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DUSP1 promotes pancreatic cancer cell proliferation and invasion by upregulating nephronectin expression

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Abstract

Objectives: To explore the role of dual-specific phosphatase 1 (DUSP1) in the proliferation, migration and invasion of pancreatic cancer (PC).
Methods: TCGA and GTEx databases were used to investigate the relationship between DUSP1 expression and prognosis of PC patients. Expression efficiency of DUSP1 was validated by qPCR and western blotting. The proliferation of SW1990 and PANC-1 cells with DUSP1 overexpression or knockout was detected by EdU assays. The migratory and invasive abilities of cells were detected by wound healing and transwell assays.
Results: DUSP1 was highly expressed in PC and associated with poor prognosis of patients. Overexpression of DUSP1 promoted the proliferation, migration and invasion of PC cells by regulating nephronectin (NPNT), whereas knockout of DUSP1 exhibited the opposite effects. NPNT expression was positively correlated with DUSP1, and the overall survival of PC patients with high levels of NPNT was shorter.
Conclusions: DUSP1 promoted the proliferation, migration and invasion of PC cells by upregulating NPNT, suggesting DUSP1 may be a potential target for PC treatment.

Keywords: DUSP1; invasion; migration; NPNT; pancreatic cancer

Introduction

The survival rate of patients with pancreatic cancer (PC) is very lower (12 %) [1]. By the time PC is diagnosed, it is already typically in the advanced stage due to lack of specific tumor markers. Moreover, PC cells have strong invasive and metastatic abilities; and in metastatic patients, surgical resection is difficult [2]. In addition, the pathogenesis of PC involves complex genetic factors and a complex tumor microenvironment that make the PC prone to develop resistance to chemotherapy and radiotherapy [3]. To improve the survival rates of PC patients, it is important to elucidate the mechanism of PC metastasis.

Dual-specific phosphatase-1 (DUSP1) is the most widely studied protein among the 25 members of the DUSPs. It acts on the threonine/serine or tyrosine residues to dephosphorylate the substrate and negatively regulate MAPK kinase activity and has different functions in various cancers [4, 5]. DUSP1 was upregulated in a series of cancers, including PC [6], non-small cell lung cancer [7], breast cancer [8], ovarian cancer [9], and gastric cancer [10]. In breast cancer, DUSP1 overexpression enhances the chemotherapy resistance by inhibiting JNK activity [11]. In contrast, DUSP1 negatively regulates the ERK signaling pathway in hepatocellular cancer (HCC) cells to inhibit proliferation. In PC, shRNA-mediated inhibition of DUSP1 has been found to enhance gemcitabine-induced activation of JNK and p38 MAPK and to make PC cells sensitive to gemcitabine [12]. However, the roles of DUSP1 in PC have not yet been completely clarified.

In this study, we found that DUSP1 and DUSP6 were highly expressed in the patients with PC and related to poor survival. Overexpression of DUSP1 significantly promoted the proliferation, migration, and invasion of PC cells by up-regulating NPNT, whereas knockout of DUSP1 exhibited the opposite effects. NPNT expression was positively correlated with DUSP1 in PC patients. These results suggest DUSP1 might be a potential target for PC treatment.

Materials and methods

Cell culture

The PC cell lines (SW1990, PANC-1) were purchased from the American Type Culture Collection (ATCC, Manzas, Virginia, USA). PANC-1 and SW1990 cells were cultured in DMEM (Gibco, New York, USA)

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supplemented with 10 % fetal bovine serum (Yeasen, Shanghai, China) and 1 % penicillin/streptomycin (NCM Biotech, Suzhou, China). All cells were maintained in a saturated humidity atmosphere at 37 °C supplied with 5 % CO_2.

RNA isolation and quantitative RT-PCR

Total RNA from PC cells was isolated with TRIzol reagent (Vazyme, Nanjing, China). Hifair ®III Supermix (Yeasen) was used to synthesis cDNA, following the manufacturer's instructions. Then, qRT-PCR was performed using SYBR Green qPCR Master Mix (Yeasen). Normalization of the *DUSP1*/*NPNT* expression fold change were calculated using the formula $2^{-\Delta\Delta CT}$ (*β-actin* as a control). The primer sequences are shown in Table 1.

CRISPR-Cas9 gene editing

The sequences of synthetic guide RNAs (sgRNAs) that targeting *DUSP1* are listed in Table 2. We diluted the synthesized paired oligos (Tsingke, Shanghai, China) with sterile water. Then, the oligos were annealed in a thermal cycler (DSBio, Guangzhou, China). After *BsmBI* (NEB, Massachusetts, USA) digestion, the oligos were cloned into the LentiCRISPR v2 (Addgene, Massachusetts, USA) sgRNA backbone. To produce viral particles, HEK 293T cells were co-transfected with lentiCRISPR v2, psPAX2 and pVSV-G using the Neofect™ DNA transfection reagent (NEOFECT, Beijing, China).

Wound healing assay

The cells were planted onto a 35 mm plate and grown to confluence, after which they were serum-starved for 24 h. Cells were scratched manually with pipette tips. Photos of the central wound edges were taken at 0 and 24 h using a light microscope (Leica, Wetzlar, Germany).

