

## Original Article

# PTEN is involved in Kanglaite<sup>®</sup> injection-induced apoptosis of human pancreatic cancer cells

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Received January 1, 2018; Accepted September 5, 2018; Epub February 15, 2019; Published February 28, 2019

**Abstract:** Background: Kanglaite<sup>®</sup> injection (KLT) exhibits anti-cancer activities in a variety of cancers. The main ingredient is the traditional Chinese medicine Coix seed oil, which has been previously used in the adjunctive therapy of cancer in China. The PI3K/Akt/mTOR signaling pathway be able to progress cell survival and proliferation in various cancers. PTEN (Phosphatase and tensin homolog deleted on the chromosome 10) is a major inhibitory controller of the PI3K/Akt/mTOR signaling and a critical cancer-suppressor protein. In this study, we examined the mechanisms underlying the effects of KLT in pancreatic cancer cells and the potential role of PTEN. Methods: Flow cytometry and CCK-8 assay were used to examine apoptosis and proliferation, respectively, of PANC-1 cells. RT-PCR and western blotting were performed to measure the expression of genes and proteins, respectively, in the PI3K/Akt/mTOR signaling pathway. Results: KLT induced growth inhibition and apoptosis in pancreatic cancer cells, and these activities were weakened in PTEN-knockdown cells. KLT treatment also resulted in decreased levels of p-Akt and p-mTOR, along with enhanced PTEN. Conclusions: Our results revealed that KLT can induce apoptosis and suppress proliferation of pancreatic cancer cells through the induction of PTEN and downregulation of pAkt and p-mTOR and the PI3K/Akt/mTOR pathway. Our study shows that KLT could be a potential treatment strategy for pancreatic cancer that harbors functional PTEN.

**Keywords:** Coix, oncogene protein v-akt, pancreatic neoplasms, PTEN phosphohydrolase, TOR serine-threonine kinases

## Introduction

Pancreatic cancer is one of the most intractable cancers in humans, with a 5-year survival rate that is lower than 5% [1]. In China, about 80% pancreatic patients are inoperable at diagnosis because of it can develop imperceptible, therefore, resulting in metastasis [2]. Unfortunately, the incidence of pancreatic cancer is increasing. In the United States, approximately 33,000 patients died of pancreatic cancer annually, meanwhile, nearly 37,000 new cases are diagnosed [3]. Consequently, effective anti-pancreatic-cancer medicines and therapeutic methods are desperately needed to improve the survival rate of patients.

In recent years, some traditional Chinese medicine has been used as a complementary and alternative medicine in cancer treatment in the United States and Europe [4]. Coix seed, a tra-

ditional Chinese medicine, which is used to treat immunological disorders, arthritis, hypertension, cancer metastasis and asthma in China [5]. Coix seed oil is the main ingredient of Kanglaite<sup>®</sup> injection (KLT) [6], which has been widely used in cancer treatment in China [7]. KLT is mainly used as an adjuvant therapy for gastric cancer, liver cancer and non-small cell lung cancer in China [4, 7, 13]. KLT can also reverse multiple drug resistance of cancers when combined with chemotherapy or radiotherapy; furthermore, it can improve the life quality of cancer patients to some degree and dramatically decrease cancer cachexy [5, 6]. Some studies have shown that KLT can stimulate an anticancer immune response and block cells at G2/M phase [8]. KLT can also inhibit the MAPK signaling pathway [9], which can induce cancer cell apoptosis through downregulating the expression of Bcl-2 and COX-2 and upregulating the expression of Fas/Apo-1 [4-7, 10-12].

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We also previously found that KLT suppressed growth and induced apoptosis of pancreatic cancer xenografts [17]. In addition, we found that KLT regulates the PI3K/Akt/mTOR signaling pathway by downregulating the levels of pAkt and p-mTOR in these xenografts.

The PI3K/Akt/mTOR signaling pathway modulates cellular migration, proliferation and differentiation processes [14] and studies have shown that the PI3K/Akt/mTOR signaling pathway can promote tumor proliferation, angiogenesis, invasion and metastasis; moreover, it can prevent cell apoptosis of cancer cells [16]. Many cancers, including pancreatic cancer, show dysregulation of this pathway through aberrations in these factors or the regulators of this pathway [15]. The PTEN is a critical negative regulator of the PI3K/Akt/mTOR signaling pathway. Indeed, several studies showed that knockdown of PTEN resulted in increased cell proliferation and invasion in several cancer cells, including gastric cancer and pancreatic cancer cells.

