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PD-L1 and PD-L2 expression in pancreatic ductal adenocarcinoma and their correlation with immune infiltrates and DNA damage response molecules

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Abstract

Immunotherapy targeting programmed cell death-1 (PD-1) has considerably improved the prognosis of patients with advanced cancers; however, its efficacy in the treatment of pancreatic ductal adenocarcinoma (PDAC) is unfavourable. To address the issue of PDAC immunotherapy, we investigated the expression of two PD-1 ligands, PD-L1 and PD-L2, in PDAC, analysed their role in survival, and explored their correlation with clinicopathological features, immune infiltration, and DNA damage response molecules. Immunohistochemistry was performed on 291 surgically resected PDAC samples. In tumour cells (TCs) and immune cells (ICs), the positivity of PD-L1 expression was 30 and 20% and that of PD-L2 expression was 40 and 20%, respectively. Moreover, PD-L1 expression on TCs correlated with its expression on ICs ($p < 0.0001$); a similar result was observed for PD-L2 ($p < 0.0001$). Nonetheless, no correlation was observed between PD-L1 and PD-L2 expression. Positive PD-L1 expression on TCs was related to N1 stage ($p = 0.011$) and AJCC II stage ($p = 0.002$), whereas positive PD-L2 expression on TCs was associated with high FOXP3^{+} cell infiltration ($p = 0.001$) and high BRCA2 expression ($p < 0.0001$). Survival analysis revealed that positive PD-L1 ($p = 0.046$) and PD-L2 ($p = 0.028$) expression on TCs was an independent risk factor for unfavourable disease-specific survival (DSS). Furthermore, positive PD-L2 expression on TCs was an independent risk factor for lower DSS in the pN0 ($p = 0.023$), moderate and well tumour differentiation ($p = 0.004$), low BRCA1 ($p = 0.017$), wild-type p53 ($p = 0.034$), and proficient mismatch repair ($p = 0.004$) subgroups. Moreover, post-operative adjuvant chemotherapy could significantly affect DSS, regardless of PD-L1/PD-L2 expression status (positive or negative) on TCs, while it only prolonged DSS in PDL1-ICs[(−)] ($p < 0.0001$) and PDL2-ICs[(−)] ($p < 0.0001$) subgroups. This study provides a comprehensive understanding of the roles of PD-L1 and PD-L2 in PDAC, supporting anti-PD-1 axis immunotherapy for PDAC.

Keywords: pancreatic ductal adenocarcinoma; PD-L1; PD-L2; tumour-infiltrating lymphocytes; DNA damage response

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly malignant tumour with a 5-year survival rate of about 8% [1]. Surgical resection is the only curative treatment; however, most patients continue to receive adjuvant chemotherapy to prevent disease recurrence. Compared with traditional chemotherapy, cancer immunotherapy has made significant progress in improving the prognosis of patients with various solid tumours such as lung cancer and melanomas [2,3]. However, the efficacy in PDAC is unfavourable [4–7].

Programmed cell death-1 (PD-1) is an immune checkpoint expressed on activated CD4^{+} T cells, CD8^{+} T cells, and peripheral B cells [8]. PD-1 regulates the immune response by inhibiting T-cell proliferation and IFN-γ production in T cells [9]. PD-1 has two ligands: PD-L1 and PD-L2. PD-L1 is widely expressed in various haematopoietic cells and tissue cells [10,11]. However, PD-L2 is expressed almost

exclusively on antigen-presenting cells and the ligand can be induced in other cells through multiple micro-environmental stimuli [12,13].

In many cases, immunotherapy targeting PD-1/PD-L1 has a substantial effect on advanced cancers. Several factors influence the response to anti-PD-1/PD-L1 therapy, such as PD-L1 expression level, tumour genomic stability, and tumour-infiltrating lymphocytes (TILs) [14]. High responders typically exhibit increased PD-L1 expression owing to the upregulation of ectopic promoters resulting from gene amplification and translocation [15,16]. Nonetheless, some PD-L1-positive patients have shown poor response to anti-PD-1 therapy, whereas a number of PD-L1-negative patients respond well, suggesting that other ligands of PD-1 such as PD-L2 may also be relevant to the efficacy of PD-1 axis immunotherapy [17,18]. One study on head and neck squamous cell carcinomas reported that response to pembrolizumab was higher in the PD-L1 and PD-L2 double positive groups than in the PD-L1 single positive group [19]. In addition, they reported that, in patients treated with pembrolizumab, PD-L2 was an independent predictor of progression-free survival (PFS). Overall, these results suggested that PD-L2 should also be considered for anti-PD-1 axis therapy.

Genomic stability is maintained by DNA damage response (DDR) through multiple repair pathways including mismatch repair (MMR) and homologous recombination repair [20]. Accumulating evidence suggests that tumours with mutations in the genes responsible for genomic stability could be potential targets for PD-1/PD-L1 therapy. For example, studies on colon cancer and gastroesophageal cancer showed that patients with microsatellite instability (MSI) have a significantly higher response to anti-PD-L1 therapy [21,22]. Moreover, patients with BRCA2 mutations (a major player in homologous recombination repair) showed a high response to anti-PD-1 therapy in melanoma [23]. As a classical tumour suppressor, p53 maintains genome stability by orchestrating various DDR mechanisms [24]. Nevertheless, the correlation between p53 and PD-1 ligands has rarely been reported in PDAC.

