Transcription factor DDIT3 is a potential driver in pancreatic cancer

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal and aggressive tumor that affects the digestive tract, leading to high mortality and poor survival rates. The purpose of the present study was to evaluate the expression levels of DNA damage-inducible transcript 3 (DDIT3) in pancreatic cancer and to investigate its effects in in vitro and in vivo experiments. Bioinformatics analysis indicated that DDIT3 expression was higher in pancreatic cancer tumor tissues and associated with a poor prognosis. Positive or strong positive DDIT3 expression was observed in PDAC, and no or weak expression was observed in normal pancreatic tissues. It was also highly expressed in PDAC cells, while being expressed at lower levels in normal pancreatic ductal epithelial cells. Transfection of short hairpin RNA targeting the DDIT3 gene reduced the proliferation, migration and invasion of PANC-1 cells. In vivo, in an in situ implantation tumor model with Pan02 cells, the size and weight of the tumors were reduced in the DDIT3 knockdown Pan02 cell-implanted group. These data suggested that DDIT3 represents a novel predictive biomarker for the potential treatment of patients presenting with PDAC.

Keywords: Pancreatic ductal adenocarcinoma, DNA damage-inducible transcript 3 (DDIT3), Bioinformatics, Proliferation, Invasion, In situ implantation tumor model.

1. Introduction

Pancreatic cancer is a deadly malignancy, and over the past two decades, there has been a doubling in the annual number of diagnosed cases worldwide. Pancreatic ductal adenocarcinoma (PDAC) accounts for >90% of all pancreatic cancers, and its incidence has markedly increased worldwide [1]. Despite developments in the detection and management of pancreatic cancer, the survival rate of patients has only increased from 5% in the 1990s to as high as 9% in the USA and Europe in 2019 [2, 3] of patients will live for 5 years after diagnosis. Therefore, it is necessary to understand the biological mechanisms that contribute to the development and progression of pancreatic tumors [4, 5].

The DNA damage-inducible transcript 3 (DDIT3) gene, also known as CCAAT/enhancer binding protein (C/EBP) homologous protein and GADD153 [6-10], encodes a basic leucine zipper transcription factor. This factor belongs to the dimer-forming C/EBP family, which is considered pathognomonic in the appropriate morphological and clinical context. The expression of DDIT3, also known as a key regulator of the cellular stress response, is induced in response to endoplasmic reticulum stress [11, 12], DNA damage [13], autophagy [14], cellular growth arrest or hypoxia [15, 16]. In recent years, it has been demonstrated that DDIT3 expression can be used as a clinical diagnosis for high-grade myxoid liposarcoma (MLPS) [17, 18], myeloid leukemia [19], malignant follicular thyroid carcinoma (FTC) [20] and gastric cancer [21], and may serve an important role in regulating the epidermal differentiation during the differentiation program of keratinocytes in the skin [22]. However, to the best of our knowledge, information regarding the expression and function of DDIT3 in PDAC tissues and pancreatic cancer cells is limited. The present study aimed to determine whether DDIT3 is highly expressed in pancreatic cancer and to elucidate its relationship with the proliferation and invasion of pancreatic cancer cells.

2. Materials and methods

2.1. Cell culture

The SW1990 and PANC-1 human pancreatic cancer cell lines, Pan02 mouse pancreatic cancer cell line, and HPNE normal pancreatic ductal epithelial cells were purchased from the American Type Culture Collection. DDIT3 gene-knockdown PANC-1 and Pan02 pancreatic cancer cell lines, along with their respective negative control cells, were established and stored in our laboratory. All cell lines

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were cultured in DMEM supplemented with 10% fetal calf serum at 37°C in a 5% CO₂ atmosphere.

2.2. Chemical reagents and antibodies

The antibody anti-DDIT3 (cat. no. ab179823; dilution, 1:1, 000) was purchased from Abcam. Anti-GAPDH (cat. no. 5174; dilution, 1:1, 000) and HRP-linked secondary antibody (cat. no. 7074; dilution, 1:2, 000) were purchased from Cell Signaling Technology, Inc. R-Phcoerythrin-Affini Pure F(ab')2 Fragment Goat Anti-Rabbit IgG (H+L) (cat. no. 111-116-144; dilution, 1:2, 000) was purchased from Jackson ImmunoResearch Laboratories, Inc. The Transwell chamber assay kit was purchased from BD Biosciences.

