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<https://hdl.handle.net/2324/7393655>

出版情報 : Kyushu University, 2025, 博士 (医学), 課程博士
バージョン :
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Identification of cold tumor induction–related markers in pancreatic cancer and the clinical implication of PCDH7

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Received: 28 October 2024 / Accepted: 13 January 2025
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Abstract

Purpose Pancreatic ductal adenocarcinoma (PDAC) is considered a “cold” tumor because the tumor immune microenvironment (TIME) exhibits poor intratumoral T-cell infiltration. This study aimed to identify the marker genes associated with induction of cold TIME in PDAC cells.

Methods We orthotopically transplanted 10 primary cultures of PDAC derived from KrasG12D/+; Trp53R172H/+; Pdx-1-Cre (KPC) mice into immunocompetent mice and evaluated TIME by immunohistochemistry (IHC) staining of CD8. We divided primary cultures into two groups: cold TIME group with low CD8⁺ T-cell infiltration and a hot TIME group with high infiltration. RNA sequencing was performed to identify specific genes in the cold TIME group, and single-cell RNA sequencing (scRNA-seq) data was used for validation. IHC was performed to evaluate expressions in human PDAC samples.

Results We identified six genes specific in PDAC cells to the cold TIME group by RNA sequencing; these were defined as “cold tumor induction–related genes”. Human PDAC scRNA-seq data revealed that cold tumor induction–related genes were significantly and negatively correlated with the number of CD8⁺ T-cells ($p=0.0341$). These genes included *protocadherin 7 (PCDH7)*. High expression of PCDH7 significantly and negatively correlated with the number of CD8⁺ T-cells in scRNA-seq ($p=0.0474$) and IHC ($p=0.0110$) data using human PDAC samples. PCDH7 was an independent factor for poor prognosis in PDAC (overall survival: hazard ratio = 2.07, $p=0.0367$).

Conclusion PCDH7 is a prognostic marker associated with CD8⁺ T-cell infiltration for PDAC patients.

Keywords CD8-positive T-lymphocytes · Pancreatic cancer · PCDH7 protein · Tumor escape · Tumor microenvironment

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a cancer with one of the poorest prognoses, with a 5-year survival rate of 12%, which is partly due to the lack of effective treatment (Siegel et al. 2023; Singh and O'Reilly 2020). Despite the remarkable recent success of immune checkpoint blockade (ICB) in many types of cancer, the effects of ICB in PDAC are limited (Yarchoan et al. 2017; Le et al. 2017; Bear et al. 2020). PDAC exhibits a “cold” tumor immune microenvironment (TIME), characterized by poor intratumoral T-cell infiltration (Spranger 2016; Binnewies et al. 2018). Studies have shown that low infiltration of T-cells correlates with a poor prognosis in PDAC (Ajina and Weiner 2020; Goulart et al. 2021; Raskov et al. 2021), and converting the TIME from cold to hot (high infiltration of

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T-cells) increased the sensitivity to ICB in a mouse cancer model (Mariathasan et al. 2018; Ford et al. 2020). Therefore, it is important to elucidate how the cold TIME is induced in PDAC.

Several tumor cell–intrinsic and tumor cell–extrinsic factors of the cold TIME in PDAC have been identified. The major tumor cell–intrinsic factors of PDAC are defects in tumor antigen processing and presentation: low tumor mutational burden and low MHC class I (Chen and Mellman 2013; Hegde et al. 2016; Bear et al. 2020; Hegde and Chen 2020). The cancer cell phenotype also has a critical influence on the TIME because cancer cells progress by changing the phenotype and affecting surrounding cells. Some reports have identified RNA signatures in pancreatic cancer cells present in the cold TIME (Ajina and Weiner 2020; Yang et al. 2023). However, these signatures were derived from cancer cells located in the cold TIME but not the cancer cells that induced the cold TIME. The identification of genes in cancer cells that induce the cold TIME is challenging because of the heterogeneity of cancer cells.

To overcome this obstacle, in this study, we used several primary cultures of PDAC with different gene expressions. And we orthotopically transplanted them into mice to identify primary cultures that induced a uniform TIME without heterogeneity of tumor-infiltrating CD8⁺ T-cells. We then identified the marker genes in cancer cells that may be involved in the induction of the cold TIME and correlated with poor prognosis in PDAC. The assessment of such cold TIME-related marker genes may contribute to the development of stratified ICB treatment for PDAC patients.

Materials and methods

Mice

KrasG12D/+; Trp53R172H/+; Pdx-1-Cre (KPC) mice were bred and maintained at Charles River Laboratories Japan (Kanagawa, Japan) and the Research Animal Facility at Kyushu University (Fukuoka, Japan) in accordance with Kyushu University guidelines. Four-week-old female C57BL/6 and BALB/c nude mice were purchased from CLEA (Tokyo, Japan). All mice were housed under standard housing conditions at the Research Animal Facility at Kyushu University. All animal experiments were conducted following the guidelines of the institutional animal committee of Kyushu University (approval number: A22-128, A23-026 and A24-006).

Mouse PDAC cells

Primary cultures of PDAC cells were established from KPC mouse pancreatic tumors as previously described (Okumura et al. 2019). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; D5523; Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS), 150 µg/ml streptomycin, and 100 µg/ml penicillin at 37 °C in a humidified atmosphere containing 10% CO₂.

Mouse experiments

KPC mouse–derived PDAC cells (2.0×10^5) were injected orthotopically into the pancreas of female 5-week-old syngeneic immunocompetent C57BL/6 mice ($n=3-5$). Three weeks after the injection, the tumors were removed and fixed in 4% paraformaldehyde for immunohistochemistry (IHC). In other experiments, 1.0×10^6 cells were injected, and tumors were removed 3–4 weeks later.

Human cell lines

This study used two human PDAC cell lines: AsPC-1 (American Type Culture Collection, Manassas, VA, USA) and MIA PaCa-2 (Japanese Collection of Research Bioresources Cell Bank, Ibaraki, Osaka, Japan). Cells were maintained in DMEM supplemented with 10% FBS in the same conditions as for the mouse cells.

3D-culture

Mouse cells were dissociated by digestion with trypsin and mechanical shearing. Cells (5.0×10^3) were diluted in Cultrex 3-D Culture Matrix Reduced Growth Factor Basement Membrane Extract (3445; R&D Systems, Minneapolis, MN, USA) and cultured in a 24-well plate (92124; TPP, Zollikon, Switzerland) with serum or niche medium (described below) at 37 °C in a humidified atmosphere containing 10% CO₂. The serum and niche medium were prepared using basic medium with the addition of FBS or niche factors, as previously reported (Shinkawa et al. 2022). Images of the 3-D culture were acquired by fluorescence microscopy (BZ-X700; Keyence, Osaka, Japan).