Table 1: Primer sequences for qPCR.

Gene	Sequence (5′ → 3′)
DUSP1-F	GCGAAGCATCATCTCTCCCA
DUSP1-R	CACTGTTCGTGGAGTGGACA
NPNT-F	AGTACTTGGTGGCCTCCGAAGAC
NPNT-R	CTGGTGGTGGTGGTGGAGTAGG
β-actin-F	AGATGTGATCAGCAAGCAG
β-actin-R	GCGCAAGTTAGGTTTTGTCA

Table 2: Sequences of sgRNAs that target *DUSP1*.

sgRNA	sgRNA sequence (5′ to 3′)
sgRNA1-DUSP1-F	CACCGTCAGCACCATCGTGCGGCGC
sgRNA1-DUSP1-R	AAACGCGCCGCACGATGGTGCTGAC
sgRNA2-DUSP1-F	CACCGCACCCAGATTCCGCGCTGTC
sgRNA2-DUSP1-R	AAACGACAGCGCGGAATCTGGGTGC

Transwell assay

The cells were plated onto the upper chamber (BD Biosciences, New Jersey, USA) coated with or without matrigel (BD Biosciences), and complete medium was added to the lower chambers. After 24 h of incubation, 0.5 % crystal violet was used to stain the methanol-fixed cells on the lower surface. The migration/invasion cells were counted using microscopy, and three randomly fields were picked.

Western blotting

After washing with PBS, we used RIPA buffer (Beyotime, Shanghai, China) supplemented with a protease inhibitor cocktail (Beyotime) to lysate cells. Then, the protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane (Roche, Basel, Switzerland), followed by a 2 h blocking (TBS buffer containing 5 % milk and 0.2 % tween). Membrane was then incubated with the primary antibodies overnight at 4 °C. The next day, the membrane was incubated with the secondary antibody at room temperature for 1 h and then washed with TBST. Finally, ECL kit and Gel imaging system (Tanon, Shanghai, China) were used to visualize the immunoblots. Following primary antibodies were used in this study: anti-β-actin (Proteintech, Chicago, USA), anti-NPNT (Abclonal, Wuhan, China), anti-DUSP1 (Cell Signaling Technology, Boston, USA), anti-Flag (Proteintech), anti-Bcl-2 (Proteintech) and anti-Bax (Cell Signaling Technology).

EdU assays

BeyoClick™ EdU Cell Proliferation Kit (Beyotime) was used to evaluate cell proliferation according to the manufacturer's protocol. Briefly, 4 % PFA were applied to fix cells for 15 min. After permeabilization with 0.5 % Triton X-100 at room temperature for 20 min, the cells were incubated with the click reaction cocktails in the dark for 30 min. The nuclei were counterstained with 1 × Hoechst 33,342 (1:2,000) for 30 min.

Bioinformatic analysis

GEPIA 2 was used to analyze the expression of DUSP1 and NPNT in PC. The Kaplan–Meier Plotter was used to analyze overall survival (OS) in PC. CRISPROR (http://crispor.tefor.net/crispor.py) was used to design the guide RNA sequence. GO and KEGG analysis were performed using the BGI platform (BGI, Shenzhen, China). GSE62452, GSE71729, and TCGA_PAAD survival information were obtained from the GEO and TCGA database.

Statistical analysis

Student's t-test, multiple t-test, and two-way ANOVA were performed to calculate statistical significance. All the data were presented as mean ± SD. GraphPad Prism 8 (GraphPad Software, California, USA) was used to generate the figures and statistical analyses. When the p-value <0.05, the difference was considered statistically significant.

Results

Significant upregulation of DUSP1 expression in pancreatic cancer

Members of the DUSP protein family play key roles in tumor progression and may be developed as potential cancer therapeutic targets [13]. To investigate DUSPs in PC, mRNA expressions of DUSP1, DUSP2, DUSP4, DUSP6, DUSP10, and DUSP16 in PC and normal tissues were investigated by combining the TCGA and GTEx databases. DUSP1, DUSP2, DUSP6, and DUSP10 were significantly upregulated in the PC tissues (Figure 1A). High expressions of DUSP1 (p=0.016), DUSP4 (p=0.021), and DUSP6 (p=0.021) were negatively correlated with OS (Figure 1B). The ROC curve showed that the area under the curve (AUC) of DUSP1 in PC was 0.820 (Figure 1C), indicating that DUSP1 could be an independent predictor of PC. We also performed a pan-cancer analysis of DUSP1 expression in different types of cancer and found that DUSP1 expression was low in all but three types of cancer (Figure 1D).

In addition, the clinicopathological data of 178 patients with PC in the TCGA database were investigated. DUSP1 mRNA level was significantly correlated with the G1/G2 histological grade (p=0.039) but not with the age, sex, TNM grade, pathological grade (Stages I – IV), and anatomical classification (Table 3).