2.3. Bioinformatics data acquisition and analysis

All pancreatic cancer gene expression datasets were collected from The Cancer Genome Atlas (TCGA; http://tcga-data.nci.nih.gov/tcga), and microarray data were obtained from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/).

The differentially expressed DDIT3 mRNA was analyzed between pancreatic tumor and normal tissues using RNA sequencing data. The association between survival and DDIT3 expression was assessed for all patients from TCGA. Kaplan-Meier plotter was used to assess the association between the survival rate and DDIT3 mRNA expression. The hazard ratio (HR) and P-value were calculated using the 2⁻log method [23].

2.4. DDIT3 gene knock-down pancreatic cancer cell established

One recombinant lentiviral vectors GV248 (hU6-MCS-Ubiquitin-EGFP-IRES-puromycin) containing short hairpin RNAs (shRNA) targeting human DDIT3 (GeneBank: NM_001195057.1) cDNA and mice DDIT3 (GeneBank:NM_007837.4) and one negative control recombinant lentiviral vectors were produced by Genechem (Shanghai, China). The shRNA sequences for PANC-1 cell interference were: shRNA-DDIT3: 5'-GAAGGCTTGAGTAGTACAA-3', shRNA-NC: 5'-TTTCTCCGAACGAGTAGACAA-3'. The shRNA sequences for Pan02 cell interference were: shRNA-DDIT3: 5'-GATTCCAGTCAGAGTTCTATG-3', shRNA-NC: 5'-TTTCTCCGAACTGTGTCACGT-3'. After sequencing, the positive lentivirus packaging plasmids pRSV-Rev and pMD2.G. The supernatant of the lysed cells was collected and non-specific binding was blocked using goat serum.

The monoclonal rabbit anti-human DDIT3 antibody was applied at a 1:100 dilution. A universal rabbit negative control was used. Slides were then treated with goat anti-rabbit horseradish peroxidase secondary antibody, followed by the addition of peroxidase substrate. Slides were counterstained with hematoxylin, dehydrated, mounted and examined under a microscope. The quantitative criteria were as follows: Strongly positive (+++), cell shading >50%; positive (+), cell shading >30% to 50%; weakly positive (+), cell shading >5% to 30%; and negative (-), <5% or without staining.

2.5. Immunohistochemistry (IHC)

IHC was performed using a rabbit monoclonal antibody directed against the C-terminus of DDIT3 on whole sections from 40 patients with high-grade PDAC and 40 normal pancreatic ductal tissues as controls. All paraffin-embedded samples collected between April 2021 and June 2022 were preserved at the Affiliated Cancer Hospital of Nanjing Medical University. The sample collection protocols were approved by the Ethics Committee of Nanjing Medical University (approval no. 2022514). Tissues were sectioned (5 μm) onto charged slides. All slides were deparaffinized and rehydrated. Endogenous peroxidase activity was inactivated with 3% H₂O₂. Heat-induced epitope retrieval was performed with 0.01 M sodium citrate and non-specific binding was blocked using goat serum.

The SW1990, PANC-1 and Pan02 pancreatic cancer cell lines, and HPNE normal ductal epithelial cells were cultured in DMEM supplemented with 10% fetal calf serum at 37°C in a 5% CO₂ atmosphere.

2.6. Quantitative real-time PCR assay (qPCR)

The SW1990, PANC-1 and Pan02 pancreatic cancer cell lines, and HPNE normal ductal epithelial cells in the logarithmic growth period were collected. Total RNA was prepared using Triazolo reagent (Invitrogen; Thermo Fisher Scientific, Inc.), precipitated with isopropyl alcohol and washed with 70% ethanol. Single-stranded cDNA was prepared from the purified RNA using oligo (dT) primers, and then, cDNA was subjected to SYBR Green (Takara Bio, Inc.) quantitative PCR. The primers used in the experiment were as follows: DDIT3, 5'-TGAACCTGTTCTCTTGGCCTT-3' and 5'-TTTCTCCTCCTAGCGTGTCG-3'; and GAPDH, 5'-GAAGGTAGGTCAGGGAGGTCG-3' and 5'-GAAGATGGTGTAGGGATTTC-3'. The relative expression levels of the target gene were normalized to those of the control gene GAPDH. The relative expression levels were calculated using the 2⁻ΔΔCt method [23].

2.7. Flow cytometry

The SW1990, PANC-1 and Pan02 pancreatic cancer cell lines, as well as HPNE normal ductal epithelial cells in the logarithmic growth period, were collected. For the detection of intracellular molecules such as DDIT3, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 before being stained with a DDIT3 antibody for 1 h. Afterwards, the cells were incubated with goat anti-rabbit IgG conjugated with PE for 30 min. Cells were analyzed using a BD Biosciences FACSAria II flow cytometer (BD Biosciences).

