PD-L1 Expression in Pancreatic Ductal Adenocarcinoma is a Poor Prognostic Factor in Patients with High CD8^{+} Tumor-Infiltrating Lymphocytes: Highly Sensitive Detection using Phosphor-Integrated Dot Staining

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Abstract

Background. Pancreatic ductal adenocarcinoma (PDAC) has an extremely poor prognosis. For the development of more effective immunotherapies, it is first necessary to elucidate the immunological escape mechanisms. In this study, we applied our recently developed highly sensitive immunostaining method employing fluorescent phosphor-integrated dot (PID) nanoparticles to evaluate the prevalence of programmed death ligand 1 (PD-L1) in patients with PDAC.

Methods. This study included 42 patients with PDAC who underwent pancreatectomy. We evaluated PD-L1 expression in the patients with PDAC using PID staining, and correlated the PD-L1 expression level with the patients' clinico-pathological features.

Results. PD-L1 expression was detected in 61.9% (26/42) of the patients with PDAC by PID staining. There was a significant difference in overall survival between PD-L1-positive and PD-L1-negative cases (hazard ratio [HR] = 2.07; 95% confidence interval [CI] = 1.00–4.54; P = 0.049). Among $CD8^+$ tumor-infiltrating lymphocyte-positive cases, the overall survival of PD-L1-positive patients was significantly poorer than that of PD-L1-negative patients (HR = 3.84; 95% CI = 1.59–10.35; P = 0.003). Univariate and multivariate analyses indicated that PD-L1 expression was an independent predictive poor prognostic factor in patients with PDAC.

Conclusions. PD-L1 expression appears to be an important prognostic factor in patients with PDAC who underwent surgical resection.

Key word: pancreatic adenocarcinoma, PD-L1, immunohistochemistry, phosphor-integrated dots

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease with a poor prognosis; it is the fifth leading cause of cancer-related deaths in Japan and the fourth most common malignancy in the United States [1,2]. At the time of PDAC diagnosis, less than 20% of the tumors are resectable, and the actual 5-year survival rate is reported to range from 15% to 25% [3]. Current clinical treatments for PDAC have limited efficacy; thus, improved treatment strategies are required to prolong patient survival.

Expression of programmed death-1 (PD-1) is significantly upregulated on activated cancer-specific T cells. PD-1 receptor attaches to its ligand PD-L1, which is expressed by tumor cells and infiltrating immune cells. The interaction of PD-1 and PD-L1 inhibits T-cell activation and promotes tumor immune escape.[4−7] The escape mechanism acquired by tumor cells to avoid immune recognition and destruction is a major contributor to the limitations of the therapeutic efficacy. However, recently developed therapeutic antibodies against PD1/PD-L1 show promising clinical results for several tumors such as melanoma, renal cancer, and non-small cell lung cancer [8].

Although the efficacy of PD-1/PD-L1 antibody therapy should be correlated with PD-L1 protein expression in tumor cells, approximately 10–40% of PD-L1 immuno-negative cases also respond to anti-PD-1/PD-L1 therapy [9−11]. This contradiction may be caused by the performance of the PD-L1 immunostaining assay based on the color intensity visualized using the chromogen dye diaminobenzidine (DAB). We recently developed an immunohistochemistry method using fluorescence-emitting phosphor-integrated dot (PID) nanoparticles as a fluorescent dye. PID shows a higher luminance and dynamic range than those of conventional fluorescent dyes and DAB [12]. Specifically, the fluorescence intensity of the PID particles was found to be approximately 100-fold higher than that of a conventional fluorescent dye. Furthermore, the ratio of particle to antibody binding is 1:1. Thus, this technique is highly sensitive as well as quantitative as compared to the conventional DAB-based method.

The aim of the present study was to evaluate the expression of PD-L1 in patients with PDAC by immunostaining using PID technology, and to compare the results with those obtained after conventional DAB staining.

Materials and methods

Patients and samples

This study included 42 patients with PDAC, 31 of whom underwent pancreaticoduodenectomy and 11 underwent distal pancreatectomy at the Department of Surgery at Kansai Medical University Hospital (Osaka, Japan) between May 2001 and December 2007. All patients were histologically confirmed as positive for PDAC. The tumors were classified according to the TNM classification [13]. A prospectively maintained institutional PDAC database was used to collect all the clinical parameters of the patients.

