USP34 regulated human pancreatic cancer cell survival via AKT and PKC pathways

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Abstract

Pancreatic cancer is known to be a fatal disease, which is difficult to be diagnosed in its early stages. USP34 (Ubiquitin-Specific Protease 34) are closely related to human cancers in the development and progression. However, there are rarely studies about the role of USP34 in pancreatic cancer. Thus, we aimed to investigate the effect of USP34 in human pancreatic cancer. Short-hairpin RNA targeting USP34 (USP34-shRNA) and USP34 overexpression lentivirus were used in the current study. The level of USP34 in human pancreatic cancer (PANC-1) cells were then analyzed by qRT-PCR. In addition, western blotting was used to examine p-AKT, p-PKC and p-ERK protein levels. CCK-8 assay, flow cytometry, and migration assay were used to detect cell proliferation, apoptosis and migration, respectively in vitro. According to the result of qRT-PCR and western blotting, USP34-shRNA1 significantly downregulated USP34 gene level in PANC-1 cell. Subsequently, western blotting assay indicated that USP34 silencing significantly down-regulated the expression of p-AKT and p-PKC in cells. On the other hand, USP34 overexpressing remarkably up-regulated the expression of p-AKT and p-PKC in cells. In addition, USP34 overexpression promoted PANC-1 cell proliferation and migration via up-regulating the proteins of p-AKT and p-PKC. Moreover, USP34 overexpression reversed AKT inhibitor and PKC inhibitor induced PACN-1 cell apoptosis. Our results indicated USP34 regulated h PANC-1 cell survival via AKT and PKC pathways, and which played a pro-survival role in human pancreatic cancer. Therefore, we suggested USP34 could be a potential therapeutic target for pancreatic cancer.

Keywords: USP34, pancreatic cancer, apoptosis, migration
**Introduction**

Pancreatic cancer is a kind of fatal aggressive disease, which is the fourth primary cause of cancer associated death in western countries.\(^1-3\) Pancreatic cancer is an extremely serious health problem in the worldwide.\(^2\) When patients suffered with this cancer for five years, the survival rate is approximately 5%. The reason of difficult to detect pancreatic cancer at early stages was due to lacking of early warning symptoms.\(^4\) In addition, several factors triggered pancreatic cancer, such as family history of chronic pancreatitis, occupational exposure, smoking, alcohol abuse, a high-fat diet and vitamin D deficiency.\(^5-8\) Generally speaking, morbidity is almost the same as mortality.\(^5\) Therefore, it is very necessary to find better therapeutic strategies for treating this disease.

The occurrence and development of pancreatic cancers are related to gene and protein expression and the activation of signaling pathways.\(^9\) \textit{USP34} gene belongs to ubiquitin-specific protease family, which located on chromosome 2p15 and encodes a kind of deubiquitinating enzyme.\(^10\) Several studies showed that USP34 are related to ovary tumor, adenomatous polyposis coli, microbial infection and infantile autism.\(^10-12\) Protein kinase C (PKC) and protein kinase B (AKT) play important roles in control of the proliferation, migration and survival of human cancer cells.\(^4, 13\) There have been no previous reports as to whether USP34 gene affects the pancreatic tumors through the PKC and AKT pathway.

In the current study, we found USP34 was significantly upregulated in tumor tissues of patients with pancreatic cancer. Therefore, the potential function of USP34 in pancreatic cancer was investigated in the present study.

**Materials and methods**

**Tumor tissues collection**

5 patients had a clear histological diagnosis of pancreatic cancer undergo surgery at our department from January 2016 to October 2017. The pancreatic cancer tissues and the paired adjacent normal tissues (with a >2 cm distance from the edge of the
tumor) were snap-frozen in liquid nitrogen after surgical resection immediately. The informed written consent has been signed by all patients and the study was approved by The Second Affiliated Hospital of Soochow University Animal Experimental Ethics Committee. Experiments involving human subjects were in accord with the Helsinki Declaration.

