Co-expression of FOXL1 and PP2A inhibits proliferation inducing apoptosis in pancreatic cancer cells via promoting TRAIL and reducing phosphorylated MYC

YUNWEI LI, DONGYANG YU, WEIWEI SHENG, HE SONG, YUJI LI and MING DONG

Department of General Surgery, Gastrointestinal Surgery, The First Hospital of China Medical University, Shenyang 110001, Liaoning, P.R. China

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Abstract. Pancreatic cancer is usually diagnosed in the advanced stages and is sensitive to only few therapies. The forkhead box L1 (FOXL1) and protein phosphatase 2A (PP2A) have been recognized to be tumor suppressive in human pancreatic cancers. In the present study, we co-expressed the two tumor suppressive molecules with a ‘2A peptide’ linker, which guaranteed the two molecules were transcribed into one mRNA, whereas they were translated into two separate proteins, in pancreatic cancer Panc-1 cells, and investigated the inhibition of the two molecules on the proliferation and migration of Panc-1 cells. Results demonstrated that, either overexpression of FOXL1 or PP2A via adenovirus significantly inhibited the proliferation of Panc-1 cells, whereas promoted apoptosis in such cells. Moreover, the co-expression of both FOXL1 and PP2A exerted synergistic antitumor effect in Panc-1 cells, with significantly higher proliferation inhibition and tumor induction. In addition, we found that the overexpressed FOXL1 promoted the TNF-related apoptosis-inducing ligand (TRAIL), whereas the overexpressed PP2A downregulated the phosphorylation of c-MYC. The co-expression of FOXL1 and PP2A presented both functions in Panc-1 cells. In conclusion, the adenovirus-mediated co-expression of FOXL1 and PP2A with the 2A peptide linker exterts synergistic suppression of pancreatic cancer cells via inhibiting the growth and promoting apoptosis of cancer cells, probably via upregulating TRAIL and reducing the phosphorylation of MYC.

Introduction

Pancreatic cancer, mainly pancreatic ductal adenocarcinoma (PDAC), is one of the most deadly and aggressive cancers (1). It is the world-wide seventh (2), and the fourth in the United States (3) of most common cause of cancer-related death, with a poor prognosis and swift progression before death. Surgical eradication is still the only potentially curative treatment of this malignancy, but there are only 15-20% cases indicative for surgery, because of the early occurrence of local advancement or distal metastasis (4). Five-year overall survival rate ranges from 1 to 6% (3,5,6). Even after surgical eradication plus adjuvant chemotherapy, OS rates do not exceed 30% (5,6).

Various chemotherapeutic agents have failed to improve survival of pancreatic cancer patients. In recent years, FOLFIRINOX, a cocktail of 5-fluorouracil (5-FU), irinotecan and oxaliplatin has significantly improved OS of pancreatic patients with metastasis, compared with the single treatment with gemcitabine (7,8). Besides conventional chemotherapy, accumulating understanding of the biological pathogenesis of pancreatic cancer has provided a variety of targeted approaches. However, except erlotinib, which is an inhibitor to epidermal growth factor receptor (EGFR), no other targeted therapy has as yet demonstrated significant effect against pancreatic cancer (9,10). Insulin-like growth factor 1 (IGF-1) and its receptor, PI3-kinase/Akt/mTOR and mitogen-activated protein kinases/extracellular signal-regulated kinases (MEK/ERK) pathway are upregulated in the majority of PDACs (11,12). However, a phase II trial on a monoclonal antibody against IGF receptor (IGFR) indicates no significant effect in OS and progression-free survival (PFS) for PDAC patients (4). The chemical agents targeting the PACD-overexpressed vascular endothelial growth factor (VEGF) which promotes cancer angiogenesis and metastasis (13), also failed to improve PFS and OS (14). Other treatments targeting farnesyltransferase, the tumor stroma in pancreatic cancer or autophagy are on the way.