Western blotting

Cultured mouse cells and human PDAC cell lines were lysed in PRO-PREP Protein Extraction Solution (17,081, iNtRON Biotechnology, Seongnam, Gyeonggi, South Korea) following the manufacturer's instructions. The protein concentration was measured using Nabi (MicroDigital,

Seongnam, Gyeonggi, Korea). After boiling for 10 min at 95 °C, collected proteins (20 µg/30 µL protein assay dye reagent) were loaded onto 4–15% Mini-PROTEAN TGX Gels (456–1084, Bio-Rad Laboratories, Hercules, CA, USA) and transferred to Trans-Blot Turbo Transfer Packs (170–4156, Bio-Rad Laboratories) using a Trans-Blot Turbo Blotting System (Bio-Rad Laboratories). The membrane was blocked with 5% milk in TBST buffer (0.1% Tween 20) at room temperature for 1 h at 4 °C overnight. The membrane was then incubated at 4 °C overnight with anti-RAS G12D Mutant (rabbit monoclonal, 1:1000, GTX635362; Gene Tex, Irvine, CA, USA), anti-EPCAM/CD326 (rabbit polyclonal, 1:1000, 21050-1-AP; Proteintech, Rosemont, IL, USA) or anti-β-actin (rabbit monoclonal, 1:2000, 4970; Cell Signaling Technology, Danvers, MA, USA) antibodies. The membrane was probed with horseradish peroxidase-conjugated secondary antibodies and processed using the ECL Prime Western Blotting Detection Reagent (RPN2236; Cytiva, Tokyo, Japan). Bands were detected by enhanced chemiluminescence using the ChemiDoc XRS System (Bio-Rad Laboratories).

Immunohistochemistry

Formalin-fixed, paraffin-embedded human and mouse tissues were cut into slices of 4 µm thickness and subjected to hematoxylin and eosin staining. After deparaffinization with xylene and ethanol, the endogenous peroxidase activity was blocked by methanol containing 0.3% hydrogen peroxidase. Heat-induced epitope retrieval was performed with a citrate buffer (pH 6.0) or Tris-EDTA buffer (pH 9.0) using a microwave oven or pressure cooker. The sections were blocked with 3% BSA in PBS and incubated overnight at 4 °C with the following primary antibodies: anti-CD8α for mouse samples (rabbit monoclonal, 1:100, 98941; Cell Signaling Technology), anti-CD8α for human samples (mouse monoclonal, undiluted, 413201; Nichirei Biosciences, Tokyo, Japan), anti-FOXP3 (rat monoclonal, 14-5773-82, 1:100, Thermo Fisher Scientific, Waltham, MA, USA), anti-Ly-6G (rat monoclonal, 551459, 1:500, BD Biosciences, Franklin Lakes, NJ, USA), and anti-PCDH7 (rabbit polyclonal, HPA011866, 1:300, Sigma-Aldrich). The sample was then incubated with secondary antibodies, EnVision+System-HRP Labelled Polymer Anti-Rabbit (K4003; Agilent Technologies, Santa Clara, CA, USA), Anti-Mouse (K4001; Agilent Technologies) and Anti-Rat (MP-7404-50, Vector Laboratories, Newark, CA, USA), for 40 min at room temperature. A 3,3'-diaminobenzidine (DAB) Kit (D5637; Agilent Technologies) was used for visualization, and slides were counterstained with hematoxylin. Images were acquired using a BZ-X800 fluorescent microscope (Keyence).

CD8α-positive cells were counted at 200×magnification in seven random hot spots (excluding tertiary lymphoid structures), and an average was calculated based on five fields, excluding the highest and lowest counts. The expression of PCDH7 on the tumor cell membrane and cytoplasm was evaluated at 200×magnification in five random fields by two pathologists. PCDH7 expression was quantified using the H-score, calculated as the intensity score (staining intensity: 0, negative; 1, weak; 2, moderate; 3, strong) times the percentage of positive cells (0–100%) for each spot. The H-score of each spot was summed to obtain the final score (0 to 1,500) for each patient, and patients were dichotomized by the mean value of the final score. Although PCDH7 is a membrane protein, cytoplasmic staining was also defined as positive as disruption of cell polarity can lead to mislocalization of membrane proteins, which would occur in PDAC.

Multiplex immunofluorescent staining

Formalin-fixed, paraffin-embedded human tissues were cut into sections of 4 µm thickness and deparaffinized with xylene and ethanol. Endogenous peroxidase activity was blocked using Peroxidase-Blocking Solution (S2023, Agilent Technologies). Heat-induced epitope retrieval was performed with a Tris-EDTA buffer (pH 9.0) using a pressure cooker. The sections were blocked with 3% BSA in PBS and incubated overnight at 4 °C with primary antibodies: anti-PCDH7 (rabbit polyclonal, HPA011866, 1:300, Sigma-Aldrich), anti-keratin/anti-cytokeratin (AE1/AE3: mouse monoclonal, undiluted, 412811, Nichirei Biosciences), and anti-CD8α (rabbit polyclonal, 1:150, ab4055, Abcam, Cambridge, UK). Samples were incubated with DAPI for nuclear staining (1:1000, WA125, Dojindo Laboratories, Kumamoto, Japan) followed by secondary antibodies, Alexa Fluor 488 anti-rabbit (1:200, A32731, Thermo Fisher Scientific) and 546 anti-mouse (1:200, A10036, Thermo Fisher Scientific), for 1 h at room temperature. Autofluorescence was blocked using the Vector TrueVIEW Autofluorescence Quenching Kit (SP-8400, Vector Laboratories, Newark, CA, USA) following the manufacturer's instructions. Images were acquired using a BZ-X800 fluorescence microscope (Keyence).

Proliferation assay

Detailed information can be found in Doc S1.

In vivo tumorigenicity assay

Detailed information can be found in Doc S1.

RNA sequencing (RNA-seq) and analysis

Total RNA was isolated from mouse cells using TRIzol Reagent (Thermo Fisher Scientific) and purified using the SV Total RNA Isolation System (Promega, Madison, WI, USA) following the manufacturer's instructions. RNA samples were quantified by an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and the quality was confirmed with a TapeStation (Agilent Technologies). Sequencing libraries were prepared from 1 µg of total RNA with the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions. Cluster amplification and 150 bp paired-end sequencing were performed following the manufacturer's protocol (Illumina) for NovaSeq (Illumina). All sequencing reads were trimmed for low-quality bases and adapters with Trimmomatic version 0.36 (Bolger et al. 2014). For expression profiling with the RNA-seq data, paired-end reads were aligned to the GRCh39 genome assembly using STAR version 2.7.10b and RSEM version 1.3.3 (Li and Dewey 2011; Dobin et al. 2013). We used DESeq2 program version 1.83.3 (Love et al. 2014) to detect the differentially expressed genes (DEGs). Log fold-changes (logFC) and p-values were obtained from the gene-level raw counts, and the following criteria were used for DEGs: p-value < 0.05 and FDR < 15%. This paper does not report the original code.

