, 68 years). At the time of analysis, 18 patients had died. The overall 5-year survival rate was 64%. The median follow-up duration for the entire series was 36 mo (mean, 45.1 mo; range, 5–124 mo). Twenty-three patients with stages III and IV disease received chemotherapy after surgery.

PD-L1, PD-L2, CD8, and FoxP3 Localization in SBA

PD-L1 immunoreactivity was observed in both tumor cells (T-PD-L1) and tumor-infiltrating immune cells (I-PD-L1), including lymphocytes and macrophages (Figure 1A and B). PD-L1 immunostaining was membranous and cytoplasmic in tumor cells and membranous in macrophages. The clinicopathological correlation of PD-L1 and PD-L2 expression are summarized in Table 2. T-PD-L1 was positive in 34% and I-PD-L1 was positive in 54% of cases. T-PD-L1 expression was associated with deeper depth of invasion ($P = 0.001$), but not with lymph node metastasis or distant metastasis. I-PD-L1 expression was associated with deeper depth of invasion ($P = 0.024$) and less peritoneal metastasis ($P = 0.019$). Both T-PD-L1 and I-PD-L1 expression were more common in stage II tumors compared to stage I tumors. PD-L2 immunoreactivity was observed in tumor cells (T-PD-L2) and tumor-infiltrating immune cells (I-PD-L2), including lymphocytes and macrophages (Figure 1C and D). The apical membrane showed a predominant PD-L2 staining pattern in tumor cells (Figure 1C). T-PD-L2 was positive in 34% and I-PD-L2 was positive in 42% of cases. T-PD-L2 expression was significantly higher in differentiated histological type ($P = 0.015$).

Then, we assessed PD-L1 and PD-L2 expression in a mixture of tumor and immune cells using CPS. Patients with PD-L1 CPS $\geq$ 10% and PD-L2 CPS $\geq$ 10% were observed in 50% and 56% of cases, respectively. In patients with PD-L1 CPS $\geq$ 10% of tumors were associated with deeper depth of invasion ($P = 0.002$), less peritoneal metastasis ($P = 0.034$), and more common in stage II compared to stage I (Table 2). Patients with PD-L2 CPS $\geq$ 10% were significantly correlated with younger age ($P = 0.027$) and more differentiated histological type ($P = 0.030$) (Table 2).

Representative micrographs of CD8 and FoxP3 staining are shown in Figure 2. CD8-high was significantly associated with less lymph node metastasis ($P = 0.047$), less distant metastasis ($P = 0.024$), less peritoneal dissemination ($P = 0.034$), and earlier TNM stage ($P = 0.047$) (Table 3). FoxP3 immunoreactivities were not associated with any clinicopathological factors, including age, sex, localization, histological type, lymph node metastasis, distant metastasis, peritoneal dissemination, or TNM stage. FoxP3-high tumors had deeper depth of invasion, although the difference was not statistically significant ($P = 0.059$) (Table 3).

Then, we calculated and summed the ratio of FoxP3 to CD8 positive T cell counts in ten high-power fields. FoxP3/CD8-high was significantly associated with lymph node metastasis ($P = 0.002$), distant metastasis ($P = 0.004$), peritoneal dissemination ($P = 0.005$), and TNM stage ($P = 0.002$) (Table 3).

dMMR was 14% of the cases identified using MLH1, MSH2, MLH6, and PMS2 immunostaining. T-PD-L1, I-PD-L1, and PD-L1 CPS $\geq$ 10% were significantly higher in the dMMR tumors than in the pMMR tumors. No PD-L2 positivity rate was associated with MMR status (Table 2).

Mutual relationship between PD-L1, PD-L2, CD8, and FoxP3 expression

Mutual relationship between PD-L1, PD-L2, CD8, and FoxP3 expression levels are shown in Table 4. A significant positive relationship was observed between PD-L1 and PD-L2 expression in both tumor and immune cells. A significant positive relationship was observed between CD8-high and T-PD-L1, I-PD-L1, and I-PD-L2 expression. FoxP3-high expression was associated with I-PD-L1 expression, but not with T-PD-L1, T-PD-L2, or I-PD-L2 expression.

Comparative survival analysis

Both I-PD-L1 and I-PD-L2 expression were significantly associated with better CSS ($P = 0.037$ and $P = 0.015$, respectively) (Figure 3). There was no significant association between T-PD-L1 expression or PD-L1 CPS $\geq$ 10% and patients' survival. There was no significant association between T-PD-L2 expression or PD-L2 CPS $\geq$ 10% and patients' survival. Patients in

Table 1 Clinicopathological data of patients with small bowel adenocarcinoma	
	No. of cases (%)
Age	
≤ 68	27 (54.0)
> 68	23 (46.0)
Sex	
Female	15 (30.0)
Male	35 (70.0)
Site	
Duodenum and jejunum	45 (90.0)
Ileum	5 (10.0)
Histology	
WD, MD	41 (82.0)
PD, Muc	9 (18.0)
Depth of invasion	
pT1-2	14 (28.0)
pT3-4	36 (72.0)
Lymph node metastasis	
Absence	27 (54.0)
Presence	23 (46.0)
Distant metastasis	
Absence	37 (74.0)
Presence	13 (26.0)
Peritoneal seeding	
Absence	40 (80.0)
Presence	10 (20.0)
Chemotherapy	
Absence	27 (54.0)
Presence	23 (46.0)
TNM stage	
I	14 (28.0)
II	13 (26.0)
III	10 (20.0)
IV	13 (26.0)

