Down-expression of poly(ADP-ribose) polymerase in p53-regulated pancreatic cancer cells

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Received June 19, 2016; Accepted August 8, 2017

DOI: 10.3892/ol.2017.7500

Abstract. The present study investigated whether poly(ADP-ribose) polymerase (PARP) has an effect on p53-regulated pancreatic cancer. The results of the present study demonstrated that the expression of PARP affects proliferation and apoptosis of pancreatic cancer cells. Olaparib was used to suppress the expression level of PARP-1 in PanC-1 cells. Decreased expression of PARP-1 suppressed cell proliferation and induced apoptosis of PanC-1 cells when compared with controls. Furthermore, decreased expression of PARP-1 resulted in decreased levels of pro-caspase-3 expression, increased caspase-3 activity, suppressed B-cell lymphoma 2 (Bcl-2) protein expression and increased p53 protein expression in PanC-1 cells. Subsequently, ataxia telangiectasia mutated (ATM) activity was inhibited alongside down-expression of PARP-1 resulting in significantly decreased cellular viability of PanC-1 cells, increased p53 protein expression, decreased expression of pro-caspase-3, increased caspase-3 activity and suppressed Bcl-2 protein expression, when compared with PARP-1 suppression alone. Overall, the *in vitro* data confirmed that down-expression of PARP-1 suppressed cell proliferation and induced apoptosis of pancreatic cancer via ATM-deficient p53 signaling pathway.

Introduction

Pancreatic cancer is a result of malignant tumor growth in the digestive system (1). Relative to other types of cancer, pancreatic cancer involves late identification of lesions, a high degree of malignance, fast progression and poorer prognosis (1). Previous research has demonstrated that pancreatic cancer primarily presents in the head of pancreas (2). The morbidity of patients with pancreatic ductal adenocarcinoma is the

highest amongst pancreatic cancer and accounts for 95% of pancreatic cancer-associated mortality (2). The 5-year survival rate of pancreatic cancer is <5% (3), and patients with pancreatic cancer are expected to survive for ~4-6 months after diagnosis prior to succumbing to the disease (4). Patients who undergo surgery are expected to live for ~13-20 months before succumbing to pancreatic cancer (2). The annual morbidity rate of patients with pancreatic cancer is ~9/10,000, with the morbidity rate in China being ~5/10,000 (3). Annually, ~40,000 people succumb to pancreatic cancer (3). Previous research has revealed that the morbidity rate of pancreatic cancer in China is increasing (4).

Inhibition of apoptosis serves a role in the progression of pancreatic cancer. Proteins in the B-cell lymphoma 2 (Bcl-2) family serve an important role in the regulation of apoptosis (5). The Bcl-2 family includes the apoptotic inhibitors Bcl-2, B-cell lymphoma extra-large (Bcl-xL), Bcl-2-like protein 2 (Bcl-w), Bcl-2-related protein A1 (Bfl-1), brefeldin A-resistant Arf-guanine nucleotide exchange factor 1 (Brag-1) and induced myeloid leukemia cell differentiation protein (Mcl-1), and the apoptotic regulators Bcl-2-associated X protein (Bax), Bcl-2 homologous antagonist/killer (Bak), B-cell lymphoma extra-small (Bcl-xS), Bcl-2-associated death promoter (Bad), BH3 interacting-domain death agonist (Bid), Bcl-2-interacting killer (Bik) and activator of apoptosis hara-kiri (Hrk) (6). The ratio of apoptotic inhibitors to apoptotic inducers determines the response of cells to apoptosis-triggering signals. To some extent, induction and inhibition of apoptosis may be regulated by controlling the expression of various Bcl-2 family members (7). p53 is the primary regulator of the Bcl-2 family. The biological behavior of pancreatic cancer tumor cells is thus affected by the regulatory properties exhibited by p53 over the various inhibitors and inducers of apoptosis within the Bcl-2 family (8).