Surgically resected specimens were fixed with formalin and embedded in paraffin, and serial sections were stained with hematoxylin and eosin for histological evaluation. The most representative tumor areas were sampled for the tissue microarray using 2-mm-diameter samples (Azumaya, Tokyo, Japan).

This study was conducted in accordance with the Declaration of Helsinki, and the study protocol was approved by the institutional review board of our hospital (Protocol No. H151043 and 27-14).

Immunohistochemistry

Four-micrometer-thick sections were routinely deparaffinized. To block endogenous peroxidase activity, deparaffinized sections were subjected to 3% hydrogen peroxide treatment for 15 min and were then washed in deionized water for 2 to 3 min. Then, the sections were boiled in 10 mM sodium citrate buffer for 10 min at 121°C. The sections were allowed to cool at room temperature for 40 min, followed by rinsing with deionized water and washing with phosphate-buffered saline for 5 min.

Measurement of the fluorescence properties of PID

The sections were incubated with the primary antibody toward PD-L1 (E1L3N, 1:1000, Cell Signaling Technology, Danvers, MA, USA). Sections were incubated with 2 μg/mL biotinylated anti-rabbit antibody (LO-RG-1) for 30 min, and then with PID-conjugated streptavidin (0.06 nM)

for 2 h, both at room temperature. The sections were then irradiated at 580 nm, and the fluorescence intensities were measured using a BX53 fluorescence microscope (Olympus, Tokyo, Japan) and images were acquired with a DP73 CCD camera (Olympus) (Fig. 1). The number of PID particles per cell was measured with an automated PID Analyzer (Konica Minolta, Tokyo, Japan). The PD-L1 particle number was evaluated only on the tumor cells. Five fields at 400× magnification were selected randomly, and the number of PD-L1 particles on each tumor cell was counted and the average number of particles per cell was calculated for each field. The highest value among the five fields was determined to be the PID staining value. The negative control was prepared with PID staining but without the primary antibody.

Measurement of DAB intensity

The sections were incubated with the primary antibody toward PD-L1 (E1L3N, 1:200, Cell Signaling Technology) diluted in antibody diluent (Signal Stain Antibody Diluent #8112, Cell Signaling Technology). The sections were treated with peroxidase-labeled secondary antibody (EnVision/HRP system, DAKO, Carpinteria, CA, USA) after linker reagent treatment for 15 min. The sections were then rinsed in the buffer and immersed in DAB to observe color development.

For DAB-naked eye evaluation, three observers (S.Y., H.R., and K.T.) assessed the immunostaining results in a blinded manner without knowledge of the clinical or histopathological diagnoses. Intensity was graded on a 3-tier scale (1+, negative to weak; 2+, moderate; and 3+, strong). The percentage of staining was recorded, and a semi-quantitative (H-score) approach [14] was used for analysis. PD-L1 expression scores were calculated (from 0 to 300) by multiplying the percentage of the stained tumor area by the staining intensity score.

Double staining of PD-L1 using the PID method and of CD8+ lymphocytes using the DAB method

Double staining, i.e., DAB staining for CD8+ lymphocytes and PD-L1 staining with the PID method, was performed to count the tumor infiltrating lymphocytes (TILs) (primary antibody for PD-L1: E1L3N, 1:100; primary antibody for CD8: C8/114B, 1:250; DAKO; secondary antibody: EnVision/HRP system) (Fig. 2).

The correlations between PD-L1 expression, overall survival, and clinico-pathological data

were evaluated.

Statistical analysis

Statistical analysis was performed using the JMP® 10 software (SAS institute Inc., Cary, NC, USA). The Student's t-test was used to analyze continuous variables and the chi-square test was used to analyze categorical variables. Cumulative survival rates were calculated by the Kaplan–Meier method. Significant differences in survival status were evaluated using the log-rank test. The Cox proportional hazards model was used in the multivariate analysis and expressed by the hazard ratio (HR) with a 95% confidence interval (CI). $P < 0.05$ was considered statistically significant.

Results

Patient characteristics

The patient background and clinico-pathological parameters are shown in Table 1. Among all the patients, 16 were women and 26 were men. The patients' median age at the time of diagnosis was 65 years (range 50−83 years). This study comprised 3, 6, 33, and 0 patients diagnosed at the T1, T2, T3, and T4 stages, respectively. Lymph node metastases were detected in 26 of 42 patients (61.9%). Seven patients were identified as M1 because of No. 16 lymph node metastasis without other organ metastasis. Considering the pathological stage (pStage) as defined by the Union for International Cancer Control classification, 3, 4, 9, 19, and 7 cases were at pStage Ia, Ib, IIa, IIb, and IV, respectively. The median survival time (MST) of the 42 patients was 26 months (Fig. 3).

