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Programmed cell death ligand-1 (PD-L1) and CD8 expression profiling identify an immunologic subtype of pancreatic ductal adenocarcinomas with favorable survival

Ludmila Danilova, Won Jin Ho, Qingfeng Zhu, Teena Vithayathil, Ana De Jesus-Acosta, Nilofer S. Azad, Daniel A. Laheru, Elana J. Fertig, Robert Anders, Elizabeth M. Jaffee*, and Mark Yarchoan*

Bloomberg–Kimmel Institute for Cancer Immunotherapy, Johns Hopkins University School of Medicine, Baltimore, MD 21287

Abstract

Immune checkpoint therapy has failed to demonstrate meaningful clinical benefit in unselected cases of pancreatic adenocarcinoma (PDAC), but a subset of PDACs are known to upregulate pathways involved in acquired immune suppression. Further delineation of immunologic subtypes of PDAC is necessary to improve clinical trial designs and identify patients who might benefit from immune checkpoint therapy. We employed clinical survival and RNA expression data from The Cancer Genome Atlas (TCGA) to investigate the relationship between immune modulating pathways and immune subset markers and their impact on survival in PDAC patients. Of the adaptive immune resistance pathways, expression of PD-L1 and IDO1 were individually associated with poor survival. Although CD8 expression alone was not correlated with survival, the combination of PD-L1⁺ and high CD8 expression identified a subtype with favorable survival. We further extended these observations using an independent PDAC cohort from our institution via immunohistochemistry, again observing that the PD-L1⁺/CD8^{high} subtype associated with positive prognosis. Although PDAC is regarded as a poorly immunogenic cancer type, these findings infer that T-cell infiltration in the absence of adaptive immune resistance pathways is a feature of long-term survival in PDAC and imply the importance of developing future immunotherapeutic strategies based on data-supported biomarkers to refine patient selection.

Keywords

PD-1; Pancreatic Cancer; Tumor Immune Microenvironment; Immunotherapy

*To whom correspondence should be addressed: Elizabeth M. Jaffee, Bloomberg–Kimmel Institute for Cancer Immunotherapy, Johns Hopkins University School of Medicine, Baltimore, 4M07 Bunting Blaustein Cancer Research Building, 1650 Orleans Street, Baltimore, MD 21287, 410-955-2957 (phone); Mark Yarchoan, Bloomberg–Kimmel Institute for Cancer Immunotherapy, 1650 Orleans St. CRBI 4M08, Baltimore, MD 21287, (410) 955-7141 (phone).

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Introduction: Adaptive immune resistance pathways and survival in pancreatic adenocarcinoma

Pancreatic adenocarcinoma (PDAC) is the fourth most common cause of cancer-related death in the United States, and the incidence of PDAC is rising worldwide (1,2). Most cases of PDAC are metastatic disease at the time of diagnosis, and the median survival for these patients is less than one year (2). This highlights the continued need for new and effective therapies in PDAC. Systemic chemotherapy is the mainstay of treatment for PDAC. Although novel immune checkpoint inhibitors targeting programmed cell death protein 1 (PD-1) or its ligand (PD-L1) or cytotoxic T lymphocyte-associated protein 4 (CTLA-4) have activity in many different cancers, they have thus far failed to show meaningful clinical benefit in unselected patients with PDAC. The objective response rate to immune checkpoint therapy in unselected patients with PDAC has been approximately 5% in prior clinical trials (3-5). Based on its poor response rate to current systemic immunotherapies, PDAC is largely considered to be a poorly immunogenic cancer type. To optimally identify patients who may have intact antitumor immune responses and/or benefit from immunotherapeutic strategies, an improved immunologic characterization of PDACs is needed.

Despite its resistance to systemic immunotherapies, an integrated genomic analysis has identified that a subset of PDACs (the “immunogenic” subset) have upregulated pathways involved in acquired immune suppression with enhanced signaling through the PD-1 and CTLA-4 pathways (6). Within this subset of PDAC, patients with high macrophage or T-cell co-inhibition scores had shorter overall survival. PD-L1 expression in the tumor microenvironment has also been identified as a poor prognostic factor for survival in other independent cohorts of patients with PDAC (7-12). This suggests the possibility that although immune checkpoint inhibitors may be insufficient to produce clinical responses in unselected populations of PDAC, there may be subsets of PDAC where acquired immune suppression through the PD-L1 pathway is, nonetheless, an important factor for PDAC disease progression.

Subtyping of the immune microenvironment in PDAC offers the opportunity for therapeutic development through personalization of systemic immunotherapies. Mismatch repair deficiency (MMRd) is the only biomarker currently used to identify cases of PDAC that are likely to respond to immune checkpoint inhibitors (13-15). Although PD-L1 expression in tumors is one of the most established biomarkers of active immunoregulation, it is less informative without contextualization. Although PD-L1 expression has been interrogated in multiple cohorts of PDAC, only a few studies have investigated the importance of PD-L1 expression in combination with CD8 expression and other adaptive immune resistance pathways that may contribute to PDAC immunosuppression (8,16-19). Using clinical survival and RNA expression data from The Cancer Genome Atlas (TCGA), we investigated the relationship between PD-L1 expression and survival in patients with pancreatic cancer in combination with tumor-infiltrating immune subsets, and identified additional immune checkpoints that are upregulated in PDAC and may be implicated in tumor progression. We also sought to confirm the significance of these relationships using a complementary, immunohistochemical method in an independent patient cohort. Our findings provide

grounds for immune subtyping of PDAC and infer opportunities for therapeutic development of immunotherapy in PDAC.

