



# Enhanced antitumor effect via combination of triptolide with 5-fluorouracil in pancreatic cancer

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**Background:** Pancreatic cancer is one of the most aggressive types of cancer, and lack of effective treatment results with a very low 6-month survival rate. This study explores the enhancement of therapeutic effect on human pancreatic cancer cell line (AsPC-1) via combination of triptolide (TPL) and 5-fluorouracil (5-FU).

**Methods:** Cell proliferation was measured by sulforhodamine B (SRB), apoptotic cells were assessed by flow cytometry and western blot for cleaved caspase-3 and vimentin to explore the role of vimentin protein in pancreatic cancer cell line. *In vivo*, AsPC-1 xenografts were treated by TPL, 5-FU and combination respectively and tumor samples were tested by western blot.

**Results:** The results showed that TPL and 5-FU had the effects on AsPC-1 cell line with the dose dependence individually. The combination of TPL and 5-FU enhanced the cytotoxicity significantly compared to 5-FU or TPL alone. Combination index (CI) indicated the effect of TPL combined with 5-FU was highly synergistic. Furthermore, pancreatic cancer cells treated with TPL plus 5-FU exhibited increased apoptosis and higher levels of pro-apoptotic proteins including caspase-3 activity compared to cells treated with TPL or 5-FU alone. In the mechanism study, high vimentin expression was involved in the enhanced apoptosis induced by TPL plus 5-FU. It indicated that the vimentin expression was inhibited by TPL plus 5-FU significantly. *In vivo*, the tumor growth of AsPC-1 xenografts was much slower in the group treated with TPL plus 5-FU compared to the group treated with TPL or 5-FU alone.

**Conclusions:** (I) TPL has a potent therapeutic effect on pancreatic cancer cell lines; (II) the combination of TPL with 5-FU enhances the therapeutic effect *in vitro* and *in vivo* TPL showed high synergistic effect with 5-FU; (III) the inhibitory effect of TPL plus 5-FU on pancreatic cancer is mediated by the induction of apoptosis and TPL enhanced the apoptosis via inhibition of vimentin expression.

**Keywords:** Triptolide (TPL); apoptosis; 5-fluorouracil (5-FU); pancreatic cancer

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## Introduction

Pancreatic cancer is a type of malignant tumor with poor prognosis. Only about 15% to 20% of the patients were able to accept surgical treatment and the 5-year survival rate is less than 1% (1). In order to improve the therapeutic effect, a variety of chemotherapeutic drugs and molecular targeted drugs were used to treat patients with advanced pancreatic cancer (2). Combined therapy can not only reduce the toxicity of chemotherapy drugs to the body but also enhance the sensitivity of tumor cells to drugs. How to enhance the effect of chemotherapy and prevent tumor recurrence and metastasis becomes an urgent research question. Although gemcitabine is the first line chemotherapy for pancreatic cancer, the treatment effect is not satisfactory due to the drug resistance of pancreatic cancer (3). For patients who fail to gemcitabine chemotherapy, the selective use of second-line chemotherapy such as 5-fluorouracil (5-FU) may also be effective (4).

Triptolide (TPL) is one of the main active ingredients of *Tripterygium wilfordii*, which has anti infection, immunity, antitumor and other pharmacological effects (5,6). TPL has been reported with a correlation to the inhibition of nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2) expression, heat shock protein (HSP) expression, nuclear transcription factor (NF- $\kappa$ B) activation pathway and the induction the apoptosis of tumor cells (7-9). TPL can not only induce tumor cells apoptosis but also has the synergistic effect to induce apoptosis; the mechanism may be correlated with the activation of NF- $\kappa$ B, promoting the expression of p53, the activation of MAP kinase and PARP, and caspase cleavage is also involved in the induction of cell apoptosis process (10-12). TPL has been reported to significantly improve the 5-FU effect in colorectal cancer cells (13). 5-FU normally acts as the preferred anti-metabolism drugs for digestive system cancer including pancreatic cancer, which mainly inhibits S DNA synthesis and block G1-S phase as its anti-tumor mechanism (14). It was also reported that TPL combined with gemcitabine to enhance antitumor effect on pancreatic cancer cells (15). Based on the above reviewed studies, we use the TPL alone or combined with 5-FU to treat pancreatic cancer cells AsPC-1. This analysis aims to find out the key protein involved in TPL in combination with 5-FU on pancreatic cancer apoptosis which plays an important role in resistance to chemotherapeutic drugs.

