Overexpression of p16^{ink4a} regulates the Wnt/β-catenin signaling pathway in pancreatic cancer cells

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Abstract. The pathogenesis and etiology of pancreatic cancer remain to be fully elucidated; therefore, associated investigations are required to improve the outcome and prognosis of patients. In the present study, the effects of the overexpression of p16^{ink4a} on the Wnt/β-catenin signaling pathway were investigated in pancreatic cancer cell lines. Two pancreatic cancer cell lines, Bxpc-3 and Miapaca-2, characterized by low expression of p16^{ink4a}, were transfected with the pc-DNA3.0-p16^{ink4a} plasmid. After 24 h, Reverse transcription-polymerase chain reaction and western blot analyses were performed to evaluate the expression of p16^{ink4a}, β-catenin, which is a key molecule in the Wnt/β-catenin signaling pathway, c-myc and cyclin D1, which are molecules downstream of β-catenin. The expression of p16^{ink4a} was significantly upregulated in the transfected cells. Consequently, the expression of β-catenin was inhibited, whereas the expression levels of c-myc and cyclin D1 were not altered significantly. The increased expression of p16^{ink4a} may affect the activity of Wnt/β-catenin signaling through modulation of the expression of β-catenin. The results of the present study provide information for the future development of targeted treatments for pancreatic cancer.

Introduction

Pancreatic cancer is associated with high rates of morbidity worldwide. Data from the American Cancer Society indicate that, in 2016, 53,070 patients were diagnosed with pancreatic cancer, and 41,780 cases were predicted to succumb to this malignancy in the United States (1). In China, the figures are higher, as statistical data from 2015 showed that 90,100 individuals suffered from pancreatic cancer, and the associated rate of mortality was as high as 79,400 (2). The accepted therapeutic strategy for pancreatic cancer is combined treatment including surgery; however, the five-year survival rate remains poor, which may be attributed to the fact that the molecular mechanisms underlying the development of pancreatic cancer remain to be fully elucidated (1,2). The entire process of cancer development follows a complex etiology, including the abnormal activation of oncogenes and inactivation of tumor suppressor genes (2,3). The subsequent dysregulation of signaling pathways in a closed network of abnormally expressed genes, which indicates that gene-based prevention and treatment may be effective in pancreatic cancer (4,5).

P16^{ink4a}, also known as cyclin-dependent kinase inhibitor 2a, is usually considered a tumor suppressor gene. The encoded P16 protein can bind and inhibit cyclin-dependent kinase 4 with high specificity to modulate the cell cycle, which can inhibit the G1/S transition and result in tumor-suppressing activity (6). Mutation and methylation of the p16 gene are important in the development of tumors, which also show abnormal protein expression of P16, the clinical staging of pancreatic cancer, and the patient's lifespan (7). The inactivation and decreased expression of anti-oncogenes, including p16^{ink4a} and small mothers against decapentaplegic 4, and the abnormal activation of oncogenes, including K-ras, have been reported in pancreatic ductal adenocarcinoma and are also associated with the degree of malignancy (7).

The Wnt/β-catenin signaling pathway is essential in cell differentiation and the maintenance of cellular homeostasis, and decreased activity of this pathway may contribute to the processes of tumor formation and inflammation (8,9). Wnt signaling is highly conserved across a wide range of species from nematode to humans, and the name for this pathway originated from the genes wingless and int-1 in *Drosophila*. The core molecule in this pathway is β-catenin, encoded by CTNNB1, which can interact with E-cadherin at the cell membrane or shuttle between the cytoplasm and the nucleus. The Wnt/β-catenin signaling pathway is closely associated with the development of malignant tumors (10,11). A previous study showed that vitamin A was able to inhibit the activation of the Wnt/β-catenin signaling pathway through astrocytes in pancreatic cancer tissues, and this finding was attributed to the increased expression of extracellularsecreted frizzled-related protein 4 and the subsequent decrease of β-catenin in the

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nucleus (12). Another study found that the survival of melanoma cells was largely dependent on the high concentration of intracellular β-catenin. β-catenin can function as a regulator of certain genes associated with cell differentiation and proliferation, including cyclin D1 and c-myc. The knockout of β-catenin causes a sharp decrease in the expression of p16^{ink4a}, which may be associated with lymphoid enhancer factor (LEF)/T cell factor (Tcf), which serve as effectors of Wnt-β-catenin in the nucleus. β-catenin can also inhibit transcription as a translation-activating factor (13,14). The knockout of low-density lipoprotein receptor-related protein 5 (Lrp5) results in increased mRNA levels of p16^{ink4a} in the breast ductal epithelium and basal cells (15).

