Programmed Cell Death Ligand 1 Expression Is an Independent Prognostic Factor in Colorectal Cancer

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Abstract. *Background/Aim:* Programmed cell death protein 1 (PD-1)/ programmed cell death ligand 1(PD-L1) axis is associated with immune tolerance via inhibition of T cell activation. The aim of this study was to clarify the significance of PD-1 and PD-L1 expressions and analyze the relationships between PD-1, PD-L1, transforming growth factor- β (TGF- β) and Forkhead box P3 (Foxp3) expressions in colorectal cancer (CRC). *Patients and Methods:* A total of 116 patients who underwent curative colectomy for stage II/III CRC were included in the study. PD-1, PD-L1, TGF- β , and Foxp3 expressions were examined by immunohistochemistry and related to prognostic factors by Kaplan–Meier. *Results:* PD-1 expression was correlated with PD-L1, TGF- β , and Foxp3 expressions. Overall survival rates were significantly poorer in the PD-1 and PD-L1-positive groups. Multivariate analysis showed that PD-L1-positive is an independent risk factor. Disease-free survival (DFS) was tended in the PD-L1-positive group. The group with double-positive expression had significantly poorer prognosis. *Conclusion:* PD-1 and PD-L1 expressions were associated with a poor prognosis and correlated with TGF- β and Foxp3 expressions in patients with CRC.

Colorectal cancer (CRC) is one of the most common cancers worldwide and the fourth most common cancer in Japan (1, 2). More than 1 million new cases were reported annually resulting in about 600,000 deaths worldwide per year (3).

Programmed cell death protein 1 (PD-1) is one of the member of B7/CD28 family, first reported in 1992 (4, 5) and

expressed in activated immune cells (4, 6). PD-1 has two ligands, programmed death-ligand 1 (PD-L1) and programmed death-ligand 2 (PD-L2). Each of them has an additional binding partner, B7-1 for PD-L1 and RGMb for PD-L2 (7, 8). PD-1 and its ligand, programmed cell death ligand 1 (PD-L1), interact to suppress T cells activation in many conditions (9).

Recently, the significance of PD-1 or PD-L1 expressions were reported in several types of cancers including rectal cancer, skin cancer, gastric cancer, and breast cancer (10-13). Also, we previously reported PD-1 and PD-L1 expression in gastric cancer after curative resection. PD-1 correlated with poor prognosis and associated with transforming growth factor- β (TGF β) and Forkhead box P3 (FoxP3) expression. However, little is known about the significance of PD-1 and PD-L1 expression in colorectal cancer.

The aim of this study was to investigate the role of PD-1 and PD-L1 expression and analyze the relationships among PD-1, PD-L1, Foxp3, and TGF- β expressions in patients with colorectal cancer.

Patients and Methods

Patients. A total of 116 patients who had undergone curative surgical resection for stage II/III colorectal cancer at Tokushima University Hospital between 2004 and 2009 were included in this study (59 patients with stage II and 57 patients with stage III). There were 77 men and 39 women, with a mean age of 69.7 years (range=41-93 years). The mean follow-up period was 52 months (range=1-115 months). All final stage III patients underwent adjuvant chemotherapy. Factors were defined according to the 14th edition of the Japanese Classification of Colorectal Carcinoma. Japanese Society for cancer of colon and rectum (14). The Ethical Committee of Tokushima University approved the study, and all patients gave written informed allowance (No. 2349). This study was conducted in accordance with the Declaration of Helsinki. Patients' information was obtained from the medical records of the institute.

Immunohistochemistry. The method of immunohistochemistry was previously reported (11). Tissue samples were formalin fixed and paraffin embedded. Serial sections were cut at 5 μ m, dewaxed, deparaffinized in xylene, and rehydrated through a series of graded alcohols. Samples were boiled for 20 min in a microwave oven in

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citrate buffer (pH 6.0) for antigen retrieval. Endogenous peroxidases were blocked with 0.3% hydrogen peroxidase for 30 min, followed by incubation in 5% goat serum for 60 min to prevent non-specific antigen binding. The slides were then incubated with primary antibodies overnight at 4°C. The following primary antibodies and dilutions were used: mouse monoclonal antibody for PD-1 (AF1086, 1:40; R&D Systems, Minneapolis, MN, USA), rabbit monoclonal antibody for PD-L1 (ab174838, 1:100; Abcam, Cambridge, UK), and mouse monoclonal antibody for Foxp3 (ab20034, 1:100; Abcam). Secondary antibody binding was detected with Histofine SAB-PO (Nichirei, Tokyo, Japan) for PD-1 and EnVision Dual Link System-HRP (Dako, Glostrup, Denmark) for PD-L1 and Foxp3. A secondary peroxidase-labeled polymer conjugated to goat anti-mouse immunoglobulins was applied for 60 min. The sections were developed in 3,3'-diaminobenzidine and were counterstained with Mayer's hematoxylin. Each slide was dehydrated through graded alcohols and covered with a coverslip. The presence of positive cells on each slide was determined by a pathologist in a blinded manner (15). PD-1 positivity was recorded if more than 20% T cells were stained in a $\times 400$ high-power field at the center of the tumor (Figure 1A). Thirty-nine patients (33.6%) were PD-1 positive.