Forkhead box L1 (FOXL1) belongs to a forkhead/winged helix-box (FOX) family of transcription factors. All Fox members, being classified as FOXA to FOXR (15), Fox molecules play critical roles in a variety of physiologic (16) or pathologic processes such as cancer, as tumor suppressors (17-19). Particularly, FOXM1 was identified to be oncogenic in pancreatic cancer, and is associated with poor prognosis and pathologic stage of PADC (20,21), whereas, FOXL1 has...
recently been recognized as a tumor suppressor in PADC (22). Therefore, FOXL1 might be another target for the treatment of pancreatic cancers. Protein phosphatase 2A (PP2A) is a large collection of oligomeric protein serine/threonine phosphatases and accounts for a large fraction of phosphatase activity in eukaryotic cells. PP2A is a critical tumor suppressor, via controlling a number of cellular processes, including cell cycle progression (23,24). Key signaling pathways that are negatively regulated by PP2A include members of the MAPK/ERK pathways, NF-κB, and c-Myc signaling (25). In particular, PP2A suppresses the oncogenic activity (26) of c-Myc via specifically dephosphorylating the key serine 62 (S62) in c-Myc (27), stimulating its ubiquitination (28) and thus accelerating the degradation of c-Myc. The overexpression of endogenous PP2A inhibitors, such as SET (12PP2A) and cancerous inhibitor of PP2A (CIP2A) in head and neck squamous cell carcinoma, colon cancer, gastric cancer, breast cancer, and most recently, pancreatic cancer (29,30). Thus, the tumor suppressive PP2A might also facilitate pancreatic cancer therapy.

In the present study, we constructed a recombinant adenovirus, which carries the coding sequence both of FOXL1 and PP2A, with a self-cleavage sequence. Then we evaluated the regulation of the recombinant adenovirus on the proliferation of pancreatic cancer cells, on the sensitivity of pancreatic cancer cells to 5'-FU. In addition, we investigated the activation of TNF-related apoptosis-inducing ligand (TRAIL) by the recombinant virus. The present study provides a novel antitumor strategy against PADC.

Materials and methods

Cell lines and culture conditions. Human pancreatic carcinoma Panc-1 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI-1640 media (Gibco Life Technologies, Rockville, MD, USA) supplemented with GlutaMAX-I (Invitrogen, Carlsbad, CA, USA), 50 IU/ml penicillin and 50 mg/ml streptomycin (both from CSPC Zhongnuo Pharmaceutical Co., Ltd., Shijiazhuang, China) and 10% (v/v) fetal bovine serum (FBS) (Sijiqing, Hangzhou, China). Cells were incubated at 37˚C in a humidified incubator with 5% CO₂. Human FOXL1 and PP2A coding sequences (both from Sinobio, Beijing, China) were amplified, respectively, and were overlapped with with 2A peptide coding sequence as followed. Briefly as follows. Panc-1 cells were quantitatively seeded in 12-well plates with 10⁴/ml, post an inoculation for 8 h, 1 h at room temperature. The specific binding was scanned via a molecular dynamics densitometer (Imaging Technology, Ontario, Canada). ImageJ software was used to quantify band density.

Growth curve assay and colony formation assay. The proliferation of Panc-1 cells was evaluated via cell counting assay, briefly as follows. Panc-1 cells were quantitatively seeded in 12-well plates with 10⁴/ml, post an inoculation for 8 h (cells closely attached), cells were infected with 3 MOI of Ad (FOXL1), Ad (PP2A), Ad (FOXL1 + PP2A) or the Ad (con) virus with 1 or 3 multiplicities of infection (MOI) for 2 h at 37°C, and then were updated with fresh RPMI-1640 media supplemented with 2% FBS.

Quantitative assay for the mRNA level of FOXL1 or PP2A. Total cellular mRNA in Panc-1 cells was isolated with TRIzol reagent (Life Technologies, Grand Island, NY, USA) and was supplemented with RNase inhibitor (Takara, Tokyo, Japan). mRNA samples were directly quantified via real-time PCR, using the SuperScript III Platinum One-Step qRT-PCR kit (Qiagen GmbH, Hilden, Germany) on an ABI PRISM 7300 detection system. The housekeeping β-actin gene was simultaneously quantified to standardize the amount of target mRNA. Relative quantification of gene transcription level was performed by the -ΔΔCt method (31), the relative target mRNA was presented as relative level to the control group.