Poly(ADP-ribose) polymerase (PARP) requires an oxidized nicotinamide-adenine dinucleotide substrate, utilizing adenosine triphosphate in order to form a poly(ADP-ribose) chain (PAR chain) at the site of single strand breaks in DNA (9). The formation of a PAR chain signals other DNA damage repair proteins to the site of the break. PARP has exhibited involvement in a number of metabolic processes in cells including genome stabilization, cell cycle progression, gene transcription, chromosome function and cell death. The PARP family has a number of distinct members including;

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Key words: poly(ADP-ribose) polymerase, pancreatic cancer, p53, ataxia telangiectasia mutated

PARP-1, PARP-2, PARP-3, Vault-PARP and tankyrases. PARP-1 was the first member of the PARP family to be identified, with a molecular mass of 116Kd (9). It comprises three regions: A DNA-binding domain, an automodification domain and a catalytic domain (10). The primary structure of PARP-1 is highly conserved in eukaryotes; distinct catalytic regions exhibit a high degree of homology (10). Increased expression levels of PARP-1 have been identified in multiple types of tumor including breast, prostate and pancreatic cancer, indicating that PARP-1 participates in tumorigenesis and tumor progression (11). In order to develop effective novel therapeutic approaches to treat pancreatic cancer, the underlying molecular mechanisms of the involvement of PARP-1 in tumor progression require investigation.

Materials and methods

Cell lines and cell culture. Human pancreatic cancer cells (PanC-1) were provided by the Institute of Biochemistry and Cell Biology (Chinese Academy of Science Shanghai, China). PanC-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc.), penicillin (50 U/ml) and streptomycin (50 µg/ml) at 37°C in a humidified atmosphere containing 5% CO₂.

Cell proliferation assay. PanC-1 cells (1x10³ in a 96-well plate) were cultured in the presence of dimethylsulfoxide (DMSO; Amresco, LLC, Solon, OH, USA; untreated) or olaparib (1, 5 and 25 µM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 1, 2, 3 and 4 days at 37°C. A second set of experiments was performed, with PanC-1 cells (1x10³ in a 96-well plate) cultured in the presence of DMSO (untreated), olaparib (5 µM) or olaparib (5 µM) + KU55933 (5 µM) for 4 days at 37°C. Subsequently, 20 µl/well MTT solution (5 mg/ml; Amresco, LLC) was added to each well prior to further incubation at 37°C for 4 h. Subsequently, DMSO solution was added to each well for solubilization of the formazan product at 37°C for 30 min with gentle mixing. Absorbance was determined at 560 nm using an M200 plate reader (Tecan Group, Ltd., Mannedorf, Switzerland).

Analysis of apoptosis using flow cytometry (FCM). PanC-1 cells (1x10⁶ in a 6-well plate) were cultured in the presence of DMSO (untreated) or olaparib (1, 5 and 25 µM) for 4 days at 37°C. A second set of experiments was performed, with PanC-1 cells (1x10⁶ in a 6-well plate) cultured in the presence of DMSO (untreated), olaparib (5 µM) or olaparib (5 µM) + KU55933 (5 µM) for 4 days at 37°C. Subsequently PanC-1 cells were washed twice with PBS and resuspended in 500 µl binding buffer (BD Biosciences, Franklin Lakes, NJ, USA) and 5 µl annexin V-fluorescein isothiocyanate (FITC) (BD Biosciences) and subsequently in 5 µl propidium iodide (PI) (BD Biosciences) in the dark. The rate of apoptosis was analyzed using FCM (BD Biosciences).

Caspase-3 activity determination using a caspase-3 activity kit. The caspase-3 activity kit it was obtained from Beyotime Institute of Biotechnology (Haimen, China; C1115). PanC-1

cells (1x10⁶ in a 6-well plate) were cultured in the presence of DMSO (untreated) or olaparib (1, 5 and 25 µM) for 4 days at 37°C. A second set of experiments was performed, with PanC-1 cells (1x10⁶ in a 6-well plate) cultured in the presence of DMSO (untreated), olaparib (5 µM) or olaparib (5 µM) + KU55933 (5 µM) for 4 days at 37°C. Subsequently PanC-1 cells were washed twice with PBS and incubated with N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (C1115; Beyotime Institute of Biotechnology) at 37°C for 30 min. Absorbance was determined at 405 nm using an M200 plate reader.

