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Comparison of Programmed Death Ligand 1 (Pd-L1) Immunostaining for Pancreatic Ductal Adenocarcinoma (PDAC) between Paired Cytological and Surgical Samples

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Abstract

Introduction: Pancreatic ductal adenocarcinoma (PDAC) has a poor prognosis with surgery or chemotherapy. PD-L1 immunotherapy has been successful for treating lung and other cancers with PD-L1 expression. However,in many unresectable PDAC cases, cytological samples are the only available tissues for PD-L1 testing. The aim of this study is to retrospectively compare the expression of PD-L1 using cytological and surgical samples.

Materials and Methods: Paired formalin-fixed cell blocks and surgical samples from the same patients with confirmed diagnoses of PDAC (n=28) were sectioned for PD-L1 immunohistochemistry. Using tumor proportion score (TPS) and combined positive score (CPS) to evaluate paired cell blocks and surgical samples, we counted and analyzed the data.

Results: With TPS, the PD-L1 was expressed in 9/28 (32%) of PDAC surgical samples and in 9/28 (32%) of paired cytological samples. Overall, the PD-L1 expression had a correlation of 26/28 (93%). With CPS, the PD-L1 was expressed in 20/28 (71%) of PDAC surgical samples and in 16/28 (57%) of paired cytological samples. The PD-L1 expression had a correlation of 20/28 (71%) and a discrepancy of 8/28 (29%). The PD-L1 expression was significantly higher in moderately-differentiated PDAC than in well-differentiated with TPS.

Conclusions: Cytological samples are useful for evaluating PD-L1 expression with TPS because the concordant rate was 93%. With CPS, cytological samples are limited due to the scant inflammatory cells with the concordant rate of 71%. Extensive sampling of the pancreatic tumor may improve the detection of immune cells expressing PD-L1 in cytological samples. With TPS, PD-L1 expression was significantly higher in moderate-differentiation of PDAC than in poor- and well-differentiation.

Keywords: Cytological samples • pancreatic ductal adenocarcinoma • paired surgical samples • programmed death ligand 1 expression (PD-L1)

Abbreviations: CPS: combined positive score• IHC: immunohistochemistry • PDAC: pancreatic ductal adenocarcinoma • PD-1: programmed cell death protein-1 • PD-L1: programmed death ligand 1 • TMA: tissue microarray • TPS: tumor proportion score

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the third leading cause of cancer death in the United States, and the seventh leading cause of cancer-related death in both men and women worldwide [1]. The high mortality rate, with 5-year survival rates as low as 9%, illustrates the poor prognosis of PDAC [1,2]. Only 10-20% of PDAC are resectable, with the majority of diagnoses made only when non-surgical/neoadjuvant therapy is recommended. Treatment of PDAC without resection includes chemotherapy, radiotherapy, and palliative care. However, these treatments have not significantly improved the survival rates of PDAC patients [2-5].Given these low survival rates, new therapies for PDAC are urgently needed. Recent advances in PD-L1 immunotherapy have shown significant progress in the treatment of non-small cell lung carcinoma (NSCLC) and other cancers, which may improve outcomes in PDAC therapy as well.

The immune checkpoint programmed cell death protein 1 (PD-1) is expressed in tumor-infiltrating T-lymphocytes, B-lymphocytes, natural killer cells, monocytes, and dendritic cells. It is engaged by tumor-expressed PD-L1 and PD-L2, which increase the apoptosis of activated tumor-reactive T-cells and promote the growth of tumor cells in-vivo [6]. Recently, PD-L1 antibodies in multiple clinical trials were used to treat many cancer types, including melanoma, [7-9] NSCLC,[10-12] hepatocellular carcinoma, [13] esophageal cancer, [14] and bladder cancer [15,16]. Several studies also investigated the association of PD-L1 and prognosis of PDAC [17-20]. With PD-L1 antibody E1L3N, PD-L1 expression was observed in 61.9% (26/42) of PDAC by fluorescent phosphor-integrated dot nanoparticles methods [21]. Three meta-analysis data showed that PD-L1 expression was significantly associated with worse overall survival and positive lymph node metastatisis [18-20]. The percentages of PD-L1 expression ranged from 3% to 86%. All studies were based on surgical resection samples. However, in routine clinical practice, most PDAC cases do not have surgical biopsy or resection tissue for ancillary testing. Endoscopic ultrasound-guided fine needle aspiration (FNA) of pancreatic tumors has become the standard method for diagnosing PDAC. Thus, frequently the only tissue available to evaluate PD-L1 expression comes from cytological samples, underscoring the urgency to standardize these samples for biomarker analysis.

