Dipeptidyl Peptidase-4 modulates Long-chain Acyl-CoA synthetase 4 to Promote Lipid Peroxidation and Regulate Ferroptosis in Pancreatic Cancer

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Research

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Abstract

Background: The global mortality rate of pancreatic ductal adenocarcinoma (PDAC) is one of the highest among all cancers, and curative chemotherapeutics are essentially nonexistent. Dipeptidyl peptidase-4 (DPP4) plays a critical role in ferroptosis, a recently discovered regulated mechanism of necrotic cell death that has an important function in colorectal cancer. However, whether DPP4 contributes to ferroptosis in pancreatic cancer has remained unknown.

Methods: DPP4 expression status was evaluated in PDAC tissues using tissue chip immunohistochemistry. The effects of DPP4 re-expression on cell proliferation, the cell cycle and energy metabolism were determined. DPP4-related cancer pathways was identified by RNA-seq analyses. The clinical impact of DPP4 in driving ferroptosis and attenuating gemcitabine sensitivity was assessed in DPP4 knockout (KD) mice orthotopic xenograft models of PDAC.

Results: We found that DPP4 is expressed at low levels in PDAC, and that it significantly inhibited cell proliferation by causing G1–S cell cycle arrest, inhibiting energy metabolism, and promoting erastin-induced ferroptosis in vitro; it also suppressed tumor growth in transgenic orthotopic xenograft mouse models ($P < 0.001$). Importantly, the sensitivity of cells overexpressing DPP4 to gemcitabine and ferroptosis was respectively decreased and increased. Mechanistically, DPP4 facilitated ferroptosis by binding long-chain acyl-CoA synthetase 4 (ACSL4), and interaction with DPP4 stabilized ACSL4 by inhibiting its ubiquitin-mediated degradation and subsequent lipid peroxidation.

Conclusions: These findings identify a novel role of DPP4 in ferroptosis and suggest a potential therapeutic strategy with which to overcome gemcitabine resistance in PDAC.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains one of the most destructive and aggressive cancers[1] because a diagnostic biomarker and effective therapeutic options for advanced diseases are unavailable, and 5-year survival rates are $\leq 9\%$. [2] The current standard first-line therapy for terminal PDAC is gemcitabine-based,[3] but the overall survival of patients remains unsatisfactory and few options are available when such therapies fail.[3, 4] Presently, PDAC treatment focuses on identifying molecular subgroups amenable to personalized strategies that could improve survival and quality of life. [5] Conservative estimates based on deep sequencing have indicated that 93% of PDAC harbors mutations in the Kirsten rat sarcoma viral oncogene homolog (*KRAS*) gene. Studies of other possible drivers in *KRAS*-wild-type cancers have revealed that 60% of these tumors harbor alternative RAS–MAPK pathway-activating variations, emphasizing the importance of the RAS–MAPK pathway in this disease.[6]

Structurally unrelated erastin and RAS-selective lethal-3 (RSL3) are selectively toxic for tumorigenic RAS-mutant cells and are regarded as RAS-selective lethal (RSL) compounds.[7, 8] These RSL cause cell death that is quite different from other types of regulated cell death such as apoptosis, autophagy, and necrosis. Death induced by RSL includes a characteristic collection of genetic, morphological, and

biochemical features and this phenotype named, ferroptosis,[9] has recently emerged as playing an important role in tumor biology. The hallmark of ferroptosis is lipid peroxidation. One study using haploid screens identified long-chain acyl-CoA synthetase 4 (ACSL4) as an important characteristic of lipid peroxidation during ferroptosis.[10] This enzyme is responsible for the ATP-dependent esterification of CoA to free fatty acids, and acyl-CoA formation stimulates comparative fatty acids for fatty acid oxidation or lipid biosynthesis,[11] which ultimately accelerates lipid peroxidation.

The membrane glycoprotein enzyme dipeptidyl peptidase-4 (DPP4, also known as CD26), has been clinically applied as a therapeutic target for type 2 diabetes mellitus but it is in fact more important in terms of the development of human cancers.[12–14] The loss of tumor protein-53 (TP53) prevents the nuclear accumulation of DPP4 and thus regulates lipid metabolism, which finally results in lipid peroxidation.[15] Moreover, targeting DPP4, which regulates ferroptosis, offers promise as a therapeutic strategy.[15, 16] We previously found that DPP4 is associated with lipid peroxidation in severe acute pancreatitis.[17] Additionally, analysis of the Cancer Genome Atlas (TCGA) data found that DPP4 and ACSL4 expressions positively correlate in PDAC. However, whether DPP4 plays an important role in ACSL4-mediated ferroptosis remains unclear.

The present study aimed to determine the role of DPP4 in ACSL4 activation after erastin-induced ferroptosis, explain why high DPP4 expression by PDAC leads to a poor prognosis, and perhaps provide a useful clinical adjuvant therapy for pancreatic cancer.