**Cell culture**

The 293T packaging cell line and Human PANC1 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen; Thermo Fisher Scientific, Inc. Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin and incubated at 37˚C with 5% CO₂.

**Short hairpin RNA plasmids**

Lentiviral expressing short-hairpin RNA directed target human USP34 and one nontargeting sequence (negative control) were purchased from GenePharma (Shanghai, China). Each plasmid vector expressed a shRNA under the control of a CMV promoter and contained a green fluorescent protein (GFP) reporter gene. The shRNA target sequences were as described as following. USP34-shRNA1: (F) 5’-CACCGCATATAATCCTAGACCTTTCCGAAAGAAAGGTCTAGGATTATATGC-3’; (R) 5’-AAAAGCATATAATCCTAGACCTTTCTTCGGAAAGGTCTAGGATTATATGC-3’. USP34-shRNA2: (F) 5’-CACCGGATTGAACTGTTGACGAAACTTCGGTTTCGTCAACA GTTCAATCC-3’; (R) 5’-AAAAGGATTGAACTGTTGACGAAACTTCGGTTTCGTCAA CAGTTCAATCC-3’. USP34-shRNA3: (F) 5’-CACCGCTGCTGTTCATGGCACTACA GTTCTGTATGC-3’; (R) 5’-AAAAGCTGCTGTTCATGGCACTACA GTTCTGTATGC-3’.

**Construction of recombinant lentivirus**

Lentiviral vector DNAs were then transfected into 293T cells including lenti-USP34 shRNAs and lenti-USP34. The lenti-USP34, provided by Genepharm, which was cloned into the pSuper-puro-GFP vector. After transfection, the cells were incubated
to enhance viral titer at 32°C. 48 h later, the supernatant was collected, which containing the retroviral particles. The supernatant was filtered through the low protein binding syringe filter (0.45 μm) and the titer of lentiviruses was determined.

**USP34 shRNA knockdown**

PANC-1 cells were maintained in DMEM and were plated into cell plates (60 mm) at 4 x 10^5 cells/well. 24 h later, four USP34-shRNAs supernatants were added directly to PANC1 cells (with 50-60% of confluence). 24 h later, virus-containing medium was replaced with fresh complete medium. Stable PANC-1 cells were then selected by puromycin (2.5 μg/mL, Sigma Aldrich, St. Louis, MO, USA) for another 3 days. Western blotting assay and qRT-PCR assay were used to verified expression of USP34 in the stable cells.

**Exogenous USP34 overexpression**

PANC-1 cells were maintained in DMEM and were plated into cell plates (60 mm) at 4x10^5 cells/well overnight. Then, lenti-USP34 supernatants were added directly to PANC1 cells (with 50-60% of confluence) for 24 h. Later on, cells were re-plated on selection medium containing puromycin (2.5 μg/mL) for another 3 days. qRT-PCR assay were used to verified expression of USP34 (GFP-tagged) in the stable cells.

**Real-time quantitative PCR.**

Total RNA from PANC-1 cells treated with lenti-control, lenti-USP34 shRNAs or lenti-USP34 were purified by using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using the cDNA reverse transcription kit (Takara Bio Inc. Shiga, Japan) following the manufacturer’s instructions. The primer sequences used were as follows: USP34-F: 5’-CGTTTGGACATGACGCCCTA-3’; USP34-R: 5’-CATC TGCCGTTCTGTGT GA-3’, GAPDH-F: 5’-GGGAAATCGTGCGTGACATTAAG-3’ and GAPDH-R: 5’-TGTGTGGGCGTGACAGGCTTTTG-3’. QRT-PCR analysis was carried out using SYBR® Premix Ex TaqTM II (Takara Bio Inc. Shiga, Japan) on Applied Biosystems Prism 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) following the manufacturer’s instructions. The level of expression of USP34 genes was measured using comparative cycle threshold (CT)
method.