Although many studies have focused on molecular targeted therapies in human pancreatic cancer that target specific signaling pathways, no studies have clarified the precise mechanisms of KLT in cancer. In the present study, we explored the mechanisms underlying the therapeutic effects of KLT and the potential involvement of PTEN in these anti-cancer functions in human pancreatic cancer.

### Material and methods

#### *Cell culture and treatment*

The human pancreatic cancer cell line PANC-1 was obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). PANC-1 cells were incubated in a 95% air and 5% CO<sub>2</sub> humidified atmosphere in RPMI 1640 (Gibco, USA), which supplemented with 1% streptomycin-penicillin and 10% fetal bovine serum (Hyclone, USA) at 37°C. Cells in experiments were treated with 10 µl/ml of KLT (Zhejiang Kanglaite Pharmaceutical Co., Ltd, Hangzhou, China) for 48 h; cells treated with deionized water served as controls.

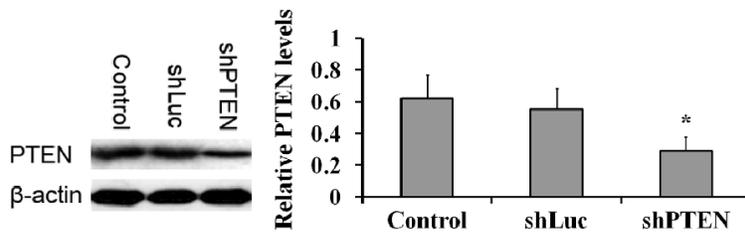
#### *Lentiviral-based RNA interference knockdown of PTEN*

PTEN knockdown in PANC-1 cells was conducted by short hairpin RNAs (shRNAs) as previously

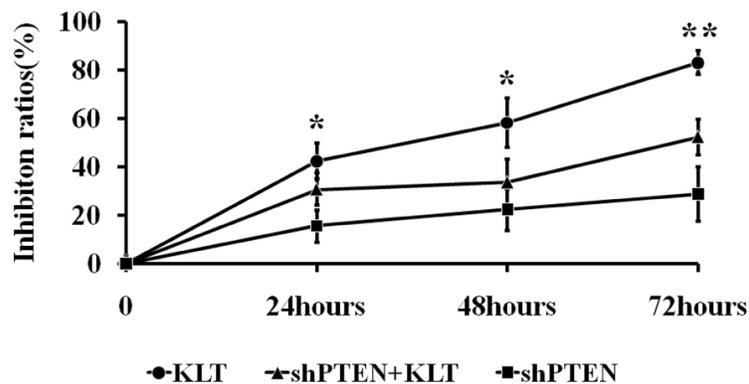
described [18]. PTEN and control shRNA constructs were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). The PTEN shRNA construct (TRCN0000219043, containing the shRNA target sequence 5'-GTA CCG GGG GCT TTA ACT GTA GTA TTT GCT CGA GCA AAT ACT ACA GTT AAA GCC CTT TTT TG-3' for human PTEN) and the luciferase shRNA construct (TRCN0000072247, containing the shRNA target sequence 5'-GAA TCG TCG TAT GCA GTG AAA-3' for a negative control) were used to generate recombinant lentiviral particles. PANC-1 cells were transduced with the viral particles comprising luciferase shRNAs or PTEN for 24 h and then cultured in fresh medium. Cell supernatants were harvested at 36, 48, 60 and 72 h after transduction and then filtered with a 0.45 µm filter. The viral particles were further concentrated by centrifugation at 20,000 × g at 4°C for 2.5 h and then resuspended in fresh medium. Lentiviral particles (shLuc and shPTEN) were used to infect PANC-1 cells with an appropriate multiplicity of infection in medium.