Currently, the efficacy of anti-PD-1 axis immunotherapy in pancreatic cancer is limited and the role of PD-L2 in PDAC has been rarely reported; thus, we investigated the influence of PD-L1 and PD-L2 expression on both tumour cells (TCs) and immune cells (ICs) in resected PDAC and analysed their correlation with T (CD3^{+}, CD4^{+}, CD8^{+}, FOXP3^{+}, and CD45RO^{+}) and B (CD20^{+}) lymphocytes, CD15^{+} neutrophils and CD68^{+} macrophage infiltration, and several DDR molecules, including major players in homologous recombination repair (BRCA1 and BRCA2), proteins associated with MMR (MLH1, MSH2, MSH6, and PMF6), as well as p53. Moreover, the role of PD-L1 and PD-L2 in predicting PDAC survival was explored. Our study helps to provide a more comprehensive understanding of the roles of PD-L1 and PD-L2 in PDAC and support immunotherapy for PDAC.

Materials and methods

Clinical information for PDAC patients

A total of 291 patients who were diagnosed with primary PDAC and underwent surgical resection at the Peking Union Medical College Hospital between June 2015 and April 2019 were included in this study. Clinical baseline information was obtained from the electronic medical record system of the hospital. Patients who died because of post-operative complications were excluded when conducting survival analysis. None of the patients received preoperative immunotherapy, chemotherapy, or radiotherapy. The follow-up ended in October 2020. This retrospective study was approved by the Institutional Review Board of the Peking Union Medical College Hospital (approval number: S-K1593; date: 2 April 2021) and was carried out in conformity to the Declaration of Helsinki. Written informed consent was obtained from all patients.

TMA construction and immunohistochemistry

Two experienced pathologists (XC and SM) independently reviewed all the original haematoxylin and eosin (HE)-stained slides to confirm that the diagnosis was correct. Subsequently, representative tumour tissues and tumour-associated stromal areas were labelled on HE-stained slides, and tissue microarray (TMA) blocks were collected. Finally, TMA instruments were used to construct 12×8 (row × column) TMAs with a 2-mm core for each case (Sakura Finetek USA, Torrance, CA, USA).

For immunohistochemistry, 4-µm TMA sections were deparaffinised with xylene and rehydrated in serial concentrations of ethanol, followed by heat-induced epitope retrieval using a citrate retrieval buffer (pH 6.0) or EDTA retrieval buffer (pH 9.0) solution, as specified by the manufacturer's protocol. The internal peroxidase activity was depleted by 3% H_2O_2, and non-specific antibody reactions were blocked with prediluted goat serum (ZLI-9056; ZSGB-BIO, Beijing, PR China). Tissue sections were incubated with primary antibodies against PD-L1, PD-L2, CD3, CD4, CD8, CD45RO, FOXP3, CD20, CD15, CD68, p53,

BRCA1, BRCA2, PMS2, MLH1, MSH2, and MSH6 at 37 °C for 1 h. Detailed antibody information is provided in supplementary material, Table S1. Negative controls were prepared without the primary antibody.

Immunostaining scoring

Evaluation of immunohistochemical (IHC) staining was conducted independently by two pathologists who were unaware of the patients' baseline information. Both pathologists re-examined the slides and reached a consensus if there were any disagreements. The staining of PD-L1 and PD-L2 on the ICs was classified as negative or positive. For the evaluation of TCs, positive expression was defined as membranous positive labelling of ≥1% of the TCs [17,25]. For CD3, CD4, CD8, CD45RO, FOXP3, CD20, CD15, and CD68 scoring, four high-power fields with relatively abundant positive staining were selected for cell counting, and the mean value was recorded. In addition, four quartile ranges were used to set the cut-off values.

For p53 immunostaining scoring, abnormal p53 expression was defined as either positive staining in ≥50% of the TC nuclei or the complete absence of nuclear staining compared with adjacent normal tissue [26]. Normal and abnormal p53 expression was marked as wild-type (WT) p53 and mutant p53, respectively. For BRCA1 and BRCA2 evaluation, a cut-off value of 50% was used to define high (≥50% of the TCs were positively stained) and low (<50% of the TCs were positively stained) expression [27]. Loss of the MMR proteins (MLH1, MSH2, MSH6, and PMS2) expression was defined as a complete loss of nuclear expression in the TCs, whereas the internal control, including acinar, ductal, islet, and ICs, showed appropriate nuclear labelling. Thus, deficient MMR (dMMR) was defined as the loss of expression of any of the MMR proteins, whereas proficient MMR (pMMR) indicates that all the MMR proteins were expressed [28].

Next-generation sequencing

DNA was extracted from formalin-fixed paraffin-embedded tissue (Qiagen, Germantown, MD, USA) and analysed by next-generation sequencing (NGS) of the *BRCA1* and *BRCA2* genes. Sequencing was performed on 60 PDAC cases with the available DNA.

Statistical analysis

All statistical analyses were conducted in SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). The Kaplan–Meier method was used to plot survival curves. Differences between the Kaplan–Meier curves were tested for significance by the log-rank test, and the figures were generated using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). Correlation analysis between PD-L1/PD-L2 and categorical variables was performed using chi-square test or Fisher's exact test. Cox proportional hazards model (Enter method) was used for univariate analysis and variables with $p < 0.25$ were selected for multivariate analysis. The statistical significance was set at $p < 0.05$.