Materials And Methods

Patients, clinical samples, and survival analysis

Fifty-six pairs of matched tumorous and nontumorous tissues from patients with PDAC (female, n = 21; male, n = 35; median age, 63.2 [35–88] years) were formalin-fixed and paraffin-embedded. The clinical characteristics of the patients were obtained from medical records. None of the patients received any form of radiotherapy, neoadjuvant chemotherapy, or radiotherapy before surgery. Blood samples were collected into coagulation tubes from 32 patients with PDAC and 24 healthy controls; thereafter, serum was separated via centrifugation at 1000g for 20 min at 4°C and stored. Serum CD26/DPP4 activity was determined using CD26/DPPIV Assay kits (Enzo Life Sciences, Farmingdale, NY, USA) as described by the manufacturer. All samples were collected from the department of Hepatobiliary Surgery of the First Affiliated Hospital of Wenzhou University, Zhejiang Province, China. The aims of the study and its protocols were explained to the patients, all of whom provided written informed consent to participate, and the study was approved by the Ethics Review Board at the First Affiliated Hospital of Wenzhou University hospital. All procedures involving patients and healthy volunteers were conducted according to the Declaration of Helsinki (2013). Survival was evaluated using Kaplan-Meier curves, and univariate and multivariate regression analyses proceeded using Cox proportional hazard models.

Quantitative real-time PCR

Total RNA was extracted from cells or samples using TRIzol Kits (15596026; Invitrogen, Carlsbad, CA, USA). Total RNA (~ 1 µg) was reverse transcribed using RevertAid First Strand cDNA Synthesis Kits (Takara Bio Inc., Kusatsu, Japan) to a final volume of 20 µL. Quantitative real-time (qRT-) PCR was conducted using SYBR-Green Master Mix kits (cat. No. A25779; Thermo Fisher Scientific Inc., Waltham, MA, USA) using a PCR Detection System (CFX96; Bio-Rad Laboratories Inc., Hercules, CA, USA) under standard conditions as described by the manufacturer. Results were analyzed using the $2^{-\Delta\Delta C_T}$ method, with the *Actin* gene as the internal standard. Table S1 lists the PCR primers.

Immunohistochemical staining

Two independent individuals graded IHC-stained samples and compared the findings with the clinical data. Formalin-fixed and paraffin-embedded tissue specimens and corresponding para-cancerous tissue from patients with pancreatic cancer are represented in tissue microarray (TMA format) in this retrospective analysis. Core samples (diameter, 2 mm) from paraffin-embedded tissue sections were dewaxed, rehydrated, and then heated in EDTA for 3 min to retrieve antigens. Endogenous peroxidase was blocked by incubation for 20 min in 0.3% H₂O₂ and then the sections were incubated with an anti-DPP4 antibody (1:200 dilution, #67138S; Cell Signaling Technology, Danvers, MA, USA) followed by anti-ACSL4 antibody (1:100 dilution, #sc-271800; Santa Cruz Biotechnology Inc., Dallas, TX, USA) overnight at 4°C. The sections were washed three times in PBS, incubated with secondary antibodies for 1 h at room temperature and then stained with DAB. The staining intensity (0, 1, 2, 3) and proportion of positive cells (0–100%) were semi-quantified, and scores ranged from 0 (no stained cells) to 3 (all cells intensely stained). According to this scoring system, 200%–300% and 0–200% were respectively regarded as high and low expression.

Cell culture and stable cell lines

The hTERT-HPNE, HEK293T, PANC-1, CFPAC-1, and PATU-8988T, and the gemcitabine-resistant PANC-1(PANC-1/GEM) cell lines (The Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai, China) were cultured in RPMI-1640 or Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific Inc.) and 1% penicillin-streptomycin (Sigma Aldrich Corp., St. Louis, MO, USA) at 37°C under a humidified 5% CO₂ atmosphere. We obtained stable cell lines overexpressing Flag-DPP4 by DPP4-Flag-GFP lentivirus transduction with Polybrene (PL9000, Public Protein/Plasmid Library; <http://www.genepl.com/>) as described by the manufacturer. We selected cells in growth medium containing appropriate antibiotics to generate stable cell lines 48 h after cell transduction.

Detection of cell proliferation

Real-time quantitative cell proliferation analysis proceeded using E-PLATE 16 (Agilent Technologies GmbH, Waldbronn, Germany) and the xCELLigence system (ACEA Biosciences, San Diego, CA, USA). Briefly, negative control or DPP4-overexpressing PANC-1, CFPAC-1, and PATU-8988T cells (1×10^4) were seeded in E-PLATE 16 overnight. Thereafter, the cells were incubated with various concentrations of erastin or gemcitabine, and the cell index (CI) was recorded automatically every 15 min.

Cell cycle analysis

Cells (3×10^5 /mL) seeded into six-well plates were incubated overnight, washed in PBS, and permeabilized with 70% ethanol at 4°C overnight. The cells were centrifuged and resuspended in 7-amino-actinomycin D (7-AAD) for 30 min in the dark at room temperature. The cell cycle was assessed by flow cytometry using a FACSCalibur (Becton Dickinson and Co., Franklin Lakes, NJ, USA), and 2×10^4 events were collected for each analysis.