Western blot analysis. PanC-1 cells (1x10⁶ in 6-well plate) were cultured in the presence of DMSO (untreated) or olaparib (1, 5 and 25 µM) for 1, 2, 3 and 4 days at 37°C. A second set of experiments was performed, with PanC-1 cells (1x10⁶ in a 6-well plate) cultured in the presence of DMSO (untreated), olaparib (5 µM) or olaparib (5 µM) + KU55933 (5 µM) for 4 days at 37°C. Subsequently, PanC-1 cells were lysed in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) with phenylmethanesulfonyl fluoride protease and phosphatase inhibitor (Beyotime Institute of Biotechnology). Total cell protein was extracted and subsequently quantified using a BCA protein quantitative assay kit (Beyotime Institute of Biotechnology). An equivalent amount of protein (50 µg) was resolved using SDS-PAGE (8-12% gel) and transferred on to a nitrocellulose membrane (Merck KGaA). Membranes were blocked with skimmed milk (5%) in TBST (with 0.1% Tween-20) for 1 h at 37°C and then incubated with anti-pro-caspase-3 (sc-98785; 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-Bcl-2 (sc-783; 1:300; Santa Cruz Biotechnology, Inc.), anti-p53 (sc-6243; 1:500; Santa Cruz Biotechnology, Inc.) and anti-GAPDH (sc-25778, 1:500; Santa Cruz Biotechnology, Inc.) at 4°C overnight. Horseradish peroxidase-labeled goat anti-rabbit secondary antibodies (sc-2004; 1:5,000, Sangon Biotech Co., Ltd., Shanghai, China) were added for 1 h at 37°C and detected using enhanced chemiluminescence reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The protein expression was measured using ImageJ 1.37 software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All data are presented as the mean ± standard deviation. Analysis of variance was used for multiple comparisons and Student's t-test was used to compare between pairs. P<0.05 was considered to indicate a statistically significant difference.

Results

Down-expression of PARP-1 suppresses the proliferation of PanC-1 cells. To investigate whether the down-expression of PARP-1 has an effect on human pancreatic cancer, an MTT assay was used to measure the proliferation of PanC-1 cells following treatment with olaparib. It was demonstrated that olaparib, a PARP-1 inhibitor, significantly suppressed the proliferation of PanC-1 cells in a time- and dose-dependent manner when compared with the DMSO control group (Fig. 1).

Down-expression of PARP-1 induces apoptosis of PanC-1 cells. To investigate whether the down-expression of PARP-1 induces apoptosis of PanC-1 cells following olaparib treatment,

Figure 1. Decreased expression of PARP-1 suppresses the viability of PanC-1 cells. ** $P < 0.01$ vs. DMSO control group. DMSO, dimethylsulfoxide.

FCM was performed. Cells treated with olaparib (5 and 10 μ M) exhibited a significant increase in the rate of apoptosis in a dose-dependent manner when compared with the DMSO control group (Fig. 2).

Down-expression of PARP-1 decreases pro-caspase-3 expression levels and increases caspase-3 activity in PanC-1 cells. Pro-caspase-3 was analyzed using western blotting and a caspase-3 activity kit. Pro-caspase-3 expression levels were significantly suppressed following down-expression of PARP-1, compared with the DMSO control group. However, caspase-3 activity was significantly increased following down-expression of PARP-1 when compared with the DMSO control group (Fig. 3).

Down-expression of PARP-1 suppresses Bcl-2 protein expression of PanC-1 cells. To quantify cell death, Bcl-2 protein expression levels in PanC-1 cells were measured using western blot analysis. The results demonstrated that olaparib significantly inhibited the expression of Bcl-2 protein in a dose-dependent manner when compared with the DMSO control group (Fig. 3).

Down-expression of PARP-1 induces p53 protein expression in PanC-1 cells. To further investigate the effect of down-expression of PARP-1 on cell death, the expression levels of p53 protein in PanC-1 cells was measured using western blot analysis. It was identified that increasing concentrations of olaparib resulted in increased expression levels of p53 protein (Fig. 3).

Inhibition of ataxia telangiectasia mutated (ATM) activity alongside down-expression of PARP-1 further suppresses the proliferation of PanC-1 cells. Functional ATM caused anchorage-independent proliferation in pancreatic cancer. KU55933 (5 μ M) was used to inhibit ATM activity. As presented in Fig. 4, following treatment with KU55933 (5 μ M) and olaparib (5 μ M) cell proliferation was decreased when compared with the olaparib (5 μ M)-treated group.