Scoring standardization between the many studies and antibodies is similarly confounding. Various clinical trials use different definitions for scoring PD-L1 as positive. Tumor Proportion Score (TPS) has been used to evaluate PD-L1 expression with DAKO 22C3 antibody and immunohistochemistry for lung cancer, which was based on evaluating the percentage of PD-L1 positive tumor cells relative to all viable tumor cells present. Of the 2222 NSCLS patients, 1475 (66.38%) had PD-L1 expression, including 623 (28.49%)

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cases with TPS ≥50% and 842 (37.89%) cases with TPS 1%–49% [22]. However, recently the new combined positive score (CPS) was developed to evaluate the PD-L1 expression in gastrointestinal cancers [23]. CPS is the number of PD-L1 stained cells (tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells and multiplied by 100. Using CPS to evaluate PD-L1 expression in gastric/gastroesophageal junction tumor (GEJ), Kulangara and colleagues reported that the prevalence of PD-L1 expression in patients with gastric/GEJ cancer was 57.6% (148 of 257 patients) [20]. To the best of our knowledge,no standard reporting criteria for scoring PD-L1 expression in pancreatic cytology samples have been published.

In our study, weused both TPS and CPS to evaluate PD-L1 expression in paired cytological and surgical samples. We used surgical samples as the gold standard to evaluate PD-L1 expression in cytological samples.

Materials and Methods

Paired cytological cell block and surgical samples

Paired formalin-fixed cell blocks of cytological and surgical samples from the same patients with confirmed diagnosis of PDAC during 2010-2016 (n=28) were found in the University of Rochester Medical Center Soft database. All the paired pancreatic FNA cytological and surgical resection samples were obtained within 1 month of each other. Both cell blocks from FNA cytological samples and resection tissues were fixed in formalin and paraffin embedded as routine standardized protocol in our laboratory **(Figure 1)**. The diagnosis was evaluated by MM and ZZ. The differentiation of PDAC was evaluated in surgical samples. For the cell block preparation, the one or two dedicated FNA passes were briefly washed in normal saline solution. The samples were centrifuged to produce a concentrated cell button (5min/2400rpm), which was re-suspended in 5 cc buffered formalin and centrifuged again (5min/2400rpm) to produce a fixed cell button. Slides containing the tissue sections of both the cytological cell blocks and surgical samples from the same patients were selected for immunostaining. All cytological cases in our study came from pancreatic FNA. No core biopsy was used. All patient identifiers were removed. This project was approved by Research Subjects Review Board at University of Rochester Medical Center.

PD-L1 immunohistochemistry (IHC)

PD-L1 IHC studies were performed on 4-μm thick sections of formalin-fixed and paraffin-embedded surgical tissues and cell blocks. After deparaffinizing and pretreating the tissue sections with PD-L1 pretreatment buffer at 99°C for 20 min, we applied ready-to-use mouse monoclonal antibody PD-L1 22C3 PharmDx IHC Kit (Dako, Carpinteria, CA, USA) following the manufacturer's instructions [9]. Appropriate positive and negative controls were evaluated by both Dako and our immunohistochemical laboratory control tissue.

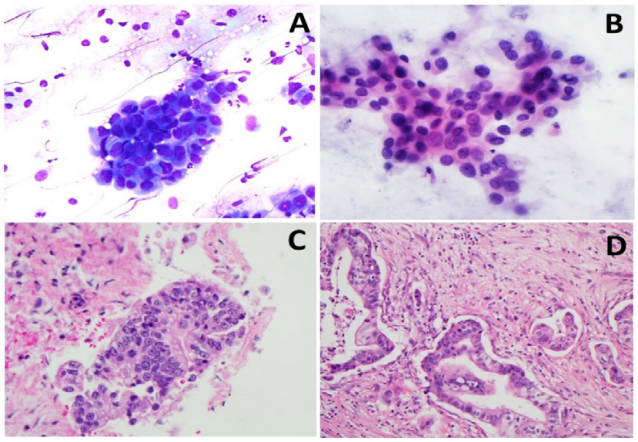

Figure 1. Paired cytological sample and surgical sample of pancreatic ductal adenocarcinoma (PDAC).

