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ORIGINAL ARTICLE

Triptolide augments the effects of 5-lipoxygenase RNA interference in suppressing pancreatic tumor growth in a xenograft mouse model

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Abstract
Purpose Pancreatic cancer has one of the highest fatality rates of all cancers, and new strategies or reagents to tackle this disease are needed. Triptolide (TL) is able to potently inhibit the growth of pancreatic tumor cells in vitro. On the other hand, blockage of 5-LOX pathway might be useful for treatment of pancreatic cancer. In the current study, we tested the effects of 5-LOX RNA interference and TL individually or in combination in suppressing human pancreatic tumor growth in xenograft mouse model.
Methods 5-LOX short hairpin RNA (shRNA) vectors were developed and screened out for their efficacy in human pancreatic cancer cell line SW1990 in vitro. Their antitumor effects were also evaluated by measuring cell proliferation and apoptosis. An effective 5-LOX shRNA was given alone or in combination with TL to treat pancreatic tumor xenograft. Expression levels of 5-LOX and VEGF were measured with Western blotting and immunohistology.
Results Knocking down 5-LOX gene suppressed cancer cell growth in vitro and intra-tumoral delivering of 5-LOX shRNA inhibited growth of transplanted tumor in vivo. TL treatment induced tumor suppression and greatly enhanced antitumor effects of 5-LOX shRNA in the mouse model. 5-LOX RNA interference or TL treatment suppresses VEGF expression in tumor tissue, and combined treatment further reduces its expression.
Conclusions Both treatments exerted antitumor effects in vivo, and combined use of the two approaches produced more powerful antitumor effects. Synergistic effects of combined treatment in VEGF expression may contribute to the mechanisms of the strong antitumor effects.

Keywords Triptolide · 5-lipoxygenase · Pancreatic tumor · RNA interference · VEGF

Introduction

The overall 5-year survival rate among patients with pancreatic cancer is <5%, and it is the fourth leading type of cancer death that affected both men and women [1]. In 2010, the incidence of pancreatic cancer was 43,140, and 36,800 patients died from the devastating disease [2]. In recent years, there have been important advances in the understanding of molecular mechanisms underlying the biology of pancreatic cancer, but minimal progress has been made in prevention and treatment of patients, especially those with advanced diseases [3, 4].

We previously reported that triptolide (TL), the main extract of the Chinese herb Tripterygium Wilfordii hook, was able to induce apoptosis in pancreatic cancer cell lines in vitro [5]. In the present study, we show that TL suppresses pancreatic tumor growth in a xenograft mouse model, suggesting that TL might be valuable as a novel anticancer agent for clinical application. Proapoptotic activities of TL were reported in various tumor cell lines [6–8], including breast, prostate and lung cancer. TL was

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also shown to sensitize tumor cells for death induction by a variety of agents, such as Apo2/Trail [9] and TNF-α [10]. Based on these observations, we expected that combined use of TL and other therapeutic approaches would provide more potent antitumor effects, while minimize TL's side effects which is a major concern for its clinical applications.

Knocking down 5-lipoxygenase (5-LOX) pathway of arachidonic acid (AA) metabolism is potentially effective to treat pancreatic cancer [11–13]. AA is a 20-carbon polyunsaturated fatty acid and major precursor of several classes of signal molecules. AA is converted to prostaglandins, prostacyclins and thromboxanes by cyclooxygenase (COX) and to hydroxyeicosatetraenoic acids (HETEs) or leukotrienes (LTs) by lipoxygenase (LOX). High levels of 5-LOX expression were found in pancreatic cancer tissue, but not in normal pancreatic ducts [14, 15]. Moreover, metabolic products of 5-LOX are able to stimulate tumor cell growth, suggesting 5-LOX pathway may be involved in carcinogenesis of pancreatic cancer [12]. In the present study, we successfully developed a 5-LOX short hairpin RNA (shRNA) vector that potently suppresses 5-LOX gene expression and pancreatic tumor growth in vivo. Furthermore, we found that combined use of TL and the RNA interference (RNAi) displayed more dramatic antitumor effects than individual treatments in a xenograft mouse model. We propose that both TL and 5-LOX RNAi are effective to suppress pancreatic tumor growth in vivo, and that the synergistic effects of their combination point to a more promising modality for treating pancreatic tumor.