Vimentin can be added to the list of intermediate filament proteins, including the nuclear lamins and

cytokeratins 18 and 19, which are cleaved by caspases during apoptotic cell death (16). The vimentin proteolysis occurs secondary to caspase pathway activation (16,17). Vimentin proteolysis releases potential pro-apoptotic proteolytic fragments that can markedly enhance apoptosis (16,17). Therefore, vimentin protein was investigated to be involved in the enhanced mechanism.

In this study, we explore the effect of TPL used alone or in combination with 5-FU and attempt to evaluate this new therapeutic approach in controlling pancreatic cancer. The underlying mechanism by which TPL exerts its enhancement effect on pancreatic cancer was also explored. This study aims to find out the key protein involved in TPL in combination with 5-FU on pancreatic cancer apoptosis which plays an important role in resistance to chemotherapeutic drugs.

## Methods

### *Cells and reagents*

AsPC-1, a human pancreatic cancer cell line, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100  $\mu$ g/mL of streptomycin and 100 U/mL of penicillin. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>.

TPL with 99.9% purity was purchased from the Institute of Medical Research (Fuzhou, China), 5-FU was purchased from Changchun Changqing Pharmaceutical Group Company, sulforhodamine B (SRB) from Sigma (Louis, MO, USA), Caspase-3 Assay Kit from Molecular Probes (OR, USA), cleaved caspase-3 and vimentin monoclonal antibodies from Cell Signaling Technology, Inc. (Beverly, MA).

### *Cell viability assay*

Viability of AsPC-1 after different treatments was evaluated by SRB assay. Briefly, AsPC-1 cells ( $1 \times 10^3$  cells/well) were plated in triplicates in 96-well plates and 24 hours later and the fresh media was added with different concentrations of TPL or 5-FU alone and TPL plus 5-FU. The control cells were treated with the same concentration of vehicle (0.01% DMSO). After further incubation for 24, 48 and 72 hours respectively, 50  $\mu$ L of 10% trichloroacetic acid was added to fix cells at 4 °C for 2 hours, stained with 70  $\mu$ L of 0.3% SRB

for 30 min, color developed with 200  $\mu$ L Tris base (10 mM, pH=10.5), and read at  $A_{490}$ .

### *Flow cytometric analysis of apoptosis*

Forty-eight hours after treatment with TPL alone, 5-FU alone or TPL plus 5-FU, cells were harvested and stained with Annexin V for 30 min and then with PI right before the analysis with flow cytometer according to the manufacturer's instructions. The percentage of cells that were Annexin V positive and PI positive was compared among the different treatment groups. The data were analyzed with the CellQuest software. Each sample was performed in duplication and the experiment was conducted with three repetitions.

### *Assays for activity of caspase-3*

The activity of caspases 3 was measured with fluorescent substrate assay according to the instruction of the manufacturer (Molecular Probes, OR, USA). Briefly,  $1 \times 10^6$  cells treated with either vehicle alone or TPL or 5-FU alone or both in combination for 24, 48 and 72 hrs were collected and resuspended in cold lysis buffer. 50  $\mu$ L of  $2 \times$  reaction buffer was added to 50  $\mu$ L of cell lysate and incubated for 2 h at 37 °C with DEVD-R110, a caspase-3 substrate, which released fluorescence after its cleavage by fluorometer.

### *Western blot for cleaved caspase-3 and vimentin*

The cleavage of caspase-3 is a characteristic marker of apoptosis. For detection this, AsPC-1 cells in 100 mm dishes at 80% confluence were treated vehicle alone, TPL or 5-FU or in combination for 24–72 hours, and then harvested with 1 mL of lysis buffer (1% Triton X-100, 0.5% Na deoxycholate, 0.5  $\mu$ g/mL leupetin, 1 mM EDTA, 1  $\mu$ g/mL pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride). The protein concentration of the lysate was determined by the bicinchoninic acid method (Pierce, Rockford, IL, USA). The protein was loaded onto a 10% SDS-PAGE, electrophoresed, and transferred to a nitrocellulose membrane. The loading and transferring of equal amounts of protein were confirmed by staining the membrane with a Ponceau S solution (Sigma). The membranes were blocked with 5% fat-free milk in PBS (pH=7.4) for 30 min and then incubated overnight with 0.2  $\mu$ g/mL of the anti-caspase-3 monoclonal antibody, which recognizes the