In the present study, p16^{ink4a} was introduced into pancreatic cancer cells using a pc-DNA3.0 plasmid as the vector. The effect of the overexpression of p16^{ink4a} on the Wnt/β-catenin signaling pathway was investigated. The results may provide evidence to support further investigations into gene therapy targeting p16^{ink4a}.

Materials and methods

Cell lines. The Bxpc-3 and Miapaca-2 human pancreatic cancer cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China).

Cell culture and treatment. The Bxpc-3 and Miapaca-2 cells were cultured in 10% FCS-containing Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The density of the cells was adjusted to 5x10^5/ml, and the culture conditions consisted of 5% CO_2 at 37˚C. Cells in the logarithmic growth phase were harvested and then inoculated onto a 6-well plate, and grown until adherence.

Pc-DNA3.0-p16^{ink4a} plasmid transfection. The Pc-DNA3.0-p16^{ink4a} plasmid (Invitrogen; Thermo Fisher Scientific, Inc.) was introduced into and replicated in *E. coli*, followed by molecular identification and adjustment of DNA concentration. The cells were seeded onto 6-well plates at a density of 1x10^6/cm^2 and incubated for 24 h. X-tremeGENE HP DNA transfection reagent (Roche Diagnostics, Basel, Switzerland), plasmid DNA was diluted in ddH_2O and incubated for 15 min at room temperature. Based on previous experiments, the X-tremeGENE HP DNA transfection reagent:pc-DNA3.0-p16^{ink4a} (1 μg/100 μl) ratio of 3:1 (X:P=3:1) was used as the concentration for transfection.

In terms of treatment groups, mock-treated specimens were considered as the control group. The specimens treated with an equal dose of pc-DNA3.0-p16^{ink4a} without transfection reagent were considered the plasmid group. The samples treated with X-tremeGENE HP without the plasmid were considered the X-tremeGENE HP group. The samples transfected with the X:P=3:1 mix were considered the X:P=3:1 group. Following incubation for 24 h post-transfection, the cells were harvested. The transfection procedure was performed based on the manufacturer's protocol of the X-tremeGENE HP DNA transfection reagent and completed on a clean bench top.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. RT-qPCR analysis was performed to evaluate the mRNA levels of p16^{ink4a}, β-catenin, c-myc and cyclin D1. The total RNA was extracted using MiniBEST Universal RNA Extraction kit (Takara Bio, Inc., Otsu, Japan). The integrity of the RNA was determined, following which the RT reaction was performed in accordance with the manufacturer's protocol (Primescript™ RT reagent kit; Takara Bio, Inc.). β-actin was used as the internal control. The cDNA was stored at -2˚C. Each 25-μl reaction sample contained 12.5 μl of SYBR® Premix Ex Taq II (Takara Bio, Inc.), 1 μl of forward primer, 1 μl of reverse primer, 1.0 μl of ROX Reference, 1 μl of cDNA and 6.0 μl of ddH_2O. The amplification conditions of RT-qPCR were set as follows: 95˚C for 30 sec, 95˚C for 5 sec, 60˚C for 34 sec, 95˚C for 15 sec and a total of 40 cycles. The primer sequences and annealing temperatures are summarized in Table I. The PCR products were analyzed by ABI Prism 7700 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.) (16). Relative expression levels of mRNA were normalized to β-actin expression in each sample, and the data were analyzed according to the comparative threshold cycle ($2^{-\Delta\Delta Cq}$) method (17). All experiments were done in triplicate.

Western blot analysis. The protein levels of β-catenin, c-myc and cyclin D1 in the p16^{ink4a}-transfected cells were determined using western blot analysis. RIPA buffer (1% Nonidet P-40), consisting of 50 mM Tris, 150 mM NaCl, 0.25% deoxycholate, 1 mM EGTA and 1 mM NaF, was precooled prior to use. The sample, with 1 μl of protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), was centrifuged at 1,000 x g at 4˚C for 10 min following incubation on ice for 20 min, and the pellet was harvested.

