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Original Article

Dickkopf-1 induces pancreatic carcinogenesis through upregulation of c-Myc and cyclin D1

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Abstract: Dickkopf-1 (DKK-1) plays an important role in tumorigenesis. It has been previously demonstrated that DKK-1 is overexpressed in human pancreatic cancer tissues. This present study aimed to investigate the roles of DKK-1 in pancreatic carcinogenesis. Knockdown of DKK-1 by siRNA inhibited proliferation, migration, and apoptosis in PANC-1 human pancreatic cancer cells. Additionally, knockdown resulted in a decrease in mRNA and protein expression levels of DKK-1, c-Myc, and cyclin D1. Positive correlation between DKK-1, c-Myc and cyclin D1 expression was observed. Present data suggests that DKK-1, c-Myc and cyclin D1 promote pancreatic carcinogenesis.

Keywords: Pancreatic cancer, Dickkopf-1, growth, invasion

Introduction

Wnt signaling plays a critical role in cell proliferation, differentiation, and tumorigenesis. Wnt signaling is classified as either canonical (β-catenin-dependent) or non-canonical (β-catenin-independent) [1]. In canonical pathways [2], Wnt binds to members of the Frizzled (Fz) family of transmembrane cell surface receptors and low-density lipoprotein receptor-related proteins, 5 and 6 (LRP5/6), to form a ternary complex (Fz-LRP5/6). This complex triggers the activation of Wnt signaling and expression of Wnt target genes, including c-Myc, cyclin D1, and c-Jun [3]. Non-canonical Wnt signaling pathways promote planar cell polarity, activation of small GTPases, and kinases, including JNK and PKC, and induces calcium mobilization [4]. Wnt signaling is regulated by both intra- and extra-cellular factors, including members of the Dickkopf (DKK) proteins family [2]. DKK proteins are secreted Wnt/β-catenin antagonists. The DKK family consists of four isoforms (DKK-1 to 4) and DKK-3-related protein Dkkl1 (soggy) [5]. DKK-1 inhibits canonical Wnt signaling by disrupting Wnt-induced Fz-LRP6 complex formation, leading to developmental abnormalities and tumorigenesis [6, 7]. Several studies [8-12] have demonstrated that DKK-1 has a role in tumorigenesis. However, DKK-1 expression and function vary depending on the histological type of the tumor and tissue microenvironment.

Pancreatic cancer accounts for over 85% of pancreatic tumors, with a 5-year survival rate of less than 5% [13]. It is characterized by rapid growth and invasion, a high degree of malignancy, advanced-stage diagnosis, and poor prognosis [14]. Several studies have demonstrated that DKK-1 is overexpressed in pancreatic cancer tissues and is a diagnostic biomarker [15-17]. It can promote pancreatic cancer aggressiveness and tumor cell migration [17]. It has been previously demonstrated that DKK-1 expression was higher in pancreatic cancer tissues, compared to benign pancreatic lesions, suggesting that it may promote tumorigenesis [18]. This present study aimed to investigate the roles of DKK-1 in pancreatic carcinogenesis. This study analyzed the effects of DKK-1 knockdown by siRNA on DKK-1, c-Myc, and cyclin D1 expression, as well as cell growth in human PANC-1 pancreatic cancer cells.

Table 1. Oligonucleotide sequences

siRNA	Sequence (5' to 3')	
siDKK-1-1	Sense	GAUGGGUAUUCCAGAAGAATT
	Anti-sense	UUCUUCUGGAAUACCCAUCTT
siDKK-1-2	Sense	GCCGGAUACAGAAAGAUCATT
	Anti-sense	UGAUCUUUCUGUAUCCGGCTT
siDKK-1-3	Sense	GUACCAAGCAUAGGAGAAATT
	Anti-sense	UUUCUCCUAUGCUUGGUACTT
NC siRNA	Sense	UUCUCCGAACGUGUCACGUTT
	Anti-sense	ACGUGACACGUUCGGAGAATT
Fluorescently-labeled siRNA	Sense	UUCUCCGAACGUGUCACGUTT
	Anti-sense	ACGUGACACGUUCGGAGAATT

