Title/Cover page

Manuscript type: Original article

Title: The relationship between the PD-L1 expression of surgically resected and fine-needle aspiration specimens for patients with pancreatic cancer.

Short Title: PD-L1 expression of resected and FNA specimens

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Conflict of interest

All authors declare no conflicts of interest.

Funding

This work was supported by JSPS KAKENHI Grant number 17K09462.

Authors' Contributions

KM, TO and AK: conception and design of the research and writing the paper. FM, MT and NT: analysis and interpretation of data. HK and SH: critical revision of the article for important intellectual content. RY, YU and TY: collection of the surgical specimens. SF: performing the external validation for pathological evaluation. MA

and HO: final approval of the article. All authors read and approved the final manuscript.

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Word count: 3076 words

ABSTRACT

Background and Aims: Recently, therapeutic antibodies against programmed death 1/programmed death ligand 1 (PD1/PD-L1) have shown promising clinical results for several solid tumors, including pancreatic cancer. In this study, we evaluated the relationship between the PD-L1 expression of surgical resected and fine-needle aspiration (FNA) specimens for patients with pancreatic cancer.

Methods: Of 121 patients who underwent endoscopic ultrasound-guided (EUS)-FNA before surgery for pancreatic cancer in an academic center, the 94 (78%) with adequate FNA specimens for a histological evaluation were retrospectively analyzed. All of the patients had undergone upfront surgery without any chemotherapy or radiotherapy. We performed immunohistochemistry (IHC) staining to investigate the PD-L1 expression in both resected and FNA specimens. The positive-stained cells were counted, and their percentage was used for the investigation.

Results: Of the 94 patients, 16 (17%) and 11 (10%) were defined as positive on resected cancer specimens using cut-off points of 5% and 10% positively stained cancer cell counts, respectively. The concordance rates for the positive frequency of PD-L1 expression between resected and FNA specimens were 44% (7/16) and

55% (6/11) when the positivity was set to ≥5% and ≥10%, respectively. The concordance rates for the negative frequency of PD-L1 expression between two specimens were 97% (76/78) and 99% (82/83) when the positivity was set to ≥5% and ≥10%, respectively.

Conclusions: Approximately half of the patients with PD-L1 expression positive and almost all of patients with PD-L1 expression negative could be diagnosed on FNA specimens.

Key words: PD-L1, pancreatic cancer, EUS-FNA, Immunohistochemistry

Acronyms and Abbreviations

PD-1: Programmed death-1

PD-L1: Programmed death-ligand 1

MMR: mismatch repair

MSI-H: high-frequency microsatellite instability

IHC: Immunohistochemistry

EUS: Endoscopic ultrasound

FNA: Fine-needle aspiration

ROSE: rapid on-site evaluation

PBS: Phosphate-buffered saline

IQR: Interquartile range

CEA: Carcinoembryonic antigen

CA19-9: Carbohydrate antigen 19-9

DUPAN-2: Duke pancreatic monoclonal antigen type 2

Span-1: Serum pancreas antigen type 1

hENT1: human equilibrative nucleoside transporter 1

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States[1] and in Japan. Due to its extremely high malignant potential, it is usually diagnosed at an advanced stage and often recurs even after curative surgical excision[2, 3]. Although significant advancements have been made in cancer therapy, including surgery, radiation, chemotherapy, or combinations thereof, the prognosis of pancreatic cancer has not dramatically changed[3-5]. Therefore, new treatment strategies are required to prolong the patient survival[6].