Single-cell RNA sequencing (scRNA-seq) analysis

We downloaded scRNA-seq data of human PDAC patients (24 primary PDAC and 11 control pancreas tissues) without any treatment (Peng et al. 2019) from the Genome Sequence Archive, a public database, under the accession number CRA001160. The downloaded data were analyzed with the R v4.2.1 package “Seurat” v4.3.0 (Hao et al. 2021). Low-quality cells (< 200 unique molecular identifiers/cell, > 6000 genes/cell, < 3 cells/gene, and > 25% mitochondrial genes) were excluded. To eliminate the potential influence of the cell cycle and batch effects on the data, “CellCycleScoring” and “SCTransform” functions were performed. For clustering estimation, after principal component analysis with the “RunPCA” function, the “FindNeighbor” function was performed. The “FindClusters” function using the Uniform Manifold Approximation and Projection (UMAP) method was used for visualization. For counting the number of CD8⁺ T-cells and regulatory T-cells (Tregs), first, among *PTPRC* (the gene encoding CD45)-positive cells, *KRT19*-positive cells were excluded to remove epithelial cells and *DCN*-positive cells were excluded to remove fibroblasts. Next, *PTPRC*-positive cells were re-clustered, and within the T-cell and NK cell clusters, *CD8A*-positive cells were

designated as CD8⁺ T-cells and *FOXP3*-positive cells were designated as Tregs.

The cancer genome atlas (TCGA) dataset analysis

We used Gene Expression Profiling Interactive Analysis 2 (Tang et al. 2019) to analyze TCGA datasets of pancreatic adenocarcinoma (PAAD). This platform does not provide detailed clinical information. We performed the following three analyses: (1) the *PCDH7* gene expression profile across tumor samples and paired normal tissues, (2) the correlation of *PCDH7* with “Treg signature score (Tsutsumi et al. 2024)” and “immunosuppressive score (Tsutsumi et al. 2024)”, and (3) the survival analysis of patients with PAAD based on the differential expression of *PCDH7*.

Human PDAC tissue samples

Pancreatic cancer tissues were obtained from patients who underwent pancreatic resection for PDAC at Kyushu University Hospital (Fukuoka, Japan). All experiments were conducted according to the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and the Helsinki Declaration.

Statistical analysis

Statistical analyses were performed using GraphPad Prism ver. 9.3.1 (GraphPad Software, San Diego, CA, USA) and JMP Pro ver. 17.0.0 (SAS Institute, Cary, NC, USA). Comparisons of groups were performed using the Mann–Whitney U test. Correlation analyses were conducted using the Spearman correlation coefficient. The χ^2 test was used to analyze correlations between *PCDH7* and clinicopathological characteristics. Survival analyses were conducted using the Kaplan–Meier method, and survival curves were evaluated using the log-rank test. To evaluate independent prognostic factors associated with survival, univariate and multivariate Cox proportional-hazards regression analyses were used. P-values < 0.05 indicated statistical significance.

Results

Primary cultures of KPC mouse-derived PDAC induced different tumor immune microenvironments in mice

The KPC mouse model is a genetically engineered and spontaneous PDAC mouse model (Hingorani et al. 2003; Mazur and Siveke 2012). We previously established 10 KPC mouse-derived primary cultures of PDAC (Fig. S1A)

(Okumura et al. 2019). In this study, we found that each primary culture exhibited a different morphological phenotype using a 3D-culture model classification on the basis of niche factor dependence that we previously reported using human PDAC samples (Fig. 1A)(Shinkawa et al. 2022). KPC490, KPC631, KPC630, and KPC619 formed well-defined ductal structures and were classified as well differentiated (Grade 1) PDAC. KPC 587, KPC662, and KPC850 formed ductal structures in niche medium but solid structures in serum medium classified as moderately differentiated (Grade 2) PDAC. KPC142, KPC180, and KPC469 formed solid structures and were classified as poorly differentiated (Grade 3) PDAC. However, how the different phenotypes of cancer cells induce different TIMEs has not been clarified.

We transplanted the 10 primary cultures of PDAC into immunocompetent C57BL/6 mice (Fig. 1B). It is important to note that the TIME of the original tumor in KPC mice and cancer cells in the original tumor were highly heterogeneous, and the transplantation of primary tumors derived from the mouse model does not necessarily reconstruct the exact microenvironment of the original tumor, unlike patient-derived organoids. Many papers defined cold tumor by the low infiltration of CD8⁺ T-cells(Hegde et al. 2016; Hegde and Chen 2020); Binnewies et al. 2018); Hornburg et al. 2021), because cytotoxic T-cells, particularly CD8⁺ T-cells, are essential to eliminate cancer cells(Raskov et al. 2021); Weigelin and Friedl 2022) and the density of CD8⁺ T-cell is a prognostic marker for various tumor(Shimizu et al. 2019); Kiryu et al. 2021). Therefore, to evaluate the TIME induced by each primary culture by CD8⁺ T-cell infiltration, we performed IHC using antibodies against CD8 α on mouse tumor tissues. We found that each primary culture induced a remarkably different TIME. However, there was no correlation between the classification of 3D culture models and the intensity of CD8⁺ T-cell infiltration. For CD8⁺ T-cell counts, 4 primary cultures exhibited high intra- or inter- tumoral heterogeneity, and the other 6 did not (Fig. S1B). Of the six with low intra- and inter- tumoral heterogeneity, three showed low infiltration of CD8⁺ T-cells and three showed high infiltration of CD8⁺ T-cells (Fig. 1C). We confirmed the reproducibility that each primary culture exhibited the same TIME pattern each time in three independent experiments, even after varying periods of observation and the number of transplanted cells (Fig. 1C, S1C). There were no significant differences in oncogenic potential between primary cultures of PDAC cells (Fig. S1D-E). We chose to study six primary cultures that exhibited low heterogeneity and confirmed reproducibility.

RNA sequencing identified specific RNA expressions in KPC mouse-derived primary cultures of PDAC that induce the cold TIME

To explore the genes expressed in cancer cells that induce low infiltration of CD8⁺ T-cells in the mouse model, we defined the two groups of primary cultures of PDAC as follows: the cold TIME group, with low infiltration of CD8⁺ T-cells ($n=3$), and the hot TIME group, with high infiltration of CD8⁺ T-cells ($n=3$) (Fig. 1C). We performed RNA-seq on these groups (Table S1). There was no difference in the expression of MHC class I in cancer cells between the two groups (Fig. S2), although low expression of MHC class I in cancer cells was reported to be one of the major factors inducing the cold TIME(Bear et al. 2020); Chen and Mellman 2013); Cornel et al. 2020). In addition, the expression of immuncheckpoint-related genes, which are also one of the factors in inducing cold TIME(Chen and Mellman 2013); Jiang et al. 2019); Ho et al. 2020), did not differ in the two groups (Fig. S2A). Therefore, we explored other genes related to the induction of poor intratumoral CD8⁺ T-cell infiltration by searching DEGs in the cold TIME group compared with the hot TIME group (Fig. 2A, Table S2). Among the DEGs in the cold TIME group, six genes (*Pcdh7*, *Itga1*, *Syt12*, *Angptl4*, *Fads1*, *Slc26a2*), which showed high expression (>800) and upregulated more than twofold in the cold TIME group (Fig. 2B), were defined as “cold tumor induction-related genes” (Table S3).