Table 2. Primer sequences

Gene	Primer sequence (5' to 3')	Amplified length (bp)
GAPDH	F: GTGAAGGTCGGAGTCAACG	300
	R: GGTGAAGACGCCAGTGGACTC	
DKK-1	F: CCAGCGTTGTTACTGTGGAG	128
	R: AGGAGTTCACTGCATTTGGA	
c-Myc	F: CACAGCAAACCTCCTCACAG	101
	R: GGATAGTCCTTCCGAGTGGA	
Cyclin D1	F: CATTGATTCAGCCTGTTTGG	103
	R: GAATTCATCGGAACCGAACT	
	R: CTCTCTGGGCTTGTTTCCTC	

Materials and methods

Cell culture and transfection

Human PANC-1 pancreatic cancer cells (CAS Shanghai Life Sciences Research Institute, China) were cultured in DMEM (HyClone, GE Healthcare, Chicago, IL, USA), supplemented with 12% fetal bovine serum (Hangzhou Evergreen, China) and penicillin/streptomycin. Cells were cultured in 6- or 96-well plates at 37°C in a humidified incubator with 5% CO_2. Once cells reached 30-50% confluence, the medium was replaced with serum- and antibiotic-free DMEM. Cells were cultured for an additional 4 hours prior to transfection. They were randomly divided into three groups: DKK-1 siRNA (transfected with siDKK-1-1, siDKK-1-2, or siDKK-1-3), NC (transfected with negative control siRNA), and blank (non-transfected) groups. Oligonucleotides were designed and synthesized by the Shanghai Jima Company (Shanghai, China). Sequences are shown in **Table 1**. Cell transfection was performed using LipofectamineTM^{2000} (Thermo Fisher Scientific Inc., Waltham, MA, USA). Transfection efficiency was compared using different ratios of siRNA to LipofectamineTM^{2000} (1:1, 1:1.5 and 1:2) using fluorescence microscopy. Relative levels of DKK-1 mRNA after transfection with indicated siRNAs were analyzed using RT-PCR. Proliferation and migration assays were performed after transfection of PANC-1 cells for 24 or 48 hours. This study analyzed mRNA and protein expression after 48 hours. Apoptosis was analyzed 48 hours after transfection. All experiments were repeated three times.

RT-PCR

Total RNA was extracted using TRIzol (Thermo Fisher Scientific Inc.) and the one-step method. Samples were digested with DNase I to degrade any DNA present in isolated RNA samples. RNA concentrations and purity were quantified using a spectrophotometer. RNA was used in experiments if the ratio of absorbance at 260 and 280 nm was between 1.8-2.0. This study reversely transcribed 2 μg of total RNA using a reverse transcription kit (Thermo Fisher Scientific Inc.), according to manufacturer protocol.

RT-PCR was performed with a PCR instrument (Dongsheng International Trade Company, China). PCR primers for DKK-1, c-Myc, cyclin D1, and GAPDH were designed based on published genomic sequences in GenBank and synthesized by the Nanjing GenScript Company (**Table 2**). Amplification conditions were as follows: denaturation at 95°C for 3 minutes, followed by amplification of the target DNA for 35 cycles (denaturation at 95°C for 30 seconds; annealing for 30 seconds at 59.9°C [DKK-1], 59.9°C [c-Myc], 58.7°C [cyclin D1], or 54.4°C [GAPDH]; extension at 72°C for 1 minute), and a final extension at 72°C for 10 minutes. The total reaction volume was 50 μL:5 μL 10 × PCR buffer, 3 μL $MgCl_2$, 1 μL 10 mM dNTPs, 1 μL forward primer, 1 μL reverse primer, 2.0 μL cDNA, and 37 μL double distilled water.

DNA products were subjected to agarose gel electrophoresis on a 2% agarose gel, contain-