**Western blotting**

Total proteins were collected from PANC-1 cells using cell lysis buffer and quantified using BCA protein quantification kit (Beyotime, Beijing, China). Proteins were separated using 10% SDS-PAGE and gels were transferred to a PVDF membrane (Millipore, Billerica, MA, USA). The PVDF membrane was blocked in 5% defatted milk and incubated for 1 h at room temperature. The membrane was subsequently incubated at 4°C overnight with the following primary antibodies: anti-AKT (Abcam ab8805) (1:1000), anti-p-AKT (Abcam ab8932) (1:1000), anti-ERK (Abcam ab54230) (1:1000), anti-p-ERK (Abcam ab192591) (1:1000), anti-PKC (Abcam ab31) (1:1000), anti-p-PKC (Abcam ab23513) (1:1000), anti-GAPDH (Abcam ab8245) (1:1000) overnight at 4 °C. After washing, the PVDF membrane was incubated with secondary antibody anti-rabbit (Abcam; ab7090) (1:5000) before determined by chemiluminescence. Finally, the PVDF membranes were incubated with ECL reagent (Santa Cruz Biotechnology) to detect the blots.

**CCK-8 assay of cell viability**

Cell viability was evaluated with Cell Counting Kit-8 (CCK8, Beyotime, Shanghai, China) according to the manufacturer’s protocols. PANC-1 cells (5×10³ cells/well) were seeded into 96-well plate overnight and treated as following for 24, 48 and 72 h: lentivirus-control (lenti-con); lentivirus-USP34 (lenti-USP34); AKT inhibitor (GSK 2110183); PKC inhibitor (Enzastaurin); lenti-USP34+AKT inhibitor; lenti-USP34+PKC inhibitor. The absorbance was determined at 450 nm using a Thermo Multiskan FC microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

**Flow cytometric analysis of cell apoptosis**

PANC-1 cells (3×10⁴ cells/well) were seeded to 6-well plates overnight and treated as following for 72 h: lentivirus-control (lenti-con); lentivirus-USP34 (lenti-USP34); AKT inhibitor (GSK 2110183); PKC inhibitor (Enzastaurin); lenti-USP34+AKT inhibitor; lenti-USP34+PKC inhibitor. Afterwards, cultured cells were stained with 10 μL Annexin V and 5 μL propidium iodide (PI) for 15 min at room temperature in the dark.
according to the manufacturer’s protocols (Thermo Fisher Scientific, Waltham, MA, USA), and measured by FCM flow cytometer (BD Bioscience, San Jose, CA, USA).

**Matrigel invasion assay**

The cell invasion assay was performed using a 24-well transwell chambers (Corning, New York, NY, USA) according to manufacturer's protocol. Briefly, PANC-1 cells (1×10^5 cells) were seeded to the upper chamber in serum-free medium, the bottom wells in the system were added into DMEM medium containing with 10% FBS. After incubation for 72 h at 37 °C, cells on the upper surface of the filter were removed by a cotton swab, and cells on the underside were fixed with 4% formaldehyde and stained with DAPI. The numbers of invaded cells were counted in 20 randomly selected fields.

**Immunohistochemistry**

The expressions of USP34 in tumor and adjacent normal tissues were detected with immunohistochemical. The specimens were cut into 5 μm sections, placed on slides, and baked at 65°C for 2 h. Then, the slices were incubated with USP34 antibody overnight at 4°C, and following incubated with second antibody for 30 minutes at room temperature. Later on the slices was observed by using a polymer immunohistochemical detection system (EnVision kit; Dako Japan).

**Statistical analysis**

The SPSS software 15.0 version was used for statistical analysis. Each group were executed at least three independent experiments and all data were expressed as the mean ± SD. The comparison between two groups was analyzed by Student’s t-test. The comparisons among multiple groups were made with one-way analysis of variance (ANOVA) followed by Dunnett’s test. P<0.05 or P<0.01 was considered to indicate a statistically significant difference (*P<0.05, ** P<0.01).

