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

Qiang-Wu Wang¹, Qi-Zhi Wang¹, Ke Zhu², Shan-Jun Yan¹, Hai-Lun Zheng¹, Jun-Chang Guan³

¹Department of Gastroenterology, The First Affiliated Hospital of Bengbu Medical College, Bengbu 233030, Anhui, PR China; ²Department of Gastroenterology, The Second People’s Hospital of Fuyang City, Fuyang 236015, Anhui, PR China; ³Anhui Key Laboratory of Infection and Immunity, Bengbu Medical College, Bengbu 233030, Anhui, PR China

Received May 22, 2018; Accepted August 1, 2018; Epub November 15, 2018; Published November 30, 2018

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 Dkk1 (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.
DKK-1 induces pancreatic carcinogenesis

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% CO₂. 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 Lipofectamine™2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). Transfection efficiency was compared using different ratios of siRNA to Lipofectamine™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 at 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 MgCl₂, 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-
DKK-1 induces pancreatic carcinogenesis

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 Lipofectamine™ 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.

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
DKK-1 induces pancreatic carcinogenesis

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™ 2000 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.
DKK-1 induces pancreatic carcinogenesis

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 mRNA expression (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-
DKK-1 induces pancreatic carcinogenesis

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 difficulty 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 mouse model.

Acknowledgements

This work was supported by grants from the General Scientific Research Foundation of the Higher Education Institutions of Anhui Province (KJ2012Z255) and the Scientific Research Foundation of Bengbu Medical College (BY0715). We would like to thank Dr. Hu JG (Anhui Key Laboratory of Tissue Transplantation, the First Affiliated Hospital of Bengbu Medical College) for his assistance with the study.

Disclosure of conflict of interest

None.

Address correspondence to: Qiang-Wu Wang, Department of Gastroenterology, The First Affiliated Hospital of Bengbu Medical College, Bengbu 233030, Anhui, PR China. E-mail: wangqiangwuyx@163.com; Jun-Chang Guan, Anhui Key Laboratory of Infection and Immunity, Bengbu Medical College, Bengbu 233030, Anhui, PR China. E-mail: guanjc2013@126.com

References

DKK-1 induces pancreatic carcinogenesis


