

Programmed death-1 (PD-1) ligand 1 and PD-1 ligand 2 expressions and their clinical relevance in colorectal cancer

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Purpose: Programmed death-1 and its ligands (PD-L1 and PD-L2) can induce T-cell apoptosis in many solid tumors, although there is limited information regarding their roles in colorectal cancer.

Methods: We used immunohistochemistry to retrospectively examine expressions of PD-L1 and PD-L2 in paraffin-embedded specimens from 104 patients with colorectal cancer.

Results: Among the 104 included patients, 31 patients (29.8%) had positive PD-L1 expression and 73 patients (70.2%) had negative PD-L1 expression. Positive PD-L2 expression was observed in 83 patients (79.8%) and negative PD-L2 expression was observed in 21 patients (20.2%). Positive PD-L1 expression group showed higher overall survival rate (OS) and disease-free survival (DFS) than negative PD-L1 expression group. However, the overall survival and DFS were not significantly different between positive and negative PD-L2 expressions group. The multivariate analyses revealed that short OS was independently associated with positive PD-L1 expression (hazard ratio [HR], 2.781; 95% confidence interval [CI], 1.284–6.026; $P=0.01$), regional lymph node status (HR, 2.611; 95% CI, 1.258–5.418; $P=0.01$), and distant metastasis (HR, 4.279; 95% CI, 1.449–12.638; $P=0.009$). In addition, short DFS was independently associated with positive PD-L1 expression (HR, 2.846; 95% CI, 1.393–5.815; $P=0.004$) and regional lymph node status (HR, 2.310; 95% CI, 1.122–4.758; $P=0.023$).

Conclusion: Although prospective multi-center studies are needed to validate our findings, we found that PD-L1 expression predicted OS and DFS among patients with colorectal cancer.

Keywords: Programmed death-1, Programmed death-ligand 1, Colorectal neoplasms, Prognosis

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer among men and women worldwide [1], and the third leading cause of death among cancer patients in the Republic of Korea [2]. The only curative treatment is currently surgery, and aggressive surgical resection plus additional treatment provides a 5-year survival rate of 9%–90%, which was improved in the early stage but still low in

the advanced stage [1]. Thus, other options are urgently needed for treating CRC. For example, targeted therapy has been developed during recent decades, and these treatments target mutations that activate or inactivate signaling pathways that drive cancer development. This approach has generated good clinical responses among patients bearing the targeted mutation, especially when CRC is treated using cetuximab (epidermal growth factor receptor inhibitor) or bevacizumab (vascular endothelial growth factor inhibitor). However, these treatments are limited by the short duration of the clinical response and increasing numbers of targets that arise through new mutations [3].

Immune check points are a topic of increasing interest in the field of cancer immunology, as molecular research has begun to explain the complex mechanisms regulating cellular immune responses [3]. For example, there are a number of inhibitory mechanisms that can be induced by the activated immune system, especially in T-cells, which can prevent an excessive immune response [4-6]. Programmed death-1 (PD-1) was initially cloned in 1992

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during a study of thymus T-cell molecules [7], and a study from 2000 revealed that one PD-1 ligand (programmed death ligand 1 [PD-L1], B7-H1, and CD274) induces T-cell apoptosis. These discoveries led to research regarding the function of PD-1 as an immune checkpoint [8], which revealed that PD-1 is only expressed in activated T-cells, and that PD-1 and CD28 regulate their response through ligand binding. The ligands of PD-1 are PD-L1 and PD-L2 (B7-DC, CD273), with PD-L1 being expressed on many cell types (e.g., immune cells, epithelial cells, and endothelial cells) and PD-L1 only being expressed on antigen-presenting cells [3]. Recent studies have indicated that tumors can evade the immune response through PD-L1 expression, and Song et al. [9] have reported that PD-L1 expression is associated with the prognosis of patients with CRC. However, no studies have examined the value of PD-L2 as a prognostic factor for CRC. Only a few reports suggested that PD-L2 may play roles in tumor immunity. Liu et al. [10] have reported that PD-L2 in the tumor cells promotes CD8 T-cell mediated rejection at both the induction and effector phase of antitumor immunity. Therefore, this retrospective study evaluated the expressions of PD-L1 and PD-L2 in CRC specimens using immunohistochemistry, and examined whether these expressions were associated with survival outcomes.