DUSP1 promotes malignant progression of PC cells

To investigate the biological function of DUSP1 in PC, we performed EdU, wound healing, and transwell assays in both SW1990 and PANC-1 cells. DUSP1 was significantly upregulated in cells transfected with pCMV3-Flag-DUSP1

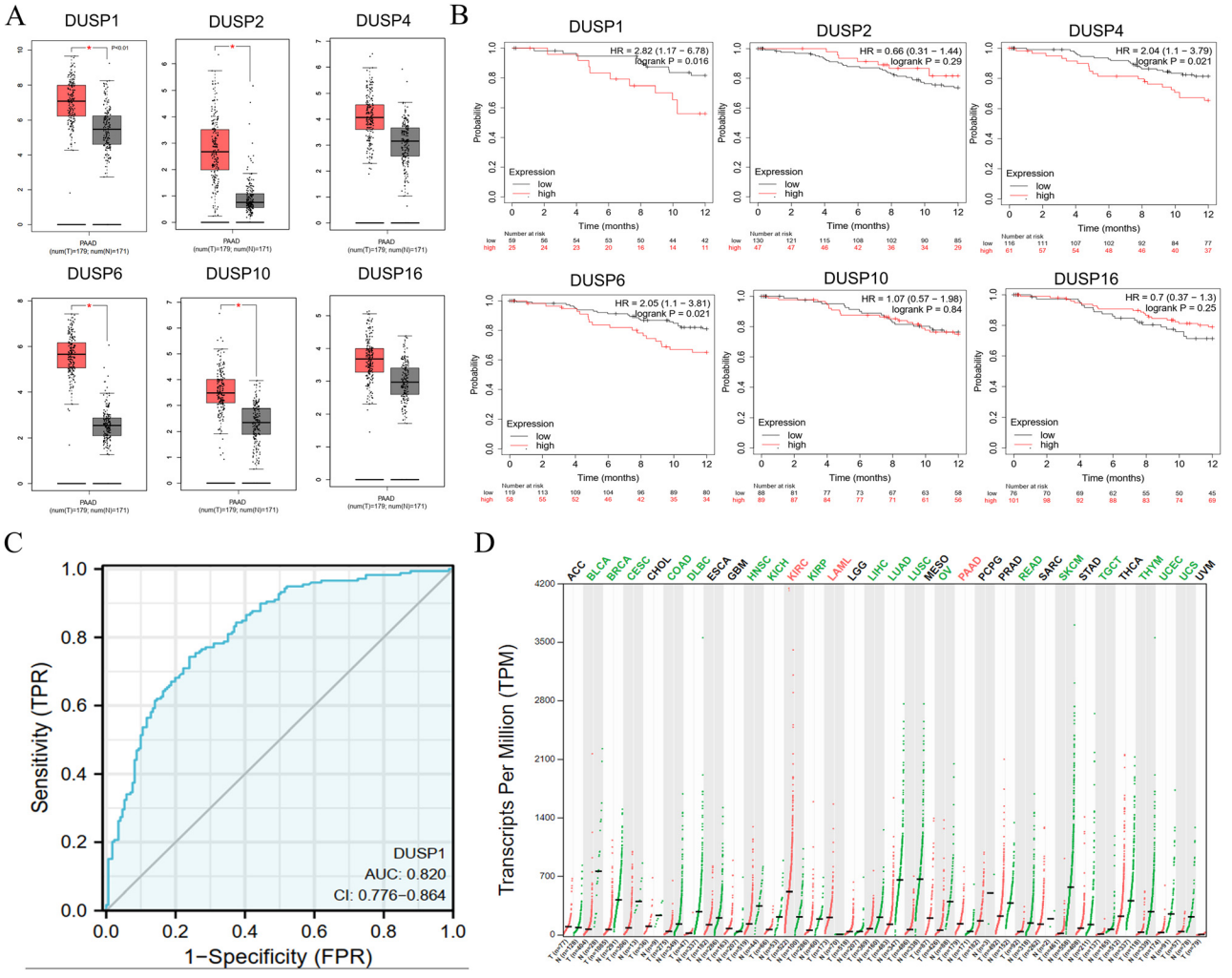

Figure 1: DUSP1 expression in pancreatic cancer. (A) Different expression levels of the DUSP family in pancreatic cancer (PC). (B) Relationship between DUSP family expression and the OS of PC patients. (C) ROC curve of DUSP1 in PC (TCGA). (D) DUSP1 expression level in pan-cancer.

Table 3: Correlation between DUSP1 expression level and the clinicopathologic features of pancreatic cancer.