The expression of programmed death-1 (PD-1) is significantly upregulated on activated cancer-specific T cells. The PD-1 receptor attaches to its ligand PD-L1, which is expressed on tumor cells and infiltrating immune cells. The interaction of PD-1 and PD-L1 inhibits T-cell activation and promotes tumor immune escape[7-10]. Recently, therapeutic antibodies against PD1/PD-L1 for several solid tumors, including pancreatic cancer, were developed, and about 20%-30% of patients achieved effective responses for their tumors[6,11]. In particular, patients with deficient DNA mismatch repair (MMR) and high-frequency microsatellite instability (MSI-H) have shown a dramatic response to immune checkpoint inhibitors[12]. Deficient MMR results in a hyper-mutated phenotype, characterized by

MSI and an increased burden of mutation-associated neoantigens that are targeted by the immune system. Tumors with a deficient MMR pathway develop high levels of microsatellite instability. Deficient MMR and MSI-H are now regarded as the best predictive markers for the therapeutic effect of immune checkpoint inhibitors. However, the frequency of MSI-H in a broad spectrum of 11,348 solid tumors was shown to be 3.0%. Furthermore, the frequency in 518 pancreatic cancer cases was only 1.2%[13]. Another study showed the frequency of deficient MMR to be only 0.8% in 833 surgically resected pancreatic ductal carcinoma specimens, all of which were found to be Lynch syndrome [14]. These reports suggest that deficient MMR and MSI-H are rare among pancreatic cancer patients. Therefore, promising markers predicting a good response to immune checkpoint inhibitors are still needed in pancreatic cancer patients. In lung cancer, PD-L1 immunohistochemistry (IHC) staining has been widely investigated and come to be defined as a viable prediction marker[15,16]. On the other hand, PD-L1 IHC expression is not a biomarker predicting the therapeutic effect of PD1 antibody drugs in pancreatic cancer until now. Whether or not this method is also useful for pancreatic cancer has not been analyzed, making this a clinical question in urgent need of an answer.

Endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) is a very sensitive tool for the diagnosis of pancreatic cancer. The overall diagnostic accuracy for a pancreatic mass was reported to be 91% in a recent meta-analysis[17], and the diagnostic ability of cytology and histology ranged from 70%-95% and 63%-80%[18-20], respectively. Using adequate FNA samples, some reports described the utility of IHC staining for several markers in patients with pancreatic cancer[20-22].

The expression of PD-L1 on pancreatic cancer has been previously reported, with a high PD-L1 expression being correlated with a poor overall survival and poor pathologic differentiation[23-27]. The PD-L1-positive rates using IHC staining mainly range from 22%-39%[13,25,28,29]. However, the previous studies concerning the PD-L1 expression were all conducted using either surgically resected or FNA specimens. In this study, we compared the PD-L1 expression in both resected and FNA specimens for patients with pancreatic cancer and investigated the relationship between the PD-L1 expression of these two samples.

MATERIALS AND METHODS

Patients

Of 190 patients with pancreatic cancer who underwent surgical resection, 121 received EUS-FNA before surgery between January 2010 and December 2017 at out institute. Of these 121 patients, the 94 (78%) with adequate FNA specimens for a histological evaluation were retrospectively analyzed. All patients had been histologically confirmed to have pancreatic cancer by both FNA and resected specimens and had undergone upfront surgery without any chemotherapy or radiotherapy. We successfully performed PD-L1 IHC staining for all samples that could be diagnosed as pancreatic cancer histologically. The success rate of IHC staining in evaluating the PD-L1 positivity was 78% (94/121). The surgical pathology from resected cancers was classified according to the International Union against Cancer (UICC) TNM Classification of Malignant Tumors 7th edition. The clinical parameters of all patients were collected from a prospectively maintained database. The overall survival time was calculated from the day of the pathological diagnosis with EUS-FNA to the date of death or loss to follow-up. This study was approved by the hospital's institutional review board for human research and was conducted in accordance with the Declaration of Helsinki.

EUS–FNA technique for the pathological diagnosis

We performed EUS using a convex linear-array endoscope (GF-UCT260 or GF-UCT240; Olympus, Medical Systems, Tokyo, Japan; EG-580UT; Fujifilm Corp., Tokyo, Japan) connected to an ultrasonography (US) device (Prosound SSD-α10; HITACHI Aloka, Tokyo, Japan; EU-ME1 or EU-ME2 PREMIER PLUS; Olympus; SU-1 ultrasound processor; Fujifilm Corp.). EUS-FNA was performed with a 22- and/or 25-gauge needle (EZ-Shot 3; Olympus Medical Systems Group, Tokyo, Japan; Acquire; Boston Scientific Corporation, Marlborough, MA, USA; EchoTip ProCore; Cook Medical, Bloomington, IN, USA; SonoTip Ⅱ; Medi-Globe GmbH, Rohrdorf, Germany; Expect; Boston Scientific Corporation).