Cell metabolism determination

Real-time oxygen consumption rates were measured in PDAC cells using a Seahorse XF96 Extracellular Flux Analyzer (Agilent Technologies GmbH.) as described by the manufacturer. Briefly, PANC1, CFPAC1, and PATU-8988T cells were seeded into 96-well plates and incubated overnight under a humidified 5% CO₂ atmosphere at 37°C (normal conditions). Calibration plates were hydrated overnight at 37°C in an atmosphere without humidified 5% CO₂. Cells were incubated with the assay medium and then the protocol was run until calibration was completed. After probe calibration, oligomycin (1 µM), FCCP (1 µM), rotenone (1 µM), and antimycin A (1 µM) that regulate mitochondrial respiration were sequentially added to the cells to determine the OCR. Protein concentrations measured using a BCA kit were normalized and are shown as means ± SD.

Annexin V apoptosis assay

Cells (2×10^4 /mL) were seeded into 6-well plates overnight for 16–20 h and then the medium was removed, and the cells were cultured with the indicated concentrations of gemcitabine for 24 h. The cells were harvested by centrifugation at $1,000 \times g$ for 5 min and resuspended in 0.5 mL of PE Annexin V binding buffer using kits (BD Pharmingen Inc., San Diego, CA, USA). The cells were then stained with Annexin V-PE-H conjugate and 7-AAD as described by the manufacturer. Cells were analyzed by flow cytometry using the FACSCalibur and at least 10,000 events were collected for analysis. Early apoptotic cells were stained only with Annexin V-PE-H conjugate, normal cells were not stained with either Annexin V-PE-H conjugate or 7-AAD, and late apoptotic cells were stained with both.

Measurement of lipid ROS and iron

For lipid ROS determination, Cells were incubated in six-well plates containing 5 µM BODIPY 581/591 C11 dye (D3861 Invitrogen). The cells were incubated for 30 min at 37°C, trypsinized and then washed with PBS. Stained cells were analyzed by flow cytometry, and FL1 channel signals were collected from live cells as shown in the column.

For iron determination, cells were collected, washed twice with PBS and then incubated with 0.2 M calcein-AM (#HY-D0041, MCE) at 37°C for 15 min. The cells were washed twice with 0.5 mL PBS and incubated with or without deferiprone (100 µM) at 37°C for 1 h. Fluorescence (FL1-H) emitted by calcein-stained cells was detected by flow cytometry. Differences in fluorescence emission between cells incubated with and without deferiprone reflected the size of the labile iron pool (LIP).

Transmission electron microscopy (TEM)

Cells were fixed for 2 h with 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4), rinsed in PBS twice and fixed in 1% osmium tetroxide in 0.1 M PBS for 1 h. The cells were dehydrated using a graded series of acetone and embedded in epoxy resin. Ultrathin sections were stained with 1% uranyl acetate and alkaline lead citrate and then examined via TEM.

Animal models

An orthotopic tumor model was established by implanting Panc02 cells (1×10^6) into the tail of the pancreas of wild-type and DPP4-knockout B6 mice. Two weeks later, those with implanted cells were randomly assigned to receive erastin (40 mg/kg, i.p., twice every other day) for 2 weeks or gemcitabine (20 mg/kg, i.p., once every other day) for 3 weeks ($n = 6$ mice/group). The tumors were excised 7 days after the end of treatment and preserved.

Bioluminescent imaging

D-luciferin (150 mg/kg, Promega, USA) was intraperitoneally administered to the mice 10 min before bioluminescence imaging in vivo using an IVIS Lumina X5 (PerkinElmer, Life and Analytical Sciences Inc., Waltham, MA, USA). After bioluminescent image acquisition, total photon flux for bioluminescent imaging plots for all organs was quantified as the P/S number using analysis software (PerkinElmer, USA).

Antibodies and western blotting

The following antibodies were obtained from the following respective suppliers: anti-DPP4 antibody (#ab222716; Abcam, Cambridge, UK, and #67138S; Cell Signaling Technology), anti-ACSL4 antibody ((#sc-271800; Santa Cruz Biotechnology Inc.), Anti Flag (#8146), actin (#3700), and anti-ubiquitin (#3936) antibodies (Cell Signaling Technology). Protein extraction buffer comprised a 1:10:100 ratio of PMSF:PI:RIPA. Protein concentrations were measured using BCA kits. The protein solution was prepared according to the obtained concentration. Heat-denatured protein (10 μ g) was separated via SDS-PAGE and then transferred to PVDF membranes. Non-specific protein binding was blocked by agitating the membranes in 5% skim milk for 2 h at room temperature and then incubating them overnight with primary antibody at 4 °C. Thereafter, the membranes were incubated with secondary antibodies at room temperature for 1 h and then bound proteins of interest were detected using Image Lab Software (Bio-Rad Laboratories Inc.).

Co-immunoprecipitation of DPP4 and ACSL4 in HEK293T and pancreatic cancer cell lines

Protease inhibitors were added to RIPA buffer to extract total protein from HEK293T and PATU-8988T cells transfected with the DPP4-Flag expression vector or an empty vector. Lysates containing 500 μ g protein were incubated with anti-DPP4, anti-ACSL4 or IgG (negative control), centrifuged, and then immune complexes were separated via SDS-PAGE. Whole cell lysates and Co-IP precipitants were

immunoblotted with either anti-ACSL4 or anti-DPP4 antibodies to confirm interactions between DPP4 and ACSL4. The lysate (10% input, 10 µg protein) also served as a control.