## Results

Expression of PD–L1 and PD–L2 in PDAC

In total, 291 patients were included in this study and the median age was 61 years (range: 25–83 years). Tumour grade and stage were defined based on the eighth edition of the American Joint Committee on Cancer (AJCC). Detailed baseline information is summarised in supplementary material, Table S2. TMA cores were excluded if they were lost during immuno-staining. Finally, 264 and 255 cases with intact PD-L1 and PD-L2 staining information, respectively, were selected for further analysis. PD-L1 and PD-L2 exhibited expression on both TCs and ICs; the positivity of PD-L1 and PD-L2 expression on ICs was 20% (54/264 and 50/255, respectively) and on TCs was 30% (80/264) and 40% (100/255), respectively. We used two different antibodies for PD-L1 IHC staining and the positivity between them was strongly correlated ($\rho = 0.977$, $p < 0.0001$). Representative images of PD-L1 and PD-L2 staining are shown in Figure 1.

Associations among PD–L1, PD–L2, and immune infiltrates

Positive PD-L1 expression on TCs was observed more frequently among the samples in which ICs were also PD-L1-positive ($p < 0.0001$) (supplementary material, Table S3). The same result was also observed for PD-L2 ($p < 0.0001$). However, positive expression of PD-L1 and PD-L2 on TCs was not correlated ($p = 0.148$). As shown in supplementary material, Table S3, 61 of the PD-L1-negative samples (64.2%) showed positive PD-L2 staining, 38 of the PD-L2-negative samples (27.0%) were PD-L1 positive, and 34 samples exhibited co-expression of PD-L1 and PD-L2.

There was no correlation between positive expression of PD-L1 on TCs and any other immune markers (supplementary material, Table S4). Nonetheless, positive PD-L1 expression on ICs was significantly related to high CD3
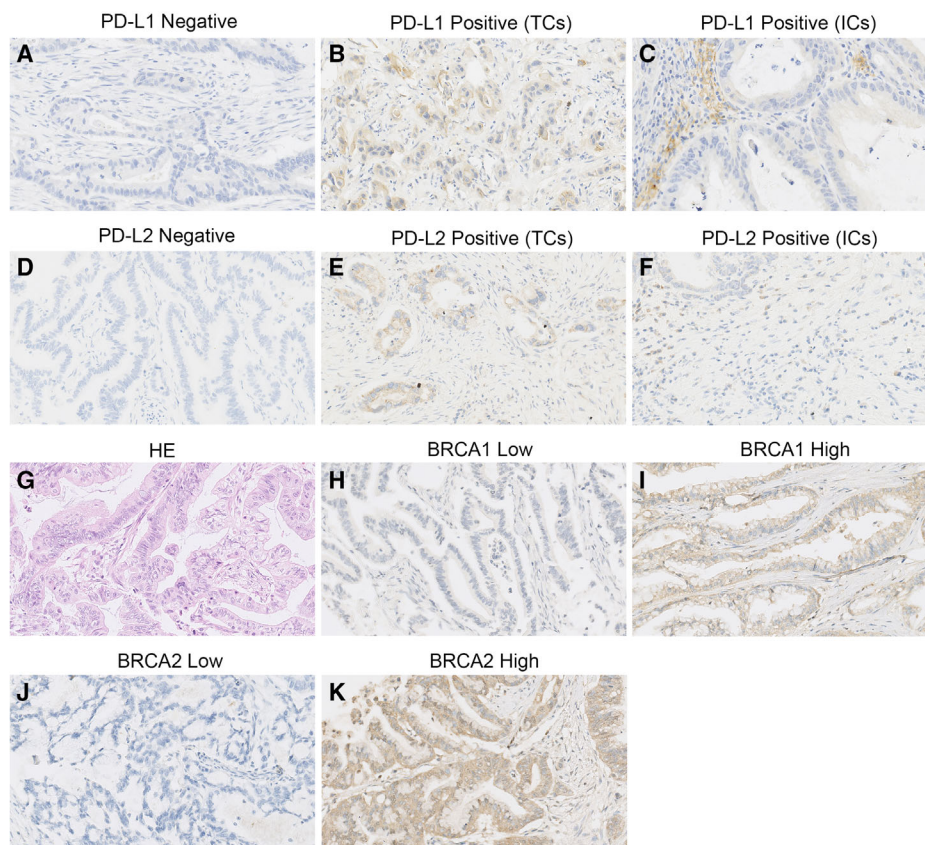
Figure 1. Representative images of PD-L1, PD-L2, and BRCA IHC staining. (A) Negative expression of PD-L1. (B, C) Positive expression of PD-L1 on TCs and ICs. (D) Negative expression of PD-L2. (E, F) Positive expression of PD-L2 on TCs and ICs. (G) HE staining of PDAC. (H, I) Low and high expression of BRCA1. (J, K) Low and high expression of BRCA2.

($p = 0.007$), CD4 ($p = 0.023$), CD8 ($p = 0.043$), FOXP3 ($p < 0.0001$), CD45RO ($p = 0.014$), and CD68 ($p = 0.011$) infiltration; in contrast, positive PD-L2 expression on ICs was only related to high Foxp3 ($p = 0.015$) infiltration (supplementary material, Table S5). In addition, positive PD-L2 expression on TCs was more frequent in the high FOXP3 expression group ($p = 0.001$), with 57.4% of the highest FOXP3 infiltration samples showing positive PD-L2 expression (supplementary material, Table S4). There was no correlation between CD20 and PD-L1/PD-L2. These results suggest a correlation between immune infiltration and PD-L1/ PD-L2 expression in PDAC.