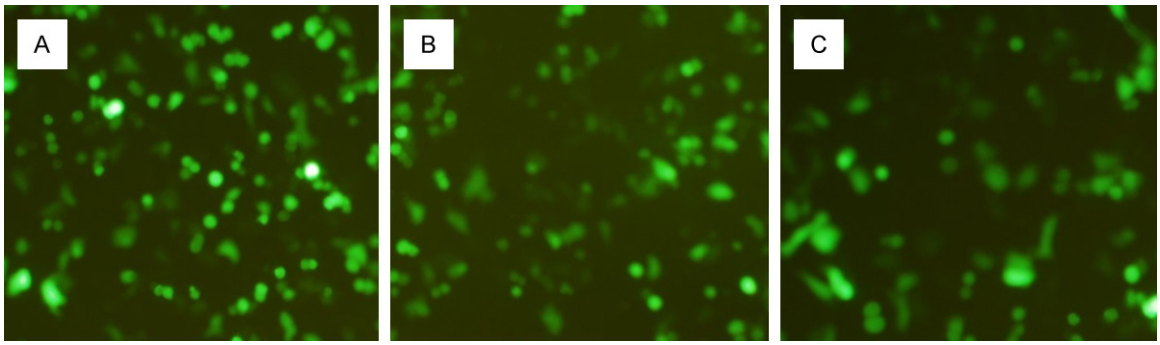

Figure 1. Analysis of transfection efficiency. Human PANC-1 pancreatic cancer cells were transfected with DDK-1-1 siRNA. Ratios of DDK-1-1 siRNA to LipofectamineTM^{2000} were as follows: (A) 1:1; (B) 1:1.5; (C) 1:2. Transfection efficiency was analyzed by fluorescence microscopy. Typical pictures are shown in a phase contrast mode. Original magnification, × 100.

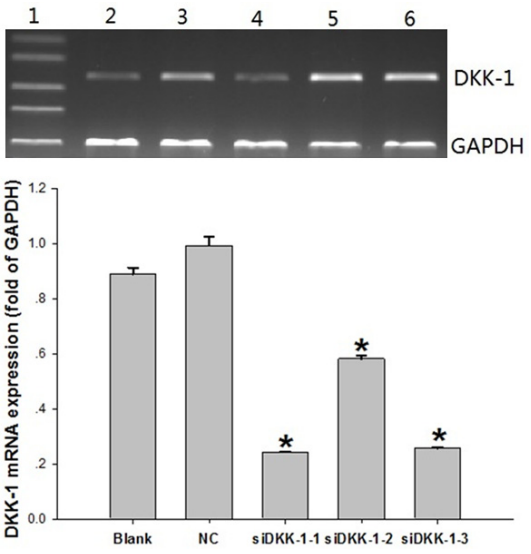

Figure 2. Effects of DKK-1 knockdown with siDKK-1-1, siDKK-1-2 and siDKK-1-3 on DKK-1 mRNA expression. PANC-1 cells were transfected with siDKK-1-1, siDKK-1-2 or siDKK-1-3 for 48 hours. Total RNA was extracted and DKK-1 mRNA expression analyzed by RT-PCR. Data are shown as mean ± SEM (n = 3). *, $P < 0.01$ *vs*. the NC group. Lane 1, marker; lane 2, siDKK-1-1; lane 3, siDKK-1-2; lane 4, siDKK-1-3; lane 5, NC; lane 6, blank.

ing 0.4 μg/mL ethidium bromide in Tris/Borate/EDTA buffer. DKK-1, c-Myc, and cyclin D1 levels were quantified by imaging the bands under UV light and quantifying band intensity in gray scale images. Target gene expression was normalized to that of GAPDH as an internal control.

MTT assays

Cells were transfected for 24 hours in 96-well plates. Following transfection, the medium was replaced with serum-containing medium. Next, 10 μL of 5 mg/mL MTT solution (Beyotime Biotechnology, Nantong, China) was added to the cells for 4 hours prior to the end of days 1, 2 and 3. Supernatant was removed and 100 μL of DMSO was added for 10 minutes in the dark to dissolve the formazan crystals. Optical densities at 490 nm were measured using a microplate reader. Each experiment was performed in triplicate.

Scratch-wound assays

Cells were transfected for 24 hours in 6-well plates. Following transfection, the medium was replaced with serum-containing medium. A sterile pipette tip was used to scratch a straight line (1 cm in length) down the middle of the cell monolayer. Cells were then incubated for 24 hours and imaged using an inverted microscope to analyze cell migration.

Flow cytometry

Cell culture media were discarded 48 hours after transfection. Cells were then washed twice with phosphate-buffered saline (PBS) and digested with 1 mL trypsin for 45 seconds. Serum-containing media were added to terminate the digestion. The cells were collected and centrifuged at 800 rpm for 4 minutes. Supernatant was discarded, then the cell pellets were washed twice and resuspended in PBS. Apoptosis was analyzed using the Annexin V-FITC Apoptosis Detection Kit (Beyotime Biotechnology). Briefly, 1 × 10^6 cells/mL in 100 μL were stained at 4°C for 30 minutes in the dark, washed three times with PBS, and fixed with 1% paraformaldehyde. Labeled cells were