Glutathione S-transferase protein pull-down assays

Purified His-tagged recombinant human DPP4 (DPP4-His; #10688-H08H; Sinobio Chemistry Co., Ltd., Dalian, China) was included in pull-down assays. Purified HIS-vector or HIS-DPP4 fusion proteins were immobilized on HIS beads (#P2210, Beyotime Biotechnology, Shanghai, China) and then mixed and incubated at 4°C for 2 h with lysates of HEK293T cells transiently transfected with ACSL4. Samples were washed five times before separation by SDS-PAGE.

Immunofluorescence analysis

Non-specific protein binding in cells seeded on culture slides was blocked with 5% BSA in PBS and then the cells were incubated with anti-DPP4 (1:200 dilution) and anti-ACSL4 (1:100 dilution) antibodies for 2 h at room temperature. Alexa Fluor 568(ab175471) or Alexa Fluor 647(ab150115; both from Abcam) secondary antibodies diluted 1:500 were applied and then cellular DNA was stained with 1 µg/mL of DAPI. Staining was visualized using a Leica TCS SP8 microscope (Leica Microsystems, CMS GmbH, Wetzlar, Germany).

Ubiquitination assay

PATU-8988T cells stably transfected with the DPP4 expression vector or empty vector were incubated with or without 30 µM MG132 for 6 h. Total protein was extracted using protease inhibitors in RIPA buffer, immunoprecipitated using anti-ACSL4 or anti-IgG antibodies, and then western blotted against corresponding antibodies and with anti-ubiquitin to detect ubiquitination levels.

Statistical analyses

Survival was analyzed using Kaplan–Meier plots and log-rank tests. All data were statistically analyzed using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). Multiple comparisons were assessed using one-way analysis of variance (ANOVA), and between-group differences were analyzed using two-tailed Student *t* tests. Tumor bioluminescence was analyzed using two-way ANOVA. Data are presented as means ± SEM, and all experiments were repeated three times.

Results

DPP4 is downregulated in primary pancreatic tumors and is an independent predictor of poor outcomes

We compared levels of mRNA expression in PDAC tissues and paired non-cancerous samples from 20 patients to clarify DPP4 expression in human PDAC. Expression was significantly downregulated in tumorous, compared non-tumorous regions from the same pancreas ($P < 0.001$; Fig. 1A). However, plasma DPP4 activity did not obviously change in patients with pancreatic cancer (Fig. 1B).

Immunostained sections of PDAC tumor and non-tumor tissues from 56 patients verified this difference in expression ($P < 0.001$; Fig. 1C and D). The median follow-up after surgery for these 56 patients was 19.5 months. Survival curves revealed that the median overall survival was 13.3 and 23.5 months in patients with tumors expressing high and low levels of DPP4, respectively (Fig. 1E). The 1- and 3-year survival rates were lower among patients expressing high, than low DPP4 (50.0% vs. 76.3% and 11.1% vs. 21.1%, respectively). Kaplan-Meier curves revealed significantly longer postoperative survival in the patients expressing low levels of DPP4 ($P = 0.067$; Fig. 1E). These results suggested that DPP4 expression is aberrantly downregulated in PDAC tissues and lower expression predicts a better prognosis for patients.

Resistance to gemcitabine is mediated by DPP4 in pancreatic cancer cells

The gemcitabine-resistant PDAC cell line PANC1 expresses more DPP4 than normal PANC1 cells (Fig. 2A) and have increased sensitivity to erastin-induced ferroptosis (Fig. 2B and Fig. 2C). We explored the role of DPP4 expression in the sensitivity of PANC1 cells to various concentrations of gemcitabine for 24 h. Figure 2D and E shows that DPP4 significantly reduced apoptosis in PANC1 cells and resulted in resistance to gemcitabine. Reflecting these results in vitro, tumors were significantly smaller in DPP4-knockout than wild-type mouse models of orthotopic tumors treated with treatment (Fig. 2F and G). These data showed that the activation and inhibition of DPP4 respectively retards and enhances the anticancer effects of gemcitabine.

Ectopic expression of DPP4 suppresses pancreatic cancer cell growth

We investigated DPP4 expression in normal pancreatic hTERT-HPNE cells, and in the PDAC cell lines PANC-1, PATU-8988T, CFPAC-1, MIA-PACA-2, and SW1990 using western blotting. The PDAC cell lines expressed less DPP4 than hTERT-HPNE cells (Figure S1A). To determine the tumor suppressive activity of DPP4 in pancreatic cancer, DPP4-downregulated PANC-1, PATU-8988T, and CFPAC-1 cells were stably transfected with a DPP4 expression vector, or an empty vector (control). The expression of DPP4 mRNA and protein in these cells was confirmed by RT-PCR and western blotting (Figure S1B and S1C). The overexpression of DPP4 significantly suppressed cell growth, as indicated by RTCA cell viability, real-time oxygen consumption rates (OCR), energy metabolism, and G1–S cell cycle arrest in PANC-1, PATU-8988T, and CFPAC-1 cells (Fig. 3A–I).