Association between clinicopathological parameters, DDR molecules, and PD-L1/PD-L2

Correlation analysis showed that positive PD-L1 expression on TCs was more frequent in the N1 stage ($p = 0.011$) and AJCC II stage ($p = 0.002$) groups; however, there was no relationship between

clinicopathological features and PD-L2 expression on TCs (supplementary material, Table S6). Conversely, positive PD-L2 expression on ICs was significantly associated with vascular invasion ($p = 0.033$) (supplementary material, Table S7). Additionally, there was no significant correlation between the expression of PD-L1/PD-L2 and post-operative liver metastasis (supplementary material, Tables S6 and S7).

IHC scoring showed that the rate of mutant p53 in our sample set was 70% (190/272) (supplementary material, Table S2), which is similar to those in previous studies [26,29]. Nonetheless, the percentage of dMMR in PDAC varies between studies and was 12% (35/291) in this study. Furthermore, NGS data revealed that the mutation rate of *BRCA1* and *BRCA2* in a subset of this cohort was 6.7% (4/60) and 5.0% (3/60), respectively. Besides, 5.9% (3/51) of low BRCA1 expression samples harboured *BRCA1* mutation, and *BRCA2* mutation was detected in 7.0% (3/43) of low BRCA2 expression samples (supplementary material, Table S8). The analysis of the correlation between PD-L2 and DDR

Table 1. Univariate analysis of DSS and PFS factors.

	DSS		PFS	
	HR (95% CI)	P value	HR (95% CI)	P value
Age (years)				
<60	Ref	0.303	Ref	0.378
≥60	1.172 (0.866–1.585)		1.133 (0.859–1.494)	
Differentiation (grade)				
Moderate and well	Ref	<0.0001	Ref	<0.0001
Poor	2.035 (1.506–2.751)		1.769 (1.339–2.338)	
Location				
Head	Ref	0.004	Ref	0.001
Body and tail	0.632 (0.460–0.867)		0.603 (0.451–0.807)	
Perineural invasion				
No	Ref	0.409	Ref	0.351
Yes	1.145 (0.830–1.579)		1.151 (0.856–1.548)	
Vascular invasion				
No	Ref	0.214	Ref	0.127
Yes	1.213 (0.895–1.646)		1.243 (0.940–1.645)	
T stage				
T1 and T2	Ref	0.001	Ref	0.002
T3 and T4	1.749 (1.266–2.418)		1.603 (1.182–2.173)	
N stage				
N0 and N1	Ref	0.002	Ref	0.002
N2	1.806 (1.249–2.612)		1.762 (1.241–2.501)	
AJCC stage				
I and II	Ref	<0.0001	Ref	<0.0001
III and IV	2.134 (1.522–2.992)		2.022 (1.466–2.788)	
PD–L1 on TCs				
Negative	Ref	0.014	Ref	0.039
Positive	1.511 (1.087–2.100)		1.319 (1.017–1.870)	
PD–L1 on ICs				
Negative	Ref	0.515	Ref	0.889
Positive	1.131 (0.781–1.636)		1.025 (0.725–1.449)	
PD–L2 on TCs				
Negative	Ref	0.038	Ref	0.065
Positive	1.404 (1.039–1.934)		1.320 (0.983–1.773)	
PD–L2 on ICs				
Negative	Ref	0.202	Ref	0.085
Positive	1.290 (0.873–1.907)		1.367 (0.958–1.951)	
p53 status				
Mutant	Ref	0.127	Ref	0.092
WT	1.311 (0.926–1.856)		1.312 (0.956–1.801)	
MMR status				
pMMR	Ref	0.109	Ref	0.078
dMMR	0.663 (0.401–1.097)		0.659 (0.414–1.049)	
BRCA1				
Low expression	Ref	0.474	Ref	0.644
High expression	0.832 (0.503–1.376)		0.899 (0.571–1.413)	
BRCA2				
Low expression	Ref	0.796	Ref	0.948
High expression	0.958 (0.690–1.329)		1.010 (0.754–1.353)	

molecules revealed positive PD-L2 expression in 53.9% ($n = 48$) of the samples with high BRCA2 expression, and PD-L2 expression on TCs was significantly related to BRCA2 expression ($p < 0.0001$) (supplementary material, Table S9). However, the correlation between PD-L2 expression on ICs and BRCA2 expression was not significant ($p = 0.115$) (supplementary material, Table S10). Representative images of BRCA staining are shown in Figure 1. Moreover, p53 status, MMR status, and BRCA1 expression were not associated with PD-L2. In addition, there was no correlation between PD-L1 and DDR molecules (supplementary material, Tables S9 and S10). These results suggest that PD-L2 expression on TCs may be associated with DDR status.

Table 2. Multivariate analysis of DSS and PFS factors.