All aspirated samples were expressed onto a glass slide by reinserting a stylet into the FNA needle. The aspirated samples were separated into a white tissue portion for a histological evaluation and other portions for a cytological evaluation. For the histological evaluation, FNA tissues were fixed in 10% paraformaldehyde and embedded in paraffin for hematoxylin and eosin (HE) and IHC staining. For the cytological evaluation, two smear preparations were made for each sample. One slide was stained with a modified Giemsa stain for rapid on-site evaluation (ROSE), whereas the other slide was alcohol-fixed and stained by the Papanicolaou method.

EUS-FNA was repeated until an adequate amount of sample had been obtained for ROSE or the endoscopist believed that further sampling was unlikely to increase the yield.

IHC

IHC staining was performed using surgically and endoscopically harvested tissues that had been fixed in 10% paraformaldehyde and embedded in paraffin. Anti-PD-L1 antibody was purchased (catalog no. 13684; Cell Signaling, Danvers, MA, USA). First, HE staining was performed to confirm the tumor. Tissues were then deparaffinized and soaked in 0.3% H_2O_2 in methanol at room temperature (RT) for 10 minutes to extinguish endogenous peroxidase activity. Antigen retrieval was performed by heating specimens in a Tris-EDTA (TE) buffer solution using a microwave. Following three 5-minute washes with PBS, tissues were incubated with primary antibody against PD-L1 in 4 ℃ for 24 h (1:200 dilution). Following another three 5-minute washes with PBS, the Polink-2 Plus HRP detection kit was used according to the manufacturer's protocol (catalog no. D39-18; GBI Labs, Bothell, WA, USA).

The evaluation of IHC findings

Immunostaining signals were evaluated independently by two experienced pathologists without access to the patient's clinical and pathological features. The proportion of tumor cells in each selected field was determined by manual counting individual tumor cells at ×10 magnification. We selected 5% and 10% as the cut-off points based on recent reports on the PD-L1 expression in human pancreatic cancer[24,25,28,29]. Resected specimens with ≥5% or ≥10% PD-L1-positive tumor cells were classified as positive. FNA specimens were stained in the same manner as resected specimens, and the percentage of PD-L1 expression at ×10 magnification was evaluated (Figure 1, 2). For the judgment of positive PD-L1 IHC staining, the external validation was performed by an individual pathologist belong to another hospital. The positive staining judgment has reached a consensus.

Statistical analyses

Continuous variables were expressed as the median and range or interquartile range (IQR). Student's *t*-test was used to analyze continuous variables, and a chi-square test was performed to analyze categorical variables. Cumulative survival rates were calculated by the Kaplan-Meier method. Significant differences in the survival

status were evaluated using the log-rank and Wilcoxon test. $P < 0.05$ was considered to be statistically significant. All analyses were performed using the JMP Pro 12 software program (SAS Institute, Cary, NC, USA).

RESULTS

Patient characteristics

Table 1 summarizes the characteristics of the 94 patients who underwent EUS-FNA before surgical resection for pancreatic cancer. The median age was 70 years (range 34–87), and the median tumor size was 25 (IQR: 20-35) mm. At the initial diagnosis, the median values of serum CEA, CA19-9, DUPAN-2 and Span-1 were 3.3 (IQR: 2-5.9) ng/mL (normal range: < 5 ng/mL), 113 (IQR: 15–324) U/mL (normal range: < 37 U/mL), 110 (IQR:25-354) U/mL (normal range: < 150 U/mL) and 53 (IQR: 17-147) U/mL (normal range: < 37 U/mL), respectively. T stages were T1 in 5 (5%), T2 in 7 (8%) and T3 in 82 (87%). Lymph nodule metastases were detected in 48 (51%) patients. The pathological stages were IA in 4 (4%), IB in 5 (5%), IIA in 37 (40%) and IIB in 48 (51%). The median interval between FNA and surgery was 15 (IQR: 11-21) days. Eighty-one (86%) patients received adjuvant chemotherapy, and the median overall survival time was 864 (IQR: 525-2462) days.

