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Original Article

Combined strategies of tumor necrosis factor-related apoptosis-inducing ligand and small molecular drugs to inhibit pancreatic cancer cell proliferation and tumor growth

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Abstract: Pancreatic cancer is one of the most lethal human cancers and remains to be a major unsolved health problem. Here we evaluated the effects of Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and combination with small molecules (resveratrol and paclitaxel) on pancreatic cancer *in vitro* and *in vivo*. The Cell proliferation in colo357 and capan-2 cell lines in cultures was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT). Trail induced apoptosis combined with small molecules was then determined by flow cytometry. Orthotopic pancreatic tumor model of capan-2 cells stably expressed RFP (RFP-capan-2) was established. Synergistic cytotoxic effects of TRAIL combined with small molecules *in vivo* were evaluated. The expressions of Caspase-3 of tissues of orthotopic pancreatic tumor were surveyed by immunohistochemistry. In contrast to colo357, cultured capan-2 cells were resistant to TRAIL-induced apoptosis and growth repression, which is reversed by small molecular drugs including resveratrol and paclitaxel. Consistently, combination of TRAIL with resveratrol or paclitaxel represses tumor growth dramatically in an orthotopic pancreatic tumor model. The data indicate novel combination methods to sensitize pancreatic cancer cell to TRAIL and suggests a strategy for pancreatic cancer treatment *in vivo*.

Keywords: Pancreatic cancer, TRAIL, resveratrol, paclitaxel, combination therapy

Introduction

As one of the most lethal cancers, pancreatic cancer is difficult to diagnose until it has severely progressed, which limits the effectivity of treatments such as surgery, chemotherapy and radiotherapy [1]. Moreover, pancreatic cancer is strongly resistant to traditional chemotherapy drugs [2]. Thus, new chemotherapeutic strategies are needed to better understand pancreatic cancer therapy. Combination antitumor drugs have been successfully used to treat diverse tumors, and such attempts are gaining attention for the treatment of pancreatic cancer.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is an apoptosis-inducing member of the TNF gene family and appears to be a relatively safe and promising agent for cancer-specific therapy [3]. TRAIL induces apoptosis in a wide range of cancer cells such as multiple myeloma, leukemia, colon, lung, and breast cancers without obvious cytotoxic effects on normal cells [4, 5]. However, some pancreatic cancer cells are resistant to TRAIL-induced apoptosis due to multiple mechanisms [6, 7]. Therefore, novel strategies to combine TRAIL with other drugs warrant further study [8, 9].

Resveratrol is a small molecule antioxidant [10], and studies suggest that it represses proliferation in various cancer cells *in vitro* [11]. Moreover, studies on animal skin tumor models revealed that resveratrol may be used as a che-

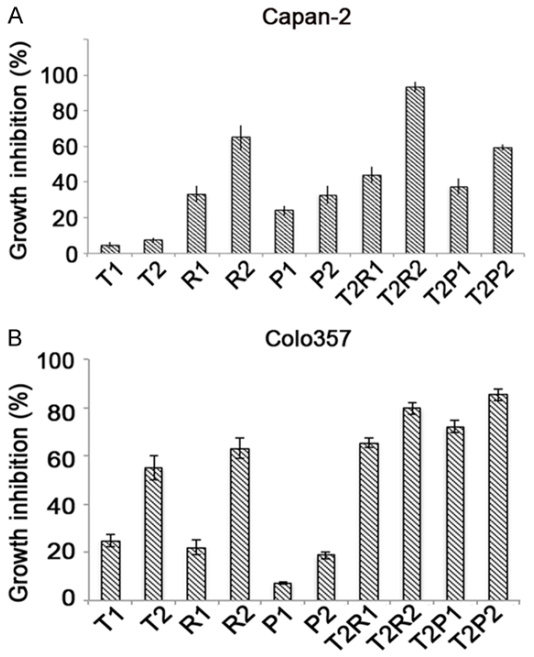

Figure 1. MTT assay. A and B. Proliferation of Capan-2 and colo357 cells in the presence of various molecules. T1: 1 ng/mL TRAIL; T2: 5 ng/mL TRAIL; R1: 20 μM resveratrol; R2: 50 μM resveratrol; P1: 20 μM paclitaxel; P2: 50 μM paclitaxel; T2R1: 5 ng/mL TRAIL plus 20 μM resveratrol; T2R2: 5 ng/mL TRAIL plus 50 μM resveratrol; T2P1: 5 ng/mL TRAIL plus 20 μM paclitaxel; T2P2: 5 ng/mL TRAIL plus 50 μM paclitaxel. Growth inhibition ratio was normalized to cells treated with DMSO (mean sd, n = 3).

mopreventive without significant side effects [12]. Resveratrol mainly inhibits cancer cell proliferation [13], and it is documented to target kinases, cyclooxygenase, and eukaryotic elongation factor [14]. Also, resveratrol inhibits the proliferation of certain pancreatic cancer cell lines [15].