Protein samples (50 μg/lane) were separated by electrophoresis on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membrane filters (EMD Millipore, Billerica, MA, USA) in a wet transfer system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The PVDF membranes were placed in methanol for 1 min and then incubated in TBS at room temperature for 15 min. The membranes were blocked with 2% BSA for 1 h at room temperature, and then incubated with anti-beta Catenin antibody (cat. no. ab32572; 1:1,000; Abcam, Cambridge, UK), Rabbit monoclonal anti-c-Myc antibody (cat. no. ab32072; 1:1,000; Abcam), Mouse monoclonal anti-cyclin D1 antibody and mouse monoclonal anti-β actin antibody (cat. no. ab6276; 1:1,000; Abcam) at 4˚C overnight. Then the membranes were incubated by horseradish peroxidase-conjugated goat anti-rabbit (cat. no. ab6721; 1:5,000; Abcam) or goat polyclonal secondary antibody to mouse (cat. no. ab6789; 1:5,000; Abcam) for 1 h at room temperature. The relative protein expression of the membranes was then normalized to the β-actin levels. The samples were developed using an enriched chemiluminescence system and then visualized (Hyperfilm ECL; GE Healthcare Life Sciences, Chalfont, England). The densities of the immunoreactive bands were quantified using a gel imaging system (VersaDoc4000; Bio-Rad Laboratories, Inc.). The mean values were obtained from three independent experiments.

Statistical analysis. Measurement data are presented as the mean ± standard deviation and were processed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Student's t-test (paired) was

Table I. Primer sequences of genes.

Primer	Sequence	Annealing temperature (°C)
$p16^{ink4a}$ CDKN2a	Forward 5'-CAGACATCCCCGATTGAAAGAAC-3' Reverse 5'-GGTAGTGGGGGAAGGCATATATCT-3'	56
β-catenin	Forward 5'-GAGTGCTGAAGGTGCTATCTGTCTG-3' Reverse 5'-GTTCTGAACAAGACGTTGACTTGGA-3'	56
C-myc	Forward 5'-CCTGGTGCTCCATGAGGAGA-3' Reverse 5'-CTCCAGCAGAAGGTGATCCAGA-3'	56
Cyclin D1	Forward 5'-ATGTTCGTGGCCTCTAAGATGA-3' Reverse 5'-CAGGTTCCACTTGAGCTTGTTC-3'	57
β-actin	Forward 5'-TGGCACCCAGCACAATGAA-3' Reverse 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'	56

used for comparison between groups and one-way analysis of variance was used for comparison among groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

mRNA expression of $p16^{ink4a}$. The expression of $p16^{ink4a}$ in the X:P=3:1 group was significantly higher, compared with that in the control, plasmid and pc-DNA3.0-$p16^{ink4a}$ groups. The lack of intergroup differences between these three groups ($P>0.05$) confirmed successful transfection (Fig. 1).

mRNA expression of key molecules in the Wnt signaling pathway. In the cells transfected with $p16^{ink4a}$, the expression of β-catenin, a key molecule in the Wnt/β-catenin signaling pathway, was significantly inhibited ($P<0.05$), whereas no significant changes were observed in the mRNA expression levels of c-myc or cyclin D1 ($P>0.05$; Fig. 2A and B).

Protein expression of β-catenin, c-myc and cyclin D1. The protein expression levels of β-catenin, a key molecule in the Wnt signaling pathway, were measured in the pcDNA3.0-$p16^{ink4a}$-transfected Bxpc-3 and Miapaca-2 cell lines. The protein level of c-myc was decreased in the Miapaca-2 cells ($P<0.05$), however, the level was not altered significantly in the Bxpc-3 cells ($P>0.05$). No significant alteration in the protein level of cyclin D1 was detected in either cell line ($P>0.05$; Fig. 3).

Discussion

$P16^{ink4a}$ is a key modulator of the cell cycle and cell aging, although the correlation between the $P16^{ink4a}$ protein and the Wnt/β-catenin signaling pathway in pancreatic cancer remains to be fully elucidated. In the present study, the overexpression of $p16^{ink4a}$ inhibited the expression of β-catenin, c-myc and cyclinD, which are key molecules in the Wnt/β-catenin signaling pathway.