**Results**

**USP34 was significantly upregulated in pancreatic cancer tissues**

Immunohistochemistry was firstly conducted to detect the expression of USP34 in
pancreatic cancer and peri-cancer tissues. As indicated in Fig. 1, the levels of UPS34 were markedly increased in cancer tissues compared with the adjacent normal tissues. This data suggested that USP34 may play important role during the tumorigensis of pancreatic cancer.

**USP34 shRNAs down-regulated USP34 gene expression in PANC-1 cells**

We first designed three different USP34 shRNAs, in order to down-regulated USP34 gene expression in PANC-1 cells effectively. QRT-PCR was used to detect the expression of USP34 following transfection with three USP34-shRNAs in PANC-1 cells. As shown in Fig.2A, the expression of USP34 was most significantly decreased after transfection with USP34-shRNA1. Therefore, USP34-shRNA1 was selected for the following experiments. Next, the data of fluorescent expression further confirmed cells were effectively transfected with lentivirus after 48 h incubation (Fig.2B). Moreover, western blotting was used to determine the expression of USP34 following transfection with USP34-shRNA1. As indicated in Fig.2C and 2D, the expression of USP34 was markedly decreased after transfection with USP34-shRNA1 compared with the shRNA-control group in cells. These data indicated that gene USP34 expression were dramatically inhibited by USP-shRNA1 in PANC-1 cells.

**Suppression of USP34 down-regulated the expression of p-AKT and p-PKC in PANC-1 cells**

For the purpose of studying the association between the USP34 gene and AKT, PKC and ERK pathways in PANC-1 cells, USP34-shRNA1 was transfected into PANC-1 cell. Scrambled shRNA transfected into PANC-1 cell and un-transfected PANC-1 cells were used as a lenti-control or a blank control, respectively. Relative proteins phosphorylated-AKT (p-AKT), phosphorylated-ERK (p-ERK) and phosphorylated-PKC (p-PKC) were detected by western blotting. As depicted in Fig. 3A, 3B, 3C, the expression of p-AKT and p-PKC in PANC-1 cells was markedly decreased following transfection with USP34-shRNA1, compared with the blank
control or lenti-control groups. However, USP34-shRNA1 had no effect on the expression of p-ERK in PANC-1 cells (Fig. 3A, 3D). These results indicated that knockdown of USP34 significantly inhibited the expression of p-AKT and p-PKC in PANC-1 cells and the function of USP34 was closely associated to these two pathways.

**USP34 overexpression promoted PANC-1 cell proliferation**

Next, we tested the effect of USP34 overexpression on PANC-1 cell proliferation. Results in Fig. 4A demonstrated that there was no difference regarding as the transfection efficiencies between lenti-control and lenti-USP34. As expected, the expression of USP34 in PANC-1 cell was significantly upregulated following transfection with lenti-USP34, compared with the blank control or lenti-control groups (Fig. 4B). Western blotting was applied next to test USP34 protein expression following transfection with lenti-USP34. As depicted in Fig. 4C and 4D, the USP34 protein expression demonstrated a notable upregulation compared with lenti-control groups. In addition, the expression of p-AKT and p-PKC in PANC-1 cells was markedly increased following transfection with lenti-USP34, compared with the blank control or lenti-control groups (Fig. 4D-4G). Moreover, CCK8 assay was applied to test the PANC-1 cell viability. As is shown in Fig. 3H, USP34 overexpression increased the proliferation of PANC-1 cells, which indicating USP34 played a pro-survival role in pancreas cancer. However, the cell growth promoted activity of USP34 was reversed by AKT or PKC inhibitor interference significantly (Fig. 3H). All these data suggested that USP34 overexpression promoted PANC-1 cell proliferation via up-regulating of AKT and PKC pathway.