Paclitaxel is a well-established chemotherapeutic used to treat patients with lung, breast, and ovarian cancers [16]. It inhibits cell cycle progression to induce apoptosis and enhances the response rate to therapy. Paclitaxel has been combined with radiotherapy to treat pancreatic cancer [17]. However, its toxicities limit its use that novel strategies for using paclitaxel should be evaluated.

The present study was undertaken to test the potential of single-use TRAIL, resveratrol, and paclitaxel in capan-2 and colo357 pancreatic cancer cell lines. Unlike colo357, capan-2 is resistant to TRAIL-induced apoptosis. Consistent with the anti-proliferative effects in other cancer cells, resveratrol inhibits proliferation in both cell lines, and colo357 had relatively higher resistance to paclitaxel than to capan-2. Furthermore, both resveratrol and paclitaxel sensitized capan-2 cells to TRAIL-induced apoptosis. A synergistic effect of TRAIL with resveratrol or paclitaxel *in vivo* has been confirmed in an orthotopic pancreatic tumor model. Thus, these combination therapies may represent novel approaches to treating pancreatic cancer.

Materials and methods

Ethics statement

All protocols were approved by the Committee on the Ethics of Animal Experiments of the Southeast University (Permit Number: 2011-0133), and the animal studies were carried out in accordance with the ethical guidelines for animal use and care established by Southeast University (Nanjing, China). Surgery was performed under sodium pentobarbital anesthesia, and efforts were made to minimize suffering.

Animals

Six- to seven-week-old male nude mice were purchased from the Laboratory Animal Center of Academy of Military Medical Sciences (Beijing, China) and housed in a specific pathogen-free (SPF) animal facility (Southeast University) that is environmentally controlled (22°C and 12:12 h light:dark cycle, with the light cycle at 08:00-20:00 and the dark cycle at 20:00-08:00) Animals were given ad libitum access to standard laboratory chow and water.

Cell proliferation assay

Cell proliferation was measured using an MTT assay. Cells (2,000/well) were plated onto each well of 96-well plates in RPMI 1640 with 10% FBS and cultured for 24 h. Cells were then treated with TRAIL, resveratrol or paclitaxel as indicated and incubated for another 72 h. Then 50 μL of MTT solution (2 mg/mL; Sigma) was added, and cells were incubated for 2 h. Media containing MTT solution was removed, and dark blue crystals were dissolved by adding 100 μL DMSO. Absorbance was measured using a microplate reader (excitation 550; reference 630 nm). Percent growth inhibition was measured relative to controls treated with DMSO. Each experiment was performed at least in triplicate.