#### *Western blotting*

Western blotting analysis of pancreatic cancer cells were carried out as previously described [22]. Cells were rinsed with ice-cold PBS (pH 7.4), and lysed in cold lysis buffer (1 mM PMSF, 1 mM EGTA, 1 mM EDTA, 100 mM Na<sub>3</sub>VO<sub>4</sub>, 150 mM NaCl, 0.5 mM NaF, 1% Triton X-100, 0.5% NP-40, and 50 mM Tris-HCl, pH 7.4) comprising fresh phosphatase inhibitor and protease cocktail (Calbiochem, USA) on ice for 30 min. The lysates were collected in microfuge tubes and centrifuged at 12,000 g for 10 min at 4°C. The supernatants were stored at -20°C or used immediately. Protein concentrations were examined by a bicinchoninic acid protein assay kit (Pierce, USA). Proteins were separated by 15% SDS-PAGE at 80 V for 2 h and transferred onto polyvinylidene difluoride membranes (Millipore, USA) at 80 V for 2 h. After hatched in blocking solution (PBS with 0.05% Tween-20 and 10% non-fat dry milk) for 1 h at 4°C, the membranes were hatched with primary antibody (rabbit anti-PTEN, rabbit anti-PI3K, rabbit anti-Akt, mouse anti-pAkt, rabbit anti-mTOR, rabbit anti-p-mTOR or mouse anti-β-actin; Cell Signaling, USA) at a dilution of 1:1000 in TBS-Tween-20 (TBST; Sigma, USA) overnight at 4°C, followed by hatched with secondary antibodies (1:2000 dilution in TBST; Cell Signaling, USA). After rinsing, the blots were examined by an



**Figure 1.** PTEN knockdown by RNA interference in pancreatic cancer cells. PANC-1 cells were transduced with viral particles containing PTEN or luciferase shRNAs for 24 h and the knockdown of PTEN was confirmed by Western blotting analysis. Left, representative Western blot image; right, quantification of PTEN protein levels normalized to  $\beta$ -actin. \* $P < 0.05$  vs. control group (untreated cells).



**Figure 2.** Role of PTEN on the effects of KLT on pancreatic cancer cell proliferation. PANC-1 cells lentivirally transduced with PTEN or control luciferase shRNA were cultured in medium with or without 10  $\mu$ l/ml KLT. CCK-8 assays were performed at 24, 48 and 72 h. The growth curves show the mean of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  vs. shPTEN group.

enhanced chemiluminescence detection system (Amersham International, UK) in accordance with the manufacturer's instructions. Protein levels were normalized using  $\beta$ -actin as the internal standard.

#### Cell proliferation assays

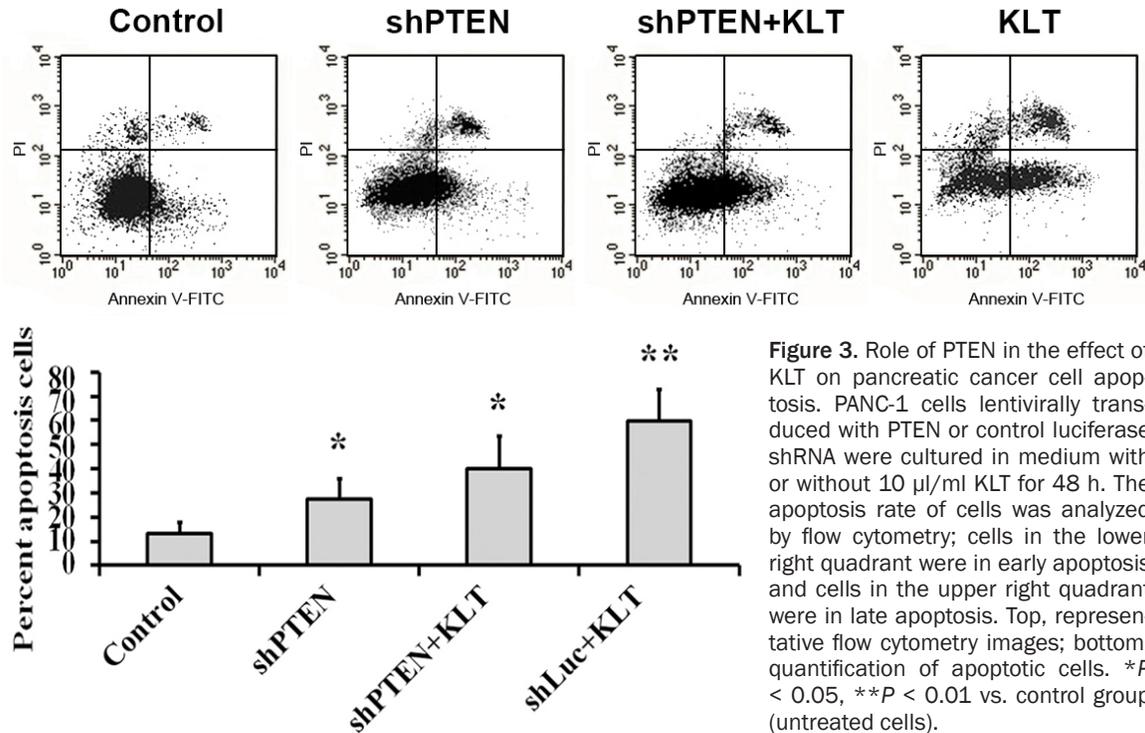
Cell proliferation was assessed by CCK-8 assays as previously described [19]. Briefly, cells were seeded in 96-well plates at a density of 5,000 cells/well, and then cells were treated with 10  $\mu$ l/ml of KLT over 24, 48 and 72 h after incubated at 37°C for 24 h. Next, CCK-8 solution (10  $\mu$ l/well) (Dojindo, Kumamoto, Japan) was added and cells were cultured for 2 h; the absorbance was then examined at 450 nm by a microplate reader (Bio Rad, USA). The cell proliferation reduction was expressed as inhibition rate (%) = (1-treated group OD values/control group OD values)  $\times$  100%.