Inhibition of ATM activity alongside down-expression of PARP-1 further increases the rate of apoptosis of PanC-1 cells. To investigate ATM activity in PanC-1 cells following olaparib treatment, the rate of apoptosis of PanC-1 cells was analyzed using FCM. It was identified that the down-expression of PARP-1 alongside inhibition of ATM activity (inhibited

Figure 2. Down-expression of PARP-1 induces apoptosis of PanC-1 cells. ** $P < 0.01$ vs. DMSO control group. DMSO, dimethylsulfoxide.

using KU55933; 5 μ M) significantly increased the rate of apoptosis of PanC-1 cells when compared with the olaparib (5 μ M)-treated group (Fig. 5).

Inhibition of ATM activity in conjunction with down-expression of PARP-1 decreases pro-caspase-3 expression levels and increases caspase-3 activity in PanC-1 cells. Following treatment with KU55933 (5 μ M) and olaparib (5 μ M), pro-caspase-3 expression was significantly decreased and caspase-3 activity was significantly increased when compared with the olaparib (5 μ M)-treated group (Fig. 6).

Inhibition of ATM activity alongside down-expression of PARP-1 suppresses Bcl-2 protein expression in PanC-1 cells. The expression levels of Bcl-2 protein in PanC-1 cells were investigated using western blot analysis following inhibition of ATM activity using KU55933 (5 μ M). It was identified that down-expression of PARP-1 in conjunction with ATM inhibition significantly decreased Bcl-2 expression levels when compared with the olaparib (5 μ M)-treated group (Fig. 6).

Inhibition of ATM activity in conjunction with down-expression of PARP-1 increases p53 protein expression levels of PanC-1 cells. Expression levels of p53 were measured using western blot analysis. Treatment with olaparib (5 μ M) and KU55933 (5 μ M) resulted in significantly increased p53 expression levels when compared with the olaparib (5 μ M)-treated group (Fig. 6).

Discussion

Pancreatic cancer presents with a malignant tumor of the digestive tract and is a serious threat to human health. Owing to a lack of typical clinical symptoms and the need for sensitive

Figure 3. Down-expression of PARP-1 decreases expression levels of pro-caspase-3, increases caspase-3 activity, decreases Bcl-2 expression levels and increases expression levels of p53 in PanC-1 cells. (A) Down-expression of PARP-1 induces degradation of pro-caspase-3, Bcl-2 and increases p53 protein expression as determined using western blotting assays. Quantification of (B) pro-caspase-3 protein expression, (C) caspase-3 activity, and (D) Bcl-2 and (E) p53 protein expression of PanC-1 cells. **P<0.01 vs. DMSO control group. DMSO, dimethylsulfoxide; Bcl-2, B-cell lymphoma 2.

Figure 4. Inhibition of ataxia telangiectasia mutated activity in conjunction with down-expression of PARP-1 suppresses the proliferation of PanC-1 cells. **P<0.01 vs. DMSO control group; ##P<0.01 vs. 5 μM olaparib-treated group. DMSO, dimethylsulfoxide.

Figure 5. Inhibition of ataxia telangiectasia mutated activity alongside down-expression of PARP-1 increases the rate of apoptosis of PanC-1 cells. **P<0.01 vs. DMSO control group; ##P<0.01 vs. 5 μM olaparib-treated group. DMSO, dimethylsulfoxide.

diagnostic tests, by the time the majority of patients receive a diagnosis, their symptoms are too severe to be treated with

surgical intervention. The 5-year survival rate of pancreatic cancer is <5% (2). An increased understanding of the etiology of pancreatic cancer may result in the development of improved diagnostic tests. It is hypothesized that pancreatic cancer is the result of multiple factors and genes. Inactivation of p53 and p16, abnormal expression of the apoptosis inhibitor gene Bcl-2, inactivation of DNA damage repair genes, overexpression of growth factors and receptors, and an increase in telomerase activity are all hypothesized to serve a function in the development of pancreatic cancer (2). PARP-1 is present in the majority of eukaryotic cells and serves an important role in DNA damage repair, genetic transcription, cell cycle progression, genome stabilization and cell death. In the present study, it was identified that PARP-1 inhibition significantly suppressed cell proliferation, suppressed pro-caspase-3 protein expression and activated caspase-3 activity in PanC-1 cells.