“Cold tumor induction-related genes” were related to cold TIME in human PDAC

To investigate whether the cold tumor induction-related genes were associated with CD8⁺ T-cell infiltration in human PDAC, we analyzed public human PDAC scRNA-seq data reported by Peng et al.(Peng et al. 2019), which contains data from 24 primary PDAC tumor and 11 control pancreas tissues. After an initial quality control, the cells were divided into 12 clusters (Fig. 3A, S3A). The number of CD8⁺ T-cells and the expression of cold tumor induction-related genes in the cancer cell clusters per patient were evaluated (Fig. 3B, S3B–F). We found that higher expression of cold tumor induction-related genes in cancer cell clusters were significantly correlated with a lower number of CD8⁺ T-cells ($R = -0.43$, $p=0.0341$) (Fig. 3C). Analysis of TCGA data revealed that cold tumor induction-related genes were associated with a poor prognosis in PDAC patients (Fig. 3D, S3G).

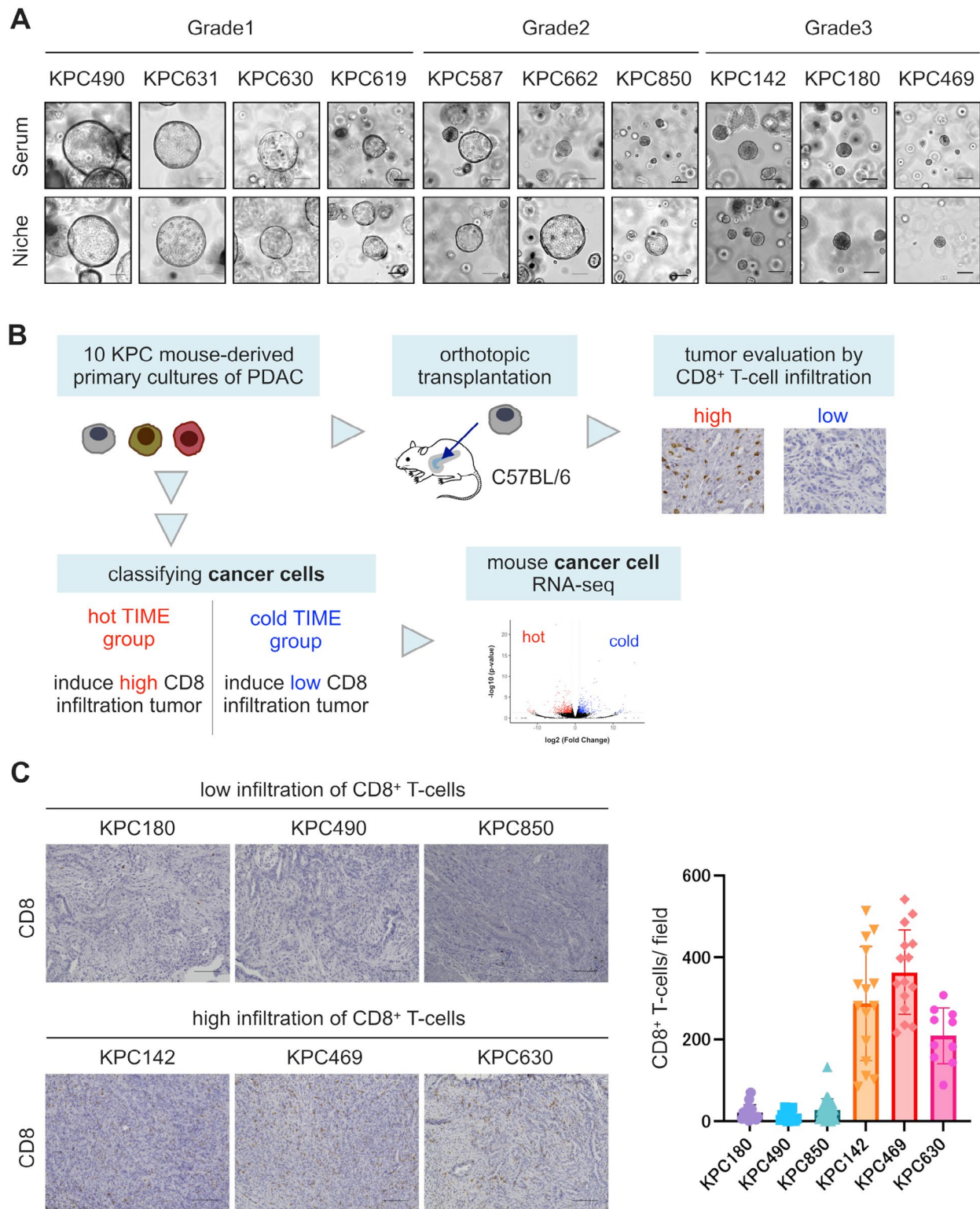


Fig. 1 Identification of KPC mouse-derived primary cultures of PDAC inducing infiltration of CD8⁺ T-cells in tumors. **(A)** Representative 3D-culture phase-contrast images of KrasG12D/+; Trp53R172H/+; Pdx-1-Cre (KPC) mouse-derived primary cultures of pancreatic ductal adenocarcinoma (PDAC) with serum or niche factor medium. We classified the morphological features of 3D-cultures based on PDAC differentiation grade. **(B)** Schematic of the study design. TIME: tumor

immune microenvironment; RNA-seq: RNA sequencing. **(C)** Primary cultures of PDAC cells (2.0×10^5) were transplanted orthotopically, and tumors were resected three weeks later. CD8 α immunohistochemistry (IHC) staining images of mouse PDAC tumors (left) and the number of CD8⁺ T-cells (CD8 α -positive cells) per field (right) for each primary culture. Scale bars, 100 μ m

