Curcumin induces apoptosis in pancreatic cancer cells through the induction of forkhead box O1 and inhibition of the PI3K/Akt pathway

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Abstract. Previous population investigations have suggested that the application of curcumin may be associated with decreased incidence and improved prognosis in certain types of cancer. Forkhead box O1 (FOXO1) has been implicated in the regulation of several biological processes, including stress resistance, metabolism, DNA repair, cell cycle and apoptosis. The aims of the present study were to investigate the effects and molecular mechanisms of curcumin on the induction of anti-proliferation, cell cycle arrest and apoptosis, by FOXO1, in pancreatic cancer cells. The MTT assay and ELISA-Brdu assay were used to assess cell proliferation. Reverse transcription-quantitative polymerase chain reaction and western blot analyses were used to detect the expression of PCNA, Ki-67, B-cell lymphoma-2 (Bcl-2), B-cell-associated X protein (Bax), cyclin D1, p21, p27 and FOXO1. Cell apoptosis was detected using a Cell Death ELISA detection kit. A Caspase-3/9 Fluorescent Assay kit was used to detect caspase activity. The findings revealed that curcumin significantly decreased cell proliferation, which was associated with increased expression of the p21(CIP1) and p27(KIP1) cyclin-dependent kinase inhibitors, and inhibited expression of cyclin D1. In addition, curcumin induced apoptosis by decreasing the Bcl-2/Bax protein ratio and increasing caspase-9/3 activation in the pancreatic cancer cells. Using siRNA against FOXO1, and Akt inhibitor and activator, the present study confirmed that curcumin induced the expression of FOXO1 by inhibition of phosphoinositide 3-kinase/Akt signaling, leading to cell cycle arrest and apoptosis. In conclusion, these findings offer support for a mechanism that may underlie the anti-neoplastic effects of curcumin and justify further investigation to examine the potential roles for activators of FOXO1 in the prevention and treatment of pancreatic cancer.

Introduction

Pancreatic cancer remains a life threatening disease with a 5-year survival rate of <5% in the United States (1) and one of the worst prognoses among all types of cancer (2). Chemotherapy regimens are not always able to improve the final outcome of patients with pancreatic cancer, as only 20% of patients with pancreatic cancer are suitable for surgical resection, which remains the only possibly curative treatment at present (3). Several cases of pancreatic cancer are not resectable at the point of diagnosis. There are limited therapeutic options available for this disease, as chemotherapy and radiotherapy are mostly ineffective and metastatic disease often relapses even if patients received surgery (2,4). The low survival rate of patients with pancreatic cancer indicates an increasing requirement for improvements in early detection, novel therapeutic methods and chemoprevention strategies.

The phosphoinositide 3-kinase (PI3K)/Akt pathway is an important survival pathway, which may mediate resistance to the apoptotic effects of chemotherapeutic drugs and radiation treatment in a variety of types of cancer, including pancreatic cancer (5). A previous study indicated that 59% of pancreatic adenocarcinomas examined exhibited hyperactivation of Akt (6). Forkhead box protein O1 (FOXO1), a member of the FOXO family of transcription factors, is characterized by a conserved forkhead domain containing a DNA binding domain (7), which functions downstream of the PI3K/Akt signaling pathway, and is also an important regulator of cell death and promotor of cell survival and resistance (8). FOXO1 has been considered as an important regulator of numerous biological events, including cell differentiation, proliferation, stress responses and DNA damage repair (9-11). FOXO1 can lead to cell cycle arrest through increases in the expression levels of p21 and p27, which are cyclin-dependent kinase inhibitors (CKIs), and a decrease in the expression of cyclin D1, a cell cycle regulator (12). Accordingly, FOXO1 has been regarded as a key tumor inhibitor. Increasing evidence has
revealed that FOXO1 may suppress cell proliferation in several types of tumor cells, including pancreatic cancer cells (13), and the regulation of FOXO1 by the PI3K/Akt pathway is receiving more attention in cancer investigations.