Clinicopathologic characteristic	Low DUSP1 expression	High DUSP1 expression	p-Value
N	89	89	
Age			0.368
≤65	43 (24.2 %)	50 (28.1 %)	
>65	46 (25.8 %)	39 (21.9 %)	
Gender			0.175
Female	35 (19.7 %)	45 (25.3 %)	
Male	54 (30.3 %)	44 (24.7 %)	
T stage			0.343
T1	3 (1.7 %)	4 (2.3 %)	
T2	14 (8 %)	10 (5.7 %)	
T3	70 (39.8 %)	72 (40.9 %)	
T4	0 (0 %)	3 (1.7 %)	
N stage			0.829
N0	26 (15 %)	24 (13.9 %)	
N1	60 (34.7 %)	63 (36.4 %)	
M stage			0.378
M0	36 (42.9 %)	43 (51.2 %)	
M1	1 (1.2 %)	4 (4.8 %)	
Pathologic stage			0.192
Stage I	12 (6.9 %)	9 (5.1 %)	
Stage II	74 (42.3 %)	72 (41.1 %)	
Stage III	0 (0 %)	3 (1.7 %)	
Stage IV	1 (0.6 %)	4 (2.3 %)	
Anatomic neoplasm subdivision			0.590
Head of pancreas	71 (39.9 %)	67 (37.6 %)	
Other	18 (10.1 %)	22 (12.4 %)	
Histologic grade			**[a]0.039**
G1	20 (15.9 %)	11 (8.7 %)	
G2	40 (31.7 %)	55 (43.7 %)	

Chi-square test was performed to calculate the statistical significance.
[a]$p<0.05$. Bold value means that DUSP1 mRNA level was significantly correlated with the histological grade and the p-Value was less than 0.05.

(Figure 2A), and DUSP1 overexpression raised the proliferative, migratory, and invasive abilities of SW1990 and PANC-1 cells (Figure 2B–D).

Moreover, we constructed KO-DUSP1 cells using the CRISPR-Cas9 system (Figure 3A and B). The KO-DUSP1 cells showed lower proliferative, migratory, and invasive abilities than KO-NC cells (Figure 3C–E). Meanwhile, we observed an anti-apoptotic effect of DUSP1 on PC cells through detection of the protein levels of BAX and Bcl-2 by western blotting. DUSP1 overexpression decreased BAX and increased Bcl-2 levels in SW1990 and PANC-1 cells, and knockout of DUSP1 exhibited the opposite effects (Figures S1 and S2). Together, these results suggest that DUSP1 may serve as an oncogene in PC cells.

NPNT is positively regulated by DUSP1

To investigate the mechanism of DUSP1 in PC progression, we performed RNA sequencing of KO-DUSP1 SW1990 cells. GO and KEGG pathways enrichment analyses of 75 DEGs ($p<0.05$, $|log2FC| > 2$) were performed. The top 12 GO terms and the top 20 KEGG pathway-enriched results were shown in Figure 4A and B.

Among the 75 DEGs, we focused on the nephronectin (NPNT) gene that was downregulated in the KO-DUSP1 SW1990 cells. The NPNT expression was associated with the metastasis of various cancers, such as breast cancer, liver cancer [14], and stomach cancer [15]. Then, we divided the patients into 2 groups (high NPNT expression and low NPNT expression) to perform survival analysis using GSE62452 and GSE71729. The high NPNT expression group showed poor OS (Figure 4C). Meanwhile, predictive value of NPNT in PC was evaluated (AUC=0.872) from the TCGA database (Figure 4D).

Next, we verified whether the expression of NPNT was regulated by DUSP1 using qPCR and WB. NPNT expression was significantly upregulated after DUSP1 overexpression in SW1990 and PANC-1 cells (Figure 4E). mRNA expression level of NPNT was also positively correlated with DUSP1 according to GEPIA2 (Figure 4F).

DUSP1 promotes malignant progression of PC cells by upregulating NPNT expression

Next, we knocked down NPNT in the PC cell lines to investigate whether it can attenuate the enhanced malignant progression of PC cells caused by DUSP1 overexpression. Figure 5A showed that NPNT was successfully knocked down by siRNA transfection. Furthermore, we found that knockdown of NPNT attenuated the enhanced proliferative, migration and invasion abilities induced by DUSP1 overexpression (Figure 5B–D). These data suggest that NPNT at least partially mediates the promoting malignant progression of DUSP1 in PC cells.

Discussion

DUSPs belong to the protein tyrosine phosphatase superfamily, which includes 10 MKPs that inhibit the MAPK signaling pathways, and 20 atypical DUSPs with different substrates [16]. DUSPs play important roles in cancer progression. In ovarian cancer, high DUSP1 expression is associated with shortened asymptomatic survival [9]. In