EUS-FNA for the pathological diagnosis

The median number of needle passes was 2 (IQR: 2-3), and the selected needle sizes for FNA were 22 G in 75 (80%) patients and 25 G in 19 (20%). For IHC of PD-L1,

the median number of counted tumor cells on FNA specimens was 507 (IQR: 234-1122).

The PD-L1 expression in resected specimens and the concordance of the PD-L1 expression between resected and FNA specimens

Of the 94 patients, 16 (17%) were positive for PD-L1 expression on resected cancer specimens with the 5% cut-off point. When these patients were considered truly positive for PD-L1 expression, the concordance rate for the positive frequency of PD-L1 expression between resected and FNA specimens was 44% (7/16), and that for the negative frequency was 97% (76/78) (Table 2a). Regarding the utility of FNA as a diagnostic examination, 7 of 9 (78%) patients with a positive PD-L1 expression showed positivity in the resected specimen.

Of the 94 patients, 11 (10%) were positive for PD-L1 expression on resected cancer specimens with the 10% cut-off point. When these patients were considered truly positive for PD-L1 expression, the concordance rate for the positive frequency of PD-L1 expression between resected and FNA specimens was 55% (6/11), while that for the negative frequency was 99% (81/82) (Table 2b). Regarding the utility of FNA as a diagnostic examination, 6 of 7 (86%) patients with positive PD-L1

expression showed positivity in the resected specimen. The diagnostic accuracy of FNA for evaluating the PD-L1 expression on resected specimens are shown in Table 2c.

Relationship between the PD-L1 expression and clinicopathological features

The relationship between the PD-L1 expression in the resected specimens and the clinicopathological features is shown in Table 3. There were no significant differences in the clinical features, such as the age, gender, tumor size, tumor location, number of FNA needle passes, selected FNA needle, number of counted tumor cells for IHC on FNA specimens and T and N stages, based on the expression of PD-L1. The CA19-9 levels in PD-L1-positive patients was significantly higher than in PD-L1-negative patients. In the survival analysis, there was no significant difference between the PD-L1-positive and PD-L1-negative patients (831 vs. 915 days, p=0.77, log-rank, p=0.95, Wilcoxon's test) (Figure 3A). However, on comparing the patients with stage IIA disease, the median survival time was significantly shorter in PD-L1-positive patients (622 days; IQR 243-1247) than in PD-L1-negative patients (1308 days: IQR 864-2462) (P = 0.10, log-rank, P=0.009, Wilcoxon's test) (Figure 3B). On comparing the patients with stage IIB disease,

there was no significant difference between the PD-L1-positive and PD-L1-negative patients (831 vs. 692 days, P=0.28).

DISCUSSION

In this study, we first evaluated the PD-L1 expression of both resected and FNA specimens for patients with pancreatic cancer and investigated the relationship between these two samples. When patients with over 10% positive cells were considered truly positive, the concordance rates for the positive and negative frequency of the PD-L1 expression were 55% (6/11) and 99% (81/82), respectively. These results indicated that approximately half of patients with positive PD-L1 expression and almost all patients with negative PD-L1 expression could be diagnosed using FNA specimens. Regarding the utility of FNA as a diagnostic examination, 6 of 7 (86%) patients with positive PD-L1 expression showed positivity in the resected specimen. When the PD-L1 expression was positive in the FNA specimen, then such resected specimens were deemed to be positive. These results have potential utility in the field of precision medicine for patients with pancreatic cancer.