WD: Well differentiated; MD: Moderately differentiated; PD: Poorly differentiated; Muc: Mucinous adenocarcinoma.

the CD8-high group had better prognosis than those in the CD8-low group (P = 0.018) (Figure 4). There was no significant association between FoxP3+ T cells and patients' survival. In contrast, the FoxP3/CD8-low group had significantly better prognosis than the FoxP3/CD8-high group (P = 0.004). We performed a survival analysis for PD-L1 and PD-L2 expression stratified by the FoxP3/CD8 ratio, and found that patients with PD-L2 CPS ≥ 10 in the FoxP3/CD8-low group had worse prognosis, although the difference was not significant (P = 0.088) (Figure 5).

In the univariate analysis using the Cox proportional hazards model for CSS, lymph node status and I-PD-L1, I-PD-L2, CD8, and FoxP3/CD8 ratios had significant prognostic value (Table 5). In the multivariate analysis performed by introducing all the above variables into the Cox proportional hazards model, lymph node status and I-PD-L2 expression retained independent prognostic significance.

Table 2 Clinicopathological correlation of programmed cell death-ligand 1 and programmed cell death-ligand 2 expression

	No.	T-PD-L1		I-PD-L1		PD-L1 CPS ≥ 10		T-PD-L2		I-PD-L2		PD-L2 CPS ≥ 10	
		N (%)	*P* value	*N* (%)	*P* value	*N* (%)	*P* value	*N* (%)	*P* value	*N* (%)	*P* value	*N* (%)	*P* value
Age													
≤ 68	27	10 (37.0)	NS	16 (59.3)	NS	14 (51.9)	NS	12 (44.4)	NS	14 (51.9)	NS	19 (70.4)	0.027
> 68	23	7 (30.4)		11 (47.8)		11 (47.8)		5 (21.7)		7 (30.4)		9 (39.1)	
Sex													
Female	15	4 (26.7)	NS	7 (46.7)	NS	7 (46.7)	NS	6 (40.0)	NS	8 (53.3)	NS	8 (53.3)	NS
Male	35	13 (37.1)		20 (57.1)		18 (51.4)		11 (31.4)		13 (37.1)		20 (57.1)	
Site													
Duodenum and jejunum	45	16 (35.6)	NS	25 (55.6)	NS	24 (53.3)	NS	16 (35.6)	NS	20 (44.4)	NS	26 (57.8)	NS
Ileum	5	1 (20.0)		2 (40.0)		1 (20.0)		1 (20.0)		1 (20.0)		2 (40.0)	
Histology													
WD, MD	41	13 (31.7)	NS	24 (58.5)	NS	21 (51.2)	NS	17 (41.5)	0.015	19 (46.3)	NS	26 (63.4)	0.030
PD, Muc	9	4 (44.4)		3 (33.3)		4 (44.4)		0 (0.0)		2 (22.2)		2 (22.2)	
Depth of invasion													
pT1-2	14	0 (0.0)	0.001	4 (28.6)	0.024	2 (14.3)	0.002	5 (35.7)	NS	6 (42.9)	NS	6 (42.9)	0.068
pT3-4	36	17 (47.2)		23 (63.9)		23 (63.9)		12 (33.3)		15 (41.7)		22 (61.1)	
Lymph node metastasis													
Absence	27	8 (29.6)	NS	16 (59.3)	NS	13 (48.1)	NS	8 (29.6)	NS	13 (48.1)	NS	15 (55.6)	NS
Presence	23	9 (39.1)		11 (47.8)		12 (52.2)		9 (39.1)		8 (34.8)		13 (56.5)	
Distant metastasis													
Absence	37	12 (32.4)	NS	22 (59.5)	NS	20 (54.1)	NS	13 (35.1)	NS	18 (48.6)	NS	23 (62.2)	0.095
Presence	13	5 (38.5)		5 (38.5)		5 (38.5)		4 (30.8)		3 (23.1)		5 (38.5)	
Peritoneal seeding													
Absence	40	14 (35.0)	NS	25 (62.5)	0.019	23 (57.5)	0.034	14 (35.0)	NS	19 (47.5)	NS	25 (62.5)	NS
Presence	10	3 (30.0)		2 (20.0)		2 (20.0)		3 (30.0)		2 (20.0)		3 (30.0)	
TNM stage													