Western blot assay. Harvested Panc-1 cells post various treatment were promptly homogenized in a Cell Lysis Buffer (Cell Signaling Technology Inc., Danvers, MA, USA), then centrifuged at 13,000 x g for 20 min at 4°C to collect the supernatant. Next, each sample was quantified with a BCA protein assay reagent kit (Pierce, Rockford, IL, USA) and was diluted to same concentration. Before being added with loading buffer and being boiled, protein samples with equal amount were separated with 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) electrophoresis and then were transferred to a PVDF membrane (Millipore, Bedford, MA, USA). After the block with 2% bovine serum albumin (BSA) (Ameresco, Framingham, MA, USA) overnight at 4°C, the membrane was incubated overnight again at 4°C with the rabbit polyclone antibody against FOXL1, PP2A, β-actin, caspase-3, poly(ADP-ribose) polymerase (PARP), TRAIL, MYC or phosphorylated MYC (Ser at 62). After triple washes with Tris-buffered saline and Tween-20 (TBST), the membrane was incubated with horseradish peroxidase-linked secondary anti-rabbit antibody (Sigma-Aldrich) for an inoculation for 1 h at room temperature. The specific binding was scanned via a molecular dynamics densitometer (Imaging Technology, Ontario, Canada). ImageJ software was used to quantify band density.
MTT assay. Panc-1 cells were seeded in 96-well plates, at >85% confluence, cells were treated with 10 µM 5'-FU, and were infected with 3 MOI of Ad (FOXL1), Ad (PP2A), or Ad (FOXL1 + PP2A) virus for 24 h. Then, the MTT solution was added and incubated for 4 h. After the MTT solution was aspirated, 100 µl dimethyl sulfoxide was added to each well. The absorbance was measured at 570 and 650 nm using a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Statistical analysis. Quantitative data are presented as the mean ± standard deviation that is calculated from three independent results. Comparison between two groups was performed with a Student's t-test. A two-way ANOVA test was used for multiple comparisons between three or more groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Overexpression of FOXL1 and PP2A in Panc-1 cells with a FOXL1- and PP2A-co-expressed adenovirus. To investigate the tumor suppressive role of FOXL1 and PP2A simultaneously in pancreatic cancer Panc-1 cells, we adopted a strategy to clone the coding sequences of both genes into one opening reading-frame (ORF), and to co-express both
proteins simultaneously. As shown in Fig. 1A, both FOXL1 and PP2A coding sequences were linked with a self-cleaved ‘2A’ peptide coding sequence (32) and were cloned into the multiple cloning sites of the shuttle plasmid, with the promoter of human cytomegalovirus (CMV) immediate early enhancer and promoter (PCMV). The Ad (con) (overexpressing EGFP), Ad (FOXL1) (overexpressing FOXL1), Ad (PP2A) (overexpressing PP2A) or Ad (FOXL1 + PP2A) (overexpressing both FOXL1 and PP2A) was rescued respectively via the co-transfection with the adenoviral genomic plasmid and the shuttle plasmid. Fig. 1B indicates that the infection with the Ad (con) virus caused EGFP expression in >85% of Panc-1 cells. The expression efficiency by the adenovirus of both FOXL1 and PP2A was evaluated in Panc-1 cells post-infection with Ad (FOXL1), Ad (PP2A), or Ad (FOXL1 + PP2A) virus for 5 or 7 days. (D and E) Difference in the number (D) and the size (E) of colonies formed by Panc-1 cells infected with the above-mentioned virus. *P<0.05, **P<0.01, or ***P<0.001; ns, no significance.

Synergistic inhibition by the co-expression of FOXL1 and PP2A to the proliferation and migration of Panc-1 cells. To investigate the regulatory role of FOXL1 or/and PP2A co-expression on the proliferation of pancreatic cancer cells, the in vitro proliferation of Panc-1 cells post the infection with Ad (con), Ad (FOXL1), Ad (PP2A) or Ad (FOXL1 + PP2A)
virus was examined with the cell counting and colony formation assays. As shown in Fig. 2A, the growth curve of Panc-1 cells infected with Ad (FOXL1) or with Ad (PP2A) was significantly retardant, compared with the Ad (con) infection. Moreover, the infection with Ad (FOXL1 + PP2A) virus caused a more retardant growth curve of Panc-1 cells (P<0.05, P<0.001 or P<0.0001). In particular, the growth efficiency of Panc-1 cells was significantly inhibited by the Ad (FOXL1 + PP2A) virus, even compared with the Ad (FOXL1) or Ad (PP2A) virus, at 5- or 7-day post-infection (DPI) (P<0.05 for 5 DPI, or P<0.01 for 7 DPI).