Methods

TCGA data

RNA sequencing (RNAseq) data Level 3 RSEM normalized data for 152 pancreatic adenocarcinoma (PAAD) patients (20) from TCGA were accessed from the Broad Institute TCGA GDAC Firehose (<https://ezid.cdlib.org/id/doi:10.7908/C11G0KM9>) and log2-transformed. Mutation annotation files (MutSig 2.0) for 146 pancreatic adenocarcinoma patients were downloaded from the Broad Institute TCGA GDAC Firehose's analysis_2015_04_02, (<http://gdac.broadinstitute.org/>). As a measure of mutational burden, we used the number of non-synonymous mutations per sample that were log10-transformed for the further analysis. Corresponding clinical data were obtained from Lui et al. (21). Data analysis was performed using R/Bioconductor software (version 3.5.0) with build-in packages and custom routines.

Johns Hopkins Cohort, Immunohistochemistry (IHC) staining, and Quantitation

This research was performed in accordance with the Declaration of Helsinki. The evaluation of archived pathology specimens described in this manuscript was approved by the Institutional Review Board (IRB) at Johns Hopkins (NA-00080582), which does not require individual written patient consent. Cases of PDAC collected from patients who had undergone a pancreatic resection with curative intent from 2008 to 2012 were identified using the Johns Hopkins Pancreas Database, a repository of patient clinical data. We excluded cases that were lost to follow-up or for whom long-term survival data were unknown. To specifically enrich for differences in tumor biology, we subsequently selected patients who died as a result of PDAC recurrence in less than 500 days from (short-term survivors, n=18) or recurred with PDAC but remained alive for at least 1000 days (long-term survivors, n=15).

The clinical diagnosis for each case was confirmed by a pathologist (RAA) through review of the corresponding H&E slides. One representative formalin-fixed paraffin-embedded (FFPE) block from each tumor specimen was selected. Consecutive 5 µm-thick sections were cut from each block and mounted on glass slides. Automated staining was performed on the Leica Bond RX (Leica Biosystems, Buffalo Grove, IL) for CD8 and IDO1. Slides were baked and dewaxed online, followed by antigen retrieval, followed by blocking for non-specific binding using Protein block (X090930, Agilent Technologies, Santa Clara, CA). Detection was performed using the Bond Polymer Refine Kit (DS9800, Leica Biosystems, Buffalo Grove, IL). Samples were counterstained and dehydrated through a graded-alcohol series into HistoClear II (508990150, ThermoFisher Scientific, Grand Island, NY) and coverslipped using Ecomount (5082832, Biocare Medical, Walnut Creek, CA). Manual staining was performed for PD-L1 staining as the protocol previously described (22). Additional staining reagent information for CD8, IDO1, and PD-L1 are listed in Supplementary Table S1.

CD8 density was determined using a quantitative image analysis approach. Whole slides were scanned at 20x objective equivalent (0.49 microns/pixel) using a digital slide scanner (Nanozoomer, Hamamatsu). Image analysis (HALO Indica Labs) was used to determine the density (# of cells/surface area analyzed) of CD8-expressing lymphocytes within the tumor compartment. Presence of PD-L1 and IDO1 expression on tumor at least 1% of tumor cells was performed manually by a trained pathologist (RAA), working independently, who was blinded to the clinical features of the case while performing the scoring. Representative PD-L1 and IDO1 IHC images are shown in Supplementary Fig. S1.

Statistical analysis

To estimate a threshold for over-expression of immune checkpoint markers, we used the Memorial Sloan Kettering (MSKCC) cBioPortal (23). We found that approximately 10% (range 5–17%)(Supplementary Fig. S2) of TCGA PDACs had expression higher than one standard deviation above the mean of the reference population used to define the cBioPortal z-score, and used this 10% cutoff to define checkpoint positivity. We defined high and low lymphocyte infiltration as values above and below median expression in tumor samples, and similarly defined high and low CD8⁺ T-cell density as above and below the median for the Johns Hopkins University samples. Data analysis was performed using R/Bioconductor software (23) with build-in packages and custom routines. Heat maps, box-and-whisker plots, and scatter plots were created using the gplots package and build-in R graphic functions.

For correlation of overexpression with 5-year survival, we split samples to high vs. low using the top 10% for each marker. Kaplan-Meier survival curves were made using the survfit function from the survival package, and hazard ratios and p-values from log-rank tests were reported. To test association of continuous expression with 5-year survival, we fitted the univariate Cox proportional hazards regression model as implemented in the coxph function from the R survival package (Supplementary Table S2).