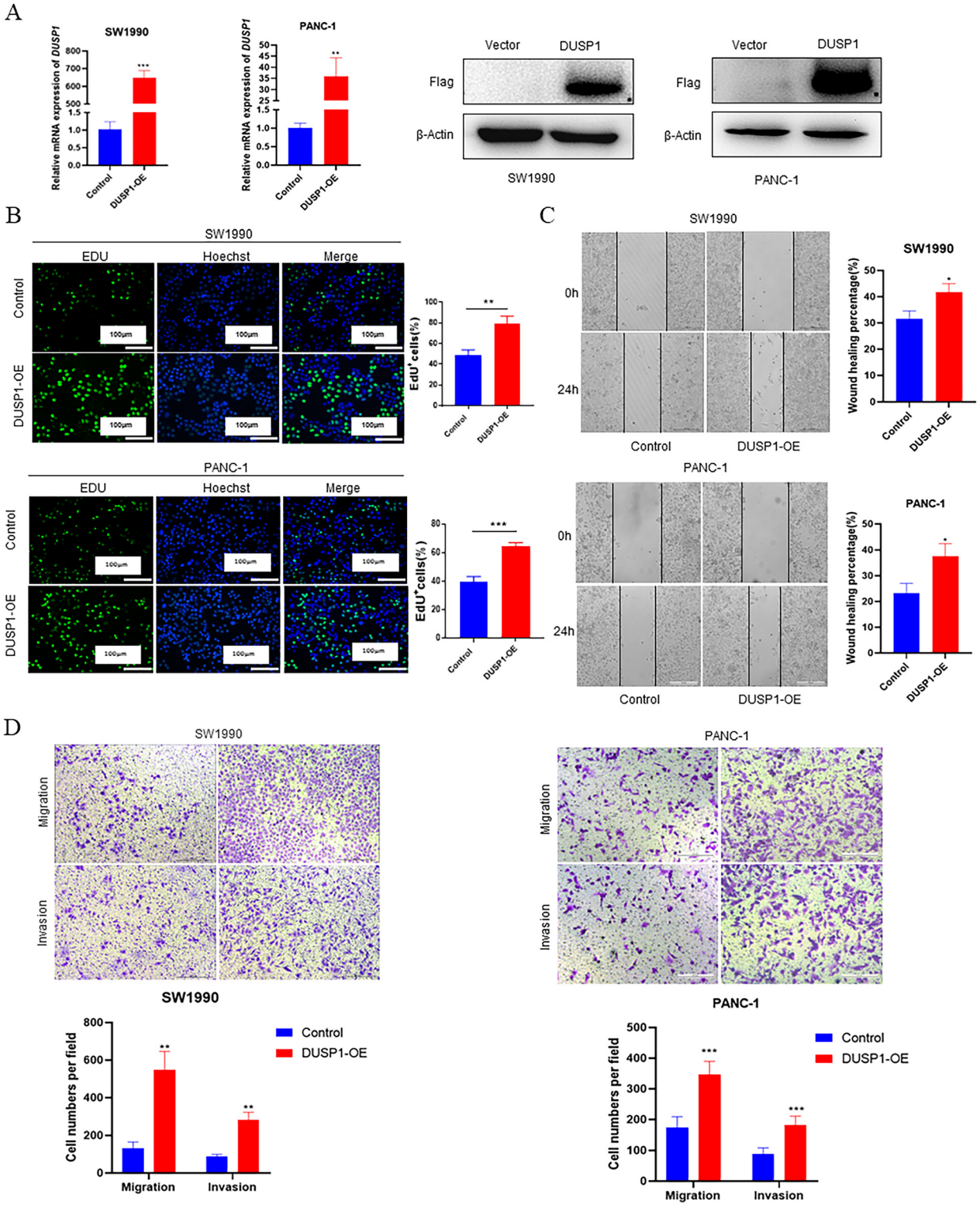

Figure 2: Overexpression of DUSP1 promotes malignant progression of PC cells. (A) The DUSP1 levels were detected in the overexpressed and control cells via qRT-PCR and western blotting. (B) Proliferative abilities of DUSP1-overexpressed SW1990 and PANC-1 cells were detected using EdU assays. Scale bar, 100 μm. (C) Migratory abilities of DUSP1-overexpressed cells were detected through wound healing assay. Scale bar, 200 μm. (D) Migration and invasion of DUSP1-overexpressed cells were detected via transwell assays. Scale bar, 200 μm $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$, student's t-test.