METHODS

Between January 2002 and August 2004, 117 patients underwent complete curative resection of pathologically-confirmed CRC and had available formalin-fixed paraffin-embedded block specimens. However, 13 patients were excluded because they died during the perioperative period or underwent preoperative chemoradiotherapy. Thus, specimens from 104 patients were included in the present study. The patients' clinicopathological characteristics and outcomes were retrospectively collected from their medical charts and pathological records. Tumor staging was performed according to the TNM classification of the American Joint Committee on Cancer. The Institutional Review Board of the Soonchunhyang University Cheonan Hospital approved the study.

We performed immunohistochemistry to evaluate the expressions of PD-L1 and PD-L2 using tissue microarray slides. The tissues were embedded in paraffin and sliced into 4- μ m sections. The sections were then deparaffinized and antigen retrieval was performed by microwaving the sections for 15 minutes in 0.01 M citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked using Dako REAL peroxidase blocking solution (Dako, Carpinteria, CA, USA) for 30 minutes at room temperature. The primary antibodies were diluted 1:100 (PD-L1: #AF156, R&D, Minneapolis, MN, USA; and PD-L2: #NBP1-88964, Novus Biologicals, Lit-

leton, CO, USA) and incubated with the slides for 2 hours at room temperature. The slides were subsequently incubated with the secondary antibodies (PD-L1: #CTS008 anti-goat-DAN cell & tissue staining kit, Novus Biological; and PD-L2: #K400311-2 envision HRP-labelled polymer anti-rabbit, Dako), and then counterstained using hematoxylin (Harri's hematoxylin solution, Merck Millipore, Billerica, MA, USA). Protein expression was subsequently assessed and scored by two pathologists using inverted light microscopy. The pathologists performed the scoring independently and were blinded to the patients' clinical outcomes.

All data were analyzed using SPSS software (ver. 18.0, SPSS Inc., Chicago, IL, USA) and P-values of < 0.05 were considered statistically significant. The chi-square and Fisher exact tests were used to evaluate the associations between the patients' clinicopathological characteristics and the expressions of PD-L1 and PD-L2. Overall survival (OS) and disease-free survival (DFS) were estimated using the Kaplan-Meier method, and differences were assessed using the log-rank test.

RESULTS

Among the 104 included patients, 31 patients (29.8%) had positive PD-L1 expression and 73 patients (70.2%) had negative PD-L1 expression. Positive PD-L2 expression was observed in 83 patients (79.8%) and negative PD-L2 expression was observed in 21 patients (20.2%) (Fig. 1). In the univariate analyses, PD-L1 expression was associated with pT status ($P = 0.009$), distant metastasis ($P = 0.009$), and stage ($P = 0.012$), although PD-L2 expression was not associated with any clinicopathological characteristics (Table 1).

Kaplan-Meier analysis and the log-rank test revealed that positive PD-L1 expression was associated with a lower 5-year survival rate, compared to negative PD-L1 expression (34.3% vs. 75.2%, $P < 0.001$) (Fig. 2A). However, no significant difference was observed when we compared positive and negative PD-L2 expressions (64% vs. 63.5%, $P = 0.961$) (Fig. 2B). The group with negative PD-L1 expression had a significantly lower recurrence rate, compared to the group with positive PD-L1 expression ($P = 0.006$), although no significant difference in recurrence was observed for PD-L2 expression ($P = 0.268$) (Fig. 3).

The univariate Cox proportional hazard analysis (Table 2) revealed that short OS was associated with positive PD-L1 expression ($P < 0.001$), advanced T status ($P = 0.04$), regional lymph node status ($P = 0.013$), distant metastasis ($P < 0.001$), and advanced stage ($P = 0.007$). Short DFS was associated with positive PD-L1 expression ($P = 0.008$) and advanced stage ($P = 0.021$). The expression of PD-L2 was not associated with the survival outcomes.