Then the regulation by the overexpression of FOXL1, PP2A or both molecules on the proliferation of Panc-1 cells was evaluated with the colony formation assay. As indicated in Fig. 2C and D, Panc-1 cells formed less colonies post-infection with Ad (FOXL1), Ad (PP2A) or Ad (FOXL1 + PP2A), compared with the infection with Ad-con (P<0.05, P<0.01 or P<0.001). Interestingly, there was also a significant difference in the colony size among the four groups. Colonies in the group of Ad (FOXL1), Ad (PP2A) and Ad (FOXL1 + PP2A) were significantly smaller than in the group of Ad (con) (Fig. 2E) (P<0.01, respectively) Thus, the co-expression of both FOXL1 and PP2A inhibited the proliferation of Panc-1 cells.

We then determined the regulation of the FOXL1- or/ and PP2A-overexpression on the migration of Panc-1 cells. The migration assay of Panc-1 cells indicated that in contrast to the Ad (con) virus infection, the infection with either Ad (FOXL1), Ad (PP2A) or Ad (FOXL1 + PP2A) at 3 MOI significantly reduced the migration of Panc-1 cells, there were less migratory cells in the three groups (Fig. 3A-C) P<0.01 for Ad (FOXL1) or Ad (PP2A); P<0.001 for Ad (FOXL1 + PP2A). In addition, the migratory cells in the Ad (FOXL1 + PP2A) group were far less than in the Ad (FOXL1) or Ad (PP2A) group (P<0.05, respectively). Thus, we confirmed the inhibition of the overexpression of FOXL1 in the migration of pancreatic cancer cells.

Co-expression of FOXL1 and PP2A sensitized Panc-1 cells to chemotherapy via enhancing the apoptosis induction. To evaluate the influence of the co-expression of FOXL1 and PP2A on the chemo-sensitivity of pancreatic cancer cells, we also examined the viability reduction and apoptosis induction of Panc-1 cells by 10 µM 5'-FU, one of widely-used anticancer agent, post-infection with Ad (con), Ad (FOXL1), Ad (PP2A) or Ad (FOXL1 + PP2A) virus. Fig. 4A indicated that the viability decreased more significantly in the 5'-FU-treated Panc-1 cells, post-infection at 3 MOI Ad (FOXL1), Ad (PP2A) or Ad (FOXL1 + PP2A) virus [P<0.05 for Ad (FOXL1), Ad (PP2A), P<0.01 for Ad (FOXL1 + PP2A)] and the viability reduction was more significant by the infection with Ad (FOXL1 + PP2A) than with Ad (FOXL1), Ad (PP2A) (P<0.05, respectively). The deteriorated apoptosis induction was also confirmed by the overexpression of FOXL1 or/ and PP2A in Panc-1 cells. As shown in Fig. 4B, compared with the Ad (con) infection, there were more apoptotic cells induced by the infection at 3 MOI Ad (FOXL1), Ad (PP2A) or Ad (FOXL1 + PP2A) at 24 (P<0.05 or P<0.01) or 48 h post-infection (HPI) (P<0.01 or P<0.001), with markedly higher apoptosis in the Ad (FOXL1 + PP2A) group (P<0.05, respectively at 24 HPI or P<0.01 at 48 HPI). In addition, we also analyzed the activation and activity of caspase-3, which is the apoptosis-executor, in each groups of cells. Fig.4C-E demonstrated that there was higher levels of activated caspase-3 (Fig. 4D) and caspase activity (Fig. 4E) induced by the Ad (FOXL1), Ad (PP2A) or Ad (FOXL1 + PP2A) than Ad (con) (P<0.05, P<0.01 or P<0.001, respectively), particularly higher by the Ad (FOXL1 + PP2A) (P<0.05, respectively). Therefore, we confirmed that the co-expression of FOXL1 and PP2A sensitized Panc-1 cells to 5'-FU via enhancing apoptosis induction.