	DSS		PFS	
	HR (95% CI)	P value	HR (95% CI)	P value
Location				
Head			Ref	0.048
Body and tail			0.703 (0.512–0.983)	
Differentiation (grade)				
Moderate and well	Ref	0.001	Ref	0.005
Poor	1.799 (1.275–2.537)		1.571 (1.146–2.155)	
AJCC stage				
I and II	Ref	<0.0001	Ref	<0.0001
III and IV	2.353 (1.601–3.459)		1.966 (1.374–2.813)	
PD–L1 on TCs				
Negative	Ref	0.046		
Positive	1.403 (1.010–2.055)			
PD–L2 on TCs				
Negative	Ref	0.028		
Positive	1.476 (1.042–2.091)			

Survival analysis of PDAC

Univariate analysis indicated that poor differentiation ($p < 0.0001$), tumour location at the head of the pancreas ($p = 0.004$), T3–T4 stage ($p = 0.001$), N2 stage ($p = 0.002$), AJCC III–IV stage ($p < 0.0001$), and positive PD-L1 ($p = 0.014$) and PD-L2 ($p = 0.038$) expression on TCs were associated with low disease-specific survival (DSS) of PDAC (Table 1). Furthermore, multivariate analysis indicated that poor tumour differentiation (hazard ratio [HR]: 1.799, 95% confidence interval [CI]: 1.275–2.537, $p = 0.001$, AJCC III and IV stage (HR: 2.353, 95% CI: 1.601–3.459, $p < 0.0001$), and positive expression of PD-L1 (HR: 1.403, 95% CI: 1.010–2.055, $p = 0.046$) and PD-L2 (HR: 1.476, 95% CI: 1.042–2.091, $p = 0.028$) on TCs were independent risk factors for unfavourable DSS in PDAC (Table 2). Besides, a positive PD-L1 expression on TCs was also associated with poor PFS in PDAC ($p = 0.039$) (Tables 1 and 2). Kaplan–Meier survival curves for PD-L1 and PD-L2 are shown in Figure 2.

In addition, survival analysis for immune infiltrates showed that high $CD3^+$ ($p = 0.016$), $CD4^+$ ($p = 0.034$), and $CD20^+$ ($p = 0.018$) lymphocyte infiltration was associated with a favourable DSS. Conversely, high $CD15^+$ neutrophils infiltration was related to poor DSS ($p = 0.014$). $FOXP3^+$ and $CD8^+$ lymphocyte infiltration was not significant for predicting patients' DSS; however, high $FOXP3^+$ lymphocyte infiltration was related to poor PFS ($p = 0.046$) (supplementary material, Figures S1 and S2).

Modification of PD–L2 by clinicopathological features and DDR molecule subgroups

It is possible that PD-L2 can be modified by several important clinicopathological features and DDR

molecules. Therefore, the stratification of PD-L2 expression on TCs by lymph node status, grade, AJCC stage, BRCA1, BRCA2, p53, and MMR status was assessed to determine a prognostic value of DSS for the various subgroups. No statistical significance was observed when PD-L2 was stratified by AJCC stage and BRCA2 (Table 3). However, multivariate analysis revealed that positive expression of PD-L2 on TCs was an independent prognostic factor for lower DSS in patients with pN0 stage (HR: 2.044, 95% CI: 1.105–3.780, $p = 0.023$), moderate and well tumour differentiation (HR: 1.965, 95% CI: 1.249–3.092, $p = 0.004$), low BRCA1 expression (HR: 1.523, 95% CI: 1.078–2.152, $p = 0.017$), WT p53 status (HR: 2.220, 95% CI: 1.061–4.647, $p = 0.034$), and pMMR status (HR: 1.692, 95% CI: 1.178–2.432, $p = 0.004$) (Table 3). Kaplan–Meier survival curves of PD-L2 expression on TCs stratified by different subgroups are shown in Figure 3 and supplementary material, Figure S3.

Associations among PD-L1, PD-L2, and chemotherapy efficacy

The relationship between PD-1 ligands and the efficacy of post-operative adjuvant chemotherapy was scarcely reported in PDAC. In our cohort, 193 (66.3%) patients received post-operative adjuvant chemotherapy. The median DSS in the $PDL1\text{-}TCs^{(-)}$ and $PDL1\text{-}TCs^{(+)}$ groups were 18.77 and 16.30 months, respectively, and adjuvant chemotherapy was significantly related to a better DSS in both groups ($p = 0.008$ and $p < 0.0001$, respectively), suggesting that the expression of PD-L1 on TCs would not affect the efficacy of adjuvant chemotherapy. However, adjuvant chemotherapy was
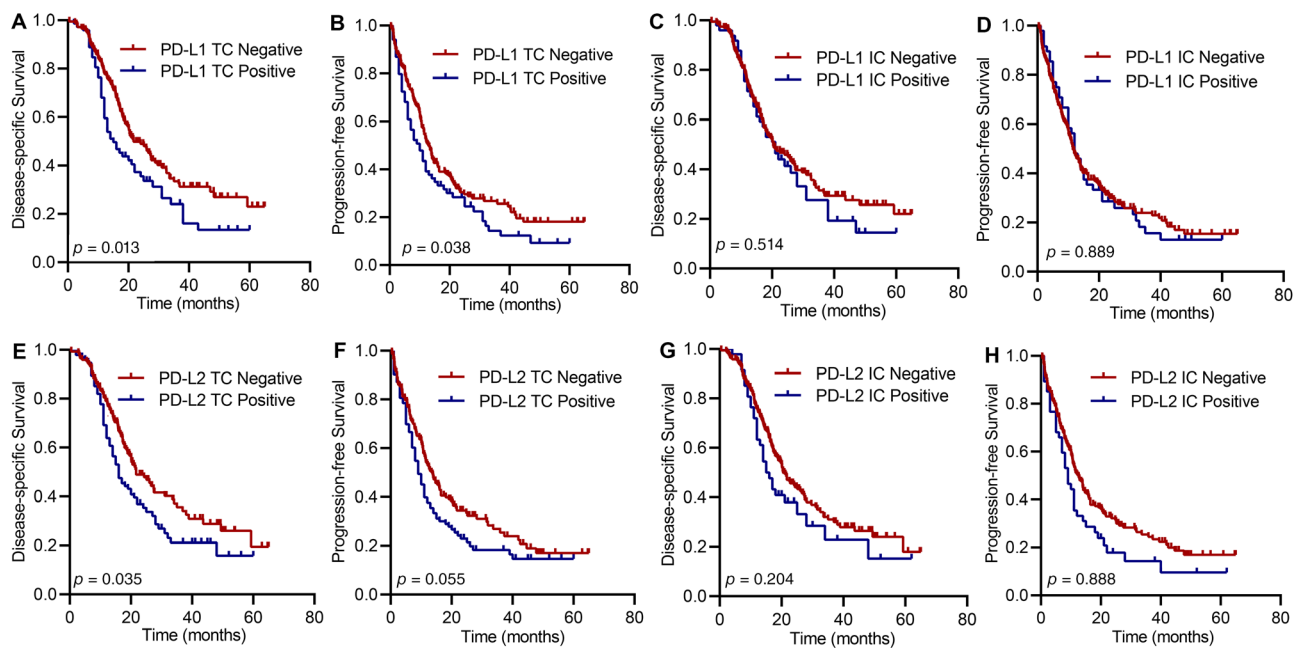
Figure 2. Kaplan–Meier curves of DSS and PFS for PD-L1 and PD-L2. (A, B) DSS and PFS curves of PD-L1 expression on TCs. (C, D) DSS and PFS curves of PD-L1 expression on ICs. (E, F) DSS and PFS curves of PD-L2 expression on TCs. (G, H) DSS and PFS curves of PD-L2 expression on ICs.