$P16^{ink4a}$, first identified in 1994, is a significant tumor suppressor gene, and is involved in cell cycle modulation, including the inhibition of cell proliferation and division (18). Therefore, this molecule is also referred to as a 'slow brake' of the cell cycle (19,20). The p16 gene also modulates the oxidative stress reaction (21). P16 gene mutation and inactivation may occur during the early phases of tumor development, which may inhibit cancer progression. In several types of malignancy, abnormal activation of the Wnt/β-catenin signaling pathway can result in the accumulation of intracellular β-catenin and its subsequent entry into the nucleus through transmembrane channels. In the nucleus, β-catenin binds to the transcriptional TCF/LEF, which contributes to the increased expression of downstream target genes or the silencing of genes (22-24); the most commonly affected genes are cyclin D1 and c-myc (24). Certain medications, including ginsenoside, ginseng extract Rg1 and vitamin E, can downregulate Wnt/β-catenin signaling, restrict the oxidative stress response, and reduce the activity of the $p16^{Ink4a}$-Rb and p53-p21Cip1/Waf1 pathways, leading to the amelioration of DNA damage and aging (25). These effects indicate a potential positive correlation between $p16^{ink4a}$ and Wnt/β-catenin. In the present study, the overexpression of $p16^{ink4a}$ in pancreatic cancer cells significantly inhibited the expression of β-catenin. Of note, no significant changes in the levels of cyclin D1 and c-myc were observed. Few studies have focused on the association between $p16^{Ink4a}$ and the Wnt/β-catenin signaling pathway. The abnormal activation of Wnt/β-catenin and the associated inhibition of malignant melanoma may be the primary mechanism of cell immortalization in malignant melanoma. In addition, the knockout of the Lrp5 gene was reported to result in a significant increase in the expression of $p16^{Ink4a}$ in breast ductal epithelium and basal cells (15). Compared with previous studies on the effects of the Wnt/β-catenin signaling pathway on $p16^{Ink4a}$, the present study focused on the effects of $p16^{Ink4a}$ on the Wnt/β-catenin signaling pathway. The results showed that the overexpression of $p16^{ink4a}$ in pancreatic cancer cells significantly inhibited the expression of β-catenin but did not affect the levels of cyclin D1 or c-myc.

With activation of the K-ras gene, the expression of cyclin D1 is increased, whereas the expression of $p16^{ink4a}$ is decreased. However, whether changes in the expression of $p16^{ink4a}$ are early events or are the result of pancreatic cancer progression remain to be elucidated (26). The expression of cyclin D1 and E2F1 has been reported to be regulated by intracellular $p16^{ink4a}$ in cells from humans and rats (27). In EH2 cells treated with actinomycin D-IPTG, the protein level of P16 was significantly increased, leading to a two-fold

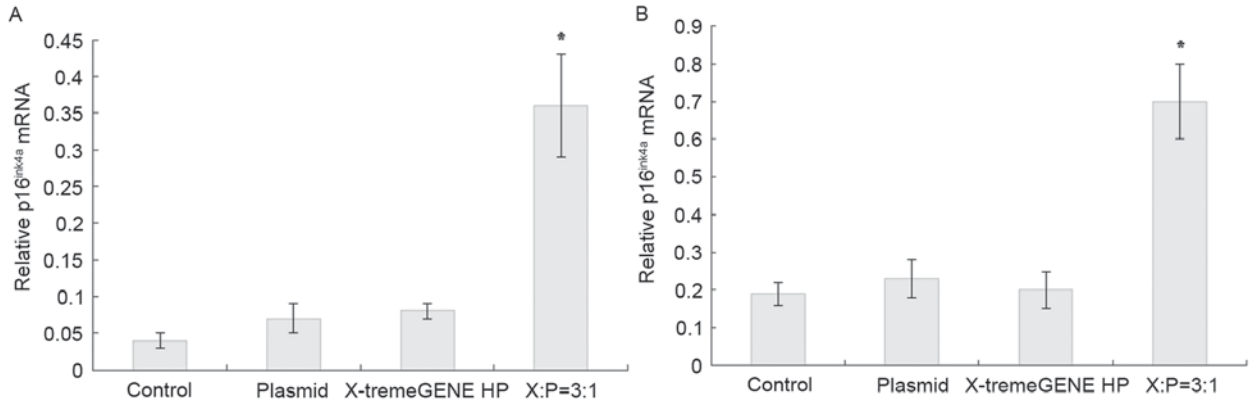

Figure 1. Expression of p16^{ink4a} in plasmid-transfected Bxpc-3 and Miapaca-2 cells based on the results of reverse transcription-quantitative polymerase chain reaction analysis. (A) Miapaca-2 cell line; (B) Bxpc-3 cell line. *P<0.05, vs. other groups. X:P, X-tremeGENE HP DNA transfection reagent:plasmid (pc-DNA3.0-p16^{ink4a}).