The multivariate analyses (Table 3) was performed with statisti-

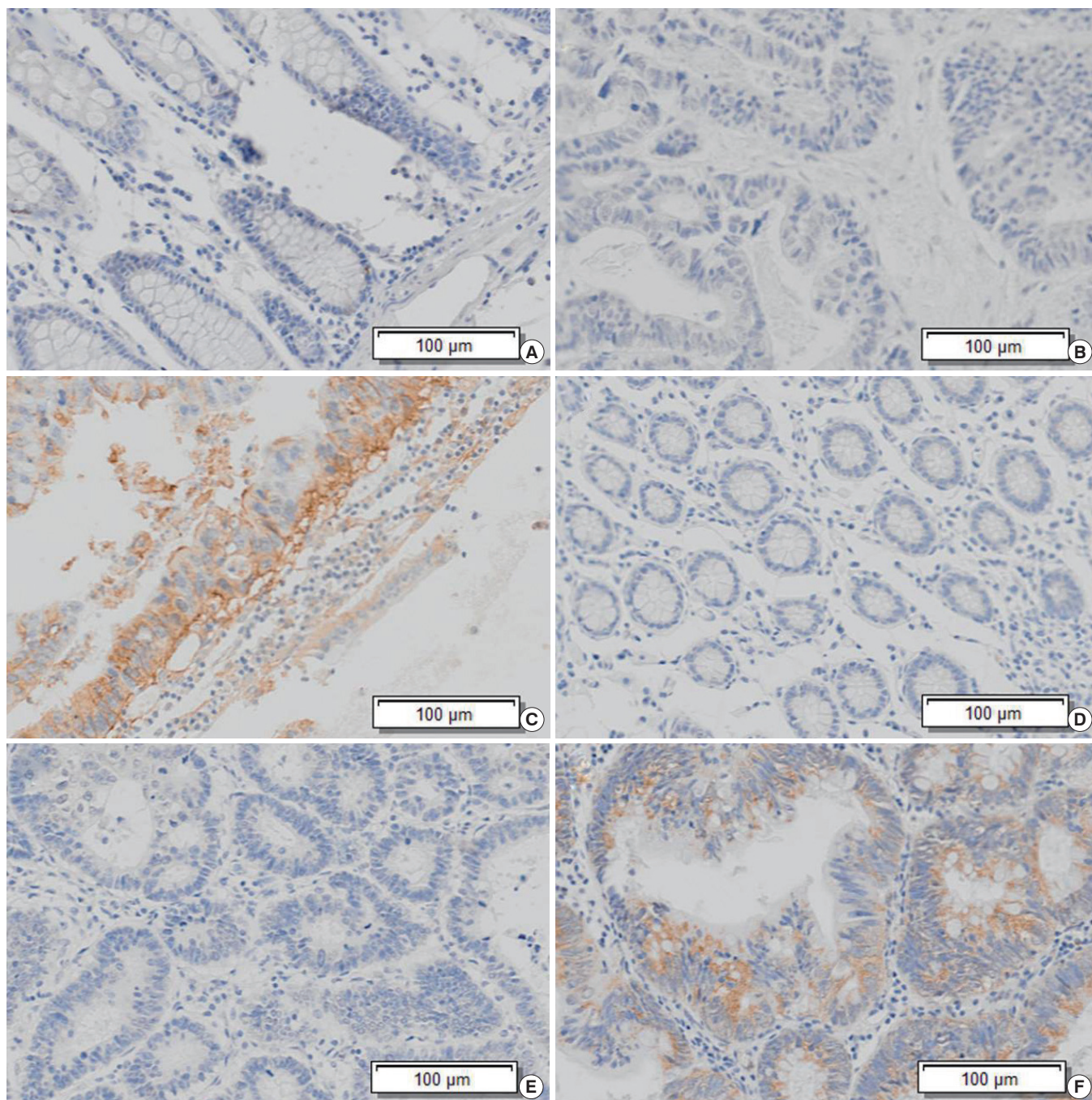


Fig. 1. Immunohistochemical staining of colorectal cancer using antibodies to programmed death (PD)-1 ligand 1 and PD-1 ligand 2-L1 and PD-L2. Representative staining patterns for the normal colon (A, D), negative expression of PD-L1 (B) and PD-L2 (E), and positive expression of PD-L1 (C) and PD-L2 (F).