For the Th1/IFN γ gene signature, we combined genes from the published Th1 signature from Bindea et al. (23) and genes from IFN γ signaling pathway from Reactome database [<http://www.reactome.org>](Supplementary Table S3). Pearson's correlation coefficients were reported for correlation of expression of PD-L1 (*CD274*) and *IDO1* with *CD8A*, *CD68*, *GZMB*, *NOS2*, and the Th1/IFN γ gene signature, and the Spearman's correlation coefficients were reported for mutational load analyses.

Results

Association between survival and immune modulating pathways in PDAC

To define candidate immune checkpoints, including PD-L1, that may induce tumor immunosuppression in the tumor microenvironment in PDAC and provide prognostic insight, we correlated tumor expression of immune modulating pathways with 5-year survival curves using RNA expression data from TCGA. Many PDAC tumors upregulated only a few, if any, adaptive immune pathways, but upregulation of select adaptive immune resistance pathways, including PD-L1, was observed in a subset of PDAC tumors. The

expression of immune checkpoints in PDAC showed limited clustering (Fig. 1, Supplementary Table S4). Upregulation of PD-L1 (*CD274*) frequently occurred with upregulation of *IDO1*, upregulation of *CTLA4* frequently co-occurred with upregulation of *TIGIT* and *TNFRSF18*, and *LAG3* upregulation frequently co-occurred with upregulation of *IDO1* and *CD40*.

When analyzing the 5-year overall survival (OS) data, tumor expression of PD-L1 (*CD274*) was associated with poor survival in PDAC [p=0.001, Hazard ratio for survival (HR)=2.55 (CI: 1.15–5.64)], consistent with prior reports (8-12)(Fig. 2A). Expression of indoleamine 2, 3-dioxygenase (*IDO1*) was also associated with a poor OS [p=0.008, HR=2.24 (0.96–5.2)]. However, no association between the expression of multiple other immune checkpoints, e.g. *LAG3*, *TIGIT*, and TIM-3 (*HAVCR2*), in the tumor microenvironment and survival in PDAC specimens was seen (Fig. 2B). Expression of CTLA-4 in the tumor microenvironment was marginally correlated with increased survival. Across many tumor types, a high density of tumor-infiltrating lymphocytes is a favorable prognostic indicator (24,25). Based on mRNA expression from the TCGA data, however, we did not find any significant relationship between expression of any canonical immune subset markers within the tumor, including *CD8A*, *CD4*, *CD19*, or *CD68*, and survival (all p>0.05).

Upon identifying that PD-L1 (*CD274*) and *IDO1* expression conferred a poor prognosis in PDAC, we subsequently investigated factors that are known to contribute to their expression. Although most pancreatic cancers have a low tumor mutational burden, a small percentage of pancreatic cancers have mismatch repair deficiency (MMRd), which results in a failure to repair errors in basepair mismatches in tumor DNA and a high tumor mutational burden (26-29). Anti-PD-1 therapy has shown clinical activity in a small subset of human cancers with MMRd, including select cases of PDAC (14,15). We hypothesized that a high tumor mutational burden might lead to an increased density of neoantigen-specific CD8⁺ lymphocytes in the tumor microenvironment and, thus, upregulation of adaptive immune pathways by the tumor cells such as PD-L1 (*CD274*) or *IDO1*. Therefore, we investigated whether the expression of PD-L1 (*CD274*) or *IDO1* was associated with tumor mutational burden in PDAC. We did not observe any relationship between the tumor mutational burden and either PD-L1 or *IDO1* expression in TCGA (Supplementary Fig. S3A-B). However, only two samples in this cohort had MMRd with a mutation in *MLH1*, *MSH2*, *MSH6* or *PMS2*. We also examined the relationship between these markers and tumor stage, hypothesizing that adaptive immune resistance might increase with increasing tumor stage. Most patients (n=131 out of 152) in TCGA had stage 2 disease, limiting the power of this analysis, but we did not observe any signal for increased expression of PD-L1 (*CD274*) or *IDO1* with increased tumor stage (Supplementary Fig. S4).

Interaction between immune cell subsets and PD-L1 and IDO1 expression in PDAC

CD8⁺ T cells in the tumor microenvironment can produce interferon-gamma (IFN γ), leading to upregulation of adaptive immune resistance pathways, including the PD-1/PD-L1 axis and *IDO1* (30,31). Therefore, we examined whether expression of *CD8A* or other immune subsets in the tumor microenvironment was associated with expression of PD-L1 (*CD274*) or *IDO1* (Fig. 3). Consistent with our hypothesis, a positive relationship between *CD8A* and

PD-L1 (*CD274*) was seen ($r=0.39$, $p=5.09\times10^{-7}$), as well as between *CD8A* and *IDO1* ($r=0.59$, $p=6.3\times10^{-16}$), and expression of *IDO1* and PD-L1 (*CD274*) were also positively correlated with one another ($r=0.54$, $p=1.03\times10^{-12}$) (Fig. 3A-C). PD-L1 (*CD274*) can also be expressed by monocyte-lineage cells, so we examined the relationship between PD-L1 (*CD274*) and *CD68*, a marker of macrophages and other monocyte lineage cells. A positive association between *CD68* and PD-L1 (*CD274*) expression in the microenvironment was also observed ($r=0.33$, $p=3.32\times10^{-5}$) (Fig. 3D). The association between PD-L1/*IDO1* expression and *CD8A/CD68* expression extended to granzyme B (*GZMB*), a marker of CD8⁺ T-cell cytolytic activity and *NOS2* (also known as iNOS), a marker for the M1 macrophage phenotype (Fig. 3E-I).