(A) PDAC cells with Diff-Quick stain in cytological sample; **(B)** PDAC cells with Papanicolaou stain in cytological sample;**(C)** PDAC cells with Hemotoxin and Eosin stain in cell block; **(D)** PDAC with Hemotoxin and Eosin stain in surgical sample.

TPS and CPS Methods

The viable tumor cells showing partial or complete membrane staining at any intensity were defined as positive PD-L1 immunostain (**Figure 2**). We used two methods to evaluate PD-L1 expression. TPS was based on evaluating the percentage of PD-L1-positive tumor cells relative to all viable tumor cells present in samples. The samples were considered to have PD-L1 expression if TPS ≥ 1%. CPS is the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells and multiplied by 100. In surgical samples, PD-L1 positive inflammatory cells surrounding tumor cells were counted. However, in cell blocks it was difficult to evaluate the relationship between tumor cells and adjacent inflammatory cells. Therefore, we counted all PD-L1 positive inflammatory cells in cell blocks. We considered samples to have PD-L1 expression if CPS ≥ 1%. Based on our experience in evaluating PD-L1 expression in lung FNA samples, two cytopathologists counted cytological cell blocks and surgical samples with at least 100 tumor cells [24]. If not in agreement, the two reviewers checked the slides again to reach a consensus.

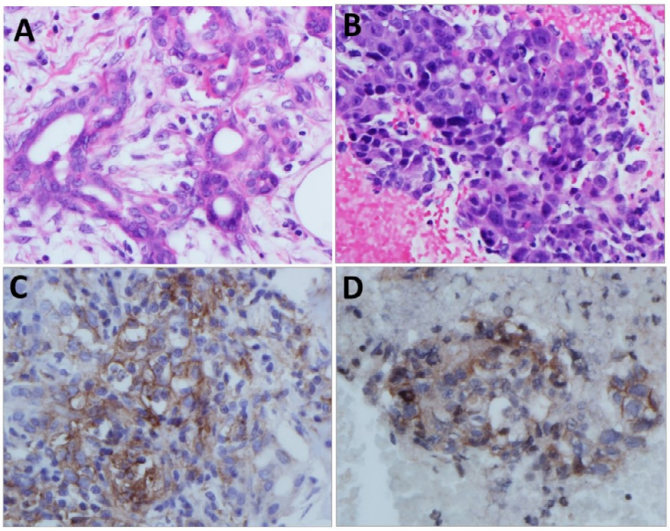

Figure 2. Programmed death ligand 1 immunostaining for pancreatic ductal adenocarcinoma (PDAC) in paired cytological cell block and surgical samples. PDAC in paired surgical samples **(A)** and PDAC in paired cytological cell block **(B)**. Adenocarcinoma cells show membrane staining. High percentage of programmed death ligand 1 expression in PDAC in both surgical biopsy samples **(C)** and n paired cell block **(D)**.

Statistical analysis

The percentages of PD-L1-positive and PD-L1-negative immunostain in both cytological and surgical samples were calculated with CPS and TPS. Fisher's exact test was used as appropriate to compare PD-L1 expression in both cytological and surgical samples. All statistical tests were two-sided. $p<0.05$ was considered to be statistically significant.