17 kDa cleavage fragment. After washing, the membranes were incubated with HRP-labeled secondary antibodies for an hour followed with ECL exposure. For re-probing-tubulin, the blots were stripped with a buffer containing 50 mM Tris-HCl (pH=6.8), 2% sodium dodecyl sulfate, and 0.1 M  $\beta$ -mercaptoethanol and detected with GAPDH antibody as loading control. Vimentin protein was performed as what we described above.

### *Xenografts in athymic mice*

AsPC-1 cells were grown in 150 mm<sup>2</sup> dishes to 80% confluence, harvested with 10 mM EDTA and resuspended in PBS for  $10^7$  cells/mL. A suspension of  $2 \times 10^6$  cells in a 0.2 mL PBS was injected subcutaneously (s.c.) into the hind leg of athymic nude mice using a 27.5-gauge needle. Tumors were allowed to grow for seven days before treatment. Thirty-two nude mice with established tumors (around 100 mm<sup>3</sup>) were divided into four groups and treated with: (I) vehicle alone; (II) 12 mg/kg of 5-FU three times weekly for 4 weeks; (III) 150  $\mu$ g/kg of TPL three times weekly for four weeks; (IV) TPL combined with 5-FU. Tumor size was measured three times per week with vernier calipers. The tumor volume was determined according to the formula (length  $\times$  width<sup>2</sup>)/2. At the end of the experiment, the mice were killed and the tumors were removed and measured. The animal protocol was approved by Animal Care and Use Committee at Fujian Medical University.

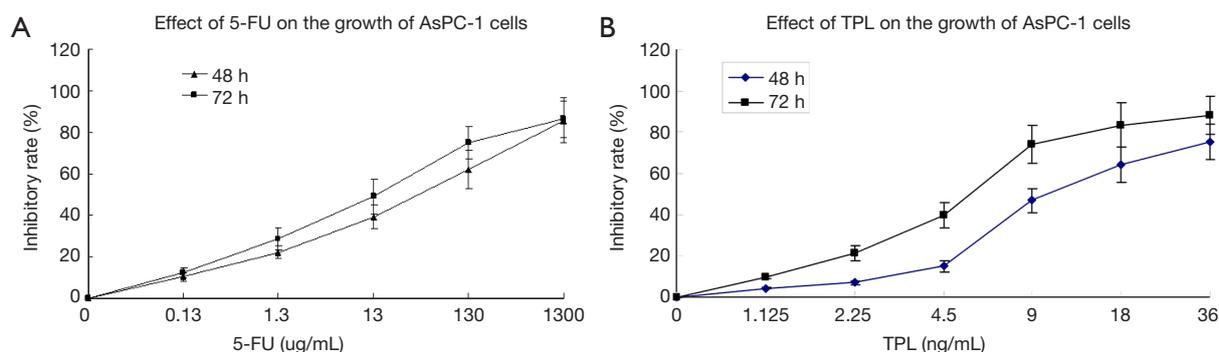
### *Statistical analyses*

All data are expressed as the mean  $\pm$  SD of at least three determinations, unless otherwise stated. The data were analyzed by ANOVAs and *t*-tests. The results were considered significant at  $P < 0.05$ .