We also examined the relationship between PD-L1 (*CD274*) and *IDO1* expression and a combined Th1/IFN γ gene signature (Fig. 4). A Th1 bias is characterized by the secretion of IFN γ and is primarily responsible for activating an antitumor immune response. If PD-L1 (*CD274*) and *IDO1* arise as a result of a cytotoxic T-cell antitumor response in the tumor immune microenvironment in PDAC, we hypothesized that expression of both molecules would be associated with a Th1/IFNG gene signature. Consistent with this hypothesis, we found that expression of both PD-L1 (*CD274*) and *IDO1* were similarly associated with the Th1/IFNG gene signature.

Association between survival and PDAC subtypes defined by PD-L1/*IDO1* and CD8 expression

Although *CD8A* expression and the Th1/IFNG gene signature were associated with PD-L1 (*CD274*) expression, some patients had elevated *CD8A* expression in the absence of PD-L1/PD-L1 expression. Because the different expression patterns of PD-L1 (*CD274*) and *CD8A* may implicate biologically and possibly clinically different behavior, we then looked at the survival of patients with elevated or normal PD-L1 expression, stratified by *CD8A* expression (Fig. 5A). Although *CD8A* expression alone did not correlate with survival in PDAC (Supplementary Table S2), patients with the best survival over five years had increased *CD8A* infiltration without expression of PD-L1, whereas patients with PD-L1 expression without increased *CD8A* infiltration had the worst survival. Similar results were obtained when the cohort was stratified by *IDO1* and *CD8A* expression, with the high *CD8A* expression and low *IDO1* expression group showing the highest survival (Fig. 5B). These data suggest that the combined analysis of *CD8A* and PD-L1 (or *IDO1*) expression yields different subtypes of PDACs; specifically, our results suggested that *CD8A* expression in the absence of PD-L1 or *IDO1* expression is associated with improved survival in PDAC.

Parallel analysis of PD-L1 and *IDO1* expression and CD8 expression

To extend our observations based on mRNA expression data from the TCGA, we sought to further confirm the relationship between PD-L1/*IDO1* expression and poor patient survival, as well as improved survival in the subtype with high CD8 expression but low PD-L1/*IDO1* expression by analyzing the protein expression in an independent cohort of PDAC from Johns Hopkins University ($n=33$). Banked PDAC tumor samples were analyzed from patients who had undergone a resection for PDAC with curative intent but died as a result of PDAC recurrence in 500 days or less (short-term survivors) or recurred with PDAC but

remained alive for at least 1000 days (long-term survivors). Most patients with PDAC recur after resection, and the selective use of patients with either poor or long survival after disease recurrence allowed us to investigate specific differences in disease biology that might contribute to the differences in survival for these patients. Samples were stained for PD-L1, IDO1, and CD8 by IHC. CD8 expression was quantified using digital image analysis to determine the density of CD8⁺ cells. Expression of PD-L1 and IDO1 on tumor cells was quantified by a pathologist (RAA) who was blinded to the clinical history of the patients. The clinicopathological characteristics of the 33 PDAC samples analyzed are shown in Table 1.

Of the samples stained, 11 (33.3%) had positive PD-L1 expression and 7 (21.2%) had positive IDO1 expression on tumor cells. No significant association between PD-L1 status or IDO1 expression and any clinical tumor features, including pathologic stage, histologic grade, resection margin, venous or perineural invasion ($p>0.05$ for all comparisons), was seen. Consistent with TCGA data, patients with positive PD-L1 expression by IHC had worse survival than patients without detectable PD-L1 expression [$p=0.01$, HR=2.61 (1.03–6.61); Fig. 6A]. A trend towards worse survival in the IDO expression group was also observed but did not meet the threshold for statistical significance ($p=0.068$; Fig. 6B). An overlap of IDO1 and PD-L1 expression occurred in this cohort, with 6/7 IDO1⁺ samples also staining positive for PD-L1. A larger separation of survival curves was seen when the six PD-L1⁺IDO1⁺ samples were compared to samples without expression of either marker ($n=21$) [$p=0.000382$, HR=4.51 (CI: 0.94–21.53); Fig. 6C].

We also sought to confirm our findings from TCGA data showing that higher *CD8A* expression in the absence of PD-L1 (*CD274*) expression was associated with improved survival in PDAC. Given the small number of PD-L1⁺ cases, we separated the cohort into three groups: PD-L1⁺, PD-L1[−]/CD8^{low}, and PD-L1[−]/CD8^{high}. Again, consistent with our prior TCGA findings, the group of patients with high CD8 expression in the absence of PD-L1 expression (PD-L1[−]/CD8^{high}) had the best survival, whereas PD-L1⁺ cases and PD-L1[−] cases with low CD8 expression had similar survival (Fig. 6D). Stratified analysis using IDO1 and CD8 expression only showed a trend toward improved prognosis for the IDO1[−]/CD8^{high} subtype ($p=0.11$; Fig. 6E).