#### Apoptosis assays

Cell apoptosis was determined using an Annexin V-FITC apoptosis detection kit (BioVision, USA) as previously described [20]. Briefly, pancreatic cancer cells were collected by trypsinization and rinsed with PBS; next, 10  $\mu$ l Annexin V-FITC (1 mg/ml) and 10  $\mu$ l propidium iodide (PI; 2.5 mg/ml) were added to the cells and it were cultured in the dark at 20°C for 10 min. Cells were analyzed by flow cytometry using a FACSCalibur (BD Biosciences, USA), with the FITC signal in FL1 and the PI signal in FL2. Cells in the upper right quadrant of the FL1/FL2 dot plot (labeled with PI and Annexin V-FITC) were considered in late apoptosis, and cells in the lower right quadrant (labeled with Annexin V-FITC) were in early apoptosis

#### RT-PCR analysis

PTEN, PI3K, Akt, and mTOR mRNA expressions were examined by RT-PCR as previously described [21]. GAPDH was employed as an internal control. Total RNA was extracted from cells by TRIzol (Invitrogen, USA). RT-PCR was carried out using the AccessQuick RT-PCR System (Promega, USA) in accordance with the manufacturer's protocol. The primer sets were: GAPDH, 5'-GGA GGT GAA GGT CGG AGT-3' and 5'-CCT GGA AGA TGG TGA TGG G-3'; PTEN, 5'-CAG AAA GAC TTG AAG GCG TAT-3' and 5'-CGT CGT GTG GGT CCT GAG TGA-3'; PI3K, 5'-AGG AGC GGT ACA GCA AAG AA-3' and 5'-GCC GAA CAC CTT TTT GAG TC-3'; AKT, 5'-TGA AAA CCT TCT GTG GGA CC-3' and 5'-TGG TCC TGG TTG TAG AAG GG-3'; mTOR, 5'-CTG GGA CTC AAA TGT GTG CAG TTC-3' and 5'-GAA CAA TAG GGT GAA TGA TCC GGG-3'. Thirty cycles of amplification were carried out under the following conditions: denaturation at 94°C for 30 s, annealing at the gene-specific temperature (55°C for PTEN, 59°C for PI3K, 58°C for AKT, and 56°C for mTOR) for 30 s, and extension at 72°C for 1 min. The PCR products were exam-



**Figure 3.** Role of PTEN in the effect of KLT on pancreatic cancer cell apoptosis. PANC-1 cells lentivirally transduced with PTEN or control luciferase shRNA were cultured in medium with or without 10  $\mu$ l/ml KLT for 48 h. The apoptosis rate of cells was analyzed by flow cytometry; cells in the lower right quadrant were in early apoptosis and cells in the upper right quadrant were in late apoptosis. Top, representative flow cytometry images; bottom, quantification of apoptotic cells. \* $P < 0.05$ , \*\* $P < 0.01$  vs. control group (untreated cells).

ined by electrophoresis on 2% agarose gel, and images were analyzed by NIH Image software (Scion, USA).