2.8. Western blotting

The SW1990, PANC-1 and Pan02 pancreatic cancer cell lines, as well as HPNE normal ductal epithelial cells in the logarithmic growth period, were collected and lysed in RIPA buffer supplemented with a 1/50 (vol./vol.) ratio. Dilution of a protease inhibitor cocktail (Calbiochem; Merck KGaA). The supernatants of the lysed cells were collected. The total protein concentration was determined, and equal amounts of samples were separated by 10% SDS-PAGE. The separated proteins were then electrotransferred to a...
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0.45-μm polyvinylidene difluoride membrane. The membranes were blocked with PBS with Tween-20 containing 5% non-fat milk for 2 h at room temperature, and incubated with DDIT3 and GAPDH antibodies overnight at 4°C. Specific bound antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit antibodies and then visualized using the ECL detection kit.

2.9. Colony formation assay
Cell proliferation was determined using a colony formation assay. Briefly, PANC-1 cells with DDIT3 gene-knockdown [short hairpin RNA (shRNA)-DDIT3] and negative control cells [shRNA-negative control (NC)] were digested with trypsin and resuspended in serum-free medium. Subsequently, 2 ml medium containing 10% FBS was added to the 6-well plates. The resuspended cells were plated into the wells (200 cells per well). The medium was replaced every 4 days. After 12 days, the cells were fixed, stained with crystal violet and counted.

2.10. Transwell assay
Migration assays were performed using 5.0-μm pore Transwell inserts. DDIT3 gene-knockdown shRNA-DDIT3 PANC-1 and negative shRNA-NC PANC-1 cells were digested with trypsin and resuspended in serum-free medium. The Transwell chamber was added to a 24-well plate to create the upper and lower chambers. Next, 800 μl medium with 10% FBS was added to the lower chamber, and 6x10^4 cells in 100 μl serum-free conditioned medium were added to the upper chamber. After 24 h, the cells in the upper chamber were wiped with cotton swabs, and the invading cells were fixed and stained with crystal violet for counting.

2.11. In situ implantation tumor model
Male C57BL/6 mice (6-8 weeks old) were used for all experiments in the present study. All mice were bred and housed in specific pathogen-free conditions at a controlled temperature (22±1°C) and exposed to a constant 12-h light-dark cycle in the animal facilities. The animal experiments were conducted according to the guidelines and with the approval of the Committees of Animal Ethics and Experimental Safety of Nanjing Medical University.

For the generation of in situ implantation models of pancreatic cancer, mice were anesthetized by intraperitoneal injection of sodium pentobarbital (45 mg/kg) [24], and a small incision was made to expose the pancreatic tissue. A total of 5x10^5 shRNA-DDIT3-transfected or shRNA-NC-transfected Pan02 cells were suspended in 40 μl PBS containing 20% ECM Gel from Engelbreth-Holm-Swarm murine sarcoma (MilliporeSigma) and injected directly into the pancreatic gland. Mice were monitored for clinical signs, and their body weight was measured daily. After 28 days, when the maximum tumor diameter was almost 15 mm, mice from each group were euthanized by CO₂ asphyxiation with a CO₂ displacement rate of 60% of the container volume per minute. Death was confirmed by examination of heartbeat and respiration. The tumors were collected for further analysis following the procedure described below: Volume = length x width^2/2. Weight was weighed by a precision balance and size was measured by a vernier caliper. Tumor size and weight were recorded. The P-value was calculated using the Wilcoxon rank-sum exact test, and P<0.05 was considered to indicate a statistically significant difference.

2.12. Statistical analysis.
Statistical results are presented as the mean ± SD of at least three repeated experiments (n=3) unless otherwise described. Experimental data were analyzed using an unpaired two-tailed Student’s t-test to compare two groups and one-way ANOVA followed by Bonferroni post hoc test for multiple comparisons. GraphPad Prism 7.0 (Diagnostics) was used for the analysis. The levels of statistical significance were marked as follows: *P<0.05; **P<0.01; ***P<0.001.