Discussion

In this study, we aimed to characterize a subtype of PDAC based on the expression of immune-modulating pathways in combination with *CD8A* expression, and explore their clinical relevance by correlation with survival. Using mRNA expression data from TCGA and protein expression data via IHC from an independent cohort, we have shown that the following: i) high PD-L1 expression is associated with poor survival in PDAC; ii) PD-L1 expression in PDAC is associated with *CD8A* expression, *IDO1* expression, and a Th1/IFN γ gene signature; and iii) although *CD8A* density alone was not predictive of improved survival in PDAC, PD-L1[−]/CD8^{high} expression identified a subtype with favorable survival in PDAC. Our findings further strengthen prior observations (6) that despite being widely described as a poorly immunogenic tumor type, the endogenous antitumor immune response is a critical factor for PDAC survival. Specifically, our data suggest that the co-assessment of

PD-L1 expression and CD8 expression density may provide a better assessment of the immunologic state of PDACs and, in turn, the patient survival than expression of PD-L1 alone. Our findings also infer potential opportunities for development of immunotherapeutic strategies based on immuno-subtyping of PDACs.

PD-L1 expression has been interrogated in multiple PDAC cohorts. Overall, prior studies based on select cohorts (8,16,18,19) are consistent with our observation, including a meta-analysis of such reports (17), demonstrating that PD-L1 expression is associated with poor prognosis in PDAC. However, these studies have not contextualized the PD-L1 expression by CD8 expression and corresponding survival outcomes. Contextualization of PD-L1 expression is not trivial. In unique clinical contexts, such as diffuse large B-cell lymphomas, PD-L1 expression can be overexpressed on tumor cells as a result of genetic events (32), but such genetic events are not thought to occur in PDAC. More often, PD-L1 expression on tumor cells can be induced by IFN γ as a consequence of an antitumor T-cell response, for which CD8⁺ T cells constitute the major effector arm of antitumor immunity (33). Accordingly, the correlation between PD-L1 expression and CD8 expression, as well as a Th1/IFN γ gene signature in our study suggest that PD-L1 expression in PDAC arises as a mechanism of adaptive immune resistance to an antitumor T-cell response. These findings, accompanied by the different survival outcomes in the PD-L1/CD8 expression subtypes, underscore the importance of considering CD8 expression when studying the PD-L1 expression in this tumor type.

Immune checkpoint inhibitors have thus far failed to demonstrate meaningful clinical benefit in PDAC. Thus, an improved understanding of the PDAC tumor microenvironment, thereby identifying subsets of patients who may derive benefit from these therapies, is needed to improve upon current therapies. The association of PD-L1 expression and poor survival suggest that PD-L1 may be a driver of disease progression in a subset of patients with PDAC. Despite a lack of compelling radiographic responses to PD-1 pathway inhibitors in unselected populations, our data suggest that there may a subset of PD-L1⁺/CD8^{high} PDAC patients for whom blocking the PD-1 pathway may reinvigorate CD8⁺ T-cell responses and lead to improved survival. Our results are consistent a recent integrated genomic analysis of PDAC that identified the existence of an “immunogenic” disease subset (6), but their analysis did not stratify PDAC based on PD-L1 or CD8 alone. Referring to the immunologic classification paradigm described by Teng et al. (34) based on the clinical experience with melanoma patients, “Type I” cancers are those with high presence of tumor-infiltrating lymphocytes and PD-L1 expression. Type I is the subtype that primarily responds to immune checkpoint inhibitor (ICI) therapies but constitutes only a minority of patients with PDAC in our study. This provides a possible explanation as to why PDACs in aggregate do not seem to respond to ICIs and again proposes the importance of refining patient selection for immunotherapy trials. This hypothesis requires clinical validation in prospective clinical trials. For patients with lower T-cell infiltration without and with PD-L1 expression, i.e. “Type II” and “Type III”, respectively, it is likely that PD-1 pathway inhibitors will need to be used in combination with other forms of immunotherapy such as vaccines to first induce a T-cell response and a compensatory upregulation of the PD-1 pathway (35). Such combinatorial approaches to recruit immune cells and enhance immune recognition may effectively convert these types into an immunotherapy-responsive state. This is an especially

critical challenge for PDACs given that most patients have relatively low CD8⁺ T-cell infiltration based on our analysis.

Our study recognized *IDO1* expression as a correlate of poor survival in PDACs consistent with previous observations (36,37), but the clinical evidence that targeting *IDO1* can improve outcomes in human cancers is lacking thus far (38). Another finding was that there was very little clustering of immune checkpoints in PDAC, with a subset of patients highly expressing at least one adaptive immune resistance pathway. This suggests that immunotherapies could eventually be paired on the basis of immune biomarkers in the tumor microenvironment, but the feasibility of a personalized approach to immunotherapy in PDAC requires prospective investigation.