EUS-FNA is a very sensitive tool for the diagnosis of pancreatic cancer, but also for obtaining adequate samples for evaluate IHC specimen. Yoshizawa et al. reported that the diagnostic accuracy rate on histology using 22- or 25-G needles for pancreatic cancer was 79.7% (122/153), and the accuracy rate on histology did

not differ markedly between 22- and 25-G needles (P=0.99)[20]. They also reported that they performed an IHC analysis of human equilibrative nucleoside transporter 1 (hENT1) using remnant cell blocks following the cytological and/or histological diagnosis of pancreatic cancer. The overall success rate of the IHC analysis was 69.3% (106/153), and the rate did not differ significantly between the 22- (67.1%, 47/70) and 25-G needle (71.1%, 59/83) groups (P=0.60). In the present study, the accuracy rate of histology was 78% (94/121). We did not perform IHC staining for the 27 (22%) FNA specimens that could not be diagnosed with pancreatic cancer histologically. It is difficult to stain all of the IHC successfully, however, the overall success rate of IHC staining in the present study was considered to be 78% (94/121). We successfully performed IHC staining for all of the samples that were able to be diagnosed with pancreatic cancer histologically. The median number of tumor cells counted for IHC on FNA specimens was 507 (IQR: 234-1122). Navina et al. reported the over 100 lesional cells in a specimen was adequate with cell blocks for ancillary studies[30]. Thus, our FNA samples were suitable for histological evaluations and IHC stain. Furthermore, in limited histologically evaluated samples, the median numbers of counted tumor cells for IHC stain using 22- and 25-G needles were 545 (IQR: 243-1123) and 369 (165-764) (P=0.33), respectively, showing no

marked differences between the two needles gauges. Our findings showed a higher rate than that of a previous study, thus suggesting that the performance of needles has been continually improving and ROSE by pathologist increased the rate of obtaining the adequate samples.

In the literature, the positive rates of PD-L1 using IHC staining in pancreatic cancer largely ranged from 22%-39%[13,25,28,29] Nomi et al. reported that, among 51 patients, 20 (39%) were interpreted as PD-L1-positive with a cut-off value of 10%[25]. In contrast, Liang et al. reported that, among 373 patients, 12 (3.2%) were interpreted as PD-L1-positive with a cut-off value of 25%. They also evaluated the PD-L1 cut-off points, and the number of PD-L1-positive cases increased to 22 (5.9%) and 33 (8.8%) with cut-off points of 5% and 10%, respectively[31]. In the present study, the numbers of PD-L1-positive cases were 16 (17%) and 11 (10%) with cut-off points of 5% and 10%, respectively. While the rate of PD-L1-positive cases was slightly low in our study, the results are considered appropriate in comparison with previous reports. Adequate cut-off points of PD-L1 positivity for pancreatic cancer should be determined in future large-scale studies.

In the present study, there were 2 and 1 false-positive FNA specimens with cut-off points of 5% and 10% for resected specimens, respectively. One reason for

these false positives may be that the time to formalin fixation of the resected specimen was longer than that of the FNA specimen, suggesting that FNA might be a better approach to obtain samples for the PD-L1 positivity evaluation. Another possible reason is that the evaluated tumor slices of resected and FNA specimens did not completely match. In this study, we included only upfront surgery cases, and the interval between FNA and the operation in the 2 false-positive cases was 11 and 13 days, respectively. Thus, the effect of chemotherapy and the time until tumor progression were not related.

In contrast, there were 9 and 5 false-negative FNA specimens with cut-off points of 5% and 10% for resected specimens, respectively. The main issue with FNA specimens is the small amount of tumor cells obtained through the procedure, and the evaluating slice did not match each specimen, and this was considered to be a limitation associated with the false-positive cases. Indeed, the mean number of tumor cells counted for IHC in the 9 false-positive cases was 399 cells, while the mean number counter for other cases was 1035. While the number of counted cells was considered sufficient for IHC staining, the counted tumor cell number was relatively small. Moreover, we added the consideration for patchy distribution of PD-L1 expression. The PD-L1 positive cells distributed patchy manner in the

resected whole cancer area. The cancer cells obtained with FNA were limited parts of the whole tumor, where we could not detect the PD-L1 positive area unless we hit the area (supplemental figure 1). These confer risks of a false-negative finding. It might be better to use the fanning technique during FNA procedure for the tumor to avoid a risk of false-negative event. The median interval between FNA and the operation was 15 days in both false-negative and other cases. Thus, the effect of tumor progression time was not related.