Materials and methods

Cell culture and materials

Human pancreatic cancer cell line SW1990 was purchased from the American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM medium (Grand Island, NY) supplemented with 10% FBS and grown as monolayers in a humidified atmosphere at 37°C. Crystalline triptolide (PG490, purity 99.5%) was obtained from Shanghai DND Pharm-Technology Co., Inc.

RNA interference

5-LOX shRNA plasmid (pGPU6/GFP/Neo-shNC) was designed and synthesized by shanghai GenePharma Co., Ltd. The sequences are: shRNA1 sense, 5′-CAC CGC ACT GAC GAC TAC ATC TAC CTT CAA GAG AGG TAG ATG TAG TCG TCA GTG CTT TTT TG-3′, shRNA1 antisense 5′-GAT CCA AAA AAG CAC TGA CGA CTA CAT CTA CCT CTC TTG AAG GTA GAT GTA GTC GTC AGT GC-3′; shRNA 2 sense, 5′-CAC CGC GCA AGT ACT GGC TGA ATG ATT CAA GAG ATC ATT CAG CCA GTA CTT GCG CTT TTT TG-3′, shRNA 2 antisense, 5′-GAT CCA AAA AAG CGC AAG TAC TGG CTG AAT GAT CTC TTG AAT CAT TCA GCC AGT ACT TGC GC-3′; shRNA 3 sense, 5′-CAC CGC TCC CAT CTG CTT GCT GTA TTT CAA GAG AAT ACA GCA AGC AGA TGG GAG CTT TTT TG-3′, shRNA 3 antisense 5′-GAT CCA AAA AAG CTC CCA TCT GCT TGC TGT ATT CTC TTG AAA TAC AGC AAG CAG ATG GGA GC-3′; shRNA 4 sense: 5′-CAC CGG GCA TGG AGA GCA AAG AAG ATT CAA GAG ATC TTC TTT GCT CTC CAT GCC CTT TTT TG-3′, shRNA 4 antisense 5′-GAT CCA AAA AAG GGC ATG GAG AGC AAA GAA GAT CTC TTG AAT CTT CTT TGC TCT CCA TGC CC-3′. Lipofectamin 2000 (Invitrogen, Carlsbad, CA) was used for in vitro transfection of shRNA vectors according to the manual. Briefly, 1×10^5 SW1990 cells were seeded into 24-well tissue culture plates and grown overnight to 80–90% confluency. For each well, cells were transfected with a complex of 1 μg plasmid plus 2 μl Lipofectamine 2000 reagents. Cells were harvested at 24 h after transfection and total RNA and protein were isolated for real-time PCR and Western blotting experiments. For in vivo experiments, nanoparticle-based In Vivo Transfection Reagent (Altogen Biosystems, Las Vegas, NV) was used for intra-tumoral injection (50 μg per mice) every 3 days for 7 times.

Xenograft tumor model and treatments

Athymic nude mice (BALB/c nu/nu, 5 week old females) were purchased from Shanghai Laboratory Animal Center of Chinese Academy of Science. 1×10^7 SW1990 cells in 100 μl phosphate-buffered saline (PBS) were injected into back of BALB/c nude mice. Once visible tumors were about 100 mm^3, the animals were treated with 5-LOX shRNA vectors or a negative control vector by intra-tumoral injection. The shRNA vectors were diluted in DMEM and the ratio of shRNA and tansfection reagent is 5 μg: 1 μl. TL was dissolved in 60% ethanol, 30% dimethyl sulfoxide, and 10% phosphate buffer (pH 6.0) at a concentration of 1 mg/ml as recommended [16], and 0.25 mg/kg TL was injected i.p. into the mice on a daily basis. The formula for calculating tumor volume was: (length) × (width) × (length + width/2) × 0.526 = volume. Three weeks postinjection, the animals were euthanized and the tumors were carefully dissected and weighted.