Strengths of our study include the use of two different disease cohorts with well-documented clinical outcomes and the use of sensitive antibodies to stain clinical samples in parallel. Limitations include the retrospective nature of our analyses and selection biases inherent in our cohort, such as a tertiary care practice referral bias. We demonstrated longer survival for PD-L1⁻/CD8^{high} patients and poor survival for PD-L1⁺ patients with PDAC. Although PDAC is widely regarded as a poorly immunogenic cancer type, these findings support the notion that an active antitumor immune response is feature of long-term survival in PDAC and infer opportunities for the development of novel immunotherapies based on data-supported biomarkers to refine patient selection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Miller KD, Siegel RL, Lin CC, Mariotto AB, Kramer JL, Rowland JH, et al. Cancer treatment and survivorship statistics, 2016. *CA Cancer J Clin.* 2016;66:271–89. [PubMed: 27253694]
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin.* 2016;66:7–30. [PubMed: 26742998]
3. Royal RE, Levy C, Turner K, Mathur A, Hughes M, Kammula US, et al. Phase 2 trial of single agent Ipilimumab (anti-CTLA-4) for locally advanced or metastatic pancreatic adenocarcinoma. *J Immunother.* 2010;33:828–33. [PubMed: 20842054]
4. Brahmer JR, Tykodi SS, Chow LQM, Hwu W-J, Topalian SL, Hwu P, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med.* 2012;366:2455–65. [PubMed: 22658128]
5. O'Reilly EM, Oh D-Y, Dhani N, Renouf DJ, Lee MA, Sun W, et al. A randomized phase 2 study of durvalumab monotherapy and in combination with tremelimumab in patients with metastatic pancreatic ductal adenocarcinoma (mPDAC): ALPS study *J Clin Oncol.* American Society of Clinical Oncology; 2018;36:217. [PubMed: 29161205]
6. Bailey P, Chang DK, Nones K, Johns AL, Patch A-M, Gingras M-C, et al. Genomic analyses identify molecular subtypes of pancreatic cancer *Nature.* Nature Publishing Group; 2016;531:47–52. [PubMed: 26909576]
7. Nomi T, Sho M, Akahori T, Hamada K, Kubo A, Kanehiro H, et al. Clinical Significance and Therapeutic Potential of the Programmed Death-1 Ligand/Programmed Death-1 Pathway in Human Pancreatic Cancer. *Clin Cancer Res.* 2007;13:2151–7. [PubMed: 17404099]
8. Birnbaum DJ, Finetti P, Lopresti A, Gilabert M, Poizat F, Turrini O, et al. Prognostic value of PDL1 expression in pancreatic cancer. *Oncotarget.* 2016;7:71198–210. [PubMed: 27589570]

9. Wang L, Ma Q, Chen X, Guo K, Li J, Zhang M. Clinical Significance of B7-H1 and B7-1 Expressions in Pancreatic Carcinoma World J Surg. Springer-Verlag; 2010;34:1059–65. [PubMed: 20145927]
10. Geng L, Huang D, Liu J, Qian Y, Deng J, Li D, et al. B7-H1 up-regulated expression in human pancreatic carcinoma tissue associates with tumor progression J Cancer Res Clin Oncol. Springer-Verlag; 2008;134:1021–7. [PubMed: 18347814]
11. Gao H-L, Liu L, Qi Z-H, Xu H-X, Wang W-Q, Wu C-T, et al. The clinicopathological and prognostic significance of PD-L1 expression in pancreatic cancer: A meta-analysis. Hepatobiliary Pancreat Dis Int. 2018;17:95–100. [PubMed: 29576277]
12. Hutcheson J, Balaji U, Porembka MR, Wachsmann MB, McCue PA, Knudsen ES, et al. Immunologic and Metabolic Features of Pancreatic Ductal Adenocarcinoma Define Prognostic Subtypes of Disease. Clin Cancer Res. 2016;22:3606–17. [PubMed: 26858311]
13. Johnson BA, Yarchoan M, Lee V, Laheru DA, Jaffee EM. Strategies for increasing pancreatic tumor immunogenicity. Clin Cancer Res. 2017;23.
14. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. N Engl J Med. 2015;372:2509–20. [PubMed: 26028255]
15. Le DT, Durham JN, Smith KN, Wang H, Bartlett BR, Aulakh LK, et al. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade Science. NIH Public Access; 2017;357:409–13. [PubMed: 28596308]
16. Wang L, Ma Q, Chen X, Guo K, Li J, Zhang M. Clinical Significance of B7-H1 and B7-1 Expressions in Pancreatic Carcinoma. World J Surg. 2010;34:1059–65. [PubMed: 20145927]
17. Zhuan-Sun Y, Huang F, Feng M, Zhao X, Chen W, Zhu Z, et al. Prognostic value of PD-L1 overexpression for pancreatic cancer: evidence from a meta-analysis. Onco Targets Ther. 2017;Volume 10:5005–12. [PubMed: 29081663]
18. Sideras K, Biermann K, Yap K, Mancham S, Boor PPC, Hansen BE, et al. Tumor cell expression of immune inhibitory molecules and tumor-infiltrating lymphocyte count predict cancer-specific survival in pancreatic and ampullary cancer. Int J Cancer. 2017;141:572–82. [PubMed: 28470686]
19. Stromnes IM, Hulbert A, Pierce RH, Greenberg PD, Hingorani SR. T-cell Localization, Activation, and Clonal Expansion in Human Pancreatic Ductal Adenocarcinoma. Cancer Immunol Res. 2017;5:978–91. [PubMed: 29066497]
20. Raphael BJ, Hruban RH, Aguirre AJ, Moffitt RA, Yeh JJ, Stewart C, et al. Integrated Genomic Characterization of Pancreatic Ductal Adenocarcinoma. Cancer Cell. 2017;32:185–203.e13. [PubMed: 28810144]
21. Liu J, Lichtenberg T, Hoadley KA, Poisson LM, Lazar AJ, Cherniack AD, et al. An Integrated TCGA Pan-Cancer Clinical Data Resource to Drive High-Quality Survival Outcome Analytics. Cell. 2018;173:400–416.e11. [PubMed: 29625055]
22. Yarchoan M, Xing D, Luan L, Xu H, Sharma RB, Popovic A, et al. Characterization of the immune microenvironment in hepatocellular carcinoma. Clin Cancer Res. 2017;23.
23. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, et al. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 2004;5:R80. [PubMed: 15461798]
24. Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci. 2005;102:18538–43. [PubMed: 16344461]
25. Adams S, Gray RJ, Demaria S, Goldstein L, Perez EA, Shulman LN, et al. Prognostic Value of Tumor-Infiltrating Lymphocytes in Triple-Negative Breast Cancers From Two Phase III Randomized Adjuvant Breast Cancer Trials: ECOG 2197 and ECOG 1199. J Clin Oncol. 2014;32:2959–66. [PubMed: 25071121]
26. Yamamoto H, Itoh F, Nakamura H, Fukushima H, Sasaki S, Perucho M, et al. Genetic and clinical features of human pancreatic ductal adenocarcinomas with widespread microsatellite instability. Cancer Res. 2001;61:3139–44. [PubMed: 11306499]