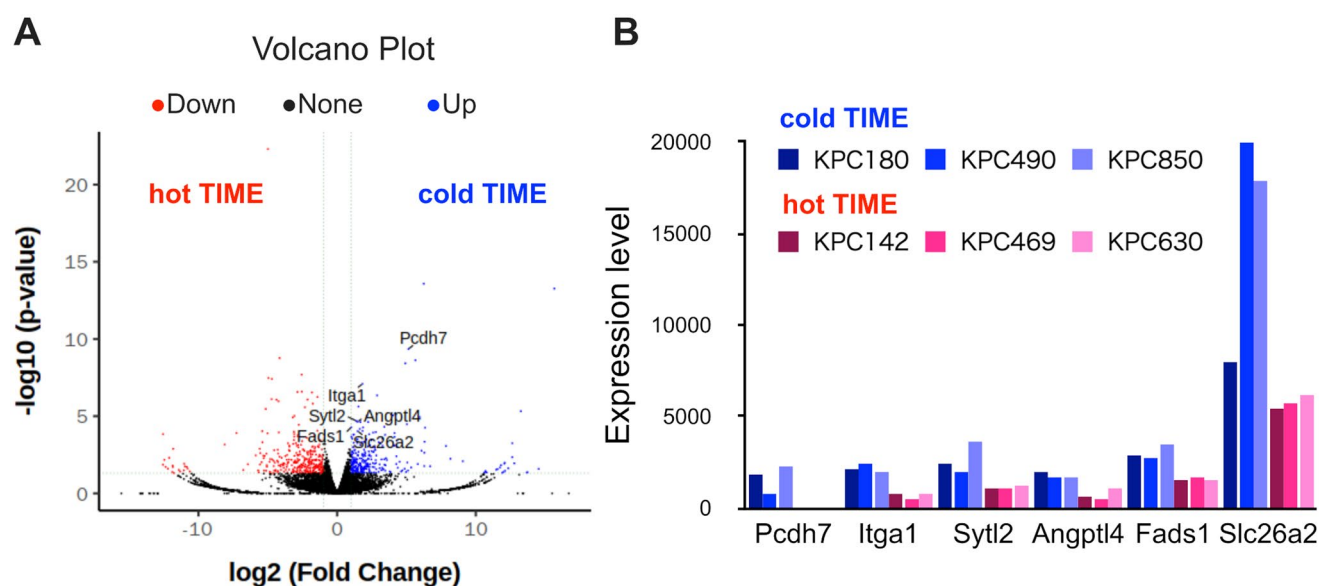


Fig. 2 RNA sequencing identified specific genes inducing cold TIME in KPC mouse-derived primary cultures of PDAC. **(A)** Volcano plot of cold and hot TIME groups. Red: hot TIME differentially expressed genes (DEGs), blue: cold TIME DEGs ($p < 0.05$). Cold tumor induc-

tion-related genes are labeled. **(B)** The expression levels of cold tumor induction-related genes in each KPC mouse-derived primary culture of PDAC

PCDH7 showed a negative correlation with CD8⁺ T-cell infiltration in human PDAC patients

We next examined the immunological significance of each cold tumor induction-related gene. We analyzed public data of human PDAC scRNA-seq (Peng et al. 2019) and found that *protocadherin 7* (*PCDH7*) expression in cancer cell clusters had a significant inverse correlation with the number of CD8⁺ T-cells ($R = -0.41$, $p = 0.0474$) (Fig. 3E). No significant correlation was found for other cold tumor induction-related genes (Fig. S4A, S4B). Analyses of TCGA data showed that *PCDH7* expression was higher in pancreatic tumor tissues than in normal pancreatic tissues (Fig. S4C). We further examined whether *PCDH7* was associated with a suppressive TIME and found that *PCDH7* expression correlated positively and significantly with the number of Tregs in human PDAC scRNA-seq ($p = 0.0098$; Fig. 3F, S3D). TCGA data of PAAD revealed that *PCDH7* expression correlated with “Treg signature score (Tsutsumi et al. 2024)” ($p < 0.0001$; Fig. 3G) and “immunosuppressive score (Tsutsumi et al. 2024)” ($p < 0.0001$; Fig. 3H) and tended to have a poor prognosis (Fig. S4D). Tregs are known as major immunosuppressive cells and correlate with poor prognosis in PDAC (Özdemir et al. 2014; Ino et al. 2013). These results suggested that *PCDH7* inhibits infiltration of CD8⁺ T-cells and induces a suppressive TIME mediated by Tregs. To evaluate the impact of *PCDH7* expression in cancer cells on Tregs and myeloid-derived suppressor cells (MDSCs), we performed IHC of FOXP3 for Tregs and Ly-6G for MDSCs using mouse tumor tissues derived from

primary cultures of PDAC cells. There was a tendency for Tregs to be more abundant in tumors derived from primary cultures with low expression of *PCDH7*, but the difference was not significant (Fig. S4E). These results may be influenced by other factors except for *PCDH7* expression.

To confirm the correlation between *PCDH7* and CD8⁺ T-cells, we performed IHC of *PCDH7* on 47 samples from PDAC patients who underwent pancreatectomy at our institution without any other treatment. We observed fewer CD8⁺ T-cells around cancer cells with high *PCDH7* expression and more CD8⁺ T-cells around cancer cells with low *PCDH7* expression (Fig. 4A). Then, the expression level of *PCDH7* was assessed by H-score, determined by the staining intensity of *PCDH7* in cancer cells (Fig. 4B) and the percentage of positive cells. We bisected samples by high and low H-score. We found significantly fewer CD8⁺ T-cells in the *PCDH7* high group compared with *PCDH7* low group ($p = 0.0110$) (Fig. 4C).

PCDH7 expression in PDAC cells showed a positive correlation with poor prognosis in human PDAC patients

To examine the clinical significance of *PCDH7* expression in PDAC cells, we performed IHC using 69 tumor samples derived from PDAC patients in our institution. We found that *PCDH7* high expression was correlated with poorer disease-free survival (DFS, $p = 0.0362$) and overall survival (OS, $p = 0.0252$) (Fig. 4D). There was no significant