#### Statistical analysis

Statistical analysis was carried out by SPSS 18.0 software. Data were expressed as mean  $\pm$  SD. Differences in both parameters were examined for significance by Student's *t*-tests. Differences among three and more parameters were examined by one-way ANOVA. A value of  $P < 0.05$  means a statistical significance.

#### Results

##### *PTEN knockdown by RNA interference in pancreatic cancer cells*

To examine the potential role of PTEN in the cellular effects of KLT in pancreatic cells, we first generated lentivirus expressing PTEN shRNA. PANC-1 cells were transduced with lentiviral particles containing PTEN or control luciferase shRNAs for 24 h, and the knockdown of PTEN was examined by Western blotting analysis (**Figure 1**). While PTEN expression levels in the shLuc control cells were similar to those of control untreated cells ( $P > 0.05$ ), PTEN expression levels were significantly lower in the shP-

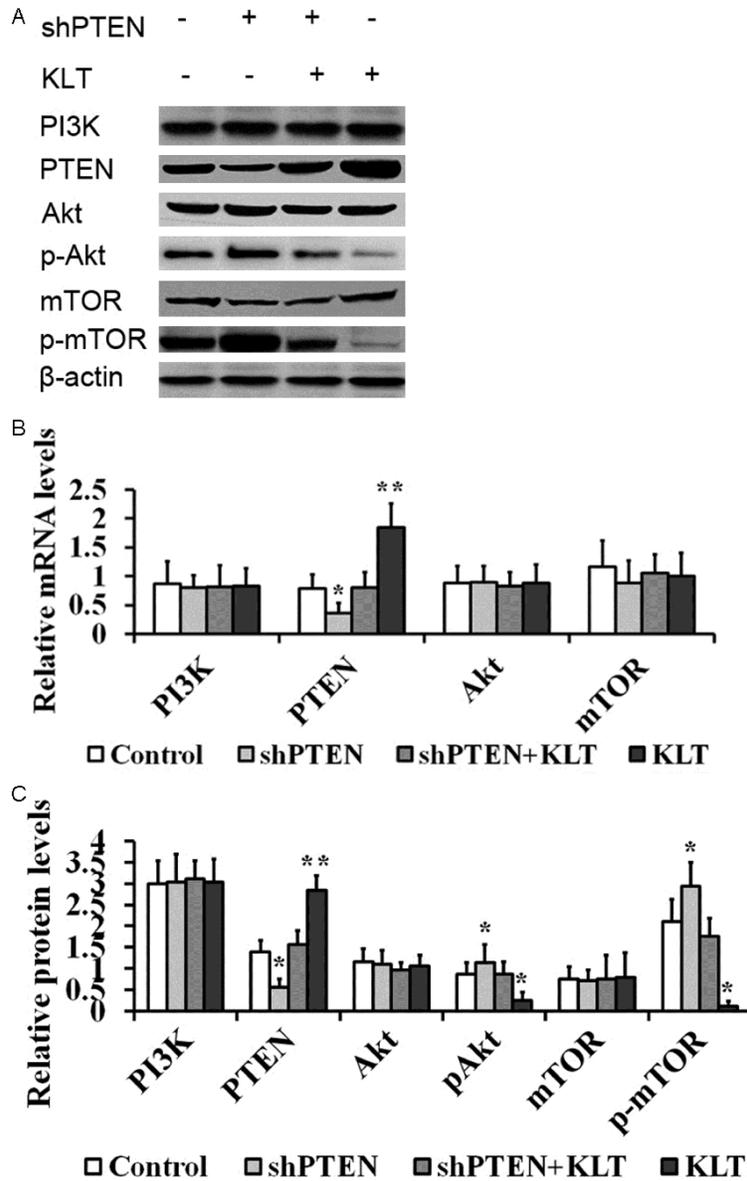
TEN groups than the controls ( $P < 0.05$ ), indicating successful knockdown of PTEN by PTEN shRNA lentivirus in PANC-1 cells.