PD-L1 has been found to be associated with a poor prognosis in pancreatic cancer[23]. In our cohort, the CA19-9 levels in PD-L1-positive patients were significantly higher than in PD-L1-negative patients (247 vs. 71 U/mL, P=0.04). Survival analyses showed no significant difference between PD-L1-positive and PD-L1-negative patients (831 vs. 915 days, p=0.77, log-rank). However, on comparing the patients with stage IIA disease, the median survival time was significantly shorter in PD-L1-positive patients (622 days; IQR 243-1247) than in PD-L1-negative patients (1308 days: IQR 864-2462) (P = 0.10, log-rank, P=0.009, Wilcoxon's test). Some reports have found that the PD-L1 expression in resected pancreatic cancer tissue was not correlated with the overall survival[26,32]. This

finding might depend on the cancer stage, ethnicity or whether or not chemotherapy is resumed after surgery.

Several limitations associated with the present study warrant mention. The main limitation is that the PD-L1 expression evaluated using IHC in FNA specimens did not match the expression in resected specimens. However, while PD-L1 heterogeneity has not been reported in pancreatic cancer, it has been described in several types of solid tumors[33,34]. Second, this was a retrospective, single-center study. Third, we did not perform genetic test to evaluate the deficient MMR and MSH, which can be used as predictive markers for immune checkpoint inhibitors, on FNA and resected samples. Fourth, we performed EUS-FNA for 121 patients with pancreatic cancer during this period but were unable to evaluate 27 (22%) specimens, thus the population of the study was not consecutive. Finally, we used several different types and sizes of needles to obtain the FNA specimen. Further studies with a prospective design, a large sample size and multicenter setting using the same method to obtain specimens are needed.

Conclusion

In conclusion, approximately half of patients with positive PD-L1 expression and almost all patients with negative PD-L1 expression were able to be diagnosed based on FNA specimens. These results have potential utility in the field of precision medicine for patients with pancreatic cancer.

Acknowledgements

The author are indebted to Dr. Toshiharu Mitsuhashi, Assistant Professor of the Center for Innovative Clinical Medicine of Okayama University Hospital for Statistical analyses.

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FIGURE LEGENDS

Figure 1. PD-L1 staining with corresponding HE staining for pancreatic cancer. PD-L1 and HE staining of a resected specimen. PD-L1 staining was primarily observed at the tumor cell membrane. Magnification, 20× HE: A, 20× PD-L1: B, 40× HE: C, 40× PD-L1: D.

Figure 2. PD-L1 and HE staining of a fine-needle aspiration specimen. PD-L1 staining was primarily observed at the tumor cell membrane, just as in the resected specimen (same case as Fig 1). Magnification, 20× HE: A, 20× PD-L1: B, 40× HE: C, 40× PD-L1: D.

Figure 3. A: The overall survival time was evaluated by the Kaplan-Meier method. There was no significant difference between the PD-L1-positive and PD-L1-negative patients (831 vs. 915 days, p=0.77, log-rank, p=0.95, Wilcoxon's test). B: The overall survival time of the stage IIA patients was evaluated by the Kaplan-Meier method. The median survival time was significantly shorter in the PD-L1-positive patients (622 days: IQR 243-1247) than in the PD-L1-negative patients (1308 days: IQR 864-2462) (P = 0.10, log-rank, P=0.009, Wilcoxon's test).

Supplemental figure 1.

PD-L1 and HE staining of a resected specimen. The PD-L1 positive cells distributed patchy in the resected whole cancer area.

HE (low magnification): A, PD-L1: B, HE (mild magnification): C, PD-L1: D.