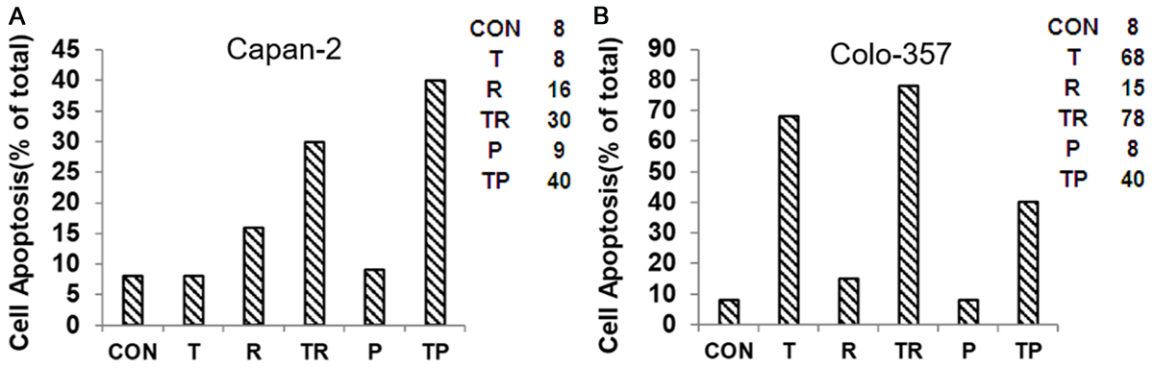

Figure 2. Apoptosis measurement. A and B. Cells were treated with molecules as indicated for 24 h. T: 5 ng/mL TRAIL; R: 50 μM resveratrol; P: 20 μM paclitaxel; TR: 5 ng/mL TRAIL plus 50 μM resveratrol; TP: 5 ng/mL TRAIL plus 20 μM paclitaxel. Cells were collected and stained with PI to measure cell death.

Apoptosis

Cells were treated as indicated, digested with trypsin, and stained with propidium iodide (PI). Cell death was measured using flow cytometry (FACS Calibur, BD Biosciences).

Cell culture and tumor model

Colo357 and capan-2 cells were purchased from American Type Culture Collection (ATCC) and cultured in DMEM or 1640 (Invitrogen) with 10% fetal bovine serum (FBS, Invitrogen). Capan-2 cells stably expressing RFP (RFP-capan-2) were established using antibiotics according to a standard protocol. Cells were subcutaneously (sc) injected into the middle flank (5×10^5 cells in 0.1 mL PBS), and RFP expression was monitored *in vivo*. An orthotopic pancreatic tumor model was established as previously described [18], and mice were anesthetized with isoflurane. Tumor volumes were measured individually with a caliper using this formula: tumor volume = length × width × width × 0.52.

Immunohistochemistry

Tissue sections (5 mm thickness) were prepared according to standard protocols for hematoxylin and eosin staining. Immunohistochemical staining using anti-cleaved caspase-3 (Cell Signaling, #9664) was conducted according to the manufacturer's instructions. Samples were counterstained with hematoxylin.

Statistical analysis

Data are presented as means ± SD. Data were analyzed with SPSS 13.0 software. Differences between two groups were analyzed by Student's t test. The survival curves were determined using the Kaplan-Meier method and estimated with the log-rank test. $P < 0.05$ was considered statistically significant.

Results

TRAIL and small molecules inhibited growth of pancreatic cancer cell lines

To evaluate the anti-tumor potential of TRAIL and small molecules, we tested the growth rate of pancreatic cancer cell lines in the presence of TRAIL combined with resveratrol or paclitaxel. TRAIL treatment offered insignificant inhibitory effects in capan-2 cells, whereas resveratrol at 50 μM (R2) inhibited cells by ~65% (**Figure 1A**). TRAIL plus resveratrol inhibited almost all (90% inhibitory) capan-2 cells (**Figure 1A**). Paclitaxel plus TRAIL gave similar results (**Figure 1A**). In contrast to capan-2, TRAIL inhibited cell proliferation in colo357 cells, and resveratrol alone (50 μM, R2) inhibited more than 60% of cell growth. TRAIL plus resveratrol treatment was not synergistic in a significant manner compared to resveratrol or TRAIL treatment alone (**Figure 1B**). In contrast, paclitaxel inhibited ~20% of colo357 cells and when combined with TRAIL inhibited 80% of cell growth (**Figure 1B**). Thus, resveratrol and paclitaxel are synergistic when combined with TRAIL for repressing pancreatic cancer cell growth.

TRAIL induced apoptosis when combined with small molecules

TRAIL induces apoptosis in many cancer cell lines including pancreatic cancer cell lines.