27. Nakata B, Wang YQ, Yashiro M, Nishioka N, Tanaka H, Ohira M, et al. Prognostic value of microsatellite instability in resectable pancreatic cancer. *Clin Cancer Res*. 2002;8:2536–40. [PubMed: 12171881]
28. Ottenhof NA, Morsink FHM, Ten Kate F, van Noorden CJF, Offerhaus GJA. Multivariate analysis of immunohistochemical evaluation of protein expression in pancreatic ductal adenocarcinoma reveals prognostic significance for persistent Smad4 expression only. *Cell Oncol (Dordr)*. 2012;35:119–26. [PubMed: 22351431]
29. Poulogiannis G, Frayling IM, Arends MJ. DNA mismatch repair deficiency in sporadic colorectal cancer and Lynch syndrome. *Histopathology*. 2010;56:167–79. [PubMed: 20102395]
30. Takikawa O, Tagawa Y, Iwakura Y, Yoshida R, Truscott RJ. Interferon-gamma-dependent/independent expression of indoleamine 2,3-dioxygenase. Studies with interferon-gamma-knockout mice. *Adv Exp Med Biol*. 1999;467:553–7. [PubMed: 10721099]
31. Garcia-Diaz A, Shin DS, Moreno BH, Saco J, Escuin-Ordinas H, Rodriguez GA, et al. Interferon Receptor Signaling Pathways Regulating PD-L1 and PD-L2 Expression. *Cell Rep*. 2017;19:1189–201. [PubMed: 28494868]
32. Georgiou K, Chen L, Berglund M, Ren W, de Miranda NFCC, Lisboa S, et al. Genetic basis of PD-L1 overexpression in diffuse large B-cell lymphomas. *Blood*. 2016;127:3026–34. [PubMed: 27030389]
33. Ribas A, Hu-Lieskovan S. What does PD-L1 positive or negative mean? *J Exp Med*. Rockefeller University Press; 2016;213:2835–40. [PubMed: 27903604]
34. Teng MWL, Ngiow SF, Ribas A, Smyth MJ. Classifying Cancers Based on T-cell Infiltration and PD-L1. *Cancer Res*. 2015;75:2139–45. [PubMed: 25977340]
35. Lutz ER, Wu AA, Bigelow E, Sharma R, Mo G, Soares K, et al. Immunotherapy converts nonimmunogenic pancreatic tumors into immunogenic foci of immune regulation. *Cancer Immunol Res*. 2014;2:616–31. [PubMed: 24942756]
36. Zhang T, Tan X-L, Xu Y, Wang Z-Z, Xiao C-H, Liu R. Expression and Prognostic Value of Indoleamine 2,3-dioxygenase in Pancreatic Cancer. *Chin Med J (Engl)*. 2017;130:710. [PubMed: 28303855]
37. Wang L, Ma Q, Li D, Mu S, Li Y, Wang Y, et al. Indoleamine 2, 3-dioxygenase and B7-H1 expressions as prognostic and follow-up markers in human pancreatic carcinoma. *Pathol - Res Pract*. 2018;214:1309–14. [PubMed: 30029936]
38. Long GV, Dummer R, Hamid O, Gajewski T, Caglevic C, Dalle S, et al. Epcadostat (E) plus pembrolizumab (P) versus pembrolizumab alone in patients (pts) with unresectable or metastatic melanoma: Results of the phase 3 ECHO-301/KEYNOTE-252 study *J Clin Oncol*. American Society of Clinical Oncology; 2018;36:108–108.