##### *KLT inhibition of pancreatic cancer cell proliferation involves PTEN*

We next examined the role of PTEN in KLT-mediated effects on cell proliferation by culturing PANC-1 cells with or without PTEN knockdown in the presence of 10  $\mu$ l/ml KLT and evaluating proliferation by CCK-8 assays (**Figure 2**). KLT treatment induced a strong inhibition of PANC-1 cell proliferation. In addition, the proliferation inhibition of KLT-treated cells was significantly higher than the shPTEN group at 24, 48 and 72 h ( $P < 0.05$ ,  $P < 0.05$ ,  $P < 0.01$ , respectively). However, in shPTEN cells, KLT treatment resulted in a reduced proliferation inhibition, which was similar to levels of the shPTEN alone group ( $P > 0.05$ ). Together this suggests that PTEN is involved in the effects of KLT on inhibiting PANC-1 cell proliferation.

##### *Effect of KLT on pancreatic cancer cell apoptosis involves PTEN*

We next examined the effects of KLT on the apoptosis of PANC-1 cells in the presence or absence of PTEN using flow cytometry (**Figure**



**Figure 4.** Role of PTEN in KLT-mediated effects on the PI3K/Akt/mTOR pathway in pancreatic cancer cells. (A-C) PANC-1 cells lentivirally transduced with PTEN or control luciferase shRNA were cultured in medium with or without 10  $\mu$ l/ml KLT for 48 h. Expression levels of the indicated proteins were detected by western blotting (A). Expression levels of the indicated genes were detected by RT-PCR (B). Protein levels from (A) normalized to those of  $\beta$ -actin are shown in the graph (C). \* $P < 0.05$ , \*\* $P < 0.01$  vs. control group (untreated cells).

3). KLT treatment induced a significantly higher apoptosis rate than control (untreated) cells ( $P < 0.01$ ). However, KLT treatment of shPTEN expressing PANC-1 cells (shPTEN+KLT group) showed reduced apoptosis compared with KLT treatment alone. Together this suggests that PTEN is involved in the effects of KLT on inducing apoptosis of PANC-1 cells.

*KLT regulates the PI3K/Akt/mTOR pathway in pancreatic cancer cells via PTEN*

We next examined changes in the PI3K/Akt/mTOR pathway in response to KLT treatment of PANC-1 cells with the presence or absence of PTEN (Figure 4). We confirmed that the expression of PTEN mRNA in shPTEN cells was significantly lower than control (untreated) cells ( $P < 0.05$ ) (Figure 4A). Interestingly, KLT-treated cells showed significantly higher PTEN mRNA levels compared with control groups ( $P < 0.01$ ). No changes in PI3K, Akt or mTOR mRNA levels were observed among the experimental groups.

We also evaluated the protein levels of PI3K, PTEN, Akt, pAkt, mTOR and p-mTOR in PANC-1 cells treated with KLT in the presence or absence of PTEN (Figure 4). As expected, the expression of PTEN in shPTEN groups was significantly lower than control cells ( $P < 0.01$ ). In addition, the expression of PTEN in response to KLT treatment was significantly higher than controls ( $P < 0.01$ ). However, the expression of PTEN in shPTEN+KLT cells was unchanged compared with the controls ( $P > 0.05$ ). While knock-down of PTEN had no impact on steady state Akt and mTOR protein levels, the pAkt and p-mTOR expression levels in shPTEN cells were significant-

ly higher than control (no treated) groups ( $P < 0.05$ ). Notably, KLT treatment cells resulted in a significant reduction of pAkt and p-mTOR levels compared with controls ( $P < 0.05$ ). However, KLT treatment of cells downregulated for PTEN (shPTEN+KLT cells) did not affect the levels of pAkt and p-mTOR compared with the controls ( $P > 0.05$ ).

## Discussion

In this study, we used RNA interference to examine the contribution and role of PTEN in the cellular effects of KLT on pancreatic cancer cell proliferation, apoptosis and signaling pathway responses.