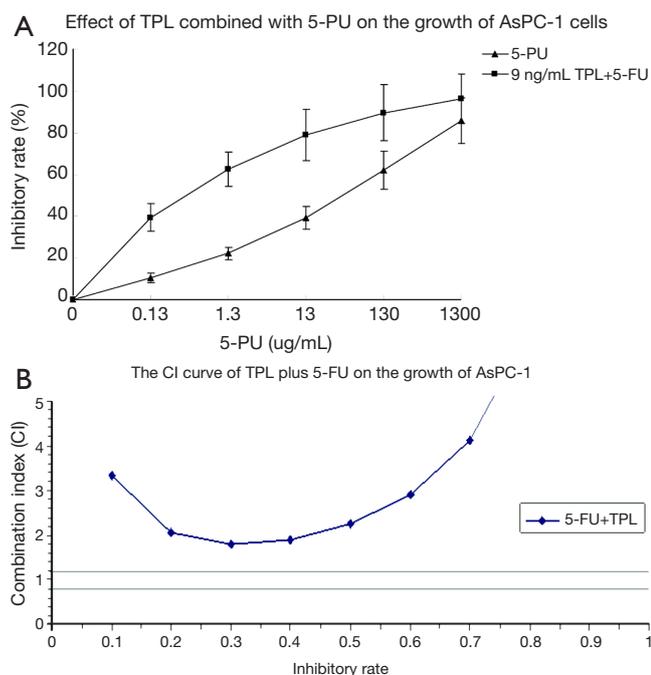
## **Results**

### *Effect of TPL or 5-FU alone on the growth of AsPC-1 cells*

The results of SRB assay showed TPL or 5-FU alone significantly inhibited AsPC-1 cell proliferation in a dose- and time-dependent manner ( $P < 0.01$ ). As shown in *Figure 1A*, 5-FU at 13  $\mu$ g/mL had an inhibitory rate of 37.15% and at 1,300  $\mu$ g/mL the inhibitory rate was 81.79% after 48 hours treatment. The results (*Figure 1B*) showed that the inhibitory rate was 40.68% at 9 ng/mL of TPL and increased to 78.43% at 36 ng/mL of TPL after 48 hours treatment.



**Figure 1** Cells proliferation inhibited by TPL or 5-FU was determined with the SRB assay. AsPC-1 cells were treated with TPL or 5-FU at different concentrations. (A) Inhibitory rate of 5-FU on ASPC-1 cells proliferation. Cells were treated with 5-FU at the indicated concentrations for 48 and 72 hrs; (B) inhibitory rate of TPL on ASPC-1 cells proliferation. Cells were treated with TPL at the indicated concentrations for 48 and 72 hrs. Cells inhibitory was determined with the SRB assay. Each point represents the mean  $\pm$  SD (error bars) from three independent experiments.



**Figure 2** The combined effects of TPL and 5-FU on the AsPC-1 cells. AsPC-1 cells were treated with TPL combined with 5-FU at a constant molar ratio: 1:360. After 48 h treatment, the inhibitory rate of cells proliferation was determined by SRB. The combination index (CI) were determined using NDST-21 software as described before. As shown in *Table 1*:  $CI > 1.2$ , combination is synergism;  $0.8 \leq CI \leq 1.2$ , combination is additive;  $CI < 0.8$ , combination is antagonism. It indicates that TPL and 5-FU had synergistic effects on inhibiting proliferation of AsPC-1 cells at different concentrations.

**Effect of TPL combined with 5-FU on the growth of AsPC-1 cells**

To define an optimal dose of TPL for the combination with 5-FU, the human AsPC-1 pancreatic cancer cells in 96 well plates were treated with different dose of 5-FU alone or combined with 9 ng/mL of TPL for 2 days and the cells viability was measured by SRB. As shown in *Figure 2A*, 13 ug/mL of 5-FU alone can inhibit 37.15% AsPC-1 cells; however, when 9 ng/mL TPL was added, the inhibitory rate increased to 78.52%, suggesting that the combination of TPL with 5-FU achieved a better treatment effect. To further determine this synergistic effect, we analyzed the interaction between TPL and 5-FU using NDST-21 software (18-20), a widely used statistical software to analyze and quantify the synergic effect of drugs in China. The results (*Figure 2B*) showed that the combined application of TPL plus 5-FU in AsPC-1 cells with different concentration has an obvious synergy. *Table 1* indicated that we calculated two-drug combination index (CI) via the inhibition rate (0.1–1). When  $CI > 1.2$ , two drugs have synergy; when  $0.8 \leq CI \leq 1.2$ , two drugs are additive; when  $CI < 0.8$ , two drugs have antagonism.