We conducted microarray analyses based on DPP4 overexpression with erastin-induced ferroptosis in PATU-8988T cells to predict the global biological roles of DPP4 in PDAC cells. To gain insight into the phenotypic changes, we analyzed our data using GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment of annotated differentially expressed genes. Significantly enriched terms and pathways were corrected based on the rigorous threshold of $P < 0.05$. The results of the GO

analysis revealed that the gene functions of the following four major processes associated with DPP4 functions were enriched in erastin-induced ferroptosis: signaling receptor binding, serine-type endopeptidase inhibitor, pancreatic polypeptide receptor, and cytokine activities (Fig. 3J). Meanwhile, KEGG analysis suggested that ferroptosis and metabolic pathways were significantly enriched in cells overexpressing DPP4 (Fig. 3K). Taken together, these results suggested that the function of DPP4 in ferroptosis is to inhibit PDAC cell proliferation.

DPP4 promotes cell death by inducing cell ferroptosis

We assessed whether erastin influences DPP4 expression in the PDAC cell lines, PANC1 and PATU-8988T. Erastin concentration-dependently decreased DPP4 protein expression in these cells (Fig. 4A). To verify whether increased DPP4 expression influences ferroptosis, we overexpressed DPP4 in PANC1 and PATU-8988T cells with relatively low baseline DPP4 expression (Figure S1). Real-time quantitative cell proliferation analysis of cell viability showed that DPP4 overexpression enhanced erastin-induced death in PANC1 and PATU-8988T cells (Fig. 4B and C). Phase-contrast (Fig. 4D) and transmission electron microscopy (TEM) findings (Fig. 4E) also supported these results, as DPP4 overexpression resulted in abnormal mitochondrial morphology, which is a typical feature of ferroptosis. The TEM findings also showed that cells overexpressing DPP4 treated with erastin had the highest rates of cell death, smaller mitochondria, and increased membrane density (Fig. 4E), suggesting ferroptosis.

Because the hallmark of ferroptosis is lipid peroxidation,[18] we examined whether DPP4-mediated death affects malondialdehyde (MDA)[9] production and lipid reactive oxygen species (ROS) in ferroptosis. In agreement with its impact on cell death, the expression of DPP4 significantly increased MDA (Fig. 4F) and lipid ROS levels (Fig. 4G). We also measured labile iron concentrations (sensitizing cells to erastin-induced ferroptosis) in control and DPP4-overexpressing PANC1 and PATU-8988T cells, which is another classical means of evaluating ferroptosis sensitivity. The results showed that DPP4 overexpression obviously increased labile iron concentrations in PDAC cells (Fig. 4H–J). Together, these data demonstrate that DPP4 is likely a positive regulator of ferroptosis in PDAC.

DPP4-knockout mice are resistant to erastin-induced ferroptosis

Orthotopic tumor models are considered more clinically relevant than subcutaneous model. We used bioluminescence imaging to further investigate whether DPP4 increases the anticancer activity of erastin in wild-type and DPP4-knockout B6 mouse models of orthotopic tumors based on established mouse PANC2 cells. Orthotopic tumors in the pancreas were significantly smaller in erastin-treated wild-type mice than erastin-treated DPP4-knockout mice ($p < 0.001$, Fig. 5A and B). The findings of Ki-67 assays showed fewer proliferating cells in erastin-treated wild-type, than erastin-treated DPP4-knockout mice ($p < 0.001$; Fig. 5C and D).

We investigated lipid peroxidation levels in tumor samples from erastin-treated wild-type and DPP4-knockout B6 mice using immunohistochemical (IHC) staining for 4-hydroxy-2-nonenal (4HNE). The

results showed increased 4HNE staining in the tumor cells from erastin-treated wild-type mice (high DPP4 expression) (Fig. 5E and F), indicating that DPP4 facilitates ferroptosis in vivo and that ferroptosis is at least partly responsible for the tumor-suppressive effects of DPP4 in vivo.

Direct interaction between ACSL4 and DPP4

Long-chain acyl-CoA synthetase 4 is an essential feature of lipid peroxidation during ferroptosis. We evaluated DPP4 and ACSL4 expression in tissues from 56 patients with pancreatic cancer using microarrays and IHC staining. Most tumor specimens expressed low levels of DPP4 and ACSL4 (Fig. 6A and B; Figure S2A and B), and these statistically correlated ($r = 0.357$, $p < 0.001$; Fig. 6C). Moreover, TCGA data analysis also revealed a positive correlation between DPP4 and ACSL4 expression in PDAC samples from patients (Figure S2C). We examined the expression and intracellular distribution of DPP4 and ACSL4 using immunofluorescence. Confocal microscopy revealed DPP4 expression throughout PATU-8988T cells stably transfected with DPP4 and ACSL4, whereas ACSL4 was located in the membrane, and co-localized in the cytoplasm with DPP4 (Fig. 6D).

We examined interactions between DPP4 and ACSL4 by co-immunoprecipitation (Co-IP) assays of anti-Flag IP products of HEK293T cells transfected with a DPP4-Flag vector or a pcDNA3.1/Flag vector with an anti-IgG IP product. In line with the above findings, DPP4 directly interacted with ACSL4 (Fig. 6E) and co-precipitated with an ACSL4 antibody in HEK293T cells (Fig. 6E), indicating physical interaction. We then addressed the specificity of ACSL4 and DPP4 interaction under cell-free conditions using purified recombinant HIS-DPP4 protein and HIS pull-down assays. Western blotting indicated ACSL4 formed complexes with HIS-DPP4 but not with HIS (Fig. 6F), confirming specific physical binding between DPP4 and ACSL4.