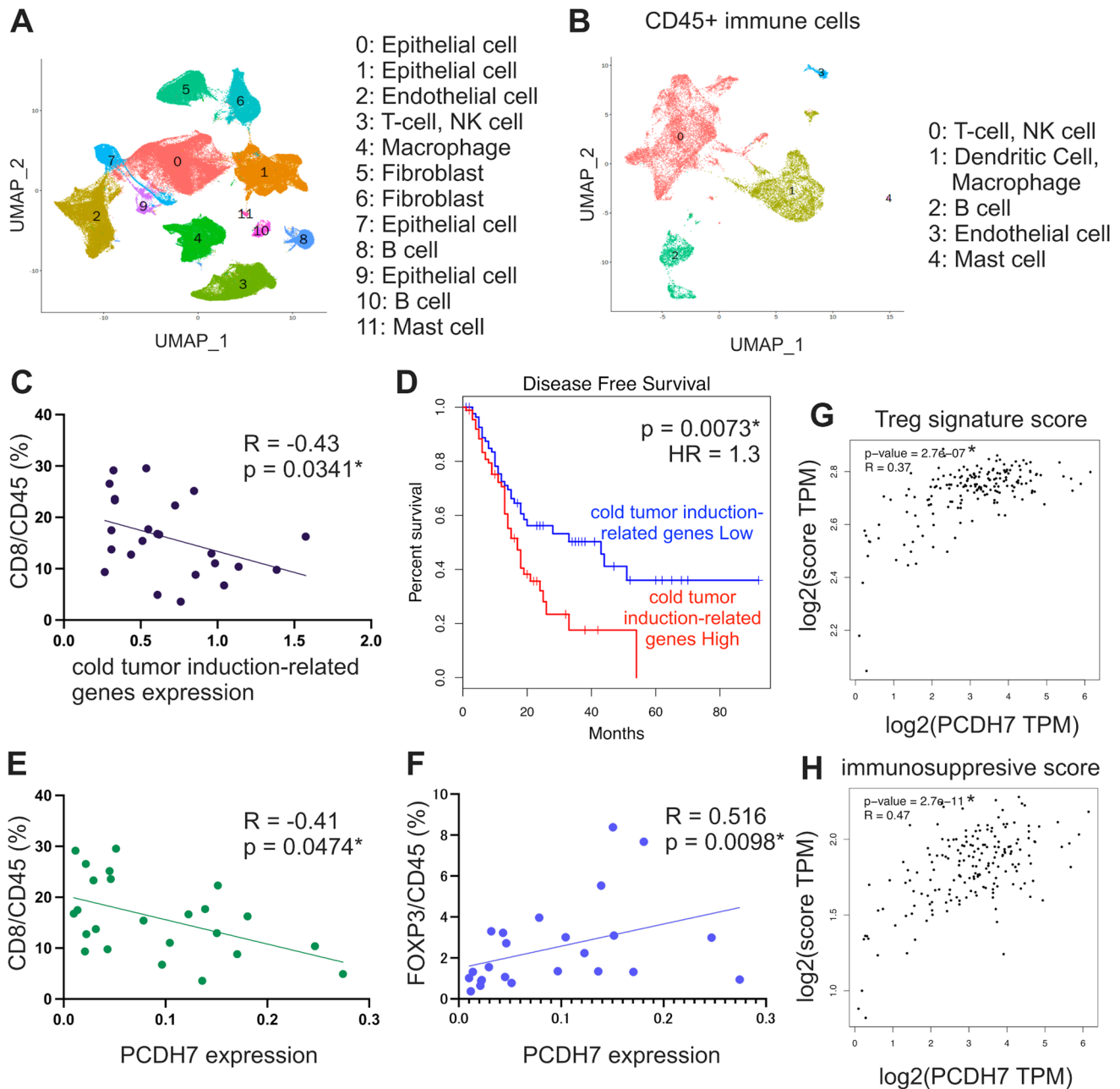


Fig. 3 “Cold tumor induction-related genes” and *PCDH7* were associated with cold TIME in human PDAC. (**A–C**, **E** and **F**) Single-cell RNA sequencing (scRNA-seq) analysis of a public database of human PDAC patients. (**A**) Uniform Manifold Approximation and Projection (UMAP) plot showing the clustering of human PDAC. (**B**) UMAP plot showing the re-clustering of *PTPRC* (CD45)-positive immune cells. (**C**) The correlation between “cold tumor induction-related genes” expression and the number of CD8-positive cells per patient. (**D**) Kaplan–Meier disease-free survival analysis of cold tumor induc-

tion-related genes from The Cancer Genome Atlas (TCGA) pancreatic adenocarcinoma samples ($n=89$ in each group). (**E** and **F**) The correlation between *PCDH7* expression and the percentage of CD8-positive cells (**E**) and Tregs (**F**) per patient. (**G** and **H**) Using TCGA data of human pancreatic adenocarcinoma patients, the correlation between *PCDH7* expression and Treg signature score (**G**) and immunosuppressive score (**H**). Spearman’s correlation test or log-rank test was used for analysis. $*p < 0.05$