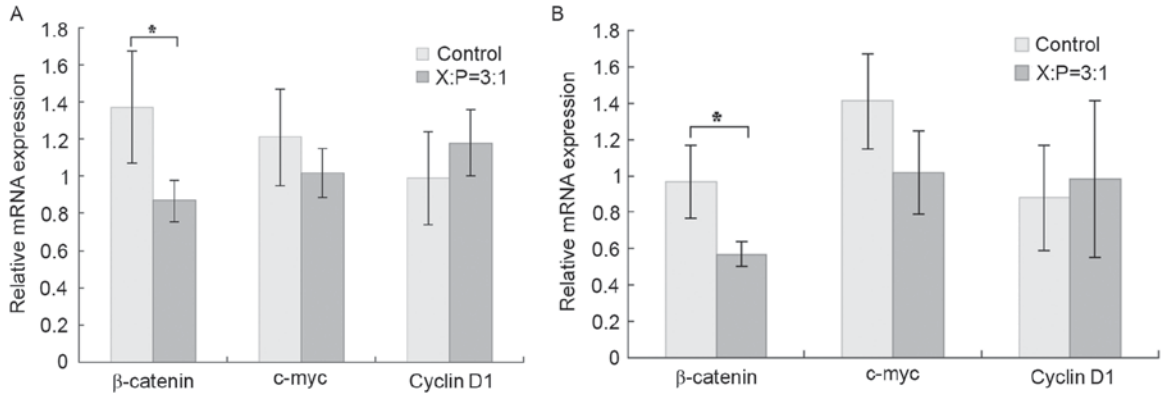

Figure 2. Evaluation of the mRNA expression levels of β-catenin, c-myc and cyclin D1 through reverse transcription-quantitative polymerase chain reaction analysis in p16^{ink4a}-transfected cells. (A) Miapaca-2 cell line; (B) Bxpc-3 cell line. *P<0.05, vs. control. X:P, X-tremeGENE HP DNA transfection reagent:plasmid (pc-DNA3.0-p16^{ink4a}).

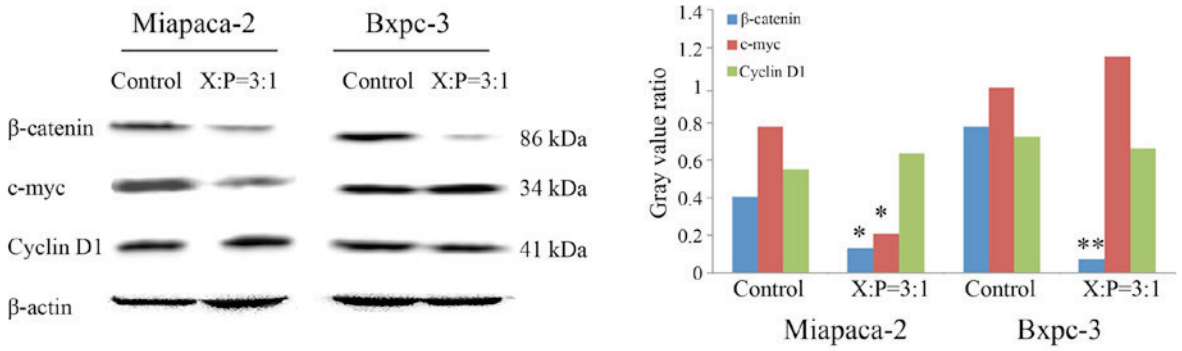

Figure 3. Protein levels of β-catenin, c-myc and cyclin D1. Protein expression was determined using western blot analysis. *P<0.05, vs. control; **P<0.01, vs. control. X:P, X-tremeGENE HP DNA transfection reagent:plasmid (pc-DNA3.0-p16^{ink4a}).

increase in the protein expression of cyclin D1 and E2F1, compared with that in the mock-treatment group; this result indicated the synergistic effect of p16 and cyclin D1 (27). As a downstream effector molecule of the Wnt/β-catenin signaling pathway, the activity of c-myc differs from that of p16. However, previous studies have found that the overexpression of c-myc induced cell aging, suggesting a potential mechanism for c-myc-associated DNA damage and increased reactive oxygen species (26,28). In the present study, the increase in the expression of p16^{ink4a} significantly inhibited the expression of β-catenin, a key molecule in the Wnt/β-catenin signaling pathway, whereas no significant changes were observed in the expression of cyclin D1 or c-myc in the pancreatic cancer cell lines examined.

In terms of the limitations of the present study, the results were derived only from *in vitro* experiments, and the external environment may have introduced bias into the results. In the development of pancreatic cancer, the abnormal expression of

genes is an early event accompanied with a series of changes, including the increased expression of oncogenes and decreased expression of anti-oncogenes. The results of the present study showed that high levels of $p16^{ink4a}$ significantly inhibited the expression of β-catenin but did not affect the expression of cyclin D1 or c-myc. The mechanism responsible for these results may involve the positive regulation of other compensatory pathways, and further investigations are required with a focus on Rb, a downstream molecule of $p16^{ink4a}$.

In conclusion, the present study demonstrated that the overexpression of $p16^{ink4a}$ inhibited the expression of β-catenin but did not affect the expression of downstream proteins c-myc and cyclin D1. The interrelation between $p16^{ink4a}$, c-myc and cyclin D1 suggests a dual-direction regulatory mechanism involving multiple pathways.

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