Curcumin is the predominant active flavonoid derived from the rhizome of Curcuma longa (Jianghuang) (14). Curcumin has been used to treat cardiovascular disease, arthritis and inflammation. Previous studies have also demonstrated that curcumin suppresses cell proliferation and survival in colon cancer, gastric cancer, prostate cancer, breast cancer, melanoma, leukemia and lymphoma (15). The inhibition of cell growth and induction of apoptosis are the common mechanisms by which curcumin exhibits its anticancer effects (16). However, the intracellular mechanisms by which curcumin suppresses growth and increases apoptosis in pancreatic cancer cells through the regulation of FOXO1 has not been investigated previously. The aims of the present study were to investigate the molecular mechanisms by which FOXO1 induces cell cycle arrest and apoptosis, and enhances the anti-proliferative effects of curcumin in pancreatic cancer cells.

Materials and methods

Reagents. Dimethylsulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco Lige Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Gibco Life Technologies (New York, USA). The bicinechonic acid assay (BCA) Protein Assay kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Fumonisin B1 was purchased from Sigma-Aldrich. Akt inhibitor IV was purchased from Merck Millipore (Darmstadt, Germany).

Cell culture and treatment. The Panc-1 human pancreatic carcinoma cell lines was obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 100 U/ml penicillin, 100 U/ml streptomycin and 10% FBS at 37°C in 5% CO₂ on 0.1% gelatin-coated culture flasks. The cells were treated with vehicle (DMSO) or curcumin (5, 10, 20, 40 or 20 µM) for 30 min, or 12, 24 or 36 h. This cell culture condition was used in the subsequent analyses.

Cell proliferation assay. The cells were plated at 1x10⁴ cells/well in 96-well culture plates. Following incubation with vehicle or curcumin in medium for 24 h, the number of viable cells was determined using MTT reagent, according to the manufacturer's instructions. In brief, MTT reagent (10 µl) was added to the 100 µl medium and incubated at 37°C for 4 h. The supernatant was removed and DMSO (150 µl) was added to solubilize the formazan crystals. The absorbance (570 nm) of the medium was measured using a Biotek Elx-800 plate reader (BioTek Instruments, Inc., Winooski, VT, USA).

To investigate the effect of curcumin on Panc-1 cell proliferation, 1x10⁴ cells were seeded into a 96-well culture plate and allowed to grow overnight in complete DMEM at 37°C. The culture medium was then removed and the cells were treated with vehicle or curcumin medium, for 24 h at 37°C. A cell Proliferation ELISA-Brdu (colorimetric) kit (Roche Diagnostics, USA) was used to determine the proliferation of the cells, according to the manufacturer's instructions.

Reverse transcription-quantitative polymerase chain reaction (R-T-PCR). The total RNA of the Panc-1 was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The RNA (2 µg) was then used for gene-specific RT-PCR using a One-Step RT-PCR kit (Qiagen, Venlo, the Netherlands), according to the manufacturer's instructions. The following primers were used: PCNA, forward 5'-AGAAAGGTGTGTTG AGGCCACT-3' and reverse 5'-GGTTTACACCGCTGG AGCTAA-3'; Ki67, forward 5'-AACTATCTCGTCTG TCCCAAAC-3' and reverse 5'-CGGCGATTGGAAAGA CAGAT-3'; p21, forward 5'-TGTCCGTCAAAACCATGC-3' and reverse 5'-AAAGTCGAAGTTCCATCGCTC-3'; p27, forward 5'-TAATTGGGCTCCGGTAACCT-3' and reverse 5'-TCGAGTGTCCGTCCTTATCCC-3'; cyclin D1, forward 5'-TGGAGAAAAAGGTTCCTACGC-3' and reverse 5'-GTA CGACTACTGTAGACAG-3'; FOXI, forward 5'-TGTGTC TATCCGTCCCACA-3' and reverse 5'-CGGCTCTCCT CCCTAGAAA-3'; β-actin, forward 5'-CATGTAGCTTGCT ATGCCAGGC-3' and reverse 5'-CTCTTTAAATGTAC CGCCAG-3'; and GAPDH, forward 5'-GAAGTGTAAGTCC GAT-3' and reverse 5'-GAAGATGGATGAGTGGATTCTC-3'.