Pathological curative resection (R0) was performed in 27 of the 42 patients (64.3%). Neo-adjuvant chemo-radiation therapy was performed in 8 of the 42 patients (19.0%), and adjuvant chemotherapy was performed in 23 of the 42 patients (54.8%).

PD-L1 expression in the 42 PDAC patients by DAB and PID staining

To judge the rate of positive PID staining, the threshold of the PID staining value was established. As the average of the highest value of the negative control of PID staining was 3.01, the threshold value for judging positive PID staining was set to 3.0. Using this threshold, PD-L1

expression was detected in 26 of the 42 patients (61.9%).

By contrast, PD-L1 expression measured by DAB-naked eye evaluation was detected in only 6 of the 42 patients (14.3%). This expression rate was measured to be between the PID positive staining value derived from the threshold set at 4.0 (28.6%, 12/42) and 5.0 (11.9%, 5/42).

Relationship between PD-L1 expression and clinico-pathological features

The correlations between pathological features and PD-L1 expression are shown in Table 2. The ratio of males was significantly higher in the PD-L1-positive group (male/female: 20/6) than in the PD-L1-negative group (male/female: 6/10; P = 0.021). There was no significant correlation between PD-L1 expression and tumor size, lymph node metastasis (including distant lymph node metastasis [M1]), pre-operative CA19-9 level, and R0/1 status. CD8$^+$ TILs counts were not significantly correlated to PD-L1 expressions.

Survival analysis

Survival curves obtained using the Kaplan-Meier method are shown in Fig 4a,b. Among the 42 patients, there was a significant difference in the overall survival rate between the PD-L1-positive cases and PD-L1-negative cases using PID staining (HR = 2.07; 95% CI = 1.00–4.54; P = 0.049). The MST was 23.5 months in the PD-L1-positive group and 51.6 months in the PDL-1-negative group. Among 29 patients who had positive CD8$^+$ TIL (more than 3 cells per 400× field), the PD-L1-positive group showed a significantly poorer overall survival rate than the PD-L1-negative group based on detection with the PID method (HR = 3.84; 95% CI = 1.59–10.35; P = 0.003).

The results of univariate and multivariate analyses in the 42 patients are shown in Table 3. Univariate and multivariate analysis revealed that PD-L1 expression determined by the PID method (PID staining value > 3.0) was an independent prognostic factor (HR = 2.34; 95% CI = 1.02–5.74; P = 0.045). In the 29 TIL-positive patients, PD-L1 expression determined by the PID method was an independent predictive poor prognostic factor (Table 4). Moreover, there was a stronger prognostic effect of PD-L1 expression among these 29 TIL-positive cases compared to the analysis including all 42 patients (HR = 4.39; 95% CI = 1.64–13.34; P = 0.003).

Discussion

In this study, we investigated PD-L1 expression in patients with PDAC. The PD-L1-positive detection rate in PID staining was higher than that in DAB staining, which was possibly due to the fact that digital immunostaining can detect proteins at lower concentrations. The higher fluorescence intensity of the nanoparticles contributes to the higher sensitivity of PID compared to DAB. In addition, quantitative analysis was possible even in cells with strong positive expression, without saturation.

In this study, the PD-L1 positive expression rate detected using the PID method in PDAC cases was higher than that reported in previous studies (4–49%) [15-17] using the DAB method. Among the current cases, no case was found to be negative for PID and positive for DAB. The PD-L1 positive expression rate was 14.3% using the DAB method and was 61.9% using the PID method when the PID staining threshold value was set to 3.0. Thus, this result supported the current PD-L1 protein expression data using PID, which was found to be more sensitive than DAB. However, the DAB staining positive rate was similar to the PID staining positive rate when using the threshold of 4.0 or 5.0. In other words, cells with slightly positive expression showing 3–5 PID particles could not be detected by conventional methods.