Real-time PCR

Total RNA was prepared with TRIzol reagent (Invitrogen) at 24 h after transfection. Real-time quantitative PCR was

performed by using a cycler (Roche Mol, IND) and SYBR green dye. For data analysis, a method designated as $2^{-\Delta\Delta}$CT was used to calculate fold changes. β-actin expression was set to be unaffected under our treatment conditions and used as a reference gene. Triplicates were set for each experiment and error bars represent the range of fold changes calculated from the three or four separate experiments. The followings are primers for β-actin, 5-LOX, and VEGF.

β-actin: sense 5′-ATTGCCGACAGGATGCAGA-3′, antisense 5′-GAGTACTTGCGCTCAGGAGGA-3′. 5-LOX: sense 5′-ACCACGGAGATGGTAGAGTGCAG-3′, antisense 5′-GCAGCTCAAAGTCCACGATGAA-3′; VEGF: sense 5′-GGGCCTCCGAAACCATGAACTT-3′, antisense 5′-TCGCATCAGGGGCACACAG-3′.

Western blotting

Western blotting was performed for whole cell lysate at 24 h after transfection. Aliquots of total protein (50 μg per lane) were electrophoresed on a 10% SDS–polyacrylamide gradient gel and transferred to nitrocellulose membranes (Millipore, Bedford). The membranes were incubated for 2 h at room temperature with anti-5-LOX monoclonal antibody (Cell Signaling Technology, MA). After washing with rinse buffer, the membranes were incubated with 1:1000 diluted horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody (Santa Cruz Biotechnology, CA) followed by developing with enhanced chemiluminescence reagents (Amershame, Little Chalfont Buckinghamshire, UK).

Cell proliferation assay

1×10^4 cells in 200 μl medium were seeded in each well in 96-well cell culture plates. After overnight incubation, cells were treated either with 5-LOX shRNA or negative control. Cell proliferation assays were performed at 24 and 48 h after incubation with Cell Counting Kit-8 reagents (CCK-8, Dojindo, Kumamoto, Japan) according to manufacturer's instructions. The cell numbers in triplicate wells were measured as absorbance at 450 nm.

Apoptosis analysis

The distribution of cells in the cell-cycle phases was determined using flow cytometric analysis of DNA content as described previously [17]. Briefly, after treatment with shRNA vectors for 24 h, cells were fixed with ice-cold 70% ethanol and stored at −20°C. Prior to flow cytometry, the cells were washed and resuspended at 1×10^7 cells/ml in PBS and incubated with 100 μg/ml RNase and 50 μg/ml propidium iodide at 37°C for 30 min. Samples were analyzed using a flow cytometer (FACScalibur, BD USA), and the sub-diploid (sub-G1) cell populations were defined as apoptotic cells.

Immunohistology

Immunohistology was performed to examine 5-LOX and VEGF expression level and localization in tissues. Briefly, tumor samples were isolated and immediately fixed in 10% pH-neutral phosphate-buffered formalin. The fixed tissues were then embedded in paraffin and kept until use. Paraffin sections (4 μm) were cut, deparaffinized and hydrated. Antigens were retrieved in 10 mM sodium citrate buffer (pH 6.0) preheated to 95°C for 10 min. Immunohistochemical staining of the protein was performed using the streptavidin-peroxidase method with anti-5-LOX or VEGF antibody at a dilution of 1:100. The stained sections were examined and scored using a microscope (Olympus).

Statistical analyses

All data were expressed as mean ± SD. The significance for the difference between groups was assessed by one-way ANOVA, and $p < 0.05$ was considered to be significant.

Results

Knocking down 5-LOX gene suppressed pancreatic cancer cell growth

Real-time PCR was performed to measure the levels