Results

PD-L1 expression in paired PDAC cytological and surgical samples using TPS

PD-L1 was expressed in 9 out of 28 (32%) PDAC surgical samples and in 9 out of 28 (32%) paired cytological samples [Figure 2 and Table 1]. Twenty-six out of 28 (93%) paired PDAC cases had correlating immunostain results, and only two cases (7%) showed PD-L1 expression discrepancy [Table 1]. The tumor cells showed positive PD-L1 in one cytological sample, but negative PD-L1 in the paired surgical samples. In one surgical sample, the

tumor cells were positive for PD-L1, but in the paired cytological sample, the tumor cells were negative **(Table 1).**

PD-L1 expression in paired PDCA cytological and surgical samples using CPS

With CPS, PD-L1 was expressed in 20 out of 28 (71%) PDAC surgical samples and in 16 out of 28 (57%) cytological samples [Figure 3 and Table 2]. Twenty out of 28 (71%) paired PDAC cases had concordant immunostain results, and 8 out of 28 (29%) had non-concordant [Table 2]. In 7 cytological cases, the tumor cells were negative for PD-L1 but positive in the paired surgical samples. In one cytological sample, the tumor cells were positive for PD-L1 but negative in the paired surgical samples **(Table 2 and Figure 3).**

Association of PD-L1 expression with the differentiation of PDAC

The relationship between PD-L1 expression and tumor differentiation was analyzed in our study. We found that in both cytological and surgical samples [Table 3] well-differentiated tumor cases were negative for PD-L1 expression (0/8) with TPS. Fisher's exact test showed that with TPS PD-L1 expression was significantly different among well- vs moderately- vs poorly-differentiated (p=0.028) cancer samples in both cytological and surgical samples. The PD-L1 expression in the well-differentiated cancer samples was significantly lower than that in the moderately-differentiated samples (p=0.0018) but not significantly different from the poorly-differentiated samples (p=0.20). The PD-L1 expression in moderately-differentiated samples was also not significantly different from poorly-differentiated samples (p=0.3742). With CPS, we found that the inflammatory cells in well-differentiated tumors showed a dramatically increased PD-L1 expression (6/8) from surgical samples with CPS but not from cytological samples (1/7) [Table 3]. Fisher's exact test showed that PD-L1 positive cases were significantly different in three separate groups (well- vs moderate- vs poorly-differentiation) with CPS only in cytological samples (p=0.004) but not in surgical samples (P=0.724) [Table 3]. In cytological samples with CPS, the PD-L1 expression in well-differentiated samples was significantly lower than that in moderately-differentiated samples (p=0.0022) but not significantly different from poorly-differentiated samples (p=0.1189). Moderately-differentiated samples were also not significantly different from poorly-differentiated samples (p=0.2898) **(Table 3).**

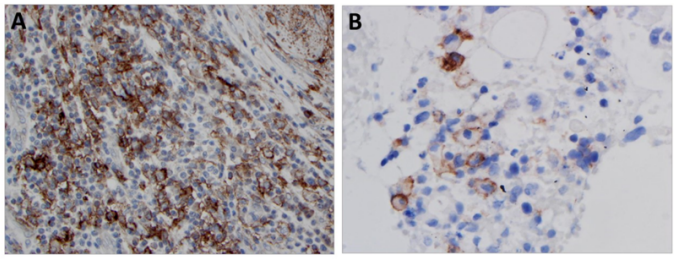

Figure 3: (A) Programmed death ligand 1 immunostaining of inflammatory cells surrounding for pancreatic ductal adenocarcinoma in paired surgical and cytological samples. **(B)** Programmed death ligand 1 expression in inflammatory cells of PDAC in both cytological samples and surgical samples.

Table 1: Using tumor proportion score, PD-L1 immunohistochemistry of tumor cells in paired surgical and cytology samples of pancreatic ductal adenocarcinoma.

Surgical specimen	PDAC cytological specimen		
	Negative	Positive	Total
Negative	18	1	19
Positive	1	8	9
Total	19	9	28

Table 2: Using Combined Positive Score, Programmed Death Ligand 1 immunohistochemistry of tumor cells in paired surgical and cytology samples of pancreatic ductal adenocarcinoma

Surgical specimen	PDAC cytological specimen		
	Negative	Positive	Total
Negative	5	1	6
Positive	7	15	22
Total	12	16	28

Table 3: Association of PD-L1 expression with three differentiations of pancreatic ductal adenocarcinoma (PDAC) group (well- vs moderate- vs poorly-differentiated PDAC).