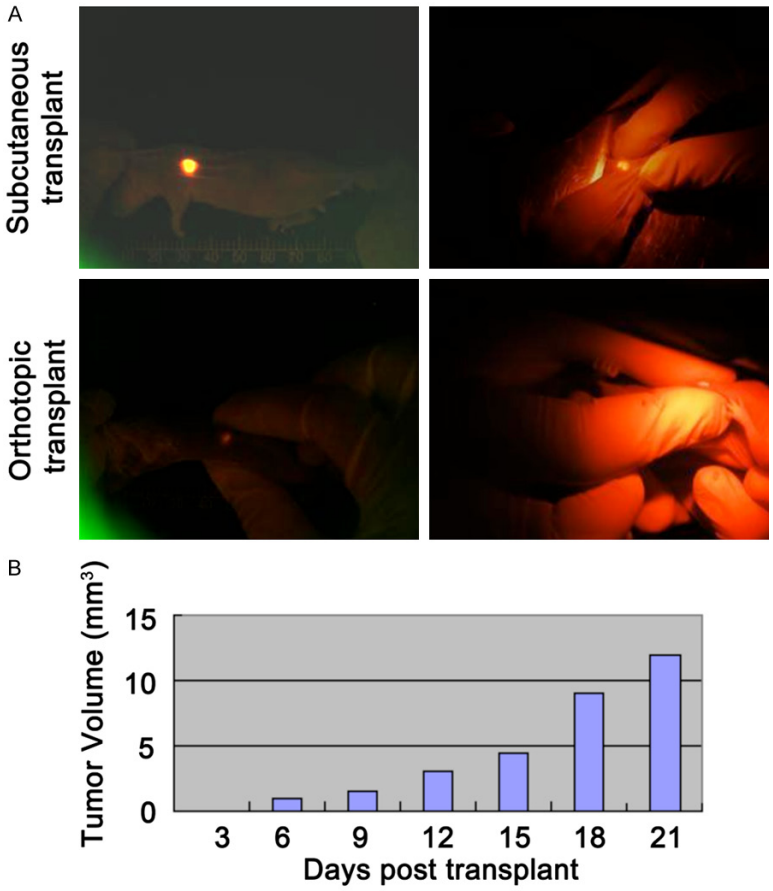

Figure 3. Orthotopic implantation of RFP-capan-2 cells. A. RFP-capan-2 cells were injected subcutaneously (top panel) or orthotopically (bottom panel). Representative images were taken from mice 7 days post-implantation. B. Tumor volume was determined at the times indicated.

However, apoptosis was not increased in capan-2 cells treated with TRAIL, indicating resistance to TRAIL-induced apoptosis (**Figure 2A**). However, twice as many apoptotic cells were observed in capan-2 cells treated with resveratrol and TRAIL compared with resveratrol alone (**Figure 2B**, 30% vs. 16%). Moreover, paclitaxel and TRAIL induced 4-times as many apoptotic cells compared with paclitaxel treatment alone (**Figure 2A**, 40% vs. 9%). In contrast, colo-357 cells were sensitive to TRAIL-induced apoptosis which is consistent with previous studies (**Figure 2B**). Therefore, synergistic effects on apoptosis were observed when TRAIL was combined with resveratrol or paclitaxel (**Figure 2B**).

Synergistic cytotoxicity of TRAIL plus small molecules on pancreatic carcinoma in vivo

We established a TRAIL-resistant capan-2 stable cell line (capan-2RFP) that constitutively expressed high-levels of red fluorescent protein (RFP) and orthotopically implanted these into animals. RFP expression confirmed successful transplantation (**Figure 3A**) and allowed us to observe these cells move to and proliferate in the pancreas of nude mice (**Figure 3B**).

We assessed tumor growth by monitoring RFP expression. TRAIL treatment alone (15 mg/kg) slightly reduced tumor size, and resveratrol (10 mg/kg) or paclitaxel (3 mg/kg) treatment alone reduced tumors to a greater extent than with TRAIL alone (**Figure 4**). Red fluorescence was reduced in mice treated with TRAIL plus resveratrol or paclitaxel. Data show that resveratrol and paclitaxel potentiate the anti-tumor activity of TRAIL *in vivo* (**Figure 4**).

Tumor size quantification confirmed these results. TRAIL plus resveratrol (TR) inhibited more than 60% of the tumor volume compared to TRAIL or resveratrol alone (**Figure 5A**), and TRAIL plus paclitaxel (TP) had less synergistic effects (**Figure 5A**). After TR administration, tumor weight was about one-third of that after TRAIL (T) or resveratrol (R) treatment alone (**Figure 5B**).

To rule out the influence of body weight, we examined the ratio of tumor to body weight. TRAIL or resveratrol alone did not change this ratio, but TR treatment changed the ratio compared to T or R used alone (**Figure 5C**). Interestingly, paclitaxel had similar synergistic effects on tumor growth as resveratrol when combined with TRAIL (**Figure 5C**). Mouse survival data show (*Kaplan-Meier* method) that resveratrol afforded better survival than the other combinations or single agents alone (**Figure 5D**).