Immunotherapeutic approaches, most notably, immune checkpoint inhibitors epitomized by antibodies directed against T-lymphocyte regulators, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and PD-1 have demonstrated efficacy in a variety of solid tumors, including metastatic melanoma and lung cancer, and have already received US Food and Drug Administration approval. PD-1/PD-Ll pathway blockade has resulted in significant and durable clinical responses in patients with several malignancies such as melanoma, renal cell carcinoma, lung cancer, mismatch repair-deficient colorectal cancer, and bladder cancer [18]. However, PDAC has generally been considered as a non-immunogenic malignancy, given that tumor-infiltrating effector T lymphocytes do not represent a histopathological hallmark of this disease [19,20]. Investigators have been actively exploring the mechanisms underlying the evasion of immune surveillance by pancreatic cancer cells, and several potential strategies have been proposed to overcome resistance to immune checkpoint inhibitors. In this study, TILs reached tumor cells in 29 of 42 surgical PDAC specimens.

In a clinical setting, patients with positive PD-L1 expression tend to show significantly unfavorable outcomes. Previous studies using DAB methods have also demonstrated that patients with PD-L1-positive PDAC showed unfavorable outcomes. Wang et al showed the correlation between B7-H1 (PD-L1) expressions and pathological grade and TNM stage [16]. Nomi et al reported that PD-L1 positive PDAC patients had significant poor prognosis than PD-L1 negative patients [17]. In their data, as same as our result, there was no significant correlation between tumor PD-L1 status and clinical indicators including tumor status, nodal status, metastatic status and pathologic stage. In the current study, among the TIL-positive patients, there was PD-L1 expression showed a stronger prognostic correlation than observed in the analysis including all patients. Univariate and multivariate analyses showed that positive PD-L1 expression (PID staining value > 3.0) was an independent poor prognostic factor in PDAC cases with positive CD8$^+$ TILs. This result suggests that an immunosuppressive tumor microenvironment with high PD-L1 expression can interfere with the attack by TILs on the tumor cells. In these cases, there is a possibility that blockage of the PD-1/PD-L1 signaling pathway can make the attack of TILs dramatically more effective. Although there have been no objective responses observed in patients with PDAC who received anti-PD-L1 antibody therapy [21], our results suggest that PD-L1-positive patients with TILs might be good candidates for anti-PD-L1 antibody therapy.

Conclusions

PID staining provided superior results as compared to those obtained by the canonical DAB-staining method. We have shown for the first time that PD-L1, detected using PID staining, is a novel prognostic marker for human PDAC. Digital immunostaining is a promising tool for companion diagnostics to evaluate the therapeutic effects of molecular-targeted drugs and immunotherapy.

Acknowledgements

Conflict of Interest Statement

The authors declare that have no conflict of interest.

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Table 1. Patients' characteristics and clinicopathological data.

	n=42
Age	65 (50-83)
Male : Female	26 : 16
Tumor location (Ph : Pbt)	31 : 11
R0 : R1	27 : 15
Neoadjuvant therapy (+:-)	8 : 34
Adjuvant chemotherapy (+:-)	23 : 19
Pre-operative CA19-9 (U/ml)	134.2 (1.6–8116)
T stage (1 : 2 : 3)	3 : 6 : 33
N stage (0 : 1)	16 : 26
M stage (0 : 1)	35 : 7
Tumor diameter (mm)	30 (16-75)

The data are expressed as median and range.
Ph: pancreatic head, Pbt: pancreatic body or tail
T and N stage were based on the TNM classification of malignant tumors, sixth edition.
All M1 cases had No.16 lymph node metastasis without other organ metastasis.

Table 2. Relationships between PD-L1 expression and pathological features

Parameter	PD-L1 expression (+) (n=26)	PD-L1 expression (-) (n=16)	P
Age	64(51-82)	66(50-78)	0.385
Male : Female	20 : 6	6 : 10	0.021
Tumor location (Ph : Pbt)	19 : 7	12 : 4	0.891
R0 : R1	15 : 11	12 : 4	0.256
Neoadjuvant therapy (+:-)	5 : 21	3 : 13	0.969
Adjuvant chemotherapy (+:-)	15 : 11	8 : 8	0.627
Pre-operative CA19-9 (U/ml)	143.0(1.6-8116)	113.0(20.3-1712)	0.421
T stage (1,2 : 3)	5 : 21	4 : 12	0.711
N stage (0 : 1)	10: 16	6 : 10	0.950
M stage (0 : 1)	21 : 5	14: 2	0.570
Tumor diameter (mm)	29.5(18-75)	32.5(16-45)	0.583

The data are expressed as median and range.