Table 3. Results of multivariate Cox regression analysis of PD-L2 per DDR molecule subgroups.

PD-L2 stratified by groups	Subgroups	DSS
Lymph node status	pN0	HR: 2.044, 95% CI: 1.105–3.780, $p = 0.023$
	pNI–N2	HR: 1.244, 95% CI: 0.835–1.852, $p = 0.283$
Differentiation (grade)	Poor	HR: 1.135, 95% CI: 0.663–1.944, $p = 0.644$
	Moderate and well	HR: 1.965, 95% CI: 1.249–3.092, $p = 0.004$
AJCC	I and II	HR: 1.410, 95% CI: 0.945–2.105, $p = 0.093$
	III and IV	HR: 1.894, 95% CI: 0.957–3.749, $p = 0.067$
BRCA1	Low	HR: 1.523, 95% CI: 1.078–2.152, $p = 0.017$
	High	HR: 1.608, 95% CI: 0.596–4.336, $p = 0.671$
BRCA2	Low	HR: 1.498, 95% CI: 0.903–2.484, $p = 0.118$
	High	HR: 1.468, 95% CI: 0.835–2.578, $p = 0.182$
p53	WT	HR: 2.220, 95% CI: 1.061–4.647, $p = 0.034$
	Mutant	HR: 1.233, 95% CI: 0.818–1.859, $p = 0.317$
MMR	pMMR	HR: 1.692, 95% CI: 1.178–2.432, $p = 0.004$
	dMMR	HR: 0.688, 95% CI: 0.189–2.496, $p = 0.569$

statistically associated with a favourable DSS ($p < 0.0001$) in the PDL1-ICs$^{(-)}$ group, which was not observed in the PDL1-ICs$^{(+)}$ group ($p = 0.061$). A similar result was also found for PD-L2 (supplementary material, Figure S4). These results suggest that the expression of PD-1 ligands on ICs, but not on TCs, may be associated with post-operative adjuvant chemotherapy efficacy.

Discussion

Although numerous clinical trials have shown favourable outcomes of PD-1/PD-L1 immunotherapy in the treatment of various tumours, their efficacy in PDAC is limited [4–7]. Unlike PD-L1, PD-L2 has been less studied in PDAC, but the role of PD-L2 in
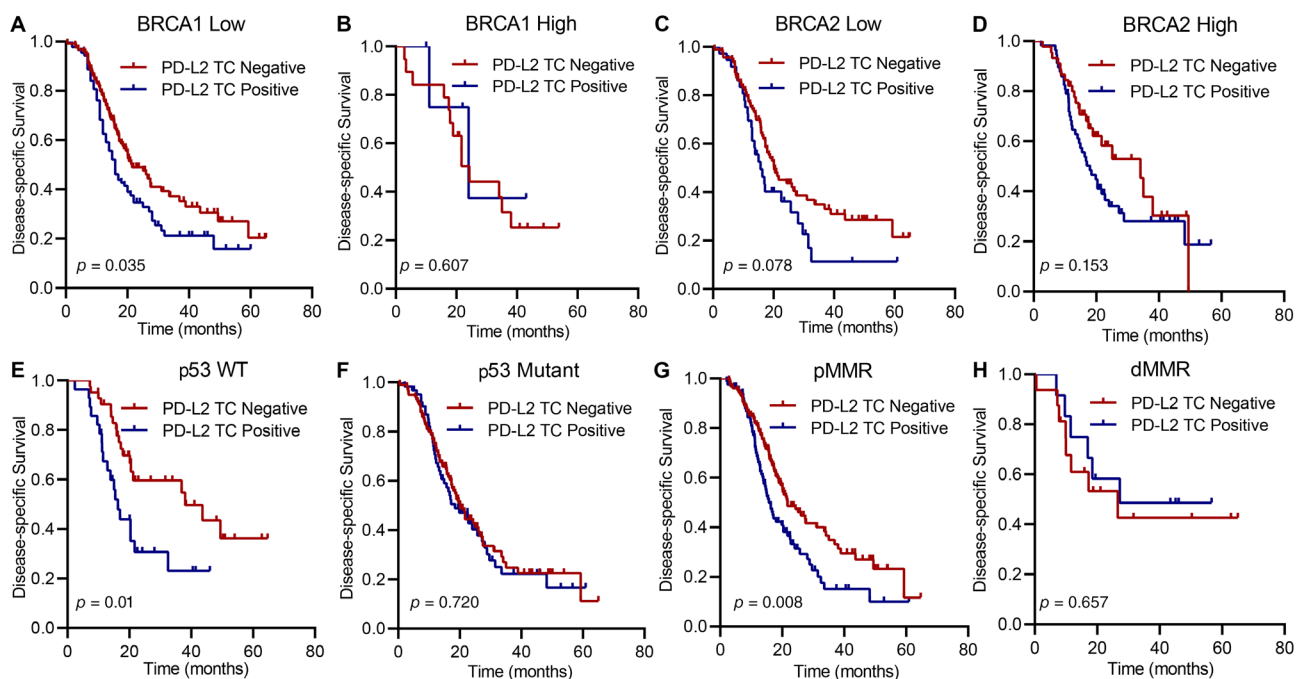
Figure 3. Kaplan–Meier curves of DSS for PD-L2 expression on TCs in low and high BRCA1 (A, B); low and high BRCA2 (C, D); p53-WT and p53-mutant (E, F); and pMMR and dMMR (G, H) subgroups.