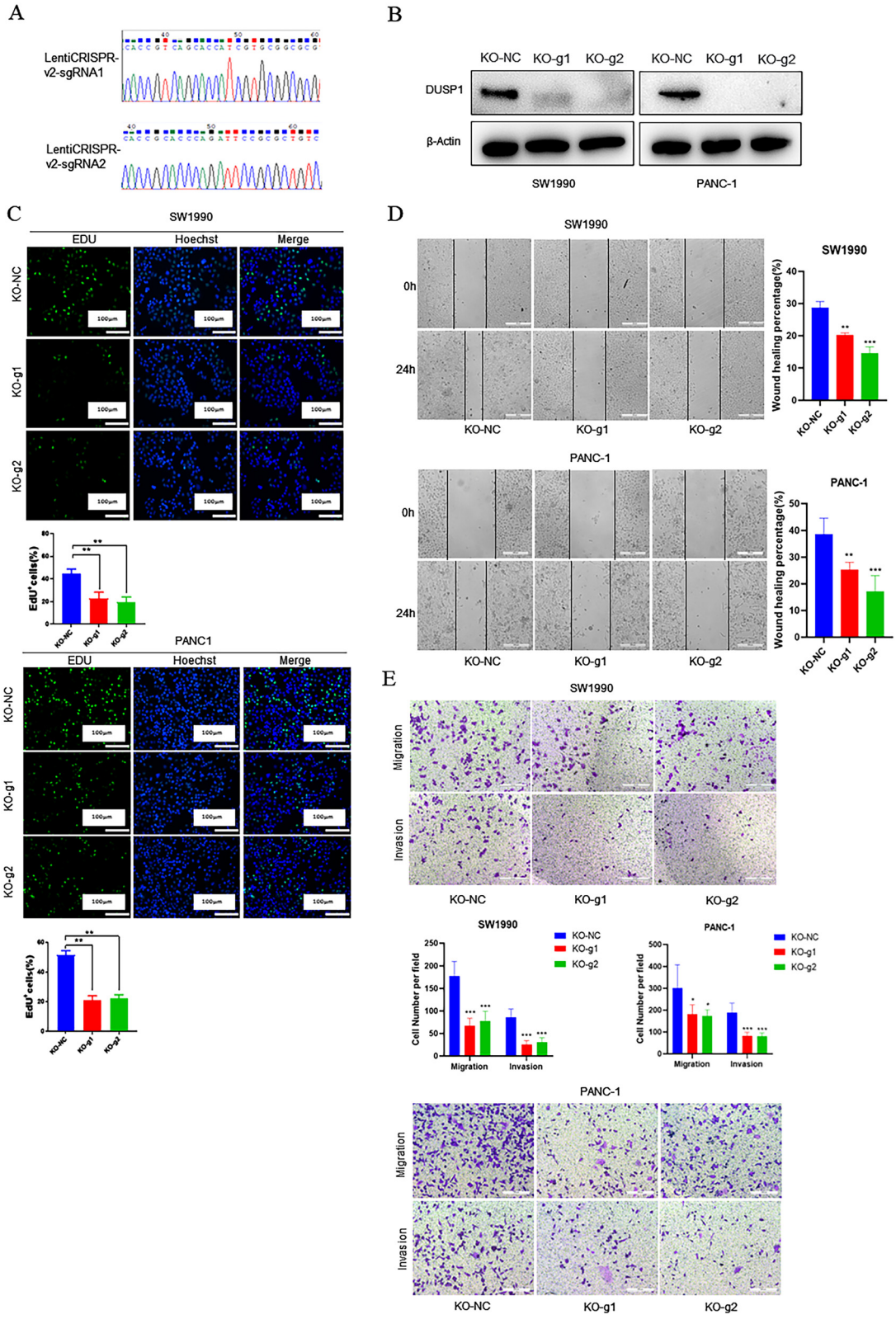

Figure 3: Knockout of DUSP1 suppresses malignant progression of PC cells. (A) LentiCRISPRv2-gRNA1/gRNA2-DUSP1 sites were validated by Sanger sequencing. (B) DUSP1 in KO-DUSP1 cells was detected via western blotting. (C) Proliferative abilities of the KO-DUSP1 cells were detected by EdU assays. Scale bar, 100 μm. (D) Migratory abilities of the KO-DUSP1 cells were detected through wound healing assay. Scale bar, 200 μm. (E) Migratory and invasive abilities of the KO-DUSP1 cells were detected using transwell assays. Scale bar, 200 μm $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$, multiple t-test.