PD-L1 expression was predominantly located in the cytoplasm, with some nuclear membrane localization at the invasive front of the tumor. Staining intensity was graded as follows: 0 for no staining, 1+ for weak staining, 2+ for moderate staining, and 3+ for strong staining. Distribution was graded according to the percentage of PD-L1-positive cancer cells and then divided into quartiles as follows: no staining, 0-5% staining; 1+, 6-25% staining; 2+, 26-50% staining; 3+, 51-75% staining; and 4+, 76-100% staining. A total score of more than 3+ was defined as PD-L1-positive expression (Figure 1B) (16). Fifty-two patients (44.8%) were PD-L1 positive.

Foxp3 positivity was recorded by counting more than ten Foxp3-stained T cells in the cancer tissue at $\times 200$ highpower field at the center of the tumor (Figure 1C) (17, 18). Seventy-three patients (62.9%) were Foxp3 positive.

TGF- β , the positive-cell percentage was scored 0 to 3 from <5%, 5% to 25%, 26% to 75%, to >75% in order. The staining intensity was scored 0 to 2 (score, 0; none, 1; weak, 2; strong), in order. The sum of positive-cell percentage and staining intensity scores was evaluated as follows: negative ≤ 3 ; positive > 3 (19). Seventy-one patients (61.2%) were TGF- β positive.

Statistical analysis. All statistical analyses were performed using SPSS Version 21 statistical software (IBM, Armonk, NY, USA). The chi-square test was used to compare categorical variables. Patients' survival was calculated by the method of Kaplan-Meier, and differences in survival rates between the groups were compared using the log-rank test. All significant factors from univariate analysis were calculated in the multivariate analysis with Cox's proportional hazard model and stepwise regression model to identify independent factors that influence patient survival. A *p*-value less than 0.05 was considered statistically significant.

Results

The characteristics of the PD-1-positive and PD-1-negative groups are shown in Table I. In terms of clinicopathological variables, PD-1 expression was positively correlated with PD-L1 expression ($p=0.029$), Foxp3 expression ($p=0.09$), and TGF- β expression ($p=0.039$) (Figure 2, Table I). PD-L1

expression was correlated with age ($p=0.034$), lymphatic invasion ($p=0.040$), tumor location ($p=0.003$), Foxp3 expression ($p<0.005$), and TGF- β expression ($p<0.005$) (Figure 2, Table II).

Overall survival (OS) rates in the PD-1-positive group were significantly poorer than those in the PD-1-negative group (5-years OS rate, 74.0% for PD-1-positive group *vs.* 92.4% for PD-1-negative group; $p=0.014$). OS rates in the PD-L1-positive group were significantly poorer than those in the PD-L1-negative group (5-years OS rate, 76.7% for PD-L1-positive patients *vs.* 93.2% for PD-L1-negative patients; $p=0.005$). Multivariate analysis showed that PD-L1-positive expression is an independent risk factor (relative risks of 3.873) (Figure 3A and B, Table III).

Disease-free survival (DFS) was similar in patients with and without PD-1 expression (5-years DFS rate, 77.9% *vs.* 78.1%, respectively; $p=0.719$). DFS in the PD-L1-positive group tended to be poorer than that in PD-L1-negative group (5-years DFS rate, 69.7% *vs.* 83.3%, respectively $p=0.07$). Univariate analysis showed that stage III tumor and positive lymph node invasion have significantly worse prognosis. Multivariate analysis showed that stage III, and lymphatic invasion are independent risk factors for recurrence (relative risks of 3.148, 1.964, respectively) (Figure 3C, D, Table IV).

The group with both PD-1-positive and PD-L1-positive expression ($n=23$) had significantly poorer OS than the group with double-negative expression (5-years OS rate, 68.6% *vs.* 97.6%, respectively; $p=0.002$), but no significant difference in DFS (5-years OS rate, 76.7% *vs.* 85.4%, respectively; $p=0.286$) (Figure 3E and F).

Discussion

This study demonstrated that PD-1 high expression was a poor prognostic factor in terms of OS and was correlated with high expressions of PD-L1, TGF- β , and Foxp3 in patients with stage II/III colorectal cancer after curative resection. This is the first report that confirmed the relationships among PD-1, PD-L1, TGF- β , and Foxp3 expression in colorectal cancer.