I	14	0 (0.0)	0.008	4 (28.6)	0.005	2 (14.3)	0.001	5 (35.7)	NS	6 (42.9)	NS	6 (42.9)	NS
II	13	8 (61.5)		12 (92.3)		11 (84.6)		3 (23.1)		7 (53.8)		9 (69.2)	
III	10	4 (40.0)		6 (60.0)		7 (70.0)		5 (50.0)		5 (50.0)		8 (80.0)	
IV	13	5 (38.5)		5 (38.5)		5 (38.5)		4 (30.8)		3 (23.1)		5 (38.5)	
MMR status													
Proficient	43	12 (27.9)	0.037	20 (46.5)	0.009	18 (41.9)	0.005	15 (34.9)	NS	16 (37.2)	NS	22 (51.2)	NS
Deficient	7	5 (71.4)		7 (100.0)		7 (100.0)		2 (28.6)		5 (71.4)		6 (85.7)	

PD-L1: Programmed cell death-ligand 1; PD-L2: Programmed cell death-ligand 2; CPS: Combined positive score; WD: Well differentiated; MD: Moderately differentiated; PD: Poorly differentiated; Muc: Mucinous adenocarcinoma; NS: Not significant.

DISCUSSION

In this study, we performed immunostaining for PD-L1 and PD-L2 to investigate their clinicopathological significance in SBA. Since PD-L1 is expressed in both tumor cells and tumor-infiltrating immune cells, we examined the clinicopathological significance of their expression separately to clarify whether there were differences in their roles in tumor progression. Although there were several similarities and correlations, some differences in association with clinicopathological factors were observed. T-PD-L1 expression was associated with deeper depth of invasion, but not with lymph node metastasis, distant metastasis, or prognosis. These results suggest that T-PD-L1 contributes to the local invasion of tumor cells but not to metastasis; consequently, it is not associated with prognosis. I-PD-L1 expression is associated with deeper depth of invasion, less peritoneal metastasis, and favorable prognosis. We speculated that I-PD-L1 expression might be influenced by peritumoral infiltrating T cells, which correlates with favorable prognosis. Indeed, we found a positive relationship between I-PD-L1 expression and the CD8+ T cell count, and a high density of CD8+ TILs was associated with favorable prognosis. The main similarity between T-PD-L1 and I-PD-L1 expression was that both were associated with deeper depth of invasion and were more common in stage II compared to stage I tumors. These results suggest that the clinical significance of PD-L1 does not differ following the cell type in which it is expressed. Based on the above findings, we adopted the CPS to evaluate PD-L1 expression. The CPS was calculated by summing the number of PD-L1–stained cells (tumor cells, lymphocytes, and macrophages) and dividing the result by the total number of viable tumor cells, and multiplying by 100. Based on the observed response rate and response durability, the U.S. Food and Drug Administration granted pembrolizumab accelerated approval for the treatment of recurrent locally advanced, metastatic gastric or gastro-esophageal junction adenocarcinoma that expresses PD-L1 CPS ≥ 1. Thus, the CPS is now becoming a standard method for the assessment of PD-L1 expression. Because the CPS is a scoring system characterized by collectively quantifying positivity for tumor cells and positivity for surrounding immune cells, it may be possible to optimize the presence or absence of PD-L1 expression in carcinomas accompanied by inflammatory or immune cell infiltration. We examined the relationship between PD-L1 expression and clinicopathological factors using the CPS ≥ 10 as a cut-off in this study, resulting in that no significant association between PD-L1 expression and prognosis was found.

It has been demonstrated that PD-L2 is a second ligand for PD-1 and can be expressed by immune, stromal, or tumor cells, mainly through Th2-associated cytokines depending on tumor microenvironmental stimuli[35]. To date, a few studies have reported the significance of PD-L2 expression in gastrointestinal carcinomas[36,37]. To our knowledge, this

Table 3 Clinicopathological correlation of CD8, FoxP3, and FoxP3/CD8							
	No.	FoxP3		CD8		FoxP3/CD8	
		N (%)	*P* value	*N* (%)	*P* value	*N* (%)	*P* value
Age							
≤ 68	27	14 (51.9)	NS	14 (51.9)	NS	14 (51.9)	NS
> 68	23	11 (47.8)		11 (47.8)		11 (47.8)	
Sex							
Female	15	7 (46.7)	NS	8 (53.3)	NS	8 (53.3)	NS
Male	35	18 (51.4)		17 (48.6)		17 (48.6)	
Site							
Duodenum and jejunum	45	22 (48.9)	NS	23 (51.1)	NS	22 (48.9)	NS
Ileum	5	3 (60.0)		2 (40.0)		3 (60.0)	
Histology							
WD, MD	41	21 (51.2)	NS	21 (51.2)	NS	20 (48.8)	NS
PD, Muc	9	4 (44.4)		4 (44.4)		5 (55.6)	
Depth							
pT1-2	14	4 (28.6)	0.059	7 (50.0)	NS	4 (28.6)	0.059
pT3-4	36	21 (58.3)		18 (50.0)		21 (58.3)	
Lymph node metastasis							
Absence	27	11 (40.7)	NS	17 (63.0)	0.047	8 (29.6)	0.002
Presence	23	14 (60.9)		8 (34.8)		17 (73.9)	
Distant metastasis							
Absence	37	16 (43.2)	NS	22 (59.5)	0.024	14 (37.8)	0.004
Presence	13	9 (69.2)		3 (23.1)		11 (84.6)	
Peritoneal seeding							
Absence	40	19 (47.5)	NS	23 (57.5)	0.034	16 (40.0)	0.005
Presence	10	6 (60.0)		2 (20.0)		9 (90.0)	
TNM stage							
I	14	4 (28.6)	NS	7 (50.0)	NS	4 (28.6)	0.011
II	13	7 (53.8)		10 (76.9)		4 (30.8)	
III	10	5 (50.0)		5 (50.0)		6 (60.0)	
IV	13	9 (69.2)		3 (23.1)		11 (84.6)	
MMR status							
Proficient	43	19 (44.2)	0.049	20 (46.5)	NS	22 (51.2)	NS
Deficient	7	6 (85.7)		5 (71.4)		3 (42.9)	