TRAIL when combined with small molecules activates caspase-3 cleavage

To confirm the apoptotic effect of treatments, we measured caspase-3 cleavage, which is cru-

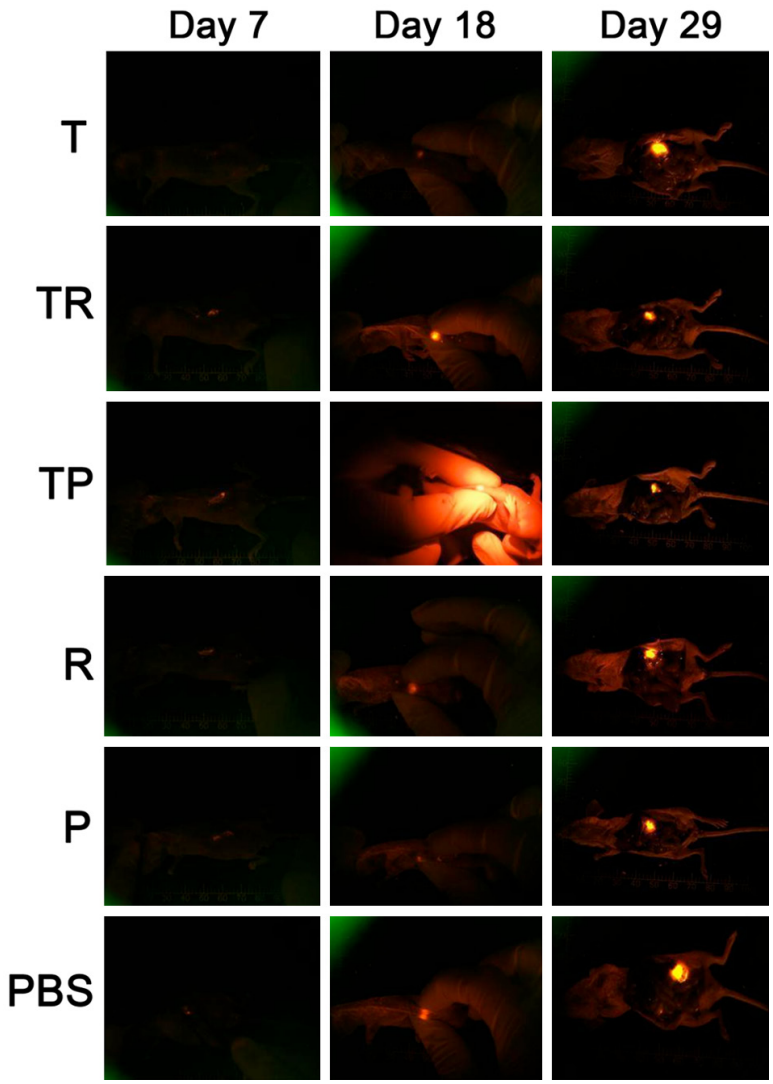

Figure 4. Anti-tumor activities of combined strategies in vivo. All molecules were introduced 7 days post-implantation. TRAIL 15 mg/kg (T); resveratrol 10 mg/kg (R); and paclitaxel (P) 3 mg/kg. Representative images were taken at the times indicated.

cial for apoptosis. TRAIL treatment alone promoted slightly more caspase-3 cleavage (**Figure 6A**) than control. Single-use resveratrol or paclitaxel had limited effects on caspase-3 cleavage (**Figure 6A**). However, TRAIL and resveratrol induced greater caspase-3 cleavage (**Figure 6A**) than single drug treatments. Quantitation of cleaved-caspase-3 positive cells indicated that TR treatment induced apoptosis about 60% greater than T or R treatment alone (**Figure 6B**).

Discussion

TRAIL has been used to inhibit pancreatic cancer, but the anti-tumor effect of TRAIL remains controversial. It successfully inhibited MIA PaCa-2 pancreatic cancer cell growth *in vivo* [19], but failed to repress other types of pancreatic cancer cell growth [20]. This discrepancy indicates variable sensitivity of pancreatic cancer cells to TRAIL. The molecular mechanisms of TRAIL resistance have been explored, identifying X-linked inhibitor of apoptosis (XIAP) and NF-κB as contributors to resistance [21, 22]. Indeed, overexpression of XIAP and induced NF-κB expression were observed in TRAIL-resistant pancreatic cancer cells. Furthermore, RNA interference of an X-linked inhibitor of apoptosis (XIAP) or NF-κB expression sensitizes pancreatic cancer cells to TRAIL-induced apoptosis in TRAIL-resistant cells [21]. Other targets of RNA interference such as FLIP and RIP have been also shown to sensitize pancreatic cells to TRAIL [23]. Although these strategies have inhibitory effects on pancreatic cancer cells, TRAIL concentrations used here are relatively high (10-300 ng/ml).