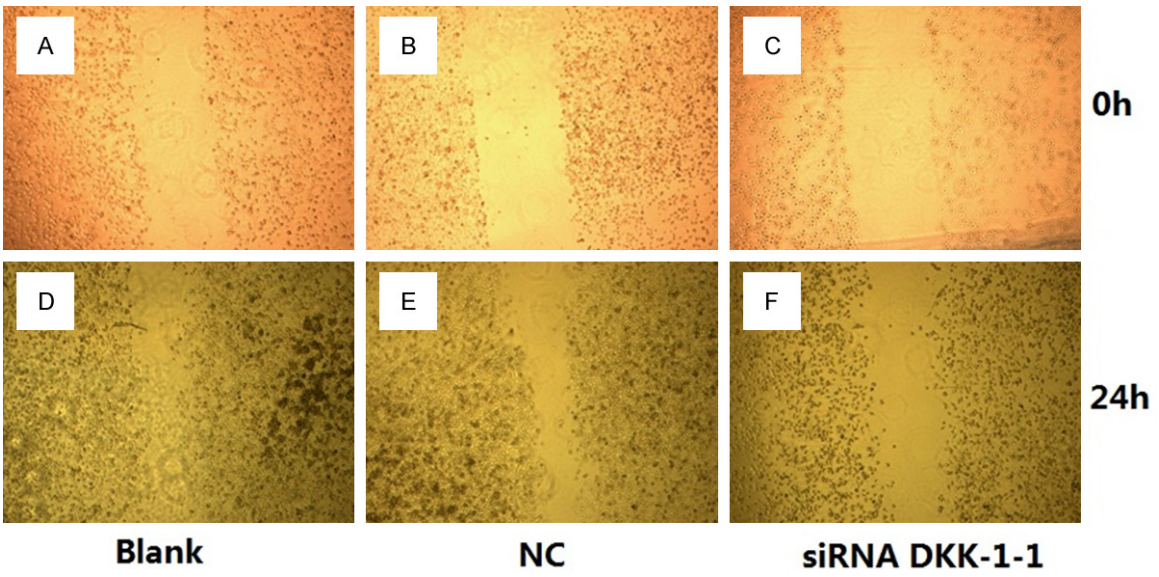

Figure 3. Effects of DKK-1 knockdown on cell migration. PANC-1 cells were transfected with siDKK-1-1 in 6-well plates for 24 hours. A sterile pipette tip was used to scratch-wound the cell monolayer in (A, D) blank group, (B, E) NC group and (C, F) siDKK-1-1 group. Cells were cultured for an additional 24 hours and then imaged using an inverted microscope. The migration ability of the cells was analyzed at 0 (A-C) and 24 hours (D-F). Original magnification, × 100.

analyzed by flow cytometry using a FACSCalibur instrument (Becton Dickinson, Germany) and CellQuest analysis software (BD Biosciences, Franklin Lakes, NJ, USA).

Western blotting

Cells were harvested 48 hours after transfection, as described above. Cells were resuspended in 150 μL of cell protein lysis buffer (RIPA buffer to PMSF ratio of 100:1). Total protein was quantified using Bradford assays and 80 μg was analyzed by western blotting with anti-DKK-1 (Cell Signaling Technology Inc, Danvers, MA, USA), anti-c-Myc (Beyotime Biotechnology), or anti-cyclin D1 (Beyotime Biotechnology) polyclonal antibodies. Immunoreactive bands were visualized using an enhanced chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ, USA), then exposed to x-ray film. Photographs were digitized and protein expression was normalized to β-actin. The magnitude of the immune signal was shown as a percentage of internal control.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed with SPSS software. Tukey's-b in one-way analysis of variance was used to assess differences in cell proliferation, mRNA, and protein levels. Correlation of DKK-1, c-Myc, and cyclin D1 gene expression was assessed using Pearson's correlation analysis. $P < 0.05$ indicates that differences are statistically significant.

Results

Transfection efficiency

This study varied the volume of Lipofectamine^{TM2000} to DKK-1 siRNA and analyzed the transfection efficiency of PANC-1 pancreatic cancer cells after 48 hours by imaging the cells using an inverted fluorescence microscope. Transfection efficiency was 70%, 30% and 50% with volume ratios of 1:1, 1:1.5, and 1:2, respectively. Thus, the optimal ratio was chosen as 1:1 (**Figure 1A-C**).