cally significant factors ($P \leq 0.05$) by univariate analyses. The multivariate analyses revealed that short OS was independently associated with positive PD-L1 expression (hazard ratio [HR], 2.781; 95% confidence interval [CI], 1.284–6.026; $P = 0.01$), regional lymph node status (HR, 2.611; 95% CI, 1.258–5.418; $P = 0.01$), and distant metastasis (HR, 4.279; 95% CI, 1.449–12.638; $P = 0.009$). In addition,

short DFS was independently associated with positive PD-L1 expression (HR, 2.846; 95% CI, 1.393–5.815; $P = 0.004$) and regional lymph node status (HR, 2.310; 95% CI, 1.122–4.758; $P = 0.023$).

Table 1. Clinicopathologic characteristics and expressions of PD-1 ligand 1 and PD-1 ligand 2 among patients with colorectal cancer

Variable	No.	PD-L1 expression		P-value	PD-L2 expression		P-value
		Negative	positive		Negative	Positive	
Age (yr)				0.319			0.728
< 60	33	21 (63.6)	12 (36.45)		27 (81.8)	6 (18.2)	
≥ 60	71	52 (73.2)	19 (26.8)		56 (78.9)	15 (21.1)	
Sex				0.278			0.217
Male	42	27 (64.3)	15 (35.7)		36 (85.7)	6 (14.3)	
Female	62	46 (74.2)	16 (25.8)		47 (75.8)	15 (24.2)	
Location				0.941			0.705
Right	23	16 (69.6)	7 (30.4)		19 (82.6)	4 (17.4)	
Left	81	57 (70.4)	24 (29.6)		64 (79.0)	17 (21.0)	
pT stage				0.009			0.883
T1	7	7 (100)	0		5 (71.4)	2 (28.6)	
T2	20	17 (85.0)	3 (15.0)		17 (85.0)	3 (15.0)	
T3	72	48 (66.7)	24 (33.3)		57 (79.2)	15 (20.8)	
T4	5	1 (20.0)	4 (80.0)		4 (80.0)	1 (20.0)	
Lymph node metastasis (N1+N2)				0.732			0.884
No	53	38 (71.7)	15 (28.3)		42 (79.2)	11 (20.8)	
Yes	51	35 (68.6)	16 (31.4)		41 (80.4)	10 (19.6)	
Distant metastasis (pM)				0.009			0.651
No	98	72 (73.5)	26 (26.5)		78 (79.6)	20 (20.4)	
Yes	6	1 (16.7)	5 (83.3)		5 (83.3)	1 (16.7)	
Lymphatic invasion				0.354			0.483
No	86	62 (72.1)	24 (27.9)		68 (79.1)	18 (20.9)	
Yes	18	11 (61.1)	7 (38.9)		15 (83.3)	3 (16.7)	
Vascular invasion				0.729			0.154
No	93	66 (71.0)	27 (29.0)		76 (81.7)	17 (18.3)	
Yes	11	7 (63.6)	4 (36.4)		7 (63.6)	4 (36.4)	
Perineural invasion				0.135			0.735
No	99	68 (68.7)	31 (31.3)		79 (79.8)	20 (20.2)	
Yes	5	5 (100)	0		4 (80.0)	1 (20.0)	
Stage				0.012			0.923
I	21	18 (85.7)	3 (14.3)		16 (76.2)	5 (23.8)	
II	30	20 (66.7)	10 (33.3)		25 (83.3)	5 (16.7)	
III	47	34 (72.3)	13 (27.7)		37 (78.7)	10 (21.3)	
IV	6	1 (16.7)	5 (83.3)		5 (83.3)	1 (16.7)	

Values are presented as number (%).
 PD-L1, programmed death-1 ligand 1.