Apoptosis is a type of programmed cell death regulated by genes in an active and orderly manner. Effective apoptosis is achieved via p53 regulation (8). Apoptosis regulation via p53 may be realized by upregulating Bax and downregulating Bcl-2 or Bcl-xL (12). Through their interaction, the permeability of cell mitochondria is regulated, affecting the function of downstream apoptosis genes. It has been demonstrated that Bcl-2 in pancreatic cancer tissue with increased expression of p53 is overexpressed, whereas pancreatic cancer tissue with decreased expression of p53 exhibits decreased expression levels of Bcl-2, indicating that Bcl-2 expression may be at least partially dependent on p53 expression levels (13,14). In the present study, it was identified that PARP-1 inhibition

Figure 6. Inhibition of ataxia telangiectasia mutated (ATM) activity alongside down-expression of PARP-1 decreases expression levels of pro-caspase-3, increases caspase-3 activity, decreases Bcl-2 expression levels and increases p53 expression levels of PanC-1 cells. (A) Western blot analysis results of levels of p53, Bcl-2, pro-caspase-3 and GAPDH in control cells, olaparib-treated cells and olaparib + KU55933-treated cells. Quantification of (B) pro-caspase-3 protein expression, (C) caspase-3 activity, and (D) Bcl-2 and (E) p53 expression levels of PanC-1 cells. ** $P < 0.01$ vs. DMSO control group; ## $P < 0.01$ vs. 5 μ M olaparib-treated group. DMSO, dimethylsulfoxide; Bcl-2, B-cell lymphoma 2.

significantly increased p53 expression levels in PanC-1 cells. Deben *et al* (15) reported that PARP inhibition induces apoptosis in non-small cell lung cancer cell lines via the p53 signaling pathway.

Bcl-2 family members exhibit the ability to form dimers or polymers by themselves or with other family members. For example, a Bax-Bax dimer may induce apoptosis, whereas a Bcl-2-Bax dimer is more stable when compared with the former (16). As a consequence of increased Bcl-2 expression, the proportion of Bcl-2-Bax dimers will be increased relative to Bax-Bax dimers, resulting in a decrease in pro-apoptotic Bax-Bax activity (17). In the presence of Bcl-xS, formation of Bcl-xS-Bcl-2 dimer results in a decrease in Bcl-2-Bax dimer levels, which subsequently increases Bax-Bax levels, so as to induce apoptosis (18). Therefore, the interaction between Bcl-2 family proteins regulates survival and apoptosis of cells. The results of the present study demonstrated that PARP inhibition significantly inhibited the expression of Bcl-2 protein in PanC-1 cells. Hao *et al* (19) provided evidence that Tubeimoside-1 inhibits lung cancer cell proliferation and induces cell apoptosis through activation of PARP and the Bcl-2/Bax signaling pathway.

The mitochondrial apoptosis pathway is triggered by the activation and release of various apoptotic factors which regulate mitochondria by releasing apoptin which targets mitochondria (20). Although regulation of apoptosis may include the Bcl-2 family, the final execution of apoptosis remains

dependent on activation of the caspase cascade reaction (21). The caspase family, particularly caspase-3, serves a key role in apoptosis (21). Various apoptotic pathways lead to apoptosis by triggering the caspase cascade reaction. Caspase-3 is the effector molecule in the apoptotic cascade signaling pathway and is the executive protein of apoptosis (22). As a DNA damage repair molecule, PARP is degraded by caspase-3 and other cysteine proteinases during apoptosis, and its degradation is regarded as an early molecular sign of apoptosis (23). In the present study, it was also identified that down-expression of PARP-1 (via olaparib) significantly decreased the cellular viability of PanC-1 cells, increased p53 protein expression, decreased expression of pro-caspase-3, increased caspase-3 activity and suppressed Bcl-2 protein expression following the inhibition of ATM activity. Liu *et al* (24) reported that PARP had an effect on sulfur mustard-induced cutaneous injuries *in vitro* and *in vivo* via the caspase-3 signaling pathway.

The results of the present study revealed that PARP-1 inhibition significantly suppressed cell proliferation, suppressed pro-caspase-3 protein expression and activated caspase-3 activity of PanC-1 cells through p53/Bcl-2 signaling. Subsequently, inhibition of ATM activity alongside down-regulation of PARP-1, further decreased cellular viability of PanC-1 cells, induced p53 protein expression, decreased expression of pro-caspase-3, increased caspase-3 activity, and suppressed Bcl-2 protein expression in PanC-1 cells. It is proposed that down-expression of PARP-1 suppresses

proliferation and induces apoptosis of pancreatic cancer cells via the ATM-deficient p53 signaling pathway.

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