Interaction between DPP4 and ACSL4 inhibits ACSL4 ubiquitin-mediated degradation

We examined the half-life of ACSL4 protein using cycloheximide chase assays to determine whether DPP4 influences ACSL4 protein degradation. The overexpression of DPP4 prolonged ACSL4 protein stabilization (Fig. 7A), and DPP4-ACSL4 interaction products significantly increased ($P < 0.05$, Fig. 7B) in PATU-8988T cells incubated with erastin, indicating that DPP4 protects against erastin-induced ACSL4 protein degradation in ferroptosis. We also analyzed whether DPP4-ACSL4 interaction affects ACSL4 protein degradation via the ubiquitin-mediated pathway. We thus measured ubiquitination and protein levels of ACSL4 in PATU-8988T cells with or without (control) DPP4 overexpression after incubated with the proteasome inhibitor MG132. The findings suggested that DPP4 overexpression increased levels of ACSL4 and decreased those of ubiquitinated ACSL4 (anti-ACSL4 IP product) (Fig. 7C). Thus, DPP4-ACSL4 interaction stabilizes ACSL4 by inhibiting its ubiquitin-dependent degradation.

Human DPP4 consists of 766 amino acids that comprise an extracellular (29–766 aa) and transmembrane regions (7–29 aa), and an intracellular tail (1–7 aa) (Fig. 7D).¹⁵ We analyzed the structural basis of the interaction between DPP4 and ACSL4. We constructed a series of vectors that

express various fragment mutants or full-length DPP4 (1–29 aa, 1–324 aa, 1–552 aa, 1–766 aa, 48–766 aa, and 324–766 aa) as described[15]. The results of Co-IP assays suggested that 1–29 aa was the only fragment that did not bind ACSL4 upon transfection into HEK293T cells (Fig. 7E).

We identified DPP4 as a potential tumor suppressor in pancreatic tumorigenesis. Based on our findings, we propose a model in which DPP4 decreases sensitivity to gemcitabine and increases sensitivity to ferroptosis (Fig. 7F). We also showed that DPP4 facilitates the resistance of pancreatic cancer cells to gemcitabine. More importantly, DPP4 binds ACSL4 to stabilize this protein by inhibiting its ubiquitin-dependent degradation, which mediates tumor-suppressive functions by promoting cell ferroptosis.

Discussion

Chemotherapeutic resistance remains a major challenge for treating PDAC with gemcitabine, which is a classical anticancer agent used to induce apoptosis.[19,20] This situation indicates that novel therapeutic strategies are needed to treat PDAC. Stockwell,[9,21] coined the word, ferroptosis, in 2012 to define a particular form of cell death that was later verified as an important non-apoptotic cell death pathway in carcinoma.[22] The efficacy of ferroptosis and selective induction of gemcitabine-resistant cell death plays an important role in achieving absolute tumor eradication and overcoming resistance to current chemotherapy.[23,24] In agreement with previous results, we also found that gemcitabine resistance in PANC1 cells was associated with upregulated levels of DPP4 protein, consequently resulting in increased cell sensitivity to ferroptosis (Figure 2). Our findings notably indicated that DPP4 overexpression inhibits gemcitabine-induced apoptosis, suggesting that it plays a critical role in pancreatic cancer chemotherapy.

The DPP4 protein[25] exists as a membrane bound form in many types of cells, and also as a soluble form in various physiological fluids that has been investigated as a disease biomarker.[26] The present study mainly focused on intratumor DPP4 expression, as soluble DPP4 in plasma did not obviously change in patients with pancreatic cancer (Figure 1B). By cleaving N-terminal dipeptides from peptides with an alanine or proline in the penultimate position, DPP4 changes the activities of chemokines, incretin hormones, and many other peptides.[27] Owing to these functions and interactions with other molecules, DPP4 promotes[12,28,29] or suppresses[30–32] tumors. In this context, we found that DPP4 is commonly silenced in primary pancreatic cancer and pancreatic cancer cell lines. We identified the effects of DPP4 in pancreatic cancer in vitro and in orthotopic xenograft transgenic mouse models in vivo. The overexpression of DPP4 significantly suppressed the viability, G1–S cell cycle transition, and OCR energy metabolism in PANC1, CFPAC-1, and PATU-8988T cells compared with those transfected with an empty vector (Figure 3). The induction of cell ferroptosis regulated the mechanism through which DPP4 inhibited pancreatic cancer cell viability (Figure 4). Accordingly, numbers of proliferative cells were obviously reduced and lipid peroxidation was increased in orthotopic xenograft tumors of erastin-treated wild-type mice compared with those in erastin-treated DPP4^{-/-} mice (Figure 5).