The qPCR analysis was performed using a 50 µl reaction volume, which contained 1 µl dNTPs (10 mM), 5 µl complementary DNA solution, 1 µl of each primer (50 PM), 1 µl Taq DNA polymerase (Invitrogen Life Technologies), 33 µl water, 3 µl MgCl₂ (15 mM) and 5 µl PCR buffer (10X). The qPCR was performed on a thermal cycler (Sorvall ST 40R; Thermo Fisher Scientific, Waltham, MA, USA). Denaturation was performed at 94°C for 1 min, annealing at 59°C for 1 min, and elongation at 72°C for 1 min for 32 cycles, followed by 72°C for 10 min. The amplified products were electrophoresed on 2% agarose gels using a Gel-Pro Analyzer 6.0 for analysis. The levels for each gene were determined by standardizing the quantified mRNA to that of GAPDH or β-actin. Each sample was assessed in triplicate.

Western blot analysis. The cells were lysed using SDS lysis buffer and protease inhibitor cocktail. The protein concentrations of the cell lysates were quantified using a BCA kit, and equal quantities of protein (50 ng) were separated by 10% SDS-PAGE and then transferred onto a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The membranes were then blocked in 5% non-fat dry milk diluted with Tris-buffered saline with Tween-20 (TBST), containing 20 mmol/l Tris-HCl, 150 mmol/l NaCl (PH 7.5) and 0.1% Tween 20 at room temperature for 1 h and probed overnight at 4°C with anti-rabbit PCNA antibody (1:1,000; cat. no. sc-7907; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-rabbit Ki67 antibody (1:1,000; cat. no. sc-15402; Santa Cruz Biotechnology, Inc.), anti-mouse p21 (1:1,000; cat. no. 2946; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-mouse p27 (1:1,000; cat. no. 3698; Cell Signaling Technology, Inc.), anti-rabbit cyclin D1 antibody (1:1,000; cat. no. 2978; Cell Signaling Technology, Inc.), anti-rabbit B-cell lymphoma (Bcl)-2 antibody (1:1,000; cat. no. 2870; Cell Signaling Technology, Inc.), anti-rabbit B-cell-associated X protein (Bax) antibody (1:1,000;
Cat. no. 5023; Cell Signaling Technology, Inc.), anti-rabbit FoxO1 antibody (1:1,000; cat. no. 9454; Cell Signaling Technology, Inc.), and then incubated for 1 h at room temperature with a goat anti-rabbit (1:1,000; cat. no. 14708; Cell Signaling Technology, Inc.) or anti-mouse (1:1,000; cat. no. 14709; Cell Signaling Technology, Inc.) IgG conjugated to horseradish peroxidase. Incubation with monoclonal mouse α-tubulin antibody (1:1,000; cat. no. T5168; Sigma-Aldrich) or monoclonal mouse β-actin (1:1,000; cat. no. 3700; Cell Signaling Technology, Inc.) was performed as a loading sample control. The proteins were visualized using ECL™ western blotting detection reagents (GE Healthcare Bio-sciences, Pittsburgh, PA, USA). The densitometry of the bands was quantified using Image J 1.38x software (National Institutes of Health, Bethesda, MA, USA).

Apoptosis. Apoptosis was determined using a Cell Death ELISA Detection kit (Roche Diagnostics), which measures cytoplasmic DNA-histone complexes generated during apoptotic DNA fragmentation. Cell apoptosis detection was performed, according to the manufacturer's instructions and monitored spectrometrically at 405 nm.

Caspase-3/9 activity assay. A Caspase-3/9 Fluorescent Assay kit (Nanjing KeyGen Biotech, Co., Ltd., Nanjing, China) was used to detect caspase activity. In brief, the cells (10⁵) were cultured in 60 mm dishes and treated with DMSO or curcumin (20 μM) for 24 h at 37°C. The cells were then lysed in the lysis buffer and centrifuged at 10,000 x g for 1 min, following which the supernatants were collected. With bovine serum albumin as the control, equal quantities of the protein samples were reacted with synthetic fluorescent substrates at 37°C for 1.5 h and the reactions were read at 405 nm on a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Fold-increases in caspase-3/9 activity were determined using the values obtained from the treatment samples, divided by those from the control samples.