**USP34 overexpression reversed AKT or PKC inhibitor-induced PANC-1 cell apoptosis**

In order to further investigate the association between the USP34 gene and AKT, PKC pathways, the apoptosis of PANC-1 cells was detected by flow cytometry. As indicated in Fig. 5A, 5B, both AKT and PKC inhibitors notably induced cell apoptosis, while USP34 overexpression had very limited effect. However, AKT or PKC...
inhibitor-induced cell apoptosis was alleviated by USP34 overexpression significantly (Fig. 5A, 5B), which further confirmed USP34 exerted its biological function via regulating AKT and PKC pathways in PACN-1 cells.

**USP34 overexpression promoted PANC-1 cell migration**

We next investigated the effect of USP34 overexpression on the migration ability of PANC-1 cells using a transwell assay. As shown in Fig. 6A and 6B, the number of penetrating cells of the lenti-USP34 transfected group was found to be notably increased in the non-basement membrane chamber compared with the lenti-control group. This result indicated USP34 overexpression promoted PANC-1 cell migration. However, this kind of effect was significantly reversed by AKT or PKC inhibitor interference compared with lenti-USP34 (Fig. 6A, 6B). All these data suggested that USP34 promoted PANC-1 cell migration via regulating AKT and PKC pathways.

**Discussion**

Pancreatic cancer is among the most aggressive of human malignancies. Although gemcitabine has proven efficacy improvement in pancreatic cancer-related symptoms, which is associated with only modest alleviation of some disease-related symptoms in patients. There is a pressing need to identify new therapeutic methods. It has been reported that USP34 is associated with chronic inflammation and oncogenesis. There have been no previous reports studied that the role of USP34 gene in the pancreatic tumors.

Recently, the lentivirus vector-mediated gene therapy has shown great prospect in pancreatic cancer including RNAi or overexpression. In this study, we identified lenti-USP34-shRNA1 most effectively down-regulate USP34 in PANC1 cells at the RNA and protein level. Our observation that silencing the USP34 gene resulted in a significant decrease of p-AKT and p-PKC level in PANC-1 cells. However, in this study we did not find that silencing of USP34 was associated with the expression of p-ERK. Previous studies have reported that PKC, AKT and ERK could promote survival and proliferation of pancreatic cancer cells. Inconsistent with this report, in
this article we showed that USP34 gene silencing only suppressed AKT and PKC activity in pancreatic cancer cells. However, our findings are consistent with a previous observation that HMGA1 (high mobility group A1) silencing suppresses AKT activity in pancreatic cancer cells.14) These results suggest that knockdown of USP34 gene performed an anti-tumor effect on pancreatic cancer via inhibiting AKT and PKC activity.

In addition, in vitro studies showed that knockdown of USP34 by USP34-shRNA1 exerted an anti-tumor effect. On the other hand, exogenous over-expression of USP34 promoted PANC-1 cell proliferation and migration. Previous studies have reported that HMGA1 overexpression also had an effect of pro-survival in pancreatic cancer cells.14) Our observation that overexpressing the USP34 gene resulted in a significant increase of p-AKT and p-PKC level in PANC-1 cells. Moreover, we confirmed in this study that AKT or PKC inhibitor-induced cell apoptosis was alleviated by USP34 overexpression significantly (Fig. 4A, B), which further confirmed USP34 exerted its biological function via regulating AKT and PKC pathways in PACN-1 cells. Furthermore, USP34 overexpression promoted PANC-1 cell proliferation and migration, which were dramatically reversed by AKT or PKC inhibitor. All these data indicates USP34 plays a pro-survival role in pancreas cancer.

Conclusion

In summary, our data demonstrate that knockdown of USP34 gene performed an antitumor effect, while USP34 overexpression in PANC-1 cells performed pro-survival and pro-migration effects. Therefore, we suggested USP34 could be a potential therapeutic target for pancreatic cancer.