**Combination of TPL and 5-FU enhances the apoptosis of AsPC-1 cells via inhibition of vimentin expression**

To characterize the apoptosis induced by TPL and 5-FU, Annexin V/PI staining was used for the quantification of pancreatic cancer cells treated by TPL, 5-FU alone or

**Table 1** Combination Index of TPL plus 5-FU on AsPC-1

Inhibitory rate	Combination Index (CI)
0.1	3.318
0.2	2.072
0.3	1.812
0.4	1.900
0.5	2.248
0.6	2.911
0.7	4.119
0.8	6.605
0.9	13.995

CI >1.2, combination is synergism;  $0.8 \leq CI \leq 1.2$ , combination is additive;  $CI < 0.8$ , combination is antagonism. TPL, triptolide; 5-FU, 5-fluorouracil.

combination for 48 hours. Assessed with flow cytometry, while the cells stained positive with Annexin V/PI were 11.39% or 37.18% with 5-FU or TPL treatment, the combined treatment induced apoptotic cells up to 61.99% (Figure 3A). Our data demonstrated that TPL and 5-FU alone significantly promoted apoptosis compared with group control ( $P < 0.01$ ), and the combined effects were stronger than the single treatment ( $P < 0.01$ , Figure 3A). The key apoptotic molecules in common path were also studied. The caspase-3 activity was enhanced to reach the peak at the 48th hour and last to the 72<sup>nd</sup> hour after the combined treatment (Figure 3B), which was consistent with the increased cleaved form of caspase-3 as demonstrated by western blot (Figure 3C). We used two-dimensional gel electrophoresis to screen out vimentin