We found that KLT treatment of PANC-1 cells for 24 h, 48 h and 72 h resulted in significantly higher inhibition of cell proliferation than shPTEN cells ( $P < 0.05$ ,  $P < 0.05$ ,  $P < 0.01$ , respectively). However, cell proliferation of shPTEN cells after KLT treatment was unchanged compared with shPTEN cells ( $P > 0.05$ ). The result suggest that the absence of PTEN may decrease the growth inhibitory effect of KLT on pancreatic cancer cells and that PTEN is involved in KLT inhibition of pancreatic cancer cell proliferation. Our results are consistent with several published studies. Chen *et al.* showed that knockdown of the PTEN gene significantly promoted cell proliferation and migration in colorectal cancer cells [23]. Zhang *et al.* demonstrated that loss of PTEN promoted pancreatic cancer cell invasion and proliferation [24]. Ma *et al.* found that knockdown of PTEN by siRNA transfection can upregulate pancreatic cancer cell proliferation and invasiveness [25].

We found that KLT treatment induced significantly high apoptosis of PANC-1 cells compared with cells ( $P < 0.01$ ); however, the apoptosis rate upon KLT treatment of cells expressing shPTEN was reduced and similar to levels observed in shPTEN cells. These data suggest that the loss of PTEN weakened the apoptosis-promoting ability of KLT in pancreatic cancer cells and that PTEN is involved in KLT promoting pancreatic cancer cell apoptosis.

PTEN is a critical negative regulator of the PI3K/Akt/mTOR signaling pathway. PTEN can dephosphorylate PIP3 and weaken upstream signals. Inactivation or deletion of PTEN will result in activated PI3K/Akt/mTOR pathway.

We performed western blot analysis to elucidate the precise changes in PI3K/Akt/mTOR signaling in response to KLT treatment. As expected, shPTEN cells showed increased pAkt and p-mTOR expression levels compared with control cells ( $P < 0.05$ ). These observations are consistent with previous studies. Zhang *et al.* reported that loss of PTEN led to significant increases in the levels of p-Akt and p-mTOR in

pancreatic cancer cells [24], and Farrow *et al.* reported that increased expression of PTEN can reduce p-Akt levels [26]. Moreover, Li *et al.* found that loss of PTEN led to significant increases in the levels of Akt, phospho-Akt, and phospho-mTOR proteins in endometrial cancer cells [27].

KLT treatment resulted in significantly lower expression of pAkt and p-mTOR compared with control cells ( $P < 0.05$ ). These results are consistent with our previous study, showing that KLT can downregulate the expressions of pAkt and p-mTOR to regulate PI3K/Akt/mTOR signaling pathway in pancreatic cancer xenografts [17]. Interestingly, however, here we found that that KLT treatment induced the expression of both PTEN mRNA and protein in PANC-1 cells. These results suggest that KLT induces the upregulation of PTEN expression, resulting in decreased pAkt and p-mTOR levels and subsequent inhibition of the PI3K/Akt/mTOR signaling pathway. Consistent with this idea, we found that the levels of pAkt and p-mTOR in shPTEN cells after KLT treatment were compromised compared with KLT-treated cells and similar to those in controls ( $P > 0.05$ ). Importantly, this data also suggests that the absence of PTEN may weaken the repressive effects of KLT in human pancreatic cancers.

In conclusion, our results show that KLT can induce apoptosis and inhibit proliferation in pancreatic cancer cells via induction of PTEN to modulate PI3K/Akt/mTOR signaling. Furthermore, the absence of PTEN may compromise the growth inhibiting and apoptosis-promoting activities of KLT on pancreatic cancer cells. Further investigation is essential to determine the effect of KLT on proteins downstream of the PI3K/Akt/mTOR pathway, and we plan to explore the growth inhibition activity, apoptosis-promoting activity and the inhibition on PI3K/Akt/mTOR signaling of KLT in PTEN knockout pancreatic cancer cells in future studies.

## Acknowledgements

This work was supported by the Science and Technology Research Grant (No 2016-KYYWF-0874) of Education Department of Heilongjiang Province, China; We thank Edanz Group ([www.edanzediting.com/ac](http://www.edanzediting.com/ac)) for editing a draft of this manuscript.

**Disclosure of conflict of interest**

None.

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