Small interfering (si)RNA transfection. siRNA against the human FOXO1 gene (Dharmacon, Pittsburgh, PA, USA) were transiently transfected into the cells using Lipofectamine 2000 reagent (Invitrogen Life Technologies), according to the manufacturer's instructions. A negative siRNA was used as a negative control. The siRNA sequences were as follows: 5'-CUGGAU CACAGUUUUCAAUG-3' (FOXO1) and 5'-GCAAGCUGA CCCUGAGUUCU-3' (negative). After 48 h, the cells were used for apoptosis, proliferation or western blot assays.

Statistical analysis. All statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The data from each group, for each parameter investigated, are presented as the mean ± standard error of the mean. Data from each group were statistically analyzed using a two-tailed Student's t-test or one-way analysis of variance. p<0.05 was considered to indicate a statistically significant difference.

Results

Curcumin decreases cell viability and proliferation in Panc-1 cells in a dose-dependent manner. The Panc-1 cells were treated with different concentrations of curcumin (5, 10, 20 and 40 μM) for 24 h, and the cell viability was evaluated using an MTT assay (Fig. 1A). Curcumin significantly inhibited cell viability in a dose-dependent manner, with a maximal effect at 20 μM. The results of the BrdU cell proliferation assay were in accordance with those of the MTT assay (Fig. 1B). From these results, the concentration of 20 μM was selected for use in the following experiments. To further confirm these results, the expression levels of PCNA and Ki67, two proliferation markers, were detected using western blot analysis. As shown in Fig. 1C and D, treatment of the Panc-1 cells with curcumin (20 μM) for 24 h markedly decreased the protein expression levels of PCNA and Ki67, compared with the control group. Accordingly, the mRNA levels of PCNA and Ki67 were also observed to decrease following treatment with curcumin (Fig. 1E and F).

Curcumin induces apoptosis in Panc-1 cells. To confirm whether curcumin inhibited growth arrest by inducing apoptosis, the present study subsequently examined nucleosomal degradation and the activation of caspase-9 and caspase-3 under the same conditions of curcumin treatment. As shown in Fig. 2A-C, the results confirmed that treatment of the Panc-1 cells with curcumin induced nucleosomal degradation, and increased the activation of caspase-9 and caspase-3, compared with control group. In addition, to further investigate the molecular basis for curcumin-induced apoptosis in pancreatic cancer, the constitutive expression levels of typical apoptosis-associated proteins, Bcl-2 and Bax were determined using western blot analysis. As shown in Fig. 2D, expression of the pro-apoptotic protein, Bax, was upregulated in the curcumin-treated cells, whereas expression levels of Bcl-2, an anti-apoptotic protein, were downregulated following treatment of the cells with curcumin for 24 h. The balance between the expression levels of Bax and Bcl-2 was, therefore, important in activating and deactivating the downstream cellular apoptosis pathway, including the activation of caspase-9 and caspase-3. These data indicated that curcumin may induce apoptosis in pancreatic cancer cells by increasing the Bax/Bcl-2 ratio, and thereby activating caspase-9 and caspase-3.

Curcumin increases the expression levels of p21WAF1/CIP1 and p27KIP1, and decreases the expression of cyclin D1. The present study demonstrated that curcumin inhibited growth in pancreatic cancer cells, which was associated with cell cycle arrest. To confirm these results, the effects of curcumin on the expression levels of p21, p27 and cyclin D1 were examined, which are known to be critical molecules involved in cell cycle arrest. As shown in Fig. 3, the mRNA and protein expression levels of the p21 and p27 CKIs were upregulated in the Panc-1 cells, which were treated with curcumin (Fig. 3A-D), compared with the control. Cyclin D1 is a nuclear protein, which is required for cell cycle regulation in the G1 phase of proliferating cells (17), and its protein and mRNA expression levels were markedly inhibited by treatment with curcumin (Fig. 3E and F). These results suggested that curcumin caused growth arrest in the Panc-1 cells by regulating the expression of cell cycle genes.