DISCUSSION

There are several pathways that allow malignant tumors to evade the host's immune response [11]. First, altered antigen presentation can cause T-cells to not recognize the tumor. Second, mutation of the MHC genes can alter the antigen recognition process. Third, immunosuppressive proteins can be produced to inhibit T-cell activation.

Many studies have recently examined the efficacy of immunological-related anticancer drugs and the patient's prognosis. The results indicate that tumor malignancy is closely related to the im-

mune response. For example, treatments targeting cytotoxic T lymphocyte-associated antigen 4 and PD-1 have been approved for treating melanoma, non-small cell lung cancer, and renal cancer. However, many questions remain regarding these drugs and their use to block checkpoint pathways.

The CD28 family plays a central role in the activation and tolerance of T cells, and PD-1 is a co-stimulatory molecule that provides a signal to inhibit T-cell activation. The ligands of PD-1 (PD-L1 and PD-L2) are cell surface glycoproteins belonging to the B7 family [8,12,13], and normal cytokine expression upregulates the expression of PD-L1 in T cells, B cells, and endothelial cells, which

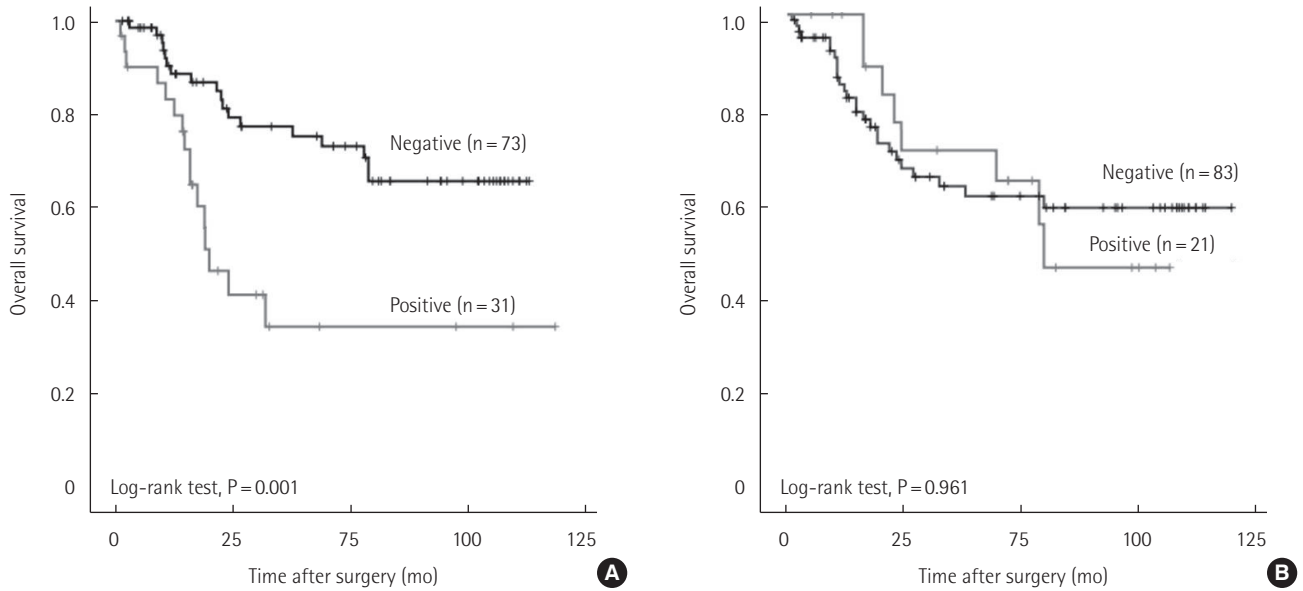


Fig. 2. Kaplan-Meier survival curves for overall survival according to programmed death (PD)-1 ligand 1 expression (A) and PD-1 ligand 2 expression (B).