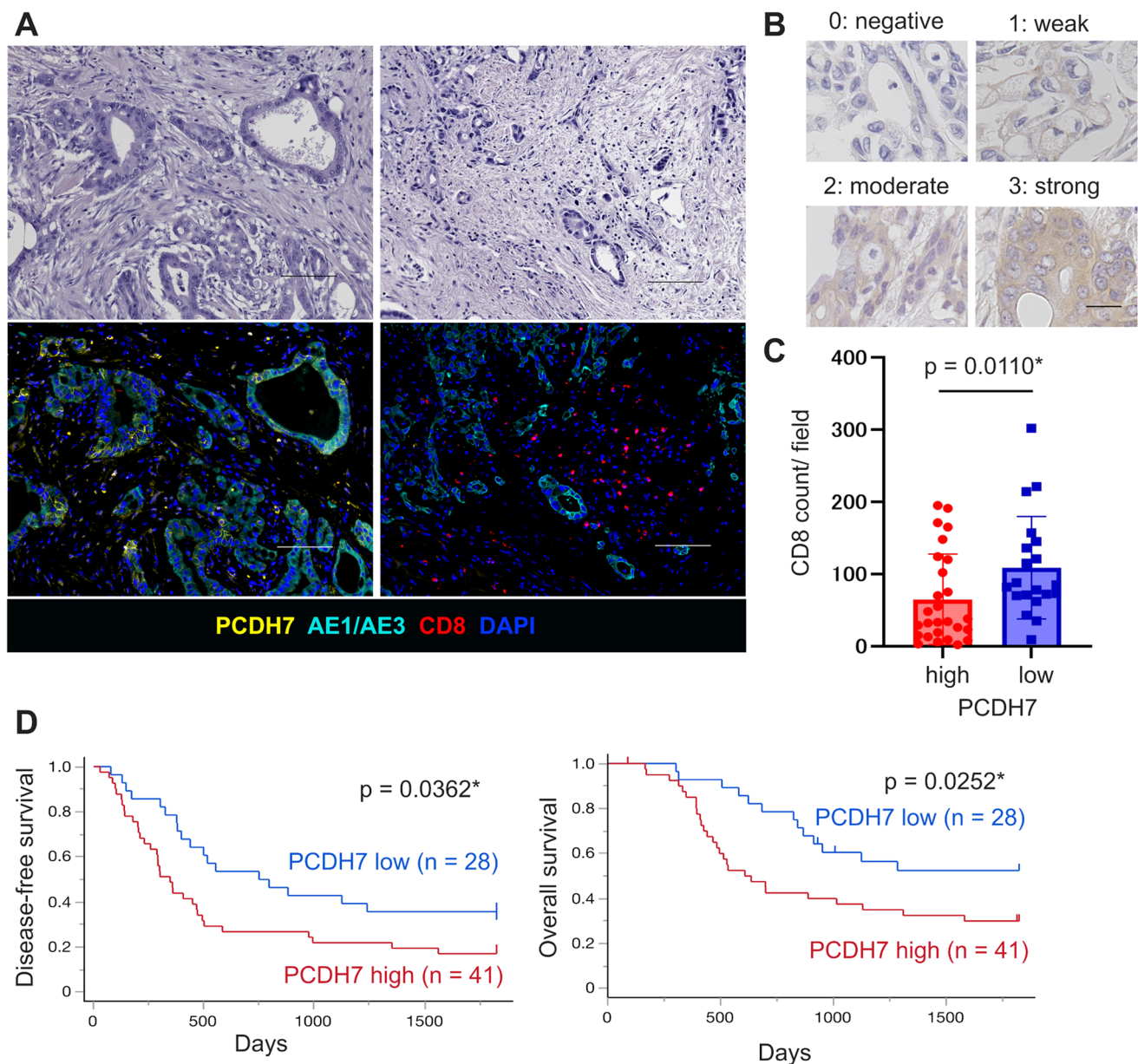


Fig. 4 PCDH7 protein negatively correlated with CD8⁺ T-cell infiltration in human PDAC patients. **(A)** Representative hematoxylin and eosin staining images of human PDAC tissues in the upper row and the corresponding immunofluorescence images of PCDH7 (yellow), AE1/AE3 (sky blue), CD8 (red), and DAPI (blue) in the bottom row. Left: high expression of PCDH7 and low number of CD8⁺ T-cell image. Right: low expression of PCDH7 and high number of CD8⁺ T-cell

image. Scale bars, 100 μ m. **(B)** Representative images of IHC staining for PCDH7. The intensity score: 0, negative; 1, weak; 2, moderate; and 3, strong. Scale bar, 20 μ m. **(C)** Quantification of the CD8⁺ cell number in PCDH7 low and high cancer cells. **(D)** Disease-free survival and overall survival analysis (performed using Kaplan–Meier analysis) of PCDH7 (high=41, low=28) in human PDAC samples. $^*p < 0.05$

difference in clinicopathological parameters between the PCDH7-low and -high groups (Table 1).

CD8 count was not a prognostic factor of OS in univariate analysis. Multivariate survival analysis using the Cox proportional hazard model (including all parameters < 0.1 in univariate analysis) revealed that high expression of PCDH7 in PDAC cells was an independent prognostic factor of OS (hazard ratio=2.07, $p = 0.0367$) in PDAC patients (Table 2).

Discussion

PDAC is well characterized as exhibiting a “cold” TIME, which is associated with reduced response to ICB treatment. Therefore, understanding the mechanism by which the cold TIME is induced is critical to improve patient prognosis. The best-known intrinsic factor of the cold TIME in PDAC is the low tumor mutational burden. Although we

Table 1 Correlations between PCDH7 expression and clinicopathological characteristics in human PDAC samples

Characteristic	PCDH7 low (n=28)	PCDH7 high (n=41)	p value ^a
Age			0.6959
<67 ^b	13 (46%)	21 (51%)	
≥67	15 (54%)	20 (49%)	
Gender			0.9315
Male	16 (57%)	23 (56%)	
Female	12 (43%)	28 (44%)	
pT category^c			0.2266
pT1/pT2	25 (11%)	32 (22%)	
pT3/pT4	3 (89%)	9 (78%)	
Lymph node metastasis			0.7810
No	8 (29%)	13 (32%)	
Yes	20 (71%)	28 (68%)	
pathological margin			0.3399
Negative	26 (93%)	35 (85%)	
Positive	2 (7%)	6 (15%)	
Neoadjuvant chemotherapy			0.9696
Yes	9 (32%)	13 (32%)	
No	19 (68%)	28 (68%)	
Adjuvant chemotherapy			0.0886
Yes	28 (100%)	37 (90%)	
No	0 (0%)	4 (10%)	

a: χ^2 test. b: Mean age. c: According to the Union for International Cancer Control (UICC) 8th. staging system. * $P < 0.05$

did not examine neo-antigen or tumor mutational burden, a recent study reported that low tumor mutational burden is not frequently observed in PDAC and other factors may be more critical in contributing to the cold TIME (Freed-Pastor et al. 2021). Cancer cells affect their surrounding microenvironment depending on their morphological phenotype or transcriptional phenotype (Moffitt et al. 2015). As the correlation between the TIME and morphological phenotype (Shinkawa et al. 2022) or Moffitt's RNA transcriptional classification (Grünwald et al. 2021); Raghavan et al. 2021) has not been clarified, we first focused on phenotypes of cancer cells. In this study, we established 10 KPC mouse-derived primary cultures of PDAC that exhibited various morphological phenotypes. We found that the tumors derived from cells transplanted into immunocompetent mice showed different TIMES. Six primary cultures of PDAC exhibited remarkably limited intra- and inter-tumoral heterogeneity. These primary cultures induced a specific TIME with reproducible uniformity in the CD8⁺ T-cell infiltration of the tumor. The other four primary cultures of PDAC showed high intra- or inter-tumoral heterogeneity in the number of tumor-infiltrating CD8⁺ T-cells. These primary cultures may be composed of various cells with different functions to induce the TIME. We did not find an association between the morphological phenotype identified using the 3D-culture model classification and the type of TIME. We thus investigated the molecular phenotypes of the cancer cells that consistently induced the poor

Table 2 Univariate and multivariate DFS and OS analysis of conventional prognostic factor and PCDH7 expression

		Univariate			Multivariate		
Characteristics		HR	95% CI	<i>p</i> value ^a	HR	95% CI	<i>p</i> value ^a
DFS							
Age	≥67 ^b	1.67	(0.97–2.87)	0.0656	1.55	(0.88–2.72)	0.1282
Gender	male	1.39	(0.80–2.40)	0.2381			
pT category ^c	pT3/pT4	2.01	(1.03–3.92)	0.0406*	1.55	(0.76–3.14)	0.2261
Lymph node metastasis	Yes	1.84	(0.98–3.44)	0.0577	1.67	(0.84–3.33)	0.1457
pathological margin	Positive	1.82	(0.85–3.89)	0.1218			
Neoadjuvant chemotherapy	No	1.03	(0.57–1.85)	0.9267			
Adjuvant chemotherapy	No	2.83	(1.01–7.91)	0.0477*	3.02	(1.02–8.97)	0.0468*
CD8 count	low	0.79	(0.46–1.36)	0.3941			
PCDH7 score	high	1.81	(1.03–3.19)	0.0390*	1.66	(0.93–2.98)	0.0873
OS							
Age	≥67 ^b	1.83	(0.99–3.40)	0.0558	1.94	(0.99–3.79)	0.0526
Gender	male	1.75	(0.93–3.32)	0.0840	1.96	(1.00–3.85)	0.0512
pT category ^c	pT3/pT4	1.99	(0.97–4.07)	0.0598	1.14	(0.49–2.67)	0.7563
Lymph node metastasis	Yes	2.04	(0.97–4.27)	0.0601	1.77	(0.79–3.95)	0.1619
pathological margin	Positive	2.31	(1.01–5.25)	0.0461*	1.21	(0.47–3.13)	0.6927
Neoadjuvant chemotherapy	No	0.93	(0.49–1.78)	0.8280			
Adjuvant chemotherapy	No	2.47	(0.76–8.04)	0.1332			
CD8 count	low	0.85	(0.46–1.58)	0.6114			
PCDH7 score	high	2.09	(1.08–4.04)	0.0287*	2.07	(1.05–4.09)	0.0367*

a: Cox regression analysis. b: Mean age. c: According to the Union for International Cancer Control (UICC) 8th. staging system. DFS, disease-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval. * $P < 0.05$

intratumoral CD8⁺ T-cell infiltration with specificity and reproducibility.