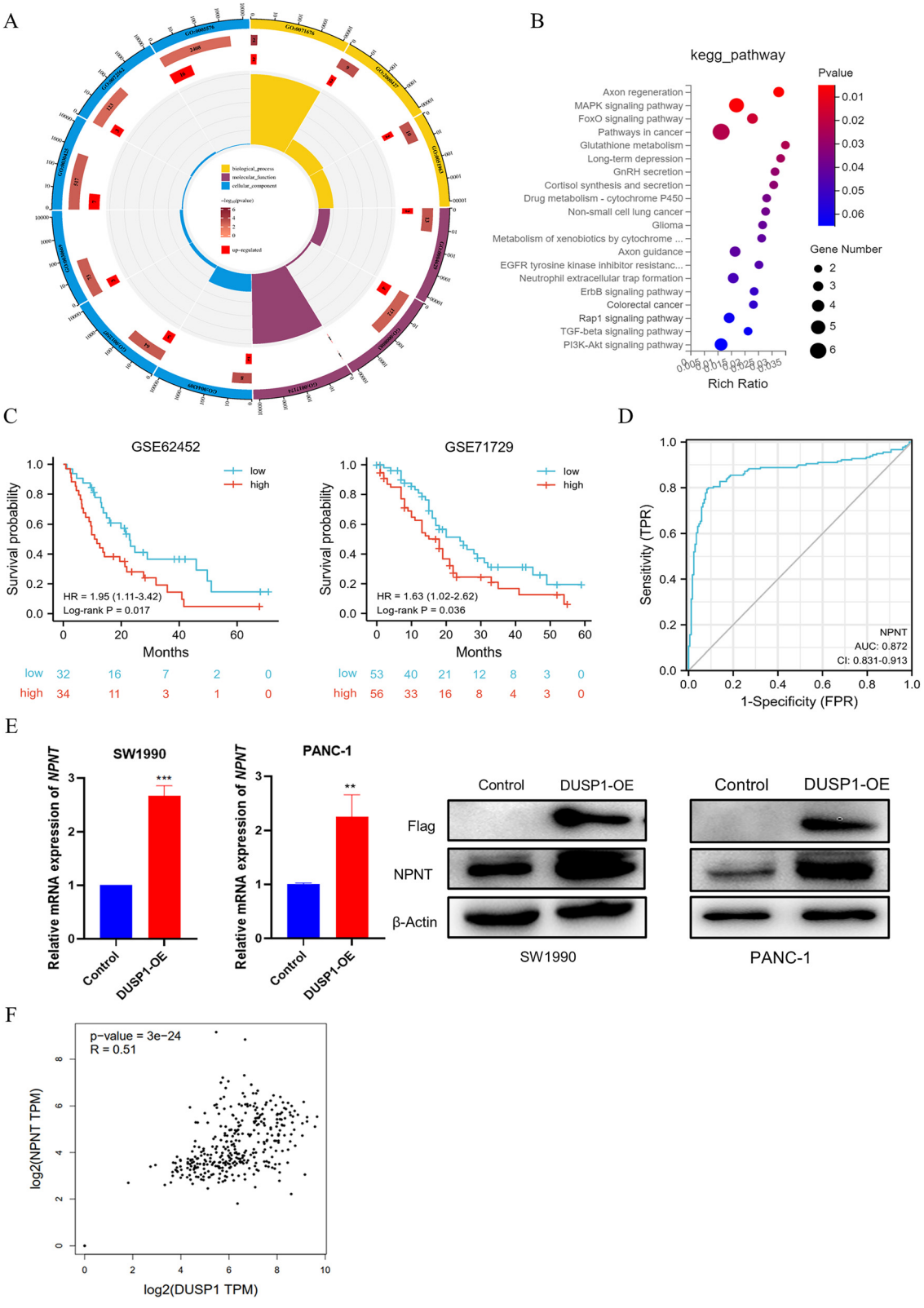

Figure 4: NPNT is positively regulated by DUSP1. (A–B) RNA sequencing of KO-DUSP1 SW1990 cells and control cells was performed. Results of the GO and KEGG enrichment analyses of 75 DEGs. (C) Relationship between the NPNT expression and the patients' overall survival in GSE62452 and GSE71729. (D) ROC curve of NPNT in PC (TCGA database). (E) NPNT levels were detected in the overexpressing-DUSP1 and control cells via qRT-PCR and western blotting. (F) The correlation between DUSP1 and NPNT mRNA expression was analysed using GEPIA 2.