Resveratrol has been successfully used in cancer therapy in several tumor models and inhibition of NF-κB has been suggested to be an important mechanism for this [24, 25]. Previous studies showed that resveratrol also inhibits XIAP expression [26]. Therefore, resveratrol plus TRAIL for treatment of pancreatic cancer is promising. Indeed, our data indicate that single-use resveratrol inhibits the proliferation but not apoptosis of TRAIL-resistant capan-2 pancreatic cancer cells. These results are consistent with previous studies, which show that resveratrol represses pancreatic cancer cell proliferation [15]. Moreover, resveratrol sensitizes TRAIL-induced cytotoxicity dramatically. The concentration of TRAIL used for combination therapy in this study was 5 ng/mL, and our observations indicate a more efficient and

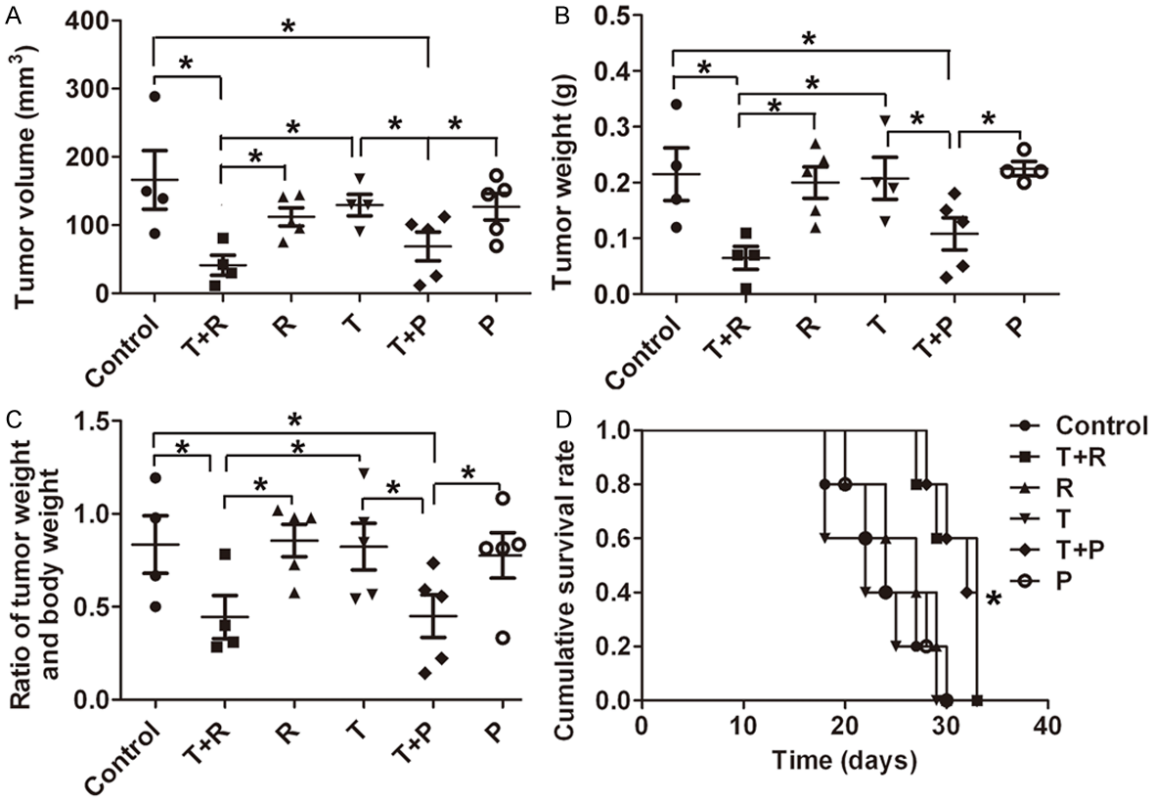

Figure 5. Evaluation of anti-tumor effect of the combined strategies. Tumor volume (A) and tumor weight (B) were measured in an orthotopic implantation model. Tumors were collected 5 weeks post-implantation. The ratio of tumor volume to body weight was measured in (C). (D) Cumulative survival (Kaplan-Meier) of mice treated with molecules as indicated. *; $p < 0.05$.

clinically viable strategy for inhibiting pancreatic cancer cell proliferation.