3. Results
3.1. DDIT3 is highly expressed in pancreatic cancer tumors and related to poor prognosis based on the GEO and TCGA databases
A total of three gene expression profile datasets were retrieved from the GEO database, including 69 pancreatic cancer samples and 61 normal samples in GSE62452, 118 pancreatic cancer samples and 13 normal samples in GSE62165, and 139 pancreatic cancer samples and 105 normal samples in GSE183795. Furthermore, data for four pancreatic cancer samples and their paired normal tissue samples were downloaded from TCGA.

The relative DDIT3 expression was significantly higher in the pancreatic tumor samples, as determined by independent-sample t-tests, in the GSE62452 (Fig. 1A), GSE62165 (Fig. 1B) and GSE183795 (Fig. 1C) datasets. In the four pancreatic cancer tumors and their paired normal tissue samples from TCGA, the relative expression of DDIT3 was significantly higher in the pancreatic tumor samples based on a paired t-test. DDIT3 expression was upregulated in pancreatic cancer samples from all four databases (Fig. 1D).

Survival analysis revealed that higher expression levels of DDIT3 were associated with lower survival in 176 patients with pancreatic cancer (HR, 1.363; 95% CI, 0.999-1.86; P<0.0491; Fig. 2A) and a lower 3-year survival period in 156 cases (HR, 1.466; 95% CI, 1.064-2.02; P<0.0171; Fig. 2B) in the cohort from TCGA. According to this analysis, it can be concluded that high DDIT3 ex-
pression was associated with a poor outcome in patients with pancreatic cancer. The pathways related to DDIT3 are shown in Fig. 2C, and the five most significant pathways included protein processing in endoplasmic reticulum, MAPK signaling pathway, pathways in cancer, PI3K-Akt signaling pathway and necroptosis.

DDIT3 was expressed in the cell cytoplasm with positive or strong positive expression in PDAC and no or weak expression in normal pancreatic ductal tissue. IHC was performed using a rabbit antibody targeting the C-terminus of DDIT3. Cytoplasmic staining in >5% of cells was considered positive. Using IHC, positive expression of DDIT3 (Fig. 3A) was observed in 38 out of 40 (95%) PDAC tissues. Of the controls, 2 out of 40 (5%) cases were positive, with no more than 10% cytoplasmic staining. An additional 95% of control cases displayed <5% nuclear staining (Fig. 3B). The differences were statistically significant.

3.2. DDIT3 is highly expressed in human pancreatic cancer cells, whereas it is not found in pancreatic normal ductal epithelial cells

To investigate the levels of DDIT3 gene and protein expression in pancreatic cells in vitro, three pancreatic cancer cell lines and one normal pancreatic cell line were utilized in the present study. As shown in Fig. 4A, the mRNA level of DDIT3 in SW1990, PANC-1 and Pan02 pancreatic cancer cells was 3.26 (P<0.01), 54.23 (P<0.001) and 15.24 (P<0.001) times higher than that in HPNE cells, respectively. Next, the expression levels of DDIT3 in different cell lines were examined by western blotting. As shown in Fig. 4B, DDIT3 expression was increased in all pancreatic cancer cell lines. Furthermore, DDIT3 expression in different cell lines was examined by flow cytometry. As shown in Fig. 5, the percentage of DDIT3-positive cells were 78.4, 97.2 and 92.4% in SW1990, PANC-1 and Pan02 pancreatic cancer cells, respectively. It was increased in all pancreatic cancer cells compared with HPNE cells.

3.3. Silencing of DDIT3 inhibits the proliferation and migration of pancreatic cancer cells

In the group of shRNA-DDIT3, the protein relative expression level is lower than that in the control shRNA-NC group (Fig. 6A and B). And DDIT3 mRNA levels was 0.00075 times and 0.00137 times, lower than those in the control shRNA-NC group of PANC-1 and Pan02 cells (Fig. 6C and D). This indicated that shRNA vector effectively inhibited the expression of DDIT3 gene in PANC-1 cell and Pan02 cell.

To confirm the tumor-promoting effect of DDIT3 in vitro, its effect was verified in stable lentiviral-mediated DDIT3-knockdown PANC-1 cells. The effect of DDIT3 on proliferation and migration was suppressed following transfection with specific shRNA. Compared with that of the control group, the colony formation of shRNA-
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DDIT3-transfected PANC-1 cells was markedly reduced (Fig. 7A and B). On the other hand, the number of migrated cells in the shRNA-DDIT3 PANC-1 group was significantly lower than that in the shRNA-NC PANC-1 group. This difference was statistically significant (Fig. 7C and D). These findings demonstrated that silencing of DDIT3 could inhibit the proliferation and migration of pancreatic cancer cells.