protein involved in the mechanism of combined treatment (data not shown). To further explore the role of vimentin in the enhanced apoptosis on AsPC-1 cells, vimentin protein expression in AsPC-1 cells with different treatments was determined by western blot. The results showed TPL alone decreased vimentin expression compared to 5-FU alone, but TPL plus 5-FU significantly decreased more compared to TPL alone (Figure 3D). The data suggest the synergistic effect of TPL combined with 5-FU might get through inhibiting vimentin expression to induce apoptosis.

#### **Combination of TPL with 5-FU enhances therapeutic effect *in vivo***

To determine whether the combination effect of pancreatic

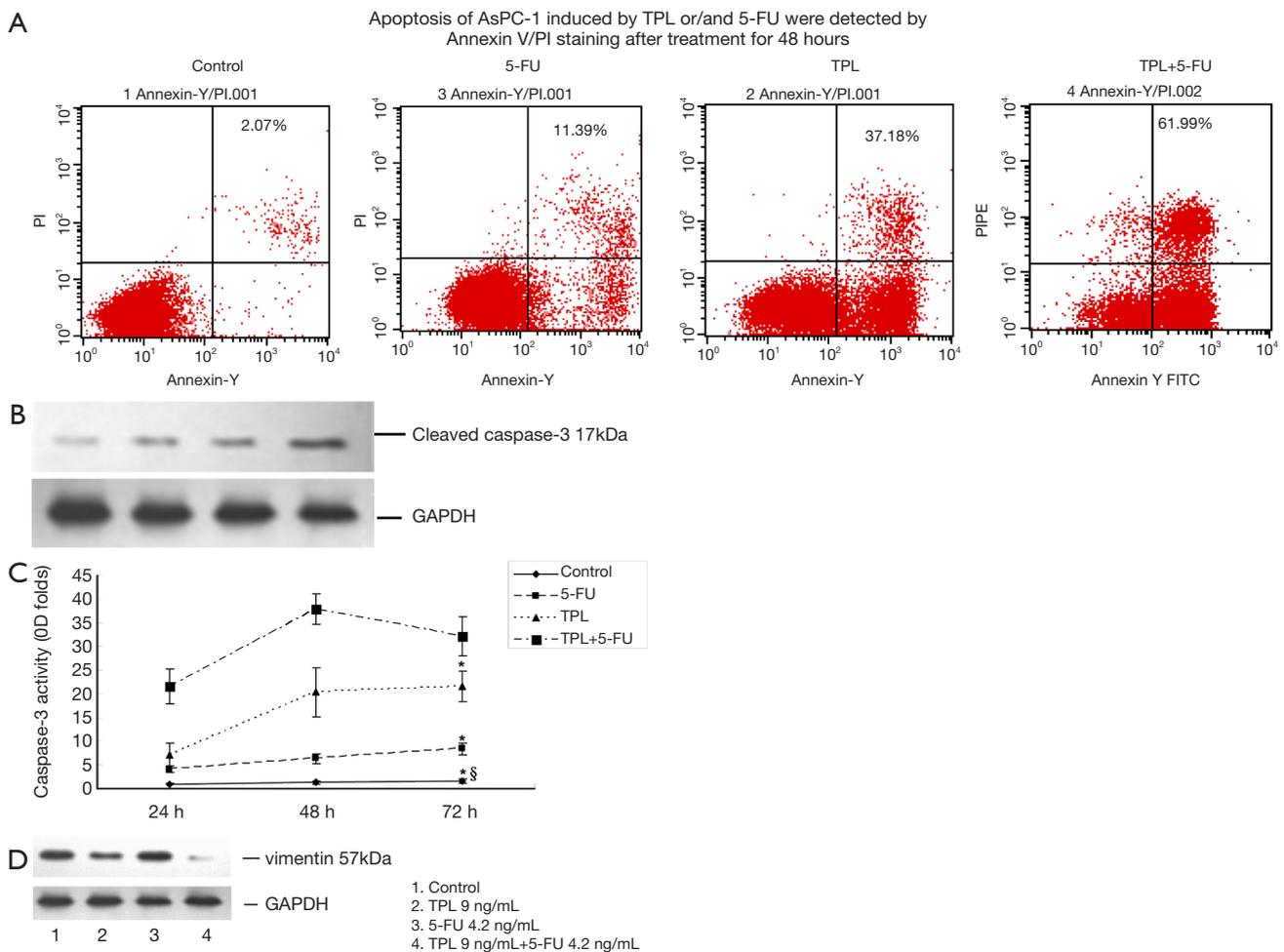
cancer cells *in vitro* was translatable *in vivo*, the AsPC-1 cells injected into the hind leg of nude mice to establish a xenograft model. The tumor growth curve measured starting on day 7 (Figure 4A) showed that the TPL and 5-FU alone could control the tumor progression ( $P < 0.01$ ), but the combined treatment was even more effective. At the end of the experiment, the mice were sacrificed and the tumors were weighted. The tumors in vehicle mock treated group reached a mean of 1.80 gram whereas 0.7–1.0 gram in the single treatment and nearly 0.4 gram in the combined treatment (Figure 4B). These results show that the antitumor effect of TPL combined with 5-FU was superior to the effect of the drugs when used individually.