PD-1 and PD-L1 play important roles in the regulation of the immune system and the maintenance of peripheral immune tolerance through T cell activation and inhibition (16, 20). PD-1 is one of the member of B7/CD28 family, expressed in activated CD4+ T cells, CD8+ T cells, B cells, natural killer T cells, activated monocytes, DCs, macrophages. Programmed death-1 (PD-1, CD279) has two ligands called programmed death-ligand 1 (PD-L1, also called B7-H1; CD274) and programmed death-ligand 2 (PD-L2, also called B7-DC; CD273). PD-1 and its ligand, PD-L1, interact to suppress the activation of T cells in many conditions including autoimmune disease, chronic infection, and cancer (4, 6).

Figure 1. Immunohistochemistry. (A) Programmed cell death protein 1 (PD-1)-positive expression in cancer tissue, (B) programmed cell death ligand 1 (PD-L1)-positive expression in cancer tissue, (C) Foxp3-positive expression in cancer tissue, (D) TGF- β -positive expression in cancer tissue.

Table I. Characteristic of programmed cell death protein 1 (PD-1)-positive and PD-1-negative.

Variables	Negative (n=77)	Positive (n=39)	p-Value
Age (<70/ $\geq$ 70)	39/38	16/23	0.327
Sex(female/male)	22/55	15/24	0.280
Differentiation (moderate, poor/well)	45/32	25/14	0.556
Depth of tumor invasion (T1, T2/T3, T4)	5/72	3/36	0.810
Stage (II/III)	39/38	20/19	0.949
Venous invasion (-/+)	38/39	16/23	0.396
Lymphatic invasion (-/+)	47/30	23/6	0.830
Tumor location (left/right)	52/25	27/12	0.853
Maximum tumor diameter (<4 cm/ $\geq$ 4 cm)	32/45	10/29	0.092

Table II. Characteristic of programmed cell death protein ligand 1 (PD-L1)-positive and PD-L1-negative.

Variables	Negative (n=64)	Positive (n=52)	p-Value
Age (<70/ $\geq$ 70)	36/28	19/33	0.034
Sex (female/male)	20/44	17/35	0.868
Differentiation (moderate, poor/well)	38/26	32/20	0.813
Depth of tumor invasion (T1, T2/T3, T4)	4/60	4/48	0.760
Stage (II/III)	34/30	25/27	0.589
Venous invasion (-/+)	29/35	25/27	0.767
Lymphatic invasion (-/+)	40/20	26/26	0.040
Tumor location (left/right)	51/13	28/24	0.003
Maximum tumor diameter (<4 cm/ $\geq$ 4 cm)	27/37	15/37	0.137

It was reported that PD-1 or PD-L1 expression status was a significant prognostic factor in epithelial-originated tumors (21). This report showed that PD-1 is expressed on immune cells in response to inflammatory cytokines stimuli, and tumor cells can express PD-L1 to suppress T-cell-mediated antitumor immunity (21). In colorectal cancer, few studies have checked PD-1 and PD-L1 status, and their roles remain unclear (13, 22, 23). A recent report proved that PD-L1-positive expression in rectal cancer was associated with poor prognosis (13), while another report defined that increased

PD-1 expression on CD4⁺ and CD8⁺ T cells was involved in immune escape (22). High-PD-1/CD8 ratio indicated the worse prognosis in stage II, III colorectal cancer (24).

There are several reports about high PD-L1 expression correlated with poor prognosis of CRC patients. However, the relationship between PD-1 and colorectal cancer has not been reported yet (25, 26).

Previous studies suggested that immune-checkpoint inhibitors of PD-1 and PD-L1 such as Nivolumab, Pembrolizumab have potential benefits for colorectal cancer

Figure 2. Correlation between (A) programmed cell death protein 1 (PD-1) and programmed cell death ligand 1 (PD-L1), (B) PD-L1 and Foxp3, (C) PD-1 and Foxp3, (D) PD-L1 and TGF- β , and (E) PD-1 and TGF- β .

Table III. Overall survival PD-1/PD-L1 in colorectal cancer (n=116).