3.4. Silencing of DDIT3 reduces the growth of tumors in an implantation tumor model

To further confirm the effect of DDIT3 in vivo, its impact was verified using stable DDIT3-knockdown Pan02 cells. The in situ implantation animal models were established by injecting the same amount of shRNA-DDIT3 Pan02 cells or shRNA-NC Pan02 cells. After the experiment, the mice were euthanized. As shown in Fig. 8A and B, the size of the tumors was significantly reduced in mice injected with shRNA-DDIT3 group cells (P<0.05). In addition, the tumor weight in the shRNA-DDIT3 group was significantly lower than that in the shRNA-NC group (P<0.01; Fig. 8C).

4. Discussion

The present study aimed to evaluate DDIT3 expression in pancreatic cancer and to elucidate its relationship with the proliferation and invasion of cancer cells. Lin-Jarnum et al evaluated a monoclonal antibody targeting the N-terminus of DDIT3 in MLPS. The authors revealed that 100% of MLPS cases exhibited nuclear positivity for DDIT3 based on IHC. The majority of cases exhibited moderate-to-strong diffuse staining in the tumor nuclei, with >80% staining, while the remaining cases showed >50% staining. This indicated that DDIT3 can be utilized to differentiate high-grade MLPS from other round-cell sarcomas [16]. Baranov et al initially reported that DDIT3 was highly sensitive and specific for MLPS among adipocytic and myxoid neoplasms. It was also highly sensitive and specific for high-grade MLPS among round-cell sarcomas. Strong, diffuse staining was 96% sensitive and 100% specific [17]. Cerutti et al reported that the DDIT3 protein was expressed in 85.2% of malignant FTC and only 9.4% of benign follicular thyroid adenoma (FTA) in differentiated thyroid cancers. This high sensitivity of DDIT3 protein expression can be used to identify FTA and FTC [20]. Another finding by Lin et al demonstrated that DDIT3 promoted the stemness of cancer stem cells by upregulating CEBPb in gastric cancer. This finding suggested that DDIT3 may serve an important role in the progression of gastric cancer [21]. By analyzing the expression levels of DDIT3 in patients with pancreatic cancer, the present study revealed that DDIT3 expression was upregulated in pancreatic cancer tissues, particularly in PDAC tumor

Fig. 5. Intracellular expression levels of DDIT3 in pancreatic cancer and normal pancreatic ductal epithelial cells examined via flow cytometry. The ratio of DDIT3-positive cells was increased in all the pancreatic cancer cell lines.

Fig. 6. The protein (A and B) and mRNA (C and D) expression of DDIT3 in stable DDIT3 gene knock-down or NC-negative pancreatic cancer cells were evaluated by western blotting and real time PCR, respectively. There was significant down-regulation in shRNA-DDIT3 group compared with shRNA-NC group (**P<0.01, n=3).

Fig. 7. Silencing of DDIT3 inhibits the proliferation and migration of pancreatic cancer cells. (A) Effect of DDIT3 suppression on proliferation following transfection with specific shRNA examined using a colony formation assay followed by nuclear staining with crystal violet. (B) Numbers of colonies in the shRNA-DDIT3 and shRNA-NC groups were calculated using Image-J software and shown in a bar graph (*P<0.05; n=3). (C) Migration and invasion of shRNA-DDIT3 and shRNA-NC group PANC-1 cells were evaluated using a Transwell assay followed by nuclear staining with crystal violet (scale bar, 100 µm; magnification, x400). (D) The number of cells able to pass through the ECM was calculated using Image-J software and shown in a bar graph (*P<0.05; n=3).

Fig. 8. Silencing of DDIT3 reduces the growth of tumors in an implantation tumor model. Silencing of DDIT3 reduces the growth of tumors in an implantation tumor model. (A) Tumor size of the mice with the indicated treatments for 28 successive days (n=6). (B) Representative tumor images of shRNA-NC group and shRNA-DDIT3 group. (C) Tumor weight of the mice with the indicated treatments for 28 successive days (n=6).
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Availability of data and materials
All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding authors.

Authors’ contributions
Conceptualization, FZL and XZ; Methodology, GYJ, BG, YFC, YCW and YTW; Bioinformatics Analysis, HH, XXC and ZQF; Investigation, XXC and ZQF; Writing -Original Draft, GYJ, YFC and YCW; Writing -Review & Editing, ZQF, FZL and XZ; Funding Acquisition, FZL and XZ. All authors have read and approved the final manuscript.

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