WD: Well differentiated; MD: Moderately differentiated; PD: Poorly differentiated; Muc: Mucinous adenocarcinoma; NS: Not significant.

is the first study to examine the relationship between PD-L2 expression and the clinicopathological characteristics of SBA patients. Since PD-L2 is expressed in both tumor cells and tumor-infiltrating immune cells, we examined the clinicopathological significance of its expression in both cell types, as we did for PD-L1. T-PD-L2 expression was not associated with any clinicopathological factors except for histological type and prognosis. I-PD-L2 expression was significantly associated with better CSS, suggesting that I-PD-L2 expression may be influenced by peritumoral infiltrating T cells that correlate with favorable prognosis. Then, we evaluated the PD-L2 expression using the CPS. PD-L2 CPS ≥ 10 was associated with younger age and was more common in differentiated histological type, but not with prognosis. It has been reported that PD-L2 expression was associated with poor prognosis in gastric and colorectal cancers[36,37]. These findings are intuitively understandable, considering that PD-L2 expression suppresses tumor immunity as well as PD-L1 does.

Table 4 Mutual relationship of programmed cell death-ligand 1, programmed cell death-ligand 2, CD8 and FoxP3 (*n* = 50)

	I-PD-L1	PD-L1 CPS ≥ 10	T-PD-L2	I-PD-L2	PD-L2 CPS ≥ 10	FoxP3	CD8	FoxP3/CD8
T-PD-L1	0.002	< 0.001	0.042	0.003	0.001	NS	0.007	NS
I-PD-L1		< 0.001	NS	0.019	0.002	0.005	0.047	NS
PD-L1 CPS ≥ 10			NS	0.010	0.004	0.048	0.011	NS
T-PD-L2				< 0.001	< 0.001	NS	NS	NS
I-PD-L2					< 0.001	NS	0.002	0.045[1]
PD-L2 CPS ≥ 10						NS	0.004	NS
FoxP3							NS	0.002
CD8								< 0.001[1]

[1]An inverse relationship.

PD-L1: Programmed cell death-ligand 1; PD-L2: Programmed cell death-ligand 2; NS: Not significant.

Table 5 Univariate and multivariate Cox proportional hazards analysis for cancer-specific survival (*n* = 50)

| Variables	Categories	Univariate analysis		Multivariate analysis	
		HR (95%CI)	*P* value	HR (95%CI)	*P* value
Histological type	PD, Muc *vs*. WD, MD	2.252 (0.799-6.348)	NS		
Depth	pT3-4 *vs*. pT1-2	39.308 (0.661-2337.048)	NS		
Lymph node metastasis	Positive *vs*. negative	42.764 (5.639-324.314)	< 0.001	66.320 (7.494-586.923)	< 0.001
T-PD-L1	Positive *vs*. negative	0.733 (0.261-2.059)	NS		
I-PD-L1	Positive *vs*. negative	0.367 (0.137-0.980)	0.046	1.156 (0.231-5.769)	NS
PD-L1 CPS ≥ 10	Positive *vs*. negative	0.621 (0.240-1.606)	NS		
T-PD-L2	Positive *vs*. negative	0.702 (0.249-1.981)	NS		
I-PD-L2	Positive *vs*. negative	0.243 (0.070-0.840)	0.025	0.113 (0.020-0.650)	0.015
PD-L2 CPS ≥ 10	Positive *vs*. negative	0.493 (0.191-1.276)	NS		
FoxP3	High *vs*. low	1.067 (0.423-2.694)	NS		
CD8	High *vs*. low	0.311 (0.111-0.872)	0.026	1.922 (0.164-22.481)	NS
FoxP3/CD8	High *vs*. low	4.490 (1.476-13.660)	0.008	1.476 (0.138-15.769)	NS

HR: Hazard ratio; 95%CI: 95% confidence interval; PD-L1: Programmed cell death-ligand 1; PD-L2: Programmed cell death-ligand 2; NS: Not significant.