Figure 3. Migration of the Panc-1 cells post-overexpression of FOXL1 or (and) PP2A. (A and B) Wound healing assay of Panc-1 cells, at 0 or 72 h post-infection at 3 MOI Ad (con) (A), Ad (FOXL1) (A), Ad (PP2A) (B), or Ad (FOXL1 + PP2A) (B) virus. (C) Difference in migratory cell numbers among groups of Ad (con), Ad (FOXL1), Ad (PP2A) and Ad (FOXL1 + PP2A). Statistical significance is shown as *P<0.05, **P<0.01, or ***P<0.001; ns, no significance.
Co-expression of FOXL1 and PP2A promotes TRAIL, whereas inhibits MYC phosphorylation. TRAIL is a member of the TNF superfamily and triggers apoptosis by recruiting the initiator caspase-8 and by directly activating downstream effector caspases (33), and FOXL1 has been indicated to inhibit the tumor aggressiveness in human pancreatic cancer via promoting TRAIL (22). The oncogenic MYC (also c-MYC) has also been deregulated in pancreatic cancers and has been confirmed to promote pancreatic cancers (34), and the targeted inhibition of MYC by antagonizing PP2A inhibitor has been indicated to inhibit the growth of breast cancers (35).

To explore the possible mechanism in apoptosis induction by the co-expression of FOXL1 and PP2A, we then determined the expression of TRAIL and the phosphorylation of MYC, in Panc-1 cells post-infection at 3 MOI adenovirus for 24 or 48 h. (A) Western blotting results reconfirmed the regulation of TRAIL and MYC.

Figure 4. Viability and apoptosis induction in Panc-1 cells post the overexpression of FOXL1 or (and) PP2A, in the presence of 10 μM 5'-FU. (A) Relative viability of Panc-1 cells treated with 10 μM 5'-FU and infected at 3 MOI Ad (con), Ad (FOXL1), Ad (PP2A) or Ad (FOXL1 + PP2A) virus for 24 or 48 h. (B) Flow cytometric analysis for apoptosis induction by the treatment with 10 μM 5'-FU and the infection at 3 MOI adenovirus for 24 or 48 h. (C) Western blot analysis for activated caspase-3 (cleaved casp-3) and its substrate poly ADP ribose polymerase (PARP) in Panc-1 cells post 5'-FU treatment and the adenovirus infection. (D) Relative level of cleaved casp-3 and lyzed PARP in Panc-1 cells post 5'-FU treatment and the adenovirus infection. (E) Relative caspase-3 activity in Panc-1 cells post 5'-FU treatment and the adenovirus infection. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001; ns, no significance.
infected with Ad (FOXL1) or Ad (FOXL1 + PP2A) at 3 MOI (P<0.01, respectively), whereas no significantly difference of MYC was found among the groups (Fig. 5C). However, the level of phosphorylated MYC (S62), was significantly downregulated by the infection with either Ad (PP2A) or Ad (FOXL1 + PP2A) (Fig. 5E; P<0.05, respectively). Taken together, the co-expression of FOXL1 and PP2A promotes TRAIL, whereas inhibits MYC phosphorylation at S62.

**Discussion**

The pathogenesis and the incurable nature of pancreatic cancer, and the rapid metastasis and the poor response to chemo-drugs might contribute to the poor prognosis (1,36). Several pathways have been recognized to regulate the pathogenesis or progression of pancreatic cancers. Hedgehog (Hh) signaling and the nuclear factor-κB (NF-κB) pathway have been implicated to involve in the pathogenesis of the disease (1,37-40). Many other pathways have also been found to be deregulated in pancreatic cancers, and to promote the growth aggression of the cancer (36). Such pathways as epidermal growth factor receptor (EGFR) and cyclooxygenase-2 (COX-2) act with an orchestrated interaction each other, and play a significant role in tumorigenesis (40). However, there is no chemo- or immuno-therapeutic agent against these pathways indicating significant effect for PDAC patients.