DKK-1 mRNA expression

DKK-1 mRNA expression was analyzed after knockdown with siDKK-1-1, siDKK-1-2, or siDKK-1-3 using RT-PCR. DKK-1 mRNA expression was lower in the siDKK-1 group, compared to the blank and negative control (NC) groups (**Figure 2**). It was found that siDKK-1-1 resulted

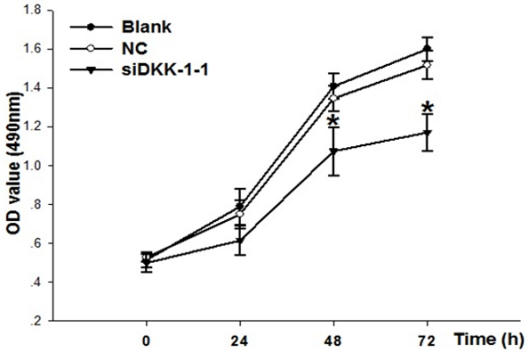

Figure 4. Effects of DKK-1 knockdown on cell proliferation. MTT assays were performed 24 hours after transfection of PANC-1 cells with siDKK-1-1. Proliferation capacity was compared between the blank, NC and siDKK-1-1 groups after 1, 2 and 3 days. Data are shown as mean ± SEM (n = 3). *, $P < 0.01$ *vs*. the NC group.

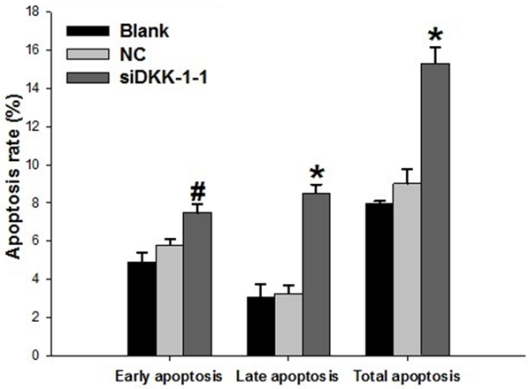

Figure 5. Effects of DKK-1 knockdown on apoptosis. Apoptosis was analyzed in the blank, NC and siDKK-1-1 groups 48 hours after transfection using flow cytometry. Living cells are observed in the lower left quadrant, cells with mechanical damage in the upper left quadrant, late apoptotic cells in the upper right quadrant, and early apoptotic cells in the right lower quadrant. Data are shown as mean ± SEM (n = 3). #, $P < 0.05$; *, $P < 0.01$ *vs*. the NC group.

in the greatest reduction in DKK-1 mRNA expression, compared to siDKK-1-2 or siDKK-1-3. Therefore, cells were transfected with siDKK-1-1 in all subsequent experiments.

DKK-1 knockdown reduces PANC-1 cell migration

Scratch-wound assay was employed to evaluate the effects of DKK-1 knockdown on PANC-1 cell migration. In all three groups, gradual migration of cells into cell-free areas was observed 1 day after scratching. However, the number of cells that migrated into the scratched area was lower in the siDKK-1-1 group, compared to the blank and NC groups (**Figure 3A-F**).

DKK-1 knockdown reduces PANC-1 cell proliferation

To investigate the effects of DKK-1 knockdown on PANC-1 cell proliferation, this study transfected PANC-1 cells with siDKK-1-1 and analyzed cell proliferation after 24, 48 and 72 hours. No differences in cell proliferation were observed between blank, NC, and siDKK-1-1 groups after 24 hours. However, cell proliferation was reduced in the siDKK-1-1 group, compared to the blank and NC groups after 48 and 72 hours (**Figure 4**).

Cell apoptosis

To study the influence of DKK-1 knockdown on PANC-1 cell apoptosis, this study analyzed early, late, and total apoptosis rates in PANC-1 cells in response to DKK-1 knockdown. Results showed that rates of early, late, and total apoptosis were higher in the siDKK-1-1 group, compared to the blank and NC groups (**Figure 5**).

Analysis of DKK-1, c-Myc and cyclin D1 mRNA expression

To investigate the effects of DKK-1 knockdown on expression of Wnt target genes, including c-Myc and cyclin D1, mRNA levels of DKK-1, c-Myc, and cyclin D1 were analyzed in PANC-1 cells 48 hours after transfection with siDKK-1-1 via RT-PCR. DKK-1, c-Myc, and cyclin D1 mRNA levels were lower in the siDKK-1-1 group, compared to the blank and NC groups (**Figure 6A**). Positive correlation was observed among DKK-1, c-Myc, and cyclin D1 expression in the siDKK-1-1 group (**Figure 6B**).