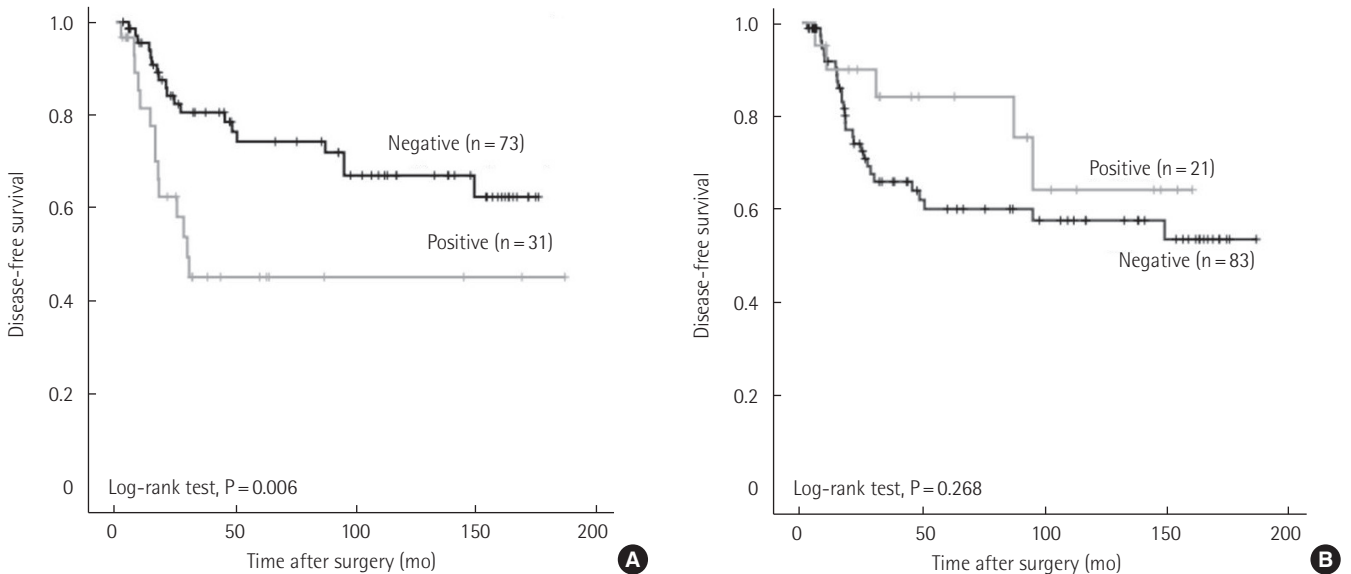


Fig. 3. Kaplan-Meier survival curves for disease-free survival according to programmed death (PD)-1 ligand 1 expression (A) and PD-1 ligand 2 expression (B).

helps maintain peripheral tolerance [14]. However, increased expression of PD-L1 on tumor cells increases the apoptosis of antigen-specific T-cells, which decreases the effectiveness of the immune response. Although the role of PD-L2 in the immune response remains unclear, one report has indicated that PD-L2 may also play a role in tumor immunity [10]. Thus, our findings may be useful in guiding research regarding the significance of PD-L1 and PD-L2 as prognostic factors for CRC.

Many studies have revealed an association between tumor ag-

gression and PD-L1 expression, although the precise mechanism for this association remains unclear. Some studies have indicated that PD-L1 expression on the tumor cell surface is controlled by interferon γ [7,13], although Song et al. [9] have reported that high PD-L1 expression was modulated by PTEN, which was associated with increased tumor staging and metastatic progression. Our study also indicates that patients with positive PD-L1 expression had more advanced T status and a higher rate of metastasis, compared to patients with negative PD-L1 expression. However, PD-

Table 2. Univariate Cox proportional hazard regression analysis of overall and disease-free survival among patients with colorectal cancer