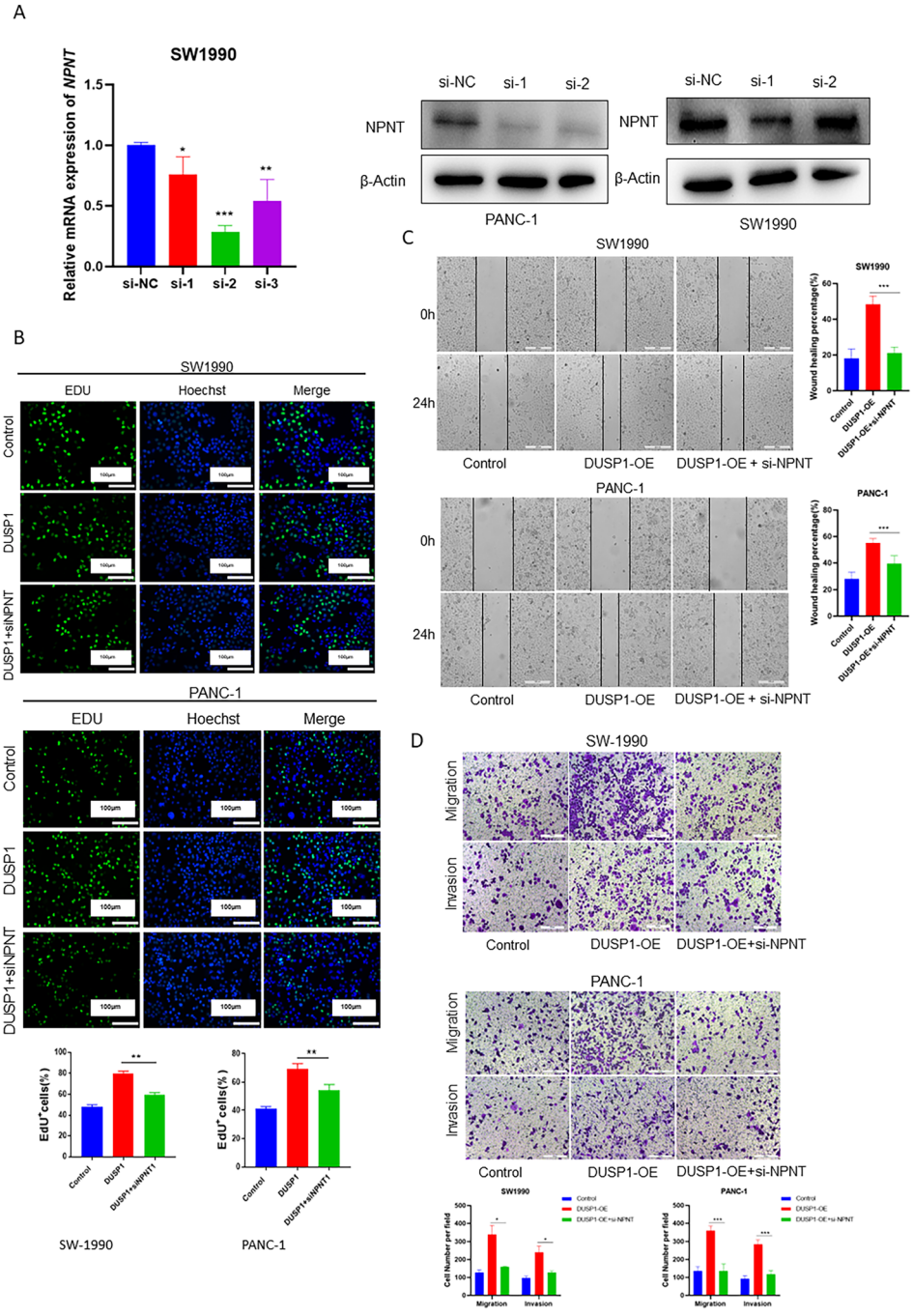

Figure 5: DUSP1 promotes malignant progression of PC cells by upregulating NPNT expression. (A) Knockdown efficiency of siRNA on NPNT in SW1990/PANC-1 cells was determined by qRT-PCR and western blotting. (B) EdU analysis of DUSP1-overexpressed SW1990/PANC-1 cells with or without NPNT knockdown. Scale bar, 100 μm. (C) Migratory abilities of DUSP1-overexpressed SW1990/PANC-1 cells with or without NPNT knockdown were detected by wound healing assays. Scale bar, 200 μm. (D) Migratory and invasive abilities of DUSP1-overexpressed cells with or without NPNT knockdown were detected by transwell assays. Scale bar, 200 μm $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$, multiple t-test.

osteosarcoma, DUSP1 inhibition significantly reduces cancer cell proliferation, migration, and invasion by regulating MAPK signaling pathway [17]. In non-small cell lung cancer, knockdown of DUSP1 inhibits cell motility, invasion, angiogenesis, and metastasis [7]. Conversely, in gallbladder carcinoma, DUSP1 overexpression inhibits cell angiogenesis through the pERK-MMP2/VEGF axis [18]. Most hepatocellular carcinoma (HCC) patients with low DUSP1 expression have a poor prognosis, as DUSP1 negatively regulates ERK signaling pathways in hepatocellular carcinoma cells to inhibit the

proliferation [19]. However, the biological function of DUSP1 in different cancers is not completely consistent. In PC cells, our results showed that DUSP1 can promote the malignant tumor progression by overexpression and knockout of DUSP1, suggesting DUSP1 may play as an oncogenic gene in PC.

The extracellular matrix (ECM) is a complex component that maintains tissue integrity through providing scaffolds while absorbing mechanical tension [20]. ECM acts as a reservoir and provides signaling cues for cell growth, proliferation, polarity, migration, and differentiation [21]. Changes in ECM composition may affect cell behavior during cancer progression [22]. NPNT is an ECM component and is associated with the metastases of breast cancer [8, 23], liver cancer [14], and gastric cancer [15]. NPNT was not only highly expressed in PC patients but was also significantly correlated with a poor prognosis. Since NPNT was upregulated by DUSP1, we speculated that NPNT may mediate the oncogenic function of DUSP1. Our results showed that knockdown of NPNT partially attenuated the proliferative, migratory, and invasive abilities of PC cells enhanced by DUSP1 overexpression.

NPNT expression is regulated by different signaling pathways [22]. In osteogenic differentiation, TGF-β1 stimulation leads to the downregulation of NPNT expression through phosphorylation of ERK1/2 and JNK [21, 24, 25]. NPNT expression is also inhibited by Oncostatin M in a dose-dependent manner via the JAK/STAT and MAPK pathways [26]. DUSP1 has an inhibitory effect on the phosphorylation of the ERK1/2, JNK, and p38 MAPK substrates [27]. Therefore, we speculate that DUSP1 may upregulate NPNT expression through the ERK/JNK signaling pathway.

Conclusions

In summary, our study revealed that DUSP1 is upregulated in PC tissues, and high DUSP1 expression is corelated with poor prognosis. DUSP1 promoted the proliferation, migration and invasion of PC cells by upregulating NPNT, suggesting that DUSP1 may be a potential target for PC treatment.

Research ethics: Not applicable.
Informed consent: Not applicable.
Author contributions: YC, YM and YT performed the experiments. YC and YM performed the statistics analysis. YC performed the bioinformatic analyses. YC and YJ reviewed the data. YC, YZ, YJ, and CM designed the research and drafted the manuscript.
Competing interests: The authors declare that they have no conflicts of interest to report regarding the present study.
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Data availability: The datasets analyzed for this study can be found as follows:TCGA-PAAD cohort https://gdc.xenahubs.net/download/GSE62452 cohort https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62452GSE71729 cohort https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE71729

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Supplementary Material: This article contains supplementary material (https://doi.org/10.1515/oncologie-2023-0215).