However, several studies have demonstrated that both PD-L2 and PD-L1 expression are favorable prognostic indicators for gastric and colorectal cancer[25,38]. To date, such discrepancies have been explained by differences in scoring methods, cutoff values of immunostaining, heterogeneities of carcinoma, and any bias originating from the inclusion of an insufficient number of cases. To resolve this, we investigated the clinical significance of PD-L1 and PD-L2 expression according to TME status. CD8+ TILs are associated with the tumor immune response and can be used to predict the response to immunotherapy and survival outcomes in CRC[39]. It has been reported a high density of CD8+ TILs is associated with favorable prognosis in CRC[40-42]. We found that CD8-high was negatively associated with lymph node metastasis, distant metastasis, and peritoneal dissemination in SBA patients. We also found that the CD8-high group had better prognosis than the CD8-low group, which is consistent with the findings of previous reports. These results suggest that CD8+ TILs have an anti-tumor effect in SBA as well as in CRC and that CD8+ TILs play an important role in improving patient survival. Then, we analyzed the association between FoxP3+ Tregs and clinicopathological factors. CD4+ Tregs expressing the transcription factor FoxP3 are highly immunosuppressive and play a central role in maintaining self-tolerance and immune homeostasis. FoxP3+ T cells promote tumor progression by suppressing effective anti-tumor immunity by inactivating or reducing the proliferation of cytotoxic CD8+ T cells and CD4+ T effector cells in tumors[27,28]. To the best of our knowledge, this is the first study to examine the relationship between the density of FoxP3+ T cells and the clinicopathological characteristics in SBA patients. We found that FoxP3-high tumors tended to have deeper depth of invasion but were not associated with lymph node metastasis, distant metastasis, and peritoneal dissemination. No association was observed between FoxP3+ T cell density and CSS. This is due to the functional hetero-
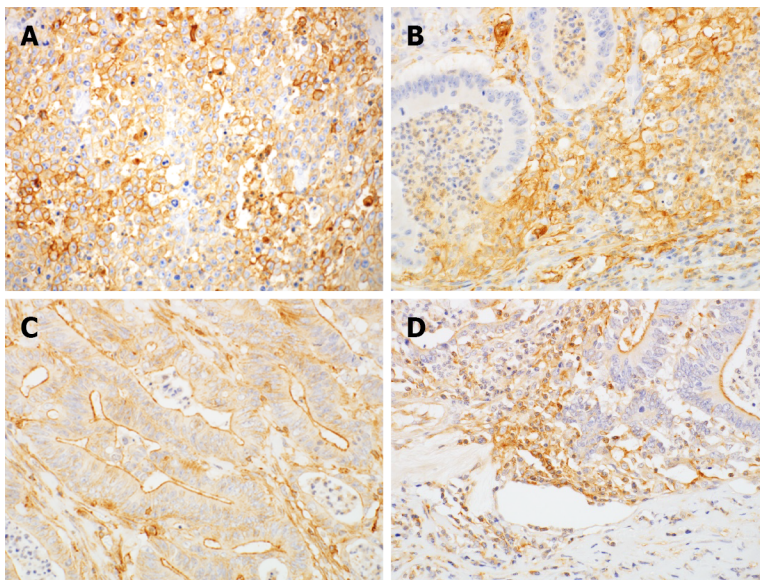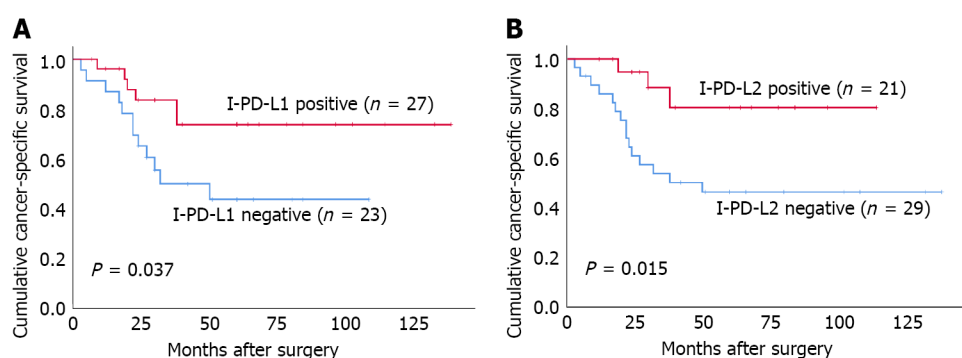
Figure 1 Immunohistochemical localization of programmed cell death-ligand 1 and programmed cell death-ligand 2 in small bowel adenocarcinoma. Magnification 400×. A: T-programmed cell death-ligand 1 expression was membranous and cytoplasmic; B: I-programmed cell death-ligand 1 (I-PD-L1) was positive in peritumoral lymphocytes and macrophages; C: The predominant pattern of T-programmed cell death-ligand 2 expression was in the apical membrane; D: I-programmed cell death-ligand 2 expression was positive in peritumoral lymphocytes and macrophages, similar to that of I-PD-L1.
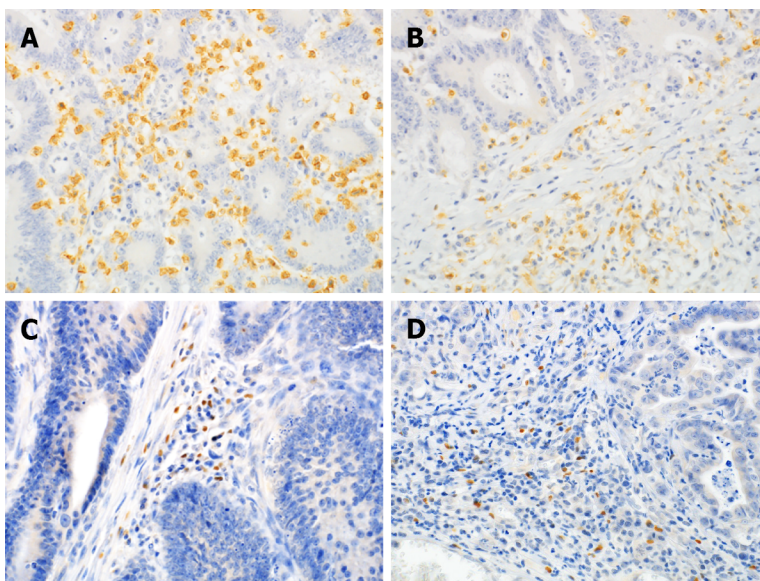
Figure 2 Immunohistochemical localization of CD8 and FoxP3 in small bowel adenocarcinoma. A: CD8+ T cells in the intratumoral stroma; B: CD8+ T cells in the tumor-surrounding stroma; C: FoxP3+ T cells in the intratumoral stroma; D: FoxP3+ T cells in tumor-surrounding stroma. Magnification 400 ×.