predicting patient survival and PD-1 axis therapy efficacy should not be neglected. To overcome the bottleneck associated with PD-1 axis immunotherapy and increase the understanding of the role of the two PD-1 ligands in predicting the survival of PDAC patients, we analysed whether the combination of PD-L1/PD-L2 and tumour-infiltrating T lymphocytes as well as molecules associated with genomic stability could indicate post-operative survival in PDAC.

We evaluated PD-L1 and PD-L2 expression on TCs and ICs separately. PD-L1 positivity was 30 and 20% and PD-L2 positivity was 40 and 20% on TCs and ICs, respectively. Furthermore, our results showed that 61 of the PD-L1-negative samples (64.2%) were PD-L2-positive, 38 of the PD-L2-negative samples (27%) showed PD-L1 staining, and 34 samples co-expressed PD-L1 and PD-L2. However, none of our patients received anti-PD-1 axis therapy; thus, we could not analyse the correlation between the single or double expression of the two PD-1 ligands and anti-PD-1 immunotherapy efficacy. Future studies exploring immunotherapy efficacy in PDAC should focus on analysing this correlation. For the survival analysis, a positive PD-L1 (HR: 1.511, 95% CI: 1.087–2.100, $p = 0.014$) or PD-L2 (HR: 1.404, 95% CI: 1.039–1.934, $p = 0.038$) expression on TCs was associated with a lower DSS in PDAC, which was consistent with the findings of previous studies [30,31].

TILs are important factors affecting survival in PDAC. Our study showed that high CD3^{+}, CD4^{+}, and CD20^{+} cell infiltration was associated with better DSS, whereas high CD15^{+} cell infiltration was related to poor DSS, which was consistent with previous studies [32–34]. Besides, high FOXP3^{+} cell infiltration was related to poor PFS possibly due to the immune suppression mediated by FOXP3^{+} T regulatory cells. Moreover, TILs could also associate with the outcomes of PD-1/PD-L1 therapy. One study using a tumour immune microenvironment model reported that PD-L1 and TIL double positive tumours showed a relatively high response to PD-1/PD-L1 blockade therapy, whereas PD-L1 single positive tumours were prone to resistant to PD-1/PD-L1 inhibitor monotherapy, suggesting that immunotherapy targeting PD-1/PD-L1 should take TILs into consideration [35]. Correlation analysis of our PDAC samples revealed that PD-L1 expression on ICs was significantly associated with high FOXP3^{+} T lymphocyte infiltration ($p < 0.0001$). As PD-L1 functions in the induction and maintenance of T regulatory cells [36], the expression of PD-L1 and FOXP3 on stromal ICs could also be consistent. Similar to PD-L1, positive PD-L2 expression on TCs was associated with high FOXP3^{+} cells infiltration ($p = 0.001$), which was consistent with the findings of a previous study [31].

Genomic instability, such as homologous recombination deficiency and MSI, could affect PD-1/PD-L1

immunotherapy in various tumours such as colon cancer, gastroesophageal cancer, and melanoma [21–23]. One study showed that BRCA1-deficient breast cancers were associated with increased PD-L1 expression in breast cancer [37]. In addition, Sato *et al* reported that BRCA2 depletion promotes PD-L1 upregulation in a Chk1-dependent manner in lung cancer, and prostate cancer cell lines and other signalling pathways, such as STAT1 and STAT3, are also required for double-strand break-dependent PD-L1 upregulation [38]. These results suggest that homologous recombination repair related proteins could regulate PD-1 ligand expression in tumours, and the regulation mechanisms are complex. However, the relationship between DDR molecules and PD-L1/PD-L2 in PDAC is not clear. In this study, we did not observe a correlation between PD-L1 and DDR molecules, whereas positive PD-L2 expression on TCs was associated with high BRCA2 expression ($p < 0.0001$). Based on previous studies, we hypothesised that, in PDAC, the regulation of homologous recombination repair associated proteins possibly occurs mainly in PD-L2. Nonetheless, the specific regulation mechanism needs to be investigated in future studies. Moreover, in the low BRCA1 expression group, positive PD-L2 expression on TCs was determined to be an independent risk indicator in terms of low DSS (HR: 1.523, 95% CI: 1.078–2.152, $p = 0.017$). Advanced PDAC patients with homologous recombination deficiency (usually caused by pathogenic BRCA mutations) harboured favourable outcomes based on poly ADP-ribose polymerase inhibitor (PARPi) treatment [39]; thus, combining PD-1 axis immunotherapy with PARPi chemotherapy may improve the prognosis of patients with both low BRCA and high PD-L2 expression.