To determine whether apoptosis was involved in TPL-enhanced regression of AsPC-1 tumors, the cleaved caspase-3 was detected by western blot in tumor sections from all groups. As shown in Figure 4C, TPL or 5-FU alone could produce some cleaved caspase-3, but the combined treatment had an increased cleaved caspase-3 expression, indicating that the TPL-enhanced anti-tumor effect was related with induction of apoptosis, which was consistent with the finding *in vitro*. The vimentin proteolysis occurs secondary to caspase pathway activation. Vimentin proteolysis releases potential pro-apoptotic proteolytic fragments that can markedly enhance apoptosis (19,20). Therefore, vimentin protein in tumor tissues was detected by western blot (Figure 4C). The results show that dual treatment decreased vimentin expression significantly compared to single treatment, which was consistent with the finding *in vitro*. Our data suggest that the enhanced killing effect of dual treatment is related to the vimentin-dependent apoptotic pathway.

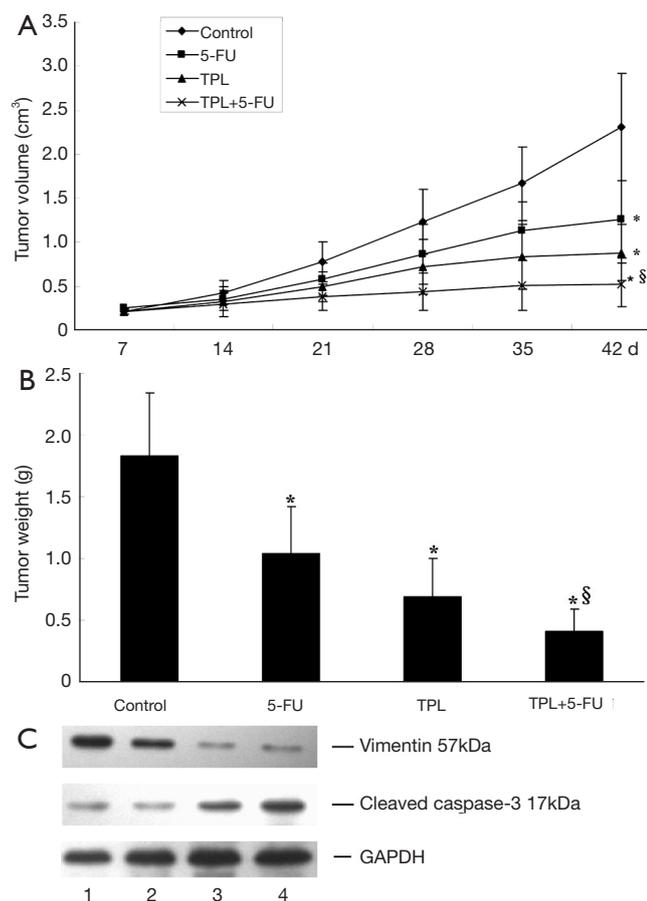
#### **Discussion**

For unresectable advanced pancreatic cancer, the main treatment is chemotherapy; chemotherapy is the main treatment to remit symptoms and provide some inoperable patients with the chance of surgery (21).

5-FU was first reported for chemotherapy in the treatment of pancreatic cancer (22). By blocking the synthesis of deoxy uridine nucleotide into DNA and RNA, 5-FU interferes tumor cell cycle and induces apoptosis of tumor cells. However, only 20–30% of the patients' symptoms can be effectively improved. The effect is not ideal and also accompanied with systemic adverse reactions (22). Clearly, the combination of chemotherapy is a promising strategy (22). In seeking of new agents, we



**Figure 3** TPL in combination with 5-FU enhances apoptosis via inhibiting vimentin expression in AsPC-1 cells. (A) Annexin V/PI staining analysis of AsPC-1 cells for the induction of apoptosis. A significant increase in apoptosis was observed with the AsPC-1 cells after in combined treatment as compared to the untreated and single treatment groups ( $P < 0.01$ ). The AsPC-1 cells showed a significant increase in apoptotic cell death after TPL or 5-FU alone as compared to the untreated group ( $P < 0.01$ ). The AsPC-1 cells showed a significant increase in apoptotic cell death after the treatment with TPL plus 5-FU as compared to the single treatment groups ( $P < 0.01$ ); (B) TPL in combination with 5-FU enhances the activity of caspases 3 in AsPC-1 cells. AsPC-1 cells were left untreated or treated with 5-FU or TPL or in combination. The enzymatic activity of cell lysates towards tetrapeptide chromogenic substrates DEVD-R110 for Caspase-3 was determined. Caspase-3 activity was expressed with OD folds; (C) TPL in combination with 5-FU enhances cleavage of caspase-3. The levels of caspase-3 cleavage in the lysate of AsPC-1 cells were examined by western blot. The result indicated that increased cleaved caspase-3 was induced by TPL or 5-FU alone, and combined treatment increased more caspase-3 cleavage; (D) TPL in combination with 5-FU inhibits vimentin expression. The levels of vimentin and caspase-3 cleavage in the lysate of tumor tissues were examined by western blot. The result indicated that vimentin expression was reduced by TPL or the combined treatment as compared to the vehicle and 5-FU alone, and the combined treatment show more inhibition on vimentin expression than TPL alone. \*,  $< 0.01$  in comparison with untreated cells; §,  $< 0.01$  in comparison with TPL or 5-FU alone. The similar result was obtained in other three separated experiments.



**Figure 4** TPL in combination with 5-FU enhances apoptosis and inhibits the growth of AsPC-1 xenografts. Each mouse of the eight in each of the three groups was injected i.v. with 250  $\mu\text{g}/\text{kg}$  TPL three times weekly or 12  $\text{mg}/\text{kg}$  5-FU three times weekly or combined treatment for 4 total weeks, respectively. All the treatments were stopped on Day 38. (A) Effect of different treatments on growth volume of AsPC-1 tumors; (B) effect of different treatments on AsPC-1 tumors weight; (C) analysis for cleaved caspase-3 and vimentin expression. The levels of vimentin and caspase-3 cleavage in the lysate of AsPC-1 tumor tissues were examined by western blot. The result indicated that vimentin expression was reduced by TPL or the combined treatments compared to the vehicle alone. It proved that increased cleaved caspase-3 depended on single and combination treatments (C). \*,  $P < 0.05$  vs. control, §,  $P < 0.01$  vs. single therapy.

found that TPL at an extremely low dose (9  $\text{ng}/\text{mL}$  *in vitro* and 0.25  $\text{mg}/\text{kg}$  *in vivo*) could exert strong inhibitory effect on the AsPC-1 pancreatic cancer cells and TPL-induced apoptosis has been examined in several types of cells (23-25).