		TPS pos	TPS neg	CPS pos	CPS neg	Total
Surgical Sample	Poorly diff	2	5	5	2	7
	Mod diff	7	6	11	2	13
	Well diff	0	8	6	2	8
P value			0.028		0.724	
Cytological Sample	Poorly diff	2	5	4	3	7
	Mod diff	7	6	11	2	13
	Well diff	0	8	1	7	8
P value			0.028		0.004	
Total						28

diff-differentiation; Mod-moderate; pos-positive; neg-negative; TPS-tumor proportion score; CPS-combined positive score

Discussion

In our study we evaluated the PD-L1 expression in cytological samples with paired surgical samples. Since there are no standard methods for determining PD-L1 expression in cytological samples, we used two common PD-L1 scoring systems: TPS and CPS. Using TPS, we found that the percentage of PD-L1 expression was excellently concordant (93%) with surgical samples from the same patients. Only two cases did not match. One was a PD-L1 negative case with a paired positive surgical sample in the cytological sample. It showed scant cellularity in cell block, barely reaching 100 tumor cells. The other PD-L1 negative case showed significant degeneration of tumor cells and surrounding inflammatory cells. This might explain the false positive PD-L1 expression in cytological samples. However, PD-L1 heterogenic expression is a common phenomenon in pancreatic tumors and has been observed in other cases. Using CPS, the concordant rate between paired surgical samples and cytological samples was 71%. Seven PD-L1 negative cytological samples with paired positive surgical samples showed scant inflammatory cells, which might have caused the false negative.

PD-L1 expression has been reported in multiple clinical trials using various antibodies, including Dako 22C3, [25,26] Dako 28–8, [27,28] Ventana SP142, [22,29] and SP263 [30-32]. The various clinical trials also used different definitions for calculatingpositive PD-L1 expression. TPS was used to evaluate Dako 22C3 antibody in a large randomized controlled trial for lung cancer. Of 2222 non-small cell lung cancer (NSCLC) patients, 1475 (66.38%) had PD-L1 expression, including 623 (28.49%) cases with TPS ≥50% and 842 (37.89%) cases with TPS ≤1%–49% [25]. Among patients with at least 50% tumor cells expressed PD-L1, overall survival (OS) and progression-free survival were significantly longer with pembrolizumab than with docetaxel [25]. Subsequently, another scoring method, the CPS, was developed to evaluate PD-L1 expression.20 Using CPS to evaluate PD-L1 expression in gastric/gastroesophageal junction tumors (GEJ), other researchers reported that the prevalence of PD-L1 expression in patients with gastric/GEJ cancer was 57.6% (148 of 257 patients) [23]. External reproducibility assessments demonstrated inter-pathologist overall agreement of 96.6% and intra-pathologist overall agreement of 97.2%. They concluded that CPS is a robust, reproducible PD-L1 scoring method that

predicts response to pembrolizumab in patients with Gastric/GEJ cancer and is approved by the FDA [23].

Therefore, in our study, we usedboth TPS and CPS to evaluate PD-L1 expression in PDAC. We found the PD-L1 positive expression with CPS was significantly higher compared to that with TPS (CPS vs TPS: 71% vs 32%; p=0.0011) in surgical samples. In cytological samples, PD-L1 expression was higher with CPS than with TPS, but not significantly higher(CPS vs TPS: 57% vs 32%, p=0.106). If CPS were used, more patients would qualify for clinical trial when surgical samples are not available. However, in cytological sampling, the disadvantage of CPS is the evaluation of inflammatory cell PD-L1 expression because many cytological samples lack significant inflammatory cell components in the cell block.