geneity of FoxP3+ T cells, as FoxP3+ non-Tregs are secreted depending on inflammatory cytokines such as TGF-β and IL-12[43]. While some studies have reported that high density of FoxP3+ T cells in the TME is associated with poor prognosis in lung cancer, others have reported that high density of FoxP3+ T cells is associated with favorable prognosis in CRC, bladder cancer, and head and neck cancers[44]. Thus, it has been reported that the amount of FoxP3+ T cells infiltration does not always serve as a poor prognostic indicator in the case of carcinoma accompanied by inflammatory cell infiltration, including CRC[43]. Then, we estimated the ratio of FoxP3+ T cells to CD8+ T cells in 10 HPF selected from the same site in the same section. FoxP3/CD8-high was significantly associated with deeper depth of invasion, lymph node metastasis, distant metastasis, peritoneal dissemination, and TNM stage progression. Patients with higher FoxP3+/CD8+ T cells ratios have poor prognosis for gastrointestinal cancers[29-31]. Similarly, we observed that the prognostic value of this ratio was better than that of FoxP3+ or CD8+ T cells alone, which partly reflects the interactions between anti-tumor CD8+ T cells and immunosuppressive FoxP3+ T cells in tumors[45,46]. These results indicate that assessing both FoxP3+ and CD8+ T cells is more useful for understanding TME status than assessing FoxP3 or CD8 + T cells alone.
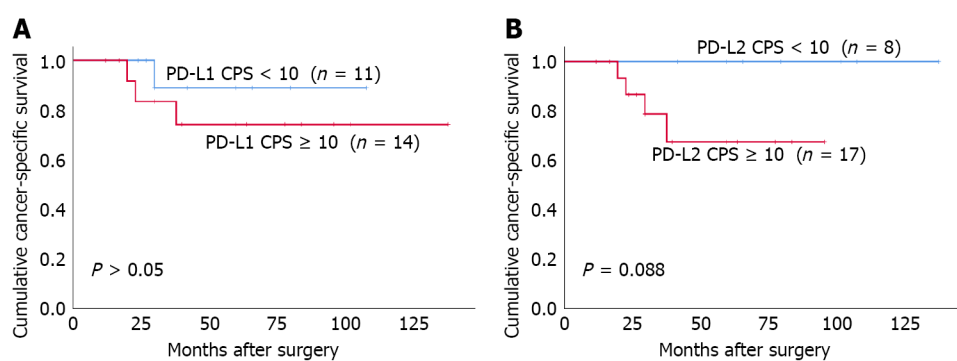
Figure 3 Kaplan–Meier analysis of cancer-specific survival according to programmed cell death-ligand 1 and programmed cell death-ligand 2 expressions in small bowel adenocarcinoma evaluated by log-rank tests. A: Survival based on I-programmed cell death-ligand 1 expression in overall cases; B: Survival based on I-programmed cell death-ligand 2 expression in overall cases. I-PD-L1: I-programmed cell death-ligand 1; I-PD-L2: I-programmed cell death-ligand 2.
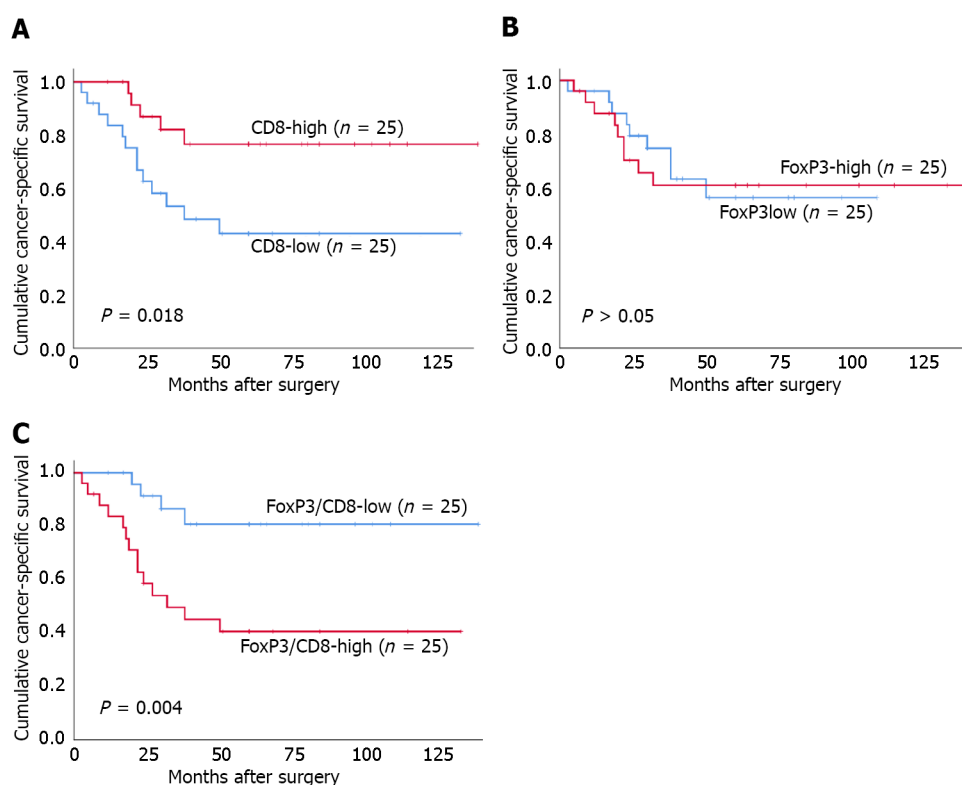
Figure 4 Kaplan–Meier analysis of cancer-specific survival according to CD8+ and FoxP3+ T cell counts in small bowel adenocarcinoma evaluated by log-rank tests. A: Survival based on CD8+ T cell counts in overall cases; B: Survival based on FoxP3+ T cell counts in overall cases; C: Survival based on FoxP3+/CD8+ T cell counts in overall cases.