Fishman et al. found that DPP4 regulates hepatic lipid metabolism independently of insulin sensitivity modulation.[33] Moreover, DPP4–NOX binding increases during the lipid peroxidation of ferroptosis.[15]

To explore the molecular basis of the role of DPP4 in pancreatic cancer ferroptosis, we evaluated the positive correlation between DPP4 and ACSL4 based on TCGA data (Figure S2C). Consistent with the above results, correlations were also statistically significant between DPP4 and ACSL4 levels in microarray tissues from 56 patients with pancreatic cancer (Figure 6). Moreover, DPP4 co-localized with ACSL4. Collectively, ACSL4 was verified as a potential functional partner that interacts with DPP4. The direct interaction between DPP4 and ACSL4 was analyzed by Co-IP assays and by HIS pull-down assays using purified DPP4 recombinant protein under cell-free conditions. We found more DPP4-ACSL4 complexes in the erastin-treated group. Given that ACSL4 is an essential regulator of lipid peroxidation in ferroptosis,[34,35] interaction between DPP4 and ACSL4 was further identified as the activation of ferroptosis by DPP4 overexpression in pancreatic cancer cells and the downregulation of ferroptosis in DPP4-knockout mice. The upregulation of ACSL4 by DPP4 was mediated by a reduction in the ubiquitination/degradation of ACSL4 in pancreatic cancer. These findings collectively showed that ACSL4 is a direct interactive partner of DPP4.

Above all, we uncovered a novel role of DPP4 in lipid metabolism and identified it as a tumor suppressor that promotes ferroptosis in pancreatic cancer by cooperating with ACSL4 to exacerbate lipid peroxidation. Accordingly, low DPP4 expression represents a good prognostic biomarker for administering gemcitabine chemotherapeutics to patients with pancreatic cancer. In contrast, erastin might be a novel therapeutic target for treating pancreatic cancer with high DPP4 expression. As DPP4 inhibitors are widely and effective in diabetes mellitus[36], whether DPP4 inhibitors have a synergistic effect with gemcitabine in PDAC is also worthy of further investigation.

Declarations

Authors' contributions

G.C. and W.M.W. designed the study; G.C. and Y.W. funded and supervised the molecular analysis; X.Z., B.F.Z., and J.G.Z. conducted experiments in vitro and analyzed the data; X.Z., W.M.W., J.Y.Z., and W.M.B. conducted experiments in vivo; Y.W. and P.Y.G. analyzed the data; X.H.C., G.R.C., and Z.P.Y. analyzed tissue staining; Y.W. and Y.F.S. conducted biologic informatics analyses; X.Z., X.H.C., Z.P.Y., and Y.W. analyzed clinical information; X.Z. and W.M.W. wrote the manuscript.

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Consent for publication

All authors have agreed with publishing this manuscript.

Competing Interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Dipeptidyl peptidase 4 is downregulated and correlates with poor prognosis in pancreatic ductal adenocarcinoma. (A) Real-time PCR shows high and low DPP4 mRNA expression in 18 pancreatic tumor and adjacent non-tumor tissues, respectively. (B) Serum DPP4 activity in 32 patients with PDAC and 24 healthy controls. (C) Scatter plot shows H-scores of DPP4 obtained from TMA containing 56 paired PDAC and non-tumor tissue samples. * $p < 0.001$ (paired Student t tests). (D) Representative immunohistochemistry (IHC) staining indicates low expression of DPP4 in pancreatic tumors. Scale bar, 100 μ m. (E) Kaplan-Meier curves suggest that postoperative survival is shorter for patients with PDAC expressing high, than low levels of DPP4.

Figure 2

Dipeptidyl peptidase reduces gemcitabine sensitivity. (A) Protein levels of DPP4 assayed in pancreatic ductal adenocarcinoma (PDAC) cells ($n = 3$; $*p < 0.05$ gemcitabine-resistant vs. control PANC1). (B, C) Gemcitabine-resistant PANC1 (PANC1/GEM) increased erastin-induced ferroptosis as indicated by RTCA (IC50, PANC1 vs. PANC1/GEM: 2.98 μ M vs. 4.98 mM; $n = 3$, $*P < 0.05$). (D) Evaluation of PANC1 cell apoptosis after 24-h incubation with 100 μ M gemcitabine. Cells were stained with Annexin V-PE-H conjugate and 7-amino-actinomycin D (7-AAD). Viable, early, and late apoptotic cells are distributed in Q4, Q3, and Q2, respectively. (E) Overexpression of DPP4 (pcDNA3.1-DPP4 cDNA) reduced gemcitabine-induced cell death in PANC1 cells at 24 h ($n = 3$; $*p < 0.05$ vs. control pcDNA3.1). (F) Representative images before and 3 weeks after administering transgenic mice with gemcitabine (20 mg/kg) i.p. once every other day. (G) Bioluminescence intensity in pancreatic tumors after gemcitabine treatment. Results are expressed as means $\pm$ SEM ($n = 6$; $*p < 0.05$).