DKK-1, c-Myc and cyclin D1 protein expression

The effects of DKK-1 knockdown on protein expression of Wnt target genes was examined, including c-Myc and cyclin D1. Western blot analysis confirmed that levels of DKK-1, c-Myc, and cyclin D1 were lower in the siDKK-1-1 group, compared to blank and NC groups (**Figure 7A**). Positive correlation among DKK-1, c-Myc, and cyclin D1 protein ex-

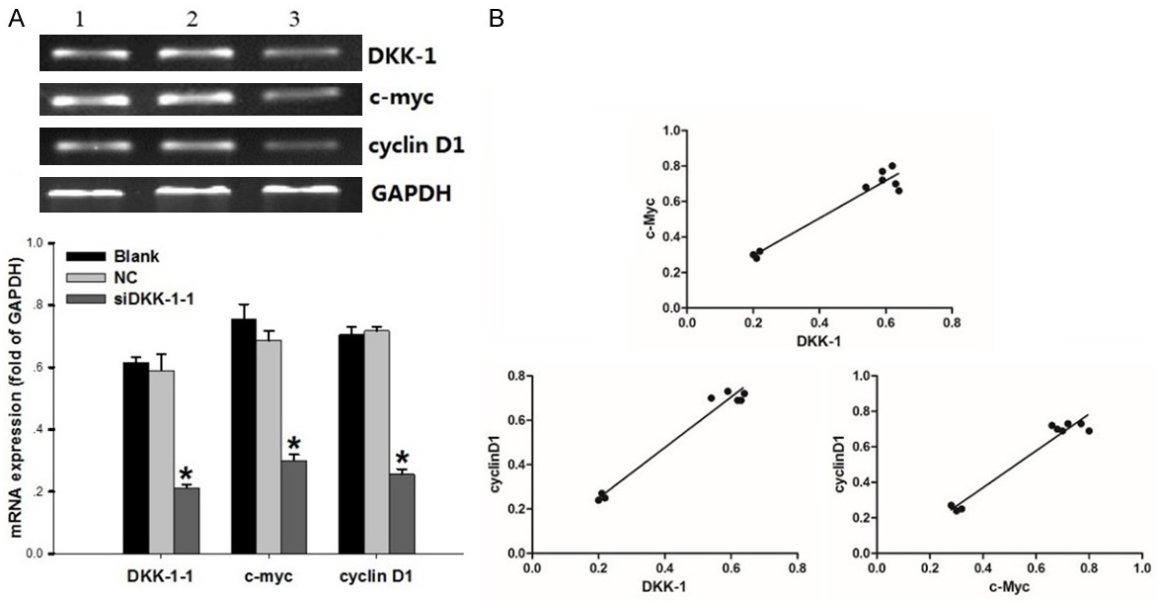

Figure 6. Effects of DKK-1 knockdown on DKK-1, c-Myc, and cyclin D1 mRNA expression. A. DKK-1, c-Myc, and cyclin D1 mRNA expression in the blank, NC, and siDKK-1-1 groups was analyzed 48 hours after transfection by RT-PCR. GAPDH was as the internal reference. Data are shown as mean ± SEM (n = 3). *, $P < 0.01$ *vs*. the NC group. Lane 1, blank; lane 2, NC; lane 3, siDKK-1-1. B. Analysis of the correlation between DKK-1, c-Myc and cyclin D1 expression using Pearson's correlation analysis.