Ph: pancreatic head, Pbt: pancreatic body or tail

T and N stage were based on the TNM classification of malignant tumors, sixth edition.

All M1 cases had No.16 lymph node metastasis without other organ metastasis.

Table 3. The results of univariate and multivariate analysis for overall survival in all cases. (n=42)

	Univariate		Multivariate	
	HR(95% CI)	P	HR(95% CI)	P
R0/1	1.81(0.87-3.64)	0.108	1.16(0.51-2.59)	0.710
Neoadjuvant therapy (+ / -)	1.75(0.74-5.18)	0.220		
Adjuvant chemotherapy (+ / -)	1.28(0.64-2.61)	0.480		
CA19-9 > 138 U/ml	1.57(0.76-3.22)	0.216		
T stage (1-2 / 3)	1.28(0.58-3.21)	0.555		
N stage (0 / 1)	2.06(1.01-4.44)	0.046	2.21(0.91-5.62)	0.081
M stage (0 / 1)	2.25(0.88-5.08)	0.087	1.09(0.38-2.86)	0.863
ly (0-1 / 2-3)	1.23(0.60-2.65)	0.573		
v (0-1 / 2-3)	1.15(0.57-2.50)	0.697		
PD-L1 expression (+) (PID)	2.07(1.00-4.54)	0.049	2.34(1.02-5.74)	0.045
PD-L1 expression (+) (DAB)	1.28(0.50-4.33)	0.633		
CD8$^+$ TILs >3.0	1.31(0.62-3.15)	0.490		

HR: Hazard Ratio, CI: confidence interval, PID: phosphor-integrated dots , DAB: diaminobenzidine
T and N stage were based on the TNM classification of malignant tumors, sixth edition.
All M1 cases had No.16 lymph node metastasis without other organ metastasis.

Table 4. The results of univariate and multivariate analysis for overall survival in patients with positive CD8$^+$TILs. (n=29)

	Univariate		Multivariate	
	HR(95% CI)	P	HR(95% CI)	P
R0/1	1.90(0.82-4.24)	0.131	1.55(0.63-3.77)	0.334
Neoadjuvant therapy (+ / -)	1.42(0.53-4.88)	0.512		
Adjuvant chemotherapy (+ / -)	1.00(0.45-2.21)	0.999		
CA19-9 > 138 U/ml	1.14(050-2.60)	0.751		
T stage (1-2 / 3)	1.08(0.39-2.60)	0.864		
N stage(0 / 1)	1.64(0.73-3.94)	0.233	1.80(0.66-5.12)	0.250
M stage(0 / 1)	3.09(0.97-8.58)	0.056	1.63(0.47-5.05)	0.420
ly (0-1 / 2-3)	1.18(0.48-2.69)	0.710		
v(0-1 / 2-3)	1.05(0.43-2.43)	0.904		
PD-L1 expression(+) (PID)	3.84(1.59-10.35)	0.003	4.39(1.64-13.34)	0.003
PD-L1 expression(+) (DAB)	1.89(0.44-5.84)	0.349		

HR: Hazard Ratio, CI: confidence interval, PID: phosphor-integrated dots , DAB: diaminobenzidine
T and N stage were based on the TNM classification of malignant tumors, sixth edition.
All M1 cases had No.16 lymph node metastasis without other organ metastasis.

Figure Captions

Fig 1. Immunohistochemistry of pancreatic ductal adenocarcinoma tissue using phosphor-integrated dot (PID) staining. Red spots on tumor cells indicate PID particles.

Fig 2. Immunohistochemical double staining for PD-L1 on tumor cells and CD8^{+} lymphocytes. Phosphor-integrated dot (PID) staining was used for PD-L1 detection and diaminobenzidine (DAB) staining was used for CD8+ lymphocytes. The number of PID particles measured by an automated PID analyzer is indicated for each tumor cell (white numbers) and lymphocytes (yellow numbers).

Fig 3. Overall survival of 42 patients with pancreatic ductal adenocarcinoma.

Fig 4. (a) Overall survival rates of 42 patients with pancreatic ductal adenocarcinoma correlated to PD-L1 expression. Overall survival in PD-L1-positive cases was significantly poorer than that in PD-L1-negative cases (P = 0.049). (b) Overall survival rate of 29 patients showing positive CD8^{+} tumor-infiltrating lymphocytes (TILs) correlated to PD-L1 expression; there was a significant difference in prognosis between PD-L1-positive and -negative cases (P = 0.003).