Role of FOXO1 in pancreatic cancer cells treated with curcumin. It has been reported that FOXO1 is responsible
for the apoptosis of cells. In the present study, curcumin was demonstrated to significantly increase the mRNA and protein expression levels of FOXO1 in the Panc-1 cells (Fig. 4A and B). Whether the induction of cell apoptosis by curcumin was mediated by FOXO1 in the pancreatic cancer cells was subsequently examined, which involved transfection of the Panc-1 cells with siRNA against FOXO1 (Fig. 4C and D). The results revealed that the knockdown of FOXO1 significantly attenuated the induction of cell apoptosis by curcumin (Fig. 4E), demonstrating that FOXO1 was essential in curcumin-induced apoptosis.

Curcumin induces the expression of FOXO1 through the PI3K/Akt pathway. In the majority of cancer cells, Akt is constitutively active and responsible for cell proliferation (18). In order to understand the association between PTEN and Akt in curcumin-induced apoptosis, the present study examined the expression of PTEN and the phosphorylation of PI3K and Akt in cells treated with curcumin (Fig. 5A-D). Curcumin increased the expression of PTEN, which is a tumor suppressor and a major negative regulator of the PI3K/Akt pathway, and inhibited the phosphorylation of PI3K and Akt in the Panc-1 cells. By contrast, curcumin had no effect on the expression of total PI3K and Akt. The present study subsequently assessed whether curcumin regulated FOXO1 through the PI3K/Akt pathway (Fig. 5E). The pancreatic cancer cells were pretreated with or without Akt inhibitor IV (Akt Inh-IV, 1 µM) or the Akt activator, fumonisin B1 (FB1, 50 µM), for 2 h and were then exposed to curcumin for another 24 h, following which the expression of FOXO1 was determined. Pretreatment with Akt...
Inh-IV further enhanced the expression of FOXO1 induced by curcumin in the Panc-1 cells. However, the expression of FOXO1 was significantly downregulated in the Panc-1 cells pretreated with FB1. In the cell proliferation assay, pretreatment with Akt Inh-IV, further inhibited the proliferation of Panc-1 cells by curcumin, whereas the inhibition of proliferation by curcumin was reversed following pretreatment with FB1 (Fig. 5F). These results confirmed that curcumin suppressed...
proliferation in Panc-1 cells by regulating the expression of FOXO1 through the PI3K/Akt pathway.

**Discussion**

Curcumin, a natural polyphenol, is derived from turmeric (*C. longa*) (19). For a number of years, curcumin has been consumed in the diet and used as a traditional medicine in several countries in the Far East, particularly for its antitumor activity, and remains of significant interest. A number of reports have identified that curcumin has effective anticancer effects on several types of cancer cell lines *in vitro*, including ovarian cancer cells, breast cancer cells, colon cancer cells, prostate cancer cells and glioblastoma cells (20-25), and chemopreventive effects in carcinoma models of muridae (26). It is also important to note that no curcumin-associated toxicity has been observed in experimental animals and humans, even at high doses (15). The signaling pathways regulating apoptosis are complicated in mammalian cells due to their mechanisms altering in accordance with cell type. A number of studies have focused on the identification of natural products, which induce apoptosis in malignant cells, and the anticancer effect of curcumin was confirmed to disturb cell cycle, induce apoptosis and inhibit the invasion of cancer (27,28). However, the molecular mechanisms...
of curcumin-induced apoptosis and anti-proliferation in pancreatic cells remains to be fully elucidated.

In the present study, the effects of curcumin on the proliferation and apoptosis in pancreatic cancer cells and its possible mechanisms, were investigated. The results indicated that curcumin treatment inhibited cell proliferation in the Panc-1 cells, and curcumin induced apoptosis of the Panc-1 cells. The mechanisms underlying the induction of anti-proliferative and apoptotic effects by curcumin may be through activation of FOXO1 and inhibition of PI3K/Akt signaling pathway in pancreatic cancer cell lines.