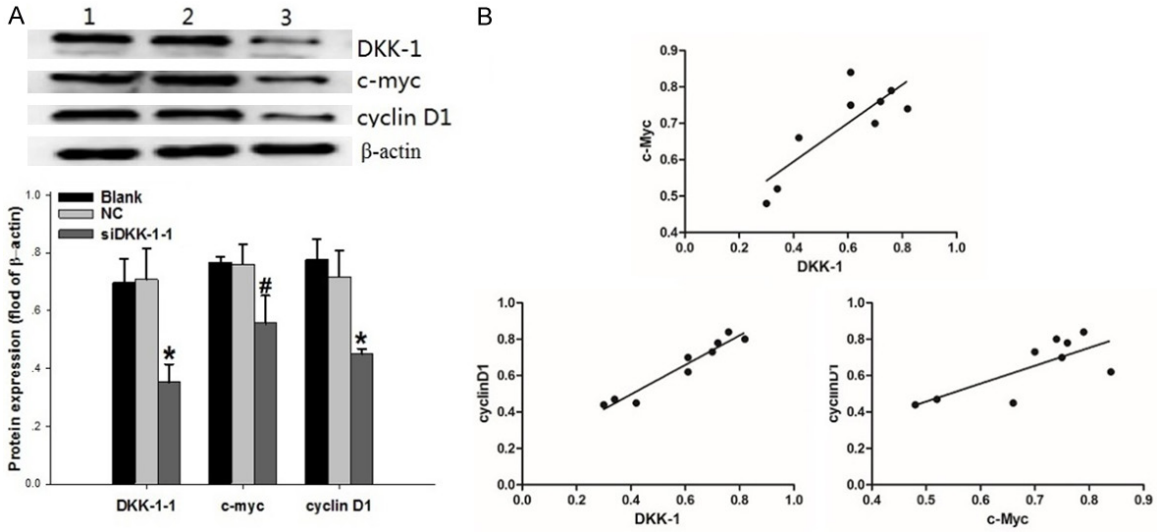

Figure 7. Effects of DKK-1 knockdown on DKK-1, c-Myc, and cyclin D1 protein levels. A. Western blot analysis of DKK-1, c-Myc, and cyclin D1 levels in the blank, NC, and siDKK-1-1 groups 48 hours after transfection. β-actin as the internal control. Data are shown as mean ± SEM (n = 3). #, $P < 0.05$; *, $P < 0.01$ *vs*. the NC group. Lane 1, blank; lane 2, NC; lane 3, siDKK-1-1. B. Analysis of the correlation between DKK-1, c-Myc and cyclin D1 expression using Pearson's correlation analysis.

pression was found in the siDKK-1-1 group (**Figure 7B**).

Discussion

The present study found that knockdown of DKK-1 in pancreatic cancer cells by siRNA inhibited proliferation and migration, resulting in a decrease in DKK-1, c-Myc and cyclin D1 mRNA and protein levels.

There were 48,960 new pancreatic cancer cases and 40,560 deaths in the United States in 2015 [5]. Although numerous studies have

investigated the etiology of pancreatic cancer, there has been limited progress in treatment of the disease, partly due to the location of the tumor within the abdominal cavity and difficultly in diagnosing the disease at an early stage. Several studies have demonstrated that DKK-1 is overexpressed and is a diagnostic biomarker of pancreatic cancer [15-17]. This study also demonstrated elevated DKK-1 expression in pancreatic cancer tissues, hypothesizing that DKK-1 could play a role in tumorigenesis [18].

Multiple roles of DKK-1 in tumorigenesis have been described, indicating there may be differences in DKK-1 activity depending on tumor histological type and native tissue microenvironment [8-12]. High DKK-1 expression has been associated with enhanced tumor cell migration and invasion in non-small cell lung and esophageal cancer [17, 19, 20]. The present study demonstrated that DKK1 knockdown reduced pancreatic cancer cell proliferation and migration.

Activation of canonical Wnt/β-catenin signaling promotes expression of Wnt target genes, including c-Myc and cyclin D1 [2, 3]. The c-Myc proto-oncogene promotes cell proliferation, differentiation, and tumorigenesis. Azmi et al. demonstrated high c-Myc expression in BxPC-3 and Colo-357 pancreatic cancer cells. They also showed that siRNA knockdown of c-Myc inhibited tumor growth [21]. Cyclin D1 is overexpressed in many tumors, including liver, lung, breast, and colon cancer [22-25]. Overexpression of cyclin D1 in pancreatic cancer cells has been correlated with reduced survival [26, 27]. DKK-1 inhibits canonical Wnt signaling to promote tumor progression [6, 7]. The present study found that DKK-1 knockdown reduced pancreatic cancer cell proliferation and migration by reducing c-Myc and cyclin D1 expression. Thus, DKK-1 may promote pancreatic carcinogenesis by inducing c-Myc and cyclin D1 expression. Further studies are necessary to investigate the regulatory function of DKK-1 on c-Myc and cyclin D1 genes in a mice model.

Acknowledgements

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Disclosure of conflict of interest

None.

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