A previous study reported that positive expression of PD-L1 is associated with p53 expression in oral squamous carcinoma [40]. Moreover, p53 could support the expression of JAK2 and thereby participate in IFN-γ-induced PD-L1 expression in melanoma cells [41]. These results suggest a correlation between p53 and PD-L1 in tumours; however, the correlation between p53 and PD-L2 in PDAC has not yet been reported. Our results showed no significant correlation between PD-L2 expression and p53 status. However, in the WT p53 group, positive PD-L2 expression on TCs was an independent risk factor (HR: 2.220, 95% CI: 1.061–4.647, $p = 0.034$) for a low DSS. Another crucial observation in this study was that PD-L2 expression on TCs was also related to a low DSS in the pMMR subgroup ($p = 0.004$). Accumulating evidence suggests that tumours with dMMR status can synthesise neoantigens and upregulate PD-L1

expression by enhancing the release of IFN-γ from TILs [42,43]. Thus, PD-1/PD-L1 immunotherapy in MSI-positive tumours showed a high response. Here, our results showed that, even in patients with a normal p53 and pMMR status, PD-L2 could serve as a significant indicator of the survival potential of a patient, which may be useful for novel risk model construction and clinical treatment decision-making for PDAC.

Another interesting result in this study was that post-operative chemotherapy could benefit patients in terms of DSS in both PDL1-TC $^{(-)}$ and PDL1-TC $^{(+)}$ groups, while it only affected survival in patients who showed negative PD-L1 expression on ICs. A similar result was also observed for PD-L2. One previous study reported that silencing of the immune checkpoint B7-H3 could promote apoptosis and increase gemcitabine sensitivity in PDAC [44]. These results suggested that there was a potential correlation between immune checkpoint and chemotherapy efficacy.

Several limitations were noted in this study. First, considering the heterogeneity within tumours, TMA cores may not accurately represent the entire tumour. Second, it was difficult to assess PD-L1 and PD-L2 expression in different subgroups of ICs and analyse the co-expression of various lymphocyte markers and PD-L1/PD-L2 using immunohistochemistry. Future studies could be performed with multiplex IHC. Third, the mechanisms of the correlations between BRCA1, BRCA2, p53 status, MMR status, and PD-L2 were not explored in this study. However, despite these limitations, our study provides novel insights into the relationship between immune checkpoints and other gene signatures, which may contribute to the development of new combination therapy regimens and personalised treatment for PDAC.

Conclusion

This study determined that both PD-L1 and PD-L2 contribute to the prediction of PDAC prognosis. Unlike PD-L1, PD-L2 has been reported less frequently in PDAC. In this study, we determined that PD-L2 was associated with Treg cell infiltration and BRCA2 status. In addition, PD-L2 could be a potential biomarker for anti-PD-1 axis immunotherapy selection in patients with normal p53 and pMMR status. Our findings further refine our understanding of the PD-1 axis, providing a reference for personalised and precise treatment.

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Author contributions statement

JC substantially contributed to the conceptualisation and design, and critical revising of the manuscript. XC, SM, HM, SY and ZL contributed substantially to analysis and interpretation of data and drafting the article. YZ provided substantial contributions to conceptualisation and design, analysis and interpretation of data, and drafting the article. All authors read and approved the final manuscript to be published.

Data availability statement

The data sets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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SUPPLEMENTARY MATERIAL ONLINE

Figure S1. Kaplan–Meier curves of DSS for CD3, CD4, CD8, FOXP3, CD45RO, CD20, CD68, and CD15 expression on ICs

Figure S2. Kaplan–Meier curves of PFS for CD3, CD4, CD8, FOXP3, CD45RO, CD20, CD68, and CD15 expression on ICs

Figure S3. Kaplan–Meier curves of DSS for PD-L2 expression on TCs

Figure S4. Univariable DSS using the Kaplan–Meier method for post-operative adjuvant chemotherapy application

Table S1. Details of antibodies for IHC staining

Table S2. Baseline information of PDAC patients

Table S3. PD-L1 in relation to PD-L2 of PDAC

Table S4. PD-L1 and PD-L2 expression on TCs in relation to immune infiltrates of PDAC

Table S5. PD-L1 and PD-L2 expression on ICs in relation to immune infiltrates of PDAC

Table S6. PD-L1 and PD-L2 expression on TCs in relation to clinicopathological characteristics of PDAC

Table S7. PD-L1 and PD-L2 expression on ICs in relation to clinicopathological characteristics of PDAC

Table S8. Comparison of NGS and IHC for BRCA1 and BRCA2 in PDAC

Table S9. PD-L1 and PD-L2 on TCs in relation to DDR molecules of PDAC

Table S10. PD-L1 and PD-L2 on ICs in relation to DDR molecules of PDAC