We also evaluated paclitaxel as a component of combination therapy. The anti-tumor effect of this combination strategy has been reported in other tumor models [27, 28]. Similarly, paclitaxel sensitizes capan-2 cells to low concentrations of TRAIL. In capan-2 cells, reduced expression of the death receptor (DR) was observed, which contributes to resistance [23]. Paclitaxel can induce DR5 in prostate cancer cells [29, 30], suggesting a mechanism for paclitaxel to promote TRAIL sensitivity of capan-2 cells. This warrants more investigation.

In our manuscript, we discuss that in contrast to colo357, cultured capan-2 cells were resistant to TRAIL-induced apoptosis and growth repression, which is reversed by small molecular drugs including resveratrol and paclitaxel. Consistently, TRAIL and resveratrol or paclitaxel repressed tumor growth dramatically in an orthotopic pancreatic tumor model. Taken together, novel methods to sensitize pancreatic cancer cell to TRAIL provides a new strategy for pancreatic cancer treatment *in vivo*.

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Disclosure of conflict of interest

None.

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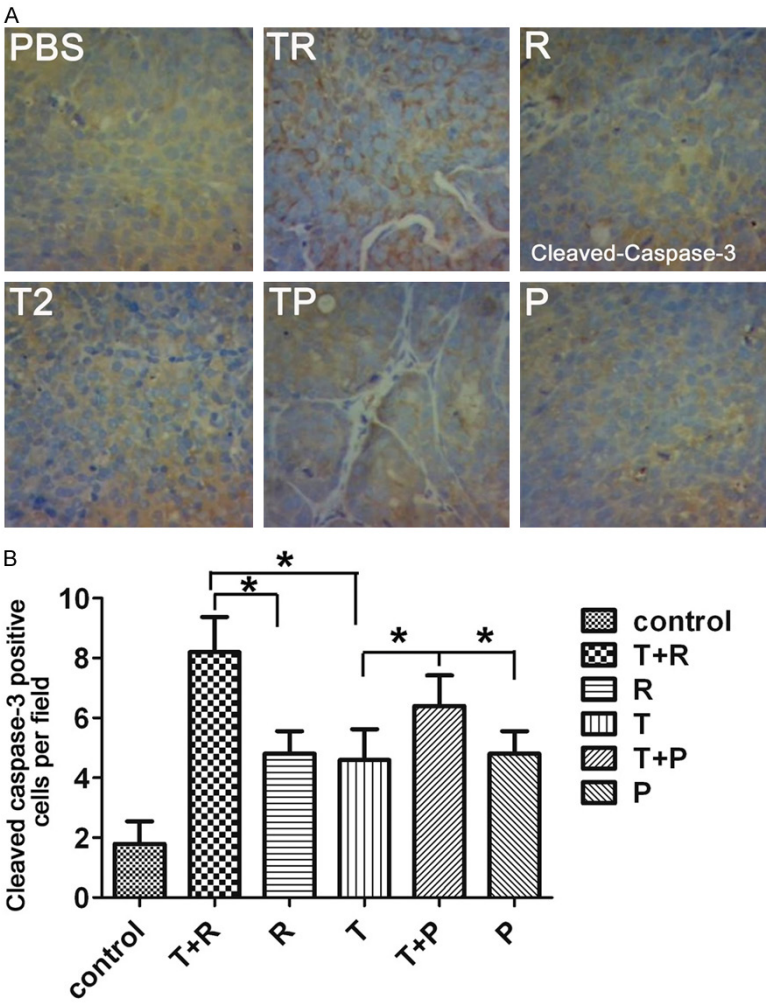

Figure 6. Caspase-3 cleavage in tumors. A. Immunohistochemistry of cleaved caspase-3 in tumor samples from mice treated as indicated. B. Ratio of cleaved caspase-3 positive cells. Nuclei were visualized with hematoxylin staining. *; $p < 0.05$.

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