There are several ongoing clinical trials for the treatment of PDAC [33-38]. In most trials, eligibility requirements mandate a tissue biopsy for biomarker testing [34-36]. However, the biomarker tests proven in trials have rarely been validated by tissue obtained by cytological methods. In routine practice, cytological samples have usually provided the diagnosis in more than half the patients with pancreatic cancer [39]. Recently, the College of American Pathologists has created guidelines for the validation of IHC on cytological preparations such as cell blocks, direct smears, and other methods before incorporating the antibodies into clinical practice [40]. In our study, we used paired cytological and surgical samples with diagnosis of PDAC to evaluate the CPS and TPS for PD-L1 expression. We found that the percentage of PD-L1 expression with TPS was excellently concordant (93%) with surgical samples from the same patients. Only two cases failed to match. One negative cytological case paired with a positive surgical sample showed scant cellularity in cell block, barely reaching 100 tumor cells. If clinical trials use TPS to evaluate the positive PD-L1 expression, the cytological samples should be similar to the surgical samples. However, the percentage of PD-L1 expression with CPS was only moderately concordant (71%) with paired surgical samples from the same patients. The major issue is the scant cellularity of inflammatory cells in cytological samples. If clinical trials use CPS to evaluate PD-L1 expression, the combined cytological sample and core biopsy should be considered to evaluate PD-L1 expression. Utilizing core needle biopsy for endoscopic pancreatic sampling could increase the yield of the tissue samples, [41,42] resolve the issue of inflammatory cell pauci-cellularity, and improve the correlation between PD-L1 expression in surgical and cytology samples calculated with CPS.

PD-L1 expression in PDAC has been published in many reports [17,18,20,21]. When the PD-L1 antibody E1L3N was used, PD-L1 was expressed in 61.9% (26/42) of PDAC by fluorescent phosphor-integrated dot nanoparticles methods.17 Univariate and multivariate analyses indicated that the PD-L1 expression was an independent, predictive, and poorly-prognostic factor in patients with PDAC [17,18,20,21]. When the PD-L1 antibody SP142 was used, PD-L1 was expressed in 34% of 252 PDAC cases [17]. In meta-analysis of the PD-L1 expression in PDAC, the PD-L1 varied from 3% to 86% [19,20]. In our study, with TPS, the PD-L1 positive rate was 32%, below the average of 45.7%. When we used CPS, our result was 71% in surgical samples, much higher than the average. The reason could be that previous studies counted only the positive PD-L1 tumor cells and not the positive PD-L1 inflammatory cells.

Based on our experience evaluating PD-L1 expression in lung FNA samples, we arbitrarily selected 100 tumor cells as the cut off for evaluating PD-L1 expressions in cell blocks and surgical samples [24]. The number of paired surgical and cytological samples was limited by the scant cellularity in pancreatic FNA cell blocks. For future studies, the quality and quantity of pancreatic tumor cells in cell block or core biopsy need to be improved, with dedicated passes directly into fixative solution.

The association of PD-L1 expression in PDAC with clinicopathological features was studied [17,18,20,21] Meanwhile, the association of PD-L1 expression with the PDAC differentiation remains controversial. In one meta-analysis study, the high-level of PD-L1 expression was significantly associated with poorly-differentiated PDAC [20]. However, in the other meta-analysis study, the pooled data indicated no significant correlation between PD-L1 expression levels and lymph node metastasis, distant metastasis, and/or differentiation [18]. In our study, using TPS, no PD-L1 expression was observed in well-differentiated PDAC in both surgical and cytological samples. Our study showed the significant difference of PD-L1 expression among well-, moderately-, and poorly-differentiated PDAC cases [Table 3] in cytological samples using both TPS and CPS. However, in surgical samples, there was a significant difference among three differentiated groups with TPS, but not with CPS. This was due to the dramatically increased PD-L1 expression in inflammatory cells in well-differentiated PDAC groups. [Table 3]. Only the moderately-differentiated group was significantly different from well-differentiated group with Fisher's exact test. PD-L1 expression seems to significantly associate with moderately-differentiated PDAC.

Conclusions

Our results showed that 93% of cytological PDAC cases were highly concordant with paired surgical samples using TPS to evaluate PD-L1 expression, which indicates that the cytological samples were useful for evaluation of PD-L1 expression in PDAC. With CPS, cytological samples were limited due to the scant inflammatory cells, with a moderate concordant rate of 71%. Extensive and widespread sampling of the pancreatic tumor and surrounding tissue, or combined FNA and core needle biopsies, may improve the detection of PD-L1 expression in cytological samples. In addition, PD-L1 expression was significantly associated with moderately-differentiated PDAC in both cytological and surgical samples except in surgical samples with CPS.

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