Figure 1. Gene expression of adaptive immune resistance markers in PDAC patients.

A heatmap of gene expression of adaptive immune resistance markers in PDAC patients from TCGA (n=152). Please also see Supplementary Fig. S2, Supplementary Table S1). Genes included in the heatmap are PD-L1 (*CD274*), *IDO1*, *CTLA4*, *LAG3*, *CD276* (B7-H3), V-set domain containing T-cell activation inhibitor 1 (*VTCN1*; also known as B7-H4), *CD70*, Hepatitis A virus cellular receptor 2 (*HAVCR2*; also known as T-cell immunoglobulin and mucin-domain containing-3 (TIM-3)), *CD40*, *CD47*, tumor necrosis factor receptor superfamily member 18 (*TNFRSF18*; also known as glucocorticoid-induced TNFR-related protein (GITR)), *TIGIT*, or LIGHT (also known as tumor necrosis factor superfamily member 14 (*TNFSF14*)). Each column is a sample.

Figure 2. Five year survival based on gene expression in TCGA cohort (n = 152).

Kaplan-Meier plots of 5-year survival based on (A) PD-L1 (*CD274*) and *IDO1* expression and (B) expression of other adaptive immune resistance markers in PDAC patients. Number of patients per group indicated in panel legend. Red curves correspond to marker-positive samples, and blue curves correspond to marker-negative samples. P-value and hazard ratio (HR) with corresponding confidence interval in the parentheses calculated by log-rank test. The p-value cutoff was 0.05.

Figure 3. Pairwise Pearson's correlation between immune markers in TCGA cohort (n=152). (A-I) Scatter plots of pairwise expression between immune markers. The Pearson's correlation coefficient (r) and corresponding p-value are shown at the top of each plot. The p-value cutoff is 0.05.

Figure 4. The correlation of PD-L1 (*CD274*) and *IDO1* with a Th1/IFN γ gene signature in TCGA cohort (n=152).

Heat map demonstrating the relationship of PD-L1/*IDO1* and *CD8A* expression with markers of a Th1/IFN γ gene signature (beyond just IFN γ), such as IL12R β 2 (*IL12RB2*), *JAK2*, and *STAT1*. Positive Pearson's correlation coefficients are represented by red, and negative correlations are represented by blue in the figure.

Figure 5. Five-year survival stratified by a combined analysis of immunomodulatory pathways and *CD8A* expression in TCGA cohort (n=152).

Survival is stratified by a combined analysis of immunomodulatory pathways and *CD8A* expression. (A) Survival based on high/low PD-L1 (*CD274*) expression and high/low *CD8A* expression. (B) Survival based on high/low *IDO1* and high/low *CD8A* expression. Number of patients per group indicated in panel legend. P-value and hazard ratio (HR) with corresponding confidence interval in the parentheses calculated by log-rank test. The p-value cutoff is 0.05.

Figure 6. Parallel analysis in an independent cohort of PDACs (n=33) using immunohistochemistry.

(A) Survival stratified by PD-L1 (*CD274*) and (B) *IDO1* expression. (C) Survival based on PD-L1 (*CD274*) expression and *IDO1* expression. (D) Survival based on PD-L1 (*CD274*) expression and high/low *CD8A* expression. (E) Survival based on *IDO1* and high/low *CD8A* expression. Representative PD-L1 and *IDO1* staining is shown in Supplementary Fig. S1. Number of patients per group indicated in panel legend. P-value and hazard ratio (HR) with corresponding confidence interval in the parentheses calculated by log-rank test. The p-value cutoff is 0.05.

Table 1:

Clinical and pathologic characteristics of the PDAC cases included in JHU cohort (n=33).

Patient Characteristics	
Age at surgery (median)	66.0 (IQR 60.0-70.5)
Sex	Male 51.5% Female 48.5%
Race/Ethnicity	Black 9.1% White 87.9% Other/mixed 3.0%
Tumor Features at Resection	
Pathologic Stage	1A: 6.1% 1B: 9.1% 2A: 15.2% 2B: 66.7% 3: 3.0%
Histologic Grade	Well: 3.0% Moderately: 60.6% Poorly: 33.3% Unknown: 3.0%
Resection Margin	Positive: 15.2% Negative: 84.8%
Venous (Large) Vessel Invasion	Present: 51.5% Absent: 36.4% Unknown: 12.1%
Perineural Invasion	Present: 81.8% Absent: 15.2% Unknown: 3.0%
Outcomes	Short-term survivors (< 500 days), n=18 Long-term survivors (> 1000 days), n=15