Both PD-L1 CPS ≥ 10 and PD-L2 CPS ≥ 10 significantly correlated with increased CD8+ T cells infiltration. Furthermore, I-PD-L2 expression was more frequent in FoxP3/CD8-low tumors. These results may explain why I-PD-L2 expression is associated with favorable outcomes. To determine the effect of PD-L1 and PD-L2 on the prognosis of patients with SBA, excluding the effect of the TME, we divided the patients into FoxP3/CD8-low and FoxP3/CD8-high groups. We investigated the correlation between PD-L1 and PD-L2 expression and the prognosis in the two patient groups. We found that patients with PD-L2 CPS ≥ 10 tended to have poorer prognosis than those with PD-L2 CPS < 10 in the FoxP3/CD8-low group, although the difference was not statistically significant. Furthermore, there were no deaths among patients with a PD-L2 CPS < 10 in the FoxP3/CD8-low group. Generally, cases in which the infiltration of CD8 is dominant over FoxP3 are predicted to have favorable prognosis due to the anti-tumor immune effect of T cells; nevertheless, patients showing poor prognosis are also included within the group. The PD-L2 pathway may contribute to poor prognosis in patients with FoxP3/CD8-low tumors. The results of this study also indicate that PD-L2 may be a better

Figure 5 Kaplan–Meier analysis of cancer-specific survival according to PD-L1 and PD-L2 expression in small bowel adenocarcinoma patients with FoxP3/CD8-low tumors. A: Survival based on PD-L1 combined positive score (CPS) among patients in the FoxP3/CD8-low group; B: Survival based on PD-L2 CPS among patients in the FoxP3/CD8-low group.

predictive factor for prognosis in these cases than PD-L1.

Recent studies have demonstrated that the tumor mutation burden (TMB) could be a biomarker in patients with cancer treated with immune checkpoint inhibitors (ICIs), in which high TMB is significantly correlated with better survival[47]. Although the efficacy of ICIs in patients with cancer with TMB-high or MSI-high/dMMR has been confirmed, patients with MSI-high have a favorable prognosis, and generally, only a small number of patients require chemotherapy. In fact, none of the patients with dMMR died in our cohort, and only two of seven patients received chemotherapy. Most patients with SBA have microsatellite stable (MSS)/pMMR or TMB-low, and it is necessary to identify useful biomarkers for ICI therapy in patients with MSS/pMMR. Previous studies have shown that TMB-high tumors are immunogenic and that TMB-high tumors are also present in SBA[48]. In small bowel cancer, TMB-high has been reported to be associated with dMMR, CD8-high, and PD-L1 expression, but it has been shown that TMB-high cases are also present in cases of pMMR [49,50]. Our results indicate that PD-L2 positive and FoxP3/CD8-low patients with pMMR may benefit from ICI therapy. Although we did not examine the TMB in this study, it may stratify the outcomes of patients with SBA with PD-L2 positive and FoxP3/CD8-low.