Figure 3

Gain-of-function assays of DPP4 in vitro. (A-C) Real-time cell growth of control and DPP4 overexpression (pcDNA3.1-DPP4 cDNA) in PANC1, CFPAC-1, and PATU-8988T cells, measured by RTCA. Data are presented as means $\pm$ SEM (* $p < 0.001$). (D-F) Real-time overall oxygen consumption rate (OCR) of control and DPP4OE (pcDNA3.1-DPP4 cDNA) by PANC1, CFPAC-1, and PATU-8988T cells. Data are presented as mean OCR $\pm$ SEM of eight replicates. (G-I) Cell cycle analysis of control and DPP4OE (pcDNA3.1-DPP4 cDNA) PANC1, CFPAC-1, and PATU-8988T cells stained with 7-amino-actinomycin D (7-AAD). Ratios of cells in Sub-G1, G0/G1, S and G2/M phases are indicated. (J) Gene Ontology analysis of target genes of differentially expressed mRNA between control and PATU-8988T cells incubated with 20

μM erastin. (K) Analysis of target genes of differentially expressed mRNA between control and DPP4OE (pcDNA3.1-DPP4 cDNA) PATU-8988T cells using Kyoto Encyclopedia of Genes and Genomes (KEGG).

Figure 4

Cell death is promoted by DPP4 through ferroptosis induction in vitro. (A) Western blots show DPP4 protein expression in PANC1 and PATU-8988T cells after incubation with erastin (5, 10, 20 μM) for 24 h (n = 3; *p < 0.05 vs. untreated cells). (B, C) Overexpression of DPP4 (DPP4 cDNA) increased erastin-induced

cell death in PANC1 and PATU-8988T cells (*p < 0.05 vs. control pcDNA3.1). (D) Representative phase-contrast images of PATU-8988T cells after incubation with 20 μ M erastin for 24 h. Scale bars, 100 μ m. (E) Representative transmission electron microscopy image of PATU-8988T cells incubated with 20 μ M erastin for 24 h. Red arrows, mitochondria with obvious cristae; blue arrows, representative mitochondrial phenotype. Scale bars, 0.5 μ m. (F) Overexpression of DPP4 cDNA enhanced erastin-induced malondialdehyde (MDA) production in PANC1 and PATU-8988T cells, whereas ferroptosis inhibitors (liproxstatin-1, 1 μ M) reversed erastin (20 μ M, 24 h)-induced MDA production (n = 3; *p < 0.05). (G) Lipid ROS in PANC1 and PATU-8988T cells incubated with or without 20 μ M erastin for 24 h, stained with C11-BODIPY, and analyzed by flow cytometry. (H) Labile iron in PANC1 and PATU-8988T cells assessed by flow cytometry. Labile iron levels quantified in PANC1 (I) and PATU-8988T (J) cells.

Figure 5

Cell death is promoted by DPP4 through ferroptosis induction in vivo. (A) Representative images of transgenic mice before and 2 weeks after i.p. injection of erastin (40 mg/kg) twice every other day. (B) Bioluminescence intensity in pancreatic tumors after erastin administration. Results are expressed as means $\pm$ SEM (n = 6; *p < 0.05). (C, E) Immunohistochemical staining of KI-67 (C) and 4-hydroxy-2-nonenal (4HNE) (E) in orthotopic tumor xenografts from wild-type and DPP4-knockout B6 mice. Scale bars, 100 μ m. (D, F) Ratios of KI-67 (D) and 4HNE (F) positively stained cells per field. Error bars, means $\pm$ SEM; n = 5 randomly selected high-power fields. All P values were calculated using two-tailed unpaired Student t-tests.

Figure 6

Interaction between DPP4 and ACSL4. (A-B) Immunohistochemical staining of DPP4 and ACSL4 ($n = 56/\text{group}$). (C) Correlations between DPP4 and ACSL4 expression ($n = 56/\text{group}$). (D) ACSL4 (red) and DPP4 (yellow) expression and localization in PATU-8988T cells. Cell nuclei are labeled with DAPI (blue). Scale bar, 25 μm . (E) Co-immunoprecipitation of DPP4 and ACSL4 from protein lysates of HEK293T cells transfected with DPP4 detected by western blotting against specific antibodies. (F) Direct interaction between DPP4 and ACSL4 under cell-free conditions determined using HIS-tag pull-down assays. All experiments were repeated three times. $*P < 0.05$.

Figure 7

DPP4 inhibits ACSL4 degradation in ferroptosis. (A) Control or DPP4-overexpressing PATU-8988T cells incubated with erastin (20 μ M) and cycloheximide (20 μ g/mL; CHX) for 0, 2, 4, 6, and 8 h. Cell lysates were analyzed against anti-ACSL4 and anti-actin antibodies. (B) Co-immunoprecipitation of DPP4 and ACSL4 from protein lysates of DPP4-transfected PATU-8988T cells. The amount of DPP4 and ACSL4 protein in the Co-IP products was enhanced during erastin-induced ferroptosis. (C) Dipeptidyl peptidase 4 upregulated ACSL4 by suppressing its ubiquitin-dependent degradation. (D) Schema of domain structure, mutants, and molecular weight of DPP4. (E) Co-immunoprecipitation of DPP4-ACSL4 complex in HEK293T cells transfected with FLAG-tagged DPP4 WT or fragments after incubation with erastin (20 μ M) for 24 h. (F) Schema of molecular mechanism underlying effects of DPP4 in pancreatic cancer. Sensitivity to gemcitabine is decreased by DPP4.

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