Members of the FOXO subfamily have been suggested as critical regulators in cancer cell biology. FOXO1 is a member of the FOXO family of transcription factors (FoxOs). As the majority of types of cancer are characterized by the inactivation of FOXO1 by hyperactivation of the PI3K/Akt pathway, reactivation of FOXO1 may be an invaluable therapeutic approach (29). Akt has been demonstrated to directly inactivate FOXO1, resulting in inactivation and inhibition of the expression of FOXO1-regulated molecules, which control a variety of biological functions, including cell proliferation, survival, cell cycle and apoptosis (30). Therefore, regulation of FOXO1 by the Akt pathway is receiving increasing attention in cancer investigations. In the present study, inhibition of FOXO1 by siRNA inhibited curcumin-induced cell cycle arrest and apoptosis. Furthermore, using an Akt inhibitor and activator revealed that curcumin induced the mRNA and protein levels of FOXO1 through the activation of PI3K/Akt signaling.

It has been reported that increased expression levels of FoxOs cause G1/S arrest, which is partially induced by upregulation of the expression of the CKI, p27KIP1, and downregulation of cyclin D (10,31). Under diverse cellular conditions, FoxOs have been observed to regulate other CKIs, including p15INK4b, p57, p19INK4d and p21CIP1, thereby contributing to G1/S arrest (32-34). From the results of the present study, it was demonstrated that curcumin caused growth arrest by promoting the expression levels of p27 and p21, and suppressing the expression of cyclin D1. Aactive FoxOs induce apoptosis in a variety of cell types through the mitochondria-dependent caspase pathway by downregulating the expression of Bcl-2 and upregulating the expression of Bax, resulting in an imbalance of Bcl-2/Bax ratio. This activates the initiator caspase-9, which eventually induces the activation of executioner caspases, including caspase-3, leading to apoptosis (35). Consistent with this, the present study found that curcumin induced apoptosis in the pancreatic cancer cells by increasing the Bax/Bcl-2 ratio, thereby activating caspase-9/3 and caspase-3, which were the downstream targets of FOXO1.

In conclusion, the present study confirmed that curcumin inhibited the proliferation of Panc-1 cells through downregulation of cyclin D1 and upregulation of p21CIP1 and p27KIP1, leading to cell cycle arrest. Furthermore, the data revealed that curcumin induced apoptosis in the Panc-1 cells by decreasing the Bcl-2/Bax ratio and increasing the activation of caspase-9/3, and the mechanisms involved curcumin increasing the expression of FOXO1 by activating PI3K/Akt signaling.

Figure 5. Curcumin regulates FOXO1 by activating PI3K/Akt signaling. The Panc-1 cells were treated with or without curcumin (20 µM) for 30 min. (A) Western-blot analysis of the intensities of PTEN, p-PI3K, PI3K, p-Akt and Akt in each group. (B-D) Bar charts indicating the different intensities of PTEN, p-PI3K, PI3K, p-Akt and Akt between groups. Panc-1 cells were pretreated with Akt inhibitor IV (Akt Inh-IV; 1 µM) or fumonisin B1 (FB1; 50 µM) for 2 h and then exposed to curcumin for another 24 h. (E) Western blot analysis of the protein levels of FOXO1 in Panc-1 cells. (F) Cell proliferation was evaluated using a BrdU-ELISA assay. Values were normalized against the control values. All data are presented as the mean ± standard error of the mean (n=6). *P<0.05 and **P<0.01 vs. control; #P<0.05, Akt Inh-IV, vs. curcumin group; &P<0.05 FB1, vs. curcumin group. FOXO1, forkhead box O1; p-, phosphorylated; t-total; PI3K, phosphoinositide 3-kinase; OD, optical density.
of PTEN and inhibiting the phosphorylation of PI3K and Akt in the Panc-1 cells. In addition, the results demonstrated that inhibition of the phosphorylation of Akt was correlated with the activation of FOXO1. These findings provide support in determining whether curcumin has potential as a novel treatment agent for pancreatic cancer therapy.

References