This study has several limitations. First, the sample size is small. In addition, there was a bias in tumor localization. Second, MMR status was determined based solely on the results of immunohistochemical staining for MMR proteins. Third, there were no deaths among the patients with dMMR tumors. Therefore, we could not analyze patients' prognosis based on the MMR status.

CONCLUSION

We elucidated a discrepancy in the previously reported clinical significance of PD-L1 expression in SBA. Several studies have reported that PD-L1 expression, which should be involved in tumorigenesis, is associated with favorable prognosis. This contradictory finding may originate from the fact that all patients are equally included in the analysis without considering the TME status and the type of cells in which PD-L1 is expressed. We demonstrated that the clinical significance of PD-L2 may be affected by TME status. Although SBA patients with high immune cell infiltration generally have better prognosis, some of these patients have poorer prognosis. PD-L2 may contribute to poorer prognosis of these patients. ICIs may improve the patients' prognosis in the FoxP3/CD8-low group through blocking the binding of the PD-1 to PD-L2 and activating locally infiltrated T cells.

ARTICLE HIGHLIGHTS

Research background

According to a recent report from the United States, the incidence of small bowel adenocarcinoma (SBA) has been increasing, with an annual increase of 1.8% between 2006 and 2015. Comprehensive genomic analyses revealed that SBA and colorectal cancer have different genomic profiles, and it is assumed that the molecular pathways leading to carcinogenesis may also be different. Therefore, it is essential to establish chemotherapeutic regimens based on the specific characteristics of SBA.

Research motivation

The clinicopathological significance of programmed cell death-ligand 1 (PD-L1) and programmed cell death-ligand 2 (PD-L2) expression in SBA is not yet fully understood. There are several conflicting reports regarding the clinicopathological significance of PD-L1 expression in gastrointestinal cancers. To resolve this discrepancy, we investigated the clinical

significance of PD-L1 and PD-L2 expression according to tumor microenvironment (TME) status stratified by the density of FoxP3+ and CD8+ T cells.

Research objectives

In this study, we investigated the clinicopathological significance of PD-L1 and PD-L2 expression in association with the infiltration of FoxP3+ and CD8+ T cells in the TME to identify PD-L/PD-1 immunotherapy candidates among patients with SBA. We elucidated the discrepancy in previously reported clinical significance of PD-L1 expression in SBA.

Research methods

The immunoreactivities of PD-L1 and PD-L2 were determined separately in tumor cells and tumor-infiltrating immune cells, such as lymphocytes and macrophages, and evaluated using the combined positive score (CPS). To our knowledge, this is the first study to examine the relationship between PD-L2 expression, and the density of FoxP3+ T cells, and the clinicopathological characteristics of patients with SBA.

Research results

PD-L1 expression was positive in 34% in tumor cells (T-PD-L1) and 54% in tumor-infiltrating immune cells (I-PD-L1) of the cases examined. T-PD-L2 was positive in 34% and I-PD-L2 was positive in 42% of the cases, respectively. PD-L1 CPS ≥ 10 and PD-L2 CPS ≥ 10 were observed in 50% and 56% of cases, respectively. I-PD-L1 and I-PD-L2 Levels were significantly associated with better prognosis. We speculated that I-PD-L1 expression might be influenced by peritumoral infiltrating T cells, which correlates with favorable prognosis. We found that patients with PD-L2 CPS ≥ 10 tended to have worse prognosis in the FoxP3/CD8-low group. Although SBA patients with high immune cell infiltration, such as those in the FoxP3/CD8-low group, generally have better prognosis, some have poorer prognosis. Therefore, PD-L2 may contribute to the poor prognosis of these patients.

Research conclusions

We identified a discrepancy in the previously reported clinical significance of PD-L1 expression in SBA. Several studies have reported that PD-L1 expression, which is involved in tumorigenesis, is associated with favorable prognosis. This contradictory finding may originate from the fact that all patients were equally included in the analysis without considering the TME status and the type of cells in which PD-L1 was expressed. To identify PD-L/PD-1 immunotherapy candidates, not only PD-L1 expression and DNA mismatch repair/microsatellite instability but also PD-L2 expression and the density of tumor-infiltrating lymphocytes, such as FoxP3+ and CD8+ T cells, in the TME should be considered.

Research perspectives

In this study, we did not consider tumor mutation burden (TMB). TMB-high has been proposed as a predictive biomarker for the response to immune checkpoint inhibitors based on the assumption that increasing the number of mutant proteins will create antigenic peptides, allowing for enhanced immunogenicity. In the future, we may be able to analyze TMB status in SBA and combine it with PD-L1/2 expression and TME status to generate powerful biomarkers for identifying immunotherapy candidates.

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FOOTNOTES

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