Variables	5-years OS rate (%)	Univariate	Multivariate	
		p-Value	HR (95%CI)	p-Value
Age (<70/≥70)	87.8/85.6	0.929		
Sex (female/male)	80.6/89.4	0.144		
Differentiation (moderate, poor/well)	88.2/84.5	0.785		
Depth of tumor invasion (T1, T2/T3, T4)	83.3/87.0	0.264		
Stage III (-/+)	90.7/81.6	0.165		
Venous invasion (-/+)	86.9/86.5	0.869		
Lymphatic invasion (-/+)	89.8/82.0	0.293		
Tumor location (left/right)	90.0/78.5	0.210		
Maximum tumor diameter (<4 cm/≥4 cm)	89.3/85.0	0.803		
PD-1 (-/+)	92.4/74.0	0.014	2.789 (0.954-8.152)	0.061
PD-L1 (-/+)	93.2/76.7	0.005	3.873 (1.193-12.571)	0.024

Table IV. Disease-free survival PD-1/PD-L1 in colorectal cancer (n=116).

Variables	5-years DFS rate (%)	Univariate	Multivariate	
		p-Value	HR (95%CI)	p-Value
Age (<70/≥70)	78.6/77.3	0.524		
Sex (female/male)	73.1/80.1	0.377		
Differentiation (moderate, poor/well)	80.8/73.5	0.458		
Depth of tumor invasion (T1, T2/T3, T4)	71.4/78.3	0.508		
Stage III (-/+)	89.2/65.7	0.003	3.148 (1.203-8.240)	0.020
Venous invasion (-/+)	83.8/72.9	0.195		
Lymphatic invasion (-/+)	85.3/65.4	0.009	1.964 (0.814-4.741)	0.133
Tumor location (left/right)	75.8/83.6	0.465		
Maximum tumor diameter (<4 cm/≥4 cm)	81.1/75.7	0.468		
PD-1 (-/+)	78.1/77.9	0.719		
PD-L1 (-/+)	83.3/69.7	0.070	1.913 (0.811-4.516)	0.139

Figure 3. Kaplan-Meier analysis of overall survival and disease-free survival for programmed cell death protein 1 (PD-1) (A, B) and programmed cell death ligand 1 (PD-L1) (C, D) after curative resection in patients with colorectal cancer. Survival for double PD-1 and PD-L1 expression (E, F).

treatment (27). Colorectal cancer is one of the cancers, which have the most MSI. Although, it was clearly reported that PD-1 blockade could decrease MSI rate and that MSI rate can be used as PD-1 blockade biomarker in many cancers, there is no report yet about in colorectal cancer (28, 29). Therefore, MSI and PD-1/PD-L1 relationship is still unknown in colorectal cancer.

In the current study, PD-1 expression status was positively correlated with expression of Foxp3, a well known transcription factor playing a role in the development and function of Tregs cells (30) and TGF- β b. Transforming growth factor beta (TGF- β) is a multifunctional cytokine that suppresses immune response (31, 32). TGF- β also

converts effector T-cells, which normally attack cancer into regulatory (suppressor) T-cells (9). Forkhead box P3 (Foxp3) is a master regulator that induces Foxp3 regulatory T cells which suppress antitumor immune response (22, 33, 34). It was already shown that Tregs have significant correlations with cancer aggression by suppressing the T cell response through membrane-bound transforming growth factor beta 1 (TGF- β 1) (9). Tregs partly express PD-1, a key regulator of immune tolerance (35). Moreover, PD-1, PD-L1, and Foxp3 have been confirmed to be associated with breast cancer progression via immunosuppressive subsets of T cells in the cancer microenvironment (10). Previous reports suggest that PD-1-positive T cells might induce

Tregs through transforming growth factor $\beta 1$ in various cancers including colorectal cancer.

Immunotherapeutic agents targeting T cell immune checkpoints such as PD-1, PD-L1, cytotoxic T-lymphocyte-associated antigen 4, and T-cell-immunoglobulin- and mucin-domain-containing molecule 3 have been investigated as potential treatments for cancer (36, 37). These immunotherapeutic agents have shown promising effects for several cancers, including non-small cell lung cancer, melanoma, and renal cell carcinoma (38-40). Our study confirmed that PD-1 expression is a favorable prognostic factor for patients' survivals after curative colectomy. Immunotherapy targeting PD-1 and PD-L1 may thus be a useful adjuvant treatment in patients with colorectal cancer.

While PD-1 and PD-L1 were independent prognostic factors for overall survival, no significant differences were seen in DFS. This leads to the possible conclusion that PD-1 and PD-L1 are related to metastasis related deaths than recurrence related deaths. Previous reports also described that PD-L1 expression was correlated with tumor aggressiveness including metastatic rates (12, 41).

Conclusion

High expressions of PD-1 and PD-L1 were associated with poor prognosis after surgery and are correlated with high expressions of TGF- β and Foxp3 in patients with colorectal cancer. Therefore, the PD-1/PD-L1 pathway may be a new useful therapeutic target for the future treatment of colorectal cancer.

Conflicts of Interest

The Authors declare that they have no conflict of interest in regard to this study.

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