Variable	OS	P-value	DFS	P-value
	HR (95% CI)		HR (95% CI)	
Age (> 60 vs. ≤ 60 yr)	1.845 (0.835–4.077)	0.130	0.692 (0.347–1.381)	0.296
Sex (male vs. female)	1.470 (0.716–3.016)	0.294	0.997 (0.499–1.990)	0.993
Tumor location (right vs. left)	0.894 (0.389–2.055)	0.792	0.856 (0.370–1.978)	0.716
T status (T1–2 vs. T3–4)	2.723 (1.048–7.074)	0.040	1.694 (0.730–3.932)	0.220
Regional lymph nodes (N0 vs. N1–N2)	2.501 (1.217–5.139)	0.013	2.090 (1.024–4.268)	0.043
Distant metastasis (M0 vs. M1)	8.619 (3.156–23.540)	<0.001	3.061 (0.718–13.058)	0.131
Staging (I–II vs. III–IV)	2.776 (1.325–5.816)	0.007	2.367 (1.142–4.906)	0.021
PD-L1 (negative vs. positive)	3.163 (1.580–6.331)	<0.001	2.583 (1.279–5.215)	0.008
PD-L2 (negative vs. positive)	0.981 (0.443–2.168)	0.961	0.591 (0.228–1.532)	0.279

OS, overall survival; DFS, disease-free survival; HR, hazard ratio; CI, confidence interval; PD-L1, programmed death-1 ligand 1.

Table 3. Multivariate Cox proportional hazard regression analysis of overall and disease-free survival among patients with colorectal cancer

Variable	OS	P-value	DFS	P-value
	HR (95% CI)		HR (95% CI)	
T status (T1–2 vs. T3–4)	1.330 (0.453–3.906)	0.604		
Regional lymph nodes (N0 vs. N1–2)	2.611 (1.258–5.418)	0.010	2.310 (1.122–4.758)	0.023
Distant metastasis (M0 vs. M1)	4.279 (1.449–12.638)	0.009		
PD-L1 (negative vs. positive)	2.781 (1.284–6.026)	0.010	2.846 (1.393–5.815)	0.004

OS, overall survival; DFS, disease-free survival; HR, hazard ratio; CI, confidence interval; PD-L1, programmed death-1 ligand 1.

L2 expression was not associated with patient age, sex, tumor status, nodal status, and metastasis. Similar to PD-L1, PD-L2 expression is associated with a poor prognosis in other types of carcinoma, such as esophageal cancer [11], although we are not aware of any reports regarding PD-L2 expression and CRC.

The present study revealed that PD-L1 expression was associated with poor survival outcomes. Poor OS was independently associated with regional lymph node metastasis, distant metastasis, and PD-L1 expression, while poor DFS was independently associated with advanced stage and PD-L1 expression. Similar results have been observed in previous studies [9,15,16]. However, T status was not associated with OS or DFS, although most patients with CRC in the present study had T3 status. Furthermore, PD-L2 expression was not significantly associated with survival among our patients with CRC. In contrast, Hamanishi et al. [17] have reported that high PD-L2 expression was non-significantly associated with poorer survival in cases of ovarian cancer. Moreover, Gao et al. [18] have reported that high PD-L2 expression is associated with poor survival, but not with the risk of recurrence. Thus, the existing evidences suggests that survival is significantly related to PD-L1 expression, although the relationship between survival and PD-L2 expression remains unclear.

The present study has several limitations. First, there appears to have been selection bias, as most of the included patients had T3 status. Second, we did not have information regarding tumor-infil-

trating lymphocytes (TILs), which are associated with PD-L expression and the host's immune response to malignancy [19]. Furthermore, several reports have indicated that TILs are a prognostic factor for malignant tumors [20–22]. Third, additional research is needed to determine whether PD-L expression is associated with mismatch repair (MMR) in CRC, which could lead to functional loss of the MMR pathway [23]. Moreover, errors in microsatellite regions can lead to microsatellite instability and an increased risk of CRC [24]. In this context, Diaz and Le [25] performed a phase II trial that revealed MMR-deficient tumors have greater expressions of TILs and PD-1 (vs. MMR-proficient tumors), which might affect the clinical response and prognosis in cases of CRC.

In conclusion, our findings indicate that positive PD-L1 expression in patients with CRC was associated with poor OS and DFS. However, further prospective multi-center studies are needed to examine the value of PD-L1 as a therapeutic target or prognostic biomarker for cases of CRC.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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