Figure 5. Expression of TRAIL and the phosphorylation of MYC in Panc-1 cells post the co-expression of FOXL1 and PP2A. (A and B) mRNA level of TRAIL (A) or MYC (B) in Panc-1 cells post-infection at 3 MOI Ad (con), Ad (FOXL1), Ad (PP2A), or Ad (FOXL1 + PP2A) virus for 24 h. (C) Western blot analysis of TRAIL, MYC or phosphorylated MYC (Ser at 62) [p-MYC(S62)] in Panc-1 cells post-infection at 3 MOI Ad (con), Ad (FOXL1), Ad (PP2A), or Ad (FOXL1 + PP2A) virus for 48 h. (D and E) Percentage of TRAIL to β-actin (D) or percent p-MYC(S62) to MYC in each type of infected Panc-1 cells (48 h). Statistical significance is shown as *P<0.05 and **P<0.01; ns, no significance.
Previous studies have indicated the tumor suppressive roles of FOXL1 (22) and PP2A (41) in pancreatic cancers. Thus, the tumor suppression mechanism of FOXL1 and PP2A might facilitate to find novel strategy or target for pancreatic cancer therapy. In the present study, we reconfirmed the tumor suppressive role of either FOXL1 or PP2A in the pancreatic cancer Panc-1 cell line. The Ad-mediated overexpression of either FOXL1 or PP2A significantly inhibited the proliferation of Panc-1 cells via multiple assays, and such overexpression sensitized Panc-1 cells to the treatment with 5'-FU via enhancing apoptosis induction. Moreover, we used a strategy of co-expression of FOXL1 and PP2A to obtain an enhanced tumor suppressive effect on pancreatic cancers. The Ad (FOXL1 + PP2A) virus not only more significantly inhibited the proliferation of Panc-1 cells, but also deteriorated the viability reduction, or enhanced the apoptosis induction in the Panc-1 cells subjected to 5'-FU. 2A peptide is encoded by foot-and-mouth disease virus (FMDV), with a 'self-cleavage' characteristic (42). This 'self-cleavage' peptide composed of 2A and 2B, both of which are translated from one mRNA molecule and function independently (42). Therefore, the 2A peptide is well used for the multiple expression of foreign proteins (43,44). In the present study, we confirmed that the adenovirus encoding both FOXL1 and PP2A with the '2A peptide' linker overexpressed both tumor suppressors in pancreatic cancer cells, and exerted synergistic growth inhibitory effect of pancreatic cancer cells.

TRAIL is a member of the tumor necrosis factor superfamily inducing apoptosis through interaction with the TRAIL-R1 and TRAILR2 receptors (alternatively known as DR4 and DR5, respectively) (45-47). TRAIL has emerged as a potential therapeutic agent due to its selective induction of apoptosis in cancer cells (48). Preliminary clinical trials with TRAIL indicate promising outcomes without obvious toxicity (49,50). The present study presents another confirmation of the antitumor effect of TRAIL via upregulating the upstream FOXL1. Significant promotion of TRAIL in both mRNA and protein levels was confirmed by the infection with either Ad (FOXL1) or Ad (FOXLI + PP2A). On the contrary, the oncogenic MYC (also namely C-MYC) has been found to be deregulated in pancreatic cancers and has been confirmed to promote pancreatic cancers (34,51). The targeted inhibition of MYC has been indicated to inhibit the growth of breast cancers (35). In mammalian cells, Ser-62 phosphorylation of MYC is associated with the MYC stabilization, and the dephosphorylation of the site by PP2A promotes its polyubiquitination and degradation (52). Previous studies confirmed that inhibited PP2A resulted in increased MYC half-life (53). The current study confirmed the inhibition to Ser-62 phosphorylation of MYC by PP2A overexpression, and it might be associated with the inhibition of pancreatic cancer cells.

In conclusion, the adenovirus-mediated co-expression of FOXL1 and PP2A with the 2A peptide linker exterts synergistic suppression of pancreatic cancer cells via inhibiting the growth and promoting apoptosis of cancer cells. The coexpressed FOXL1 and PP2A functions independently via upregulating TRAIL (by FOXL1) and reducing the phosphorylation of MYC (by PP2A). Our findings re-confirmed the tumor suppressive role of PP2A and FOXL1 in pancreatic cancer cells, with an enhanced antitumor effect via co-expression of both molecules.

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