Due to the differences of cell-intrinsic suicide

machinery among different cells, the sensitivity of cells to the apoptotic stimuli varies with cell lines (24-26). Among the three cell lines of pancreatic cancer, we found that AsPC-1 was less sensitive to chemo-drug including gemcitabine and 5-FU than other cell lines (data not shown). Therefore, AsPC-1 was chosen as the target cells for the treatment of TPL combined with 5-FU. The *in vitro* data showed the extent of apoptosis was greatly enhanced by the combined treatment compared to TPL or 5-FU alone in this drug-resistant cell line.

It was reported that TPL combined with 5-FU had a strong synergistic effect and significantly enhanced the inhibitory effect of 5-FU on the proliferation in colon cancer cell HT-29 (27). Other studies reported that TPL enhanced 5-FU-induced apoptosis in hepatocellular carcinoma cell line SMMC 7721 (28). However, the specific target of TPL-enhanced apoptosis of tumor cells is not clear.

In our study, we investigated the inhibitive effect of TPL combined with 5-FU on human pancreatic cancer cells *in vitro* and *in vivo*. We found that TPL or 5-FU alone inhibited significantly the proliferation of AsPC-1 cells in a dose- and time-dependent manner. The combined effect of TPL and 5-FU on the growth of human pancreatic cancer cells was much stronger than that of TPL or 5-FU alone *in vitro* and *in vivo* and the combined effect was synergistic at lower concentrations. We believe that the potential to translate this novel combination therapy into clinical trial is possible since 5-FU is a standard chemotherapy drug and TPL has been used to treat a number of tumors in pre-clinical trial (24-26). The use of this novel therapeutic strategy is expected to control the devastating pancreatic cancer and prolong the patient's life.

In the process of investigating the mechanism of this synergistic effect, we found vimentin expression might be correlated with the enhanced mechanism. Vimentin is the gene encoding the intermediate filament protein. It is involved in cellular structure and integrity, which is a Wnt activity-targeted gene expressed in normal mesenchymal cells (29,30). Vimentin influences cell shape and motility in the process of epithelial-to-mesenchymal transition (EMT) that occurs during metastasis (29-31). The role of vimentin in apoptosis has been elucidated. Vimentin undergoes rapid proteolysis upon diverse pro-apoptotic stimuli, including Fas, TRAIL, TNF- $\alpha$ , and tamoxifen (31). This vimentin proteolysis occurs secondary to caspase pathway activation, resulting in cleavage of vimentin by multiple caspases at several sites (Asp85, Asp259, and Asp429) (16). It is possible that vimentin proteolysis and collapse contribute to many

of the morphological manifestations of apoptosis, including cellular rounding, nuclear condensation, and packaging of the debris of dying cells into apoptotic bodies (17). Furthermore, Vimentin proteolysis releases potential pro-apoptotic proteolytic fragments that can markedly enhance apoptosis (16,17). This might explain the effect of vimentin in enhancing the apoptosis of AsPC-1 cells induced by TPL combined with 5-FU. Therefore, vimentin might be a relevant target for cancer therapeutic interventions. Since vimentin is overexpressed in a number of cancers, we think it is a potential cancer therapeutic target. Besides its known role in affecting cell structure, integrity, and reaction to stress, vimentin may influence cell signaling. It deserves further investigation.

In summary, TPL or 5-FU alone can inhibit the proliferation of AsPC-1 cells and induce cells apoptosis, and their combination has a synergistic effect on killing AsPC-1 cells. The synergistic mechanism is related with vimentin expression which may play the key role in inducing apoptosis on human pancreatic cancer cell line AsPC-1. However, the complete apoptotic signaling of combined treatment is still unclear and needs to be investigated further.

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### Footnote

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2018.01.17>). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This article was involved in animals' study and the animal protocol was approved by Animal Care and Use Committee at Fujian Medical University and all operations complied with the animal experiment regulations.

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