In this study, we demonstrate that PCDH7 in cancer cells was correlated with poor prognosis and low infiltration of CD8⁺ T-cells in PDAC patients. PCDH7 is a member of the protocadherin family, a novel cadherin superfamily member, and regulates cell adhesion (Yoshida et al. 1998). Recent studies have suggested that PCDH7 plays a cancer-promoting role in several tumor types. PCDH7 expression promoted cell growth, migration, and invasion, and induced bone metastasis in breast cancer (Li et al. 2013), and PCDH7 enhanced MAPK signaling and tumorigenesis in non-small cell lung cancer (NSCLC) and lung adenocarcinoma (Zhou et al. 2017, 2019). Some reports showed that high expression of PCDH7 was correlated with poor clinical outcomes in breast cancer, lung cancer, prostate cancer, gastric cancer, and colon cancer (Li et al. 2013; Zhou et al. 2017, 2019; Shishodia et al. 2019; Xin et al. 2022; Zheng et al. 2023). In contrast, other studies indicated that PCDH7 correlated with better prognosis in bladder cancer, cervical cancer, and gastric cancer (Lin et al. 2016; Chen et al. 2017; Zhang and Fu 2021). However, there has been no report on the expression and clinical implications of PCDH7 in pancreatic cancer. Two studies found that high expression of PCDH1, an analog of PCDH7, was associated with poor prognosis in PDAC (Ye et al. 2022; Jiang et al. 2023). Our findings revealed that high expression of PCDH7 was correlated with poor prognosis in PDAC.

In a previous study in NSCLC, PCDH7 was positively correlated with resting NK cells, M0 macrophages, and neutrophils, and was negatively correlated with CD8⁺ T-cells and activated NK cells in scRNA-seq data (Li et al. 2023). Another study reported that *PCDH7* was one of the T-cell non-infiltration markers in gastric cancer (Xin et al. 2022). These previous data are consistent with our current results in PDAC. In the present study, we did not determine the mechanism by which PCDH7 directly inhibits the infiltration of CD8⁺ T-cells. A previous study showed that PCDH7 inhibited the homotypic cell-in-cell formation in breast cancer cells (Wang et al. 2020). Homotypic cell-in-cell formation (also known as entosis) is one of the regulated forms of cell death (Overholtzer and Brugge 2008; Martins et al. 2017) and its role in the CD8⁺ T-cell infiltration remains unclear. In addition, PCDH1, the analog of PCDH7, suppresses CD8⁺ T-cell infiltration via the CCL5-CCR5 axis (Jiang et al. 2023). We also performed additional scRNA-seq analyses on the data of Peng et al. 2019 and found that *PCDH1* expression negatively correlated with CD8⁺ T-cell infiltration (Fig. S4F) as well as *PCDH7* expression. PCDH1 and PCDH7 are different molecules and there are no reports regarding the functional differences of these molecules in PDAC TIME. Therefore, further examination will be

needed to elucidate the functional impact of PCDH1 on PDAC TIME as well as PCDH7. Our data also indicated that *PCDH7* expression correlated with Tregs and immunosuppressive score. These data suggest that PCDH7 may affect not only CD8⁺ T-cells but also immunosuppressive cells. Further studies to elucidate the mechanism of PCDH7 inducing the cold TIME in PDAC are required.

A limitation of this study is the exclusive use of female mice as recipients in our experiments. While this approach minimizes variability due to gender, it may introduce a gender-specific bias that could influence the tumor microenvironment or immune response. Future investigations involving both male and female mice will be necessary to comprehensively evaluate the role of gender in the observed phenomena. Furthermore, the CD8⁺ T-cells identified by IHC possibly include exhausted T-cells, which may contribute to an immunosuppressive tumor microenvironment (Miller et al. 2019). However, further studies are needed to elucidate the functional state of these cells in cold tumors with PCDH7 expression and hot tumors without PCDH7 expression.

In conclusion, we identified six genes (“cold tumor induction-related genes”) that were associated with the cold TIME. Among the six genes, PCDH7 was significantly and inversely correlated with the number of CD8⁺ T-cells in human PDAC scRNA-seq and IHC data. Additionally, PCDH7 was an independent prognostic marker in human PDAC samples. These findings may help lead to strategies to identify PDAC patients for treatment with ICB.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00432-025-06095-z>.

Acknowledgements We would like to thank E. Manabe, S. Sadatomi (Department of Surgery and Oncology, Kyushu University Hospital) and members of Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University for their expert technical assistance. We also thank H. Hagiwara (Cell Innovator, Fukuoka, Japan) for assistance with the RNA sequencing. We thank Gabrielle White Wolf, PhD, from Edanz (<https://jp.edanz.com/ac>) for editing a draft of this manuscript.

Author contributions YM designed the experimental approach, performed the experimental work, analyzed data, coordinated projects, and wrote the manuscript. KOH and TS helped design the experimental approach. KOH also contributed to data interpretation and discussion and reviewed the manuscript. BZ and AK helped with the histological examinations. YY, NT, and YO provided and prepared human samples. YY helped evaluate pathology specimens. CT and NT helped prepare the data for analysis. CT, KOY, CI, TA, NId, NIK and KN contributed to data interpretation and discussion. MN supervised and coordinated projects, contributed to data interpretation and discussion, and helped with review and editing of the manuscript. All authors read and approved the final manuscript.

Funding This work was supported by JSPS KAKENHI Grant Numbers JP21K19531, JP22H00480, JP21K19530, JP23H02770, JP23H02982, and JP23K19636.

Data availability The RNA sequencing datasets generated and analyzed during the current study have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE255873 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE255873>).

Declarations

Ethical approval This study was approved by the Ethics Committee of Kyushu University (approval number: 22002-00). All animal experiments were conducted following the guidelines of the institutional animal committee of Kyushu University (approval number: A22-128 and A23-026).

Consent to participate Written informed consent was waived owing to the retrospective analysis of the study.

Competing interests The authors declare no competing interests.

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