Conflicts of interest

The authors declare no conflict of interest.
Reference


14) Liau SS, Ashley SW, Whang EE: Lentivirus-mediated RNA interference of HMGA1 promotes chemosensitivity to gemcitabine in pancreatic adenocarcinoma. *J Gastrointest Surg*, 10,


Figure 1. **USP34 was significantly upregulated in pancreatic cancer tissues.** 5 pair of cancer and peri-cancer tissues were isolated from the patients with pancreatic cancer. IHC staining of paraffin-embedded human tissue were pictured.
Figure 2. USP34 shRNAs down-regulated USP34 gene expression in PANC-1 cells. (A) PACN-1 were transfected with lenti-control, USP34-shRNA1, USP34-shRNA2 or USP34-shRNA3 for 72 h and the level of USP34 were detected with qRT-PCR (**P < 0.01, *P < 0.05 compared with lenti-control group). (B) The transfection efficacy of lenti-control-GFP and USP34-shRNA1-GFP plasmids in PANC-1 cells were observed under fluoresce microscope (×200 magnification). (C) PANC-1 cells were transfected with USP34-shRNA1 for 72 h. Then, the expressions of USP34 were analyzed by western blotting in cells. (D) USP34 relative expression was quantified by normalizing to GAPDH (**P < 0.01 compared with shRNA-con group).
Figure 3. Suppression of USP34 down-regulated the expressions of p-AKT and p-PKC in PANC-1 cells. Lenti-control shRNA and USP34-shRNA were transfected into PANC-1 cells. (A) PANC-1 cells were transfected with USP34-shRNA1 for 72 h. Then, the expressions of p-AKT, p-ERK and p-PKC were analyzed by western blotting in cells. (B) P-AKT relative expression was quantified by normalizing to AKT (*P < 0.05 compared with lenti-con group). (C) p-PKC relative expression was quantified by normalizing to PKC. (D) P-ERK relative expression was quantified by normalizing to ERK (**P < 0.01 compared with lenti-con group).
Figure 4. USP34 overexpression promoted PANC-1 cell proliferation. Lenti-control and lenti-USP34 were transfected into PANC-1 cells for 72 h. (A) The transfection efficacy of lenti-control-GFP and lenti-USP34-GFP plasmids in PANC-1 cells were observed under fluoresce microscope (×200 magnification). (B) USP34 gene levels in PANC-1 cells were detected with qRT-PCR (**P < 0.01 compared with lenti-con group). (C) The expressions of USP34 were analyzed by western blotting in cells. (D) USP34 relative expression was quantified by normalizing to GAPDH (**P < 0.01 compared with shRNA-con group). (E) The expressions of p-AKT and p-PKC were analyzed by western blotting in cells. (F) P-AKT relative expression was quantified by normalizing to AKT (**P < 0.01 compared with lenti-con group). (G) P-PKC relative expression was quantified by normalizing to PKC (**P < 0.01 compared with lenti-con group). (H) Cell viability was determined using CCK-8.
assay in PANC-1 cells treated with AKT inhibitor or PKC inhibitor for 24, 48 and 72 h (**P < 0.01 compared with lenti-con group; ##P < 0.01 compared with lenti-USP34 group).
Figure 5. USP34 overexpression reversed AKT or PKC inhibitor-induced PACN-1 cell apoptosis. Lenti-USP34 was transfected into PANC-1 cells with or without AKT inhibitor and PKC inhibitor for 72 h, respectively. (A) Apoptotic cells were detected with Annexin V and PI double staining. (B) The apoptosis cell rates were calculated (**P < 0.01 compared with AKT inhibitor and PKC inhibitor group, respectively).
Figure 6. USP34 overexpression promoted PANC-1 cell migration. Lenti-USP34 was transfected into PANC-1 cells with or without AKT inhibitor and PKC inhibitor for 72 h, respectively. (A) Migration in PANC-1 cells were detected using transwell invasion assays. (B) The migration cell number were calculated (**P < 0.01 compared with lenti-con group; ##P < 0.01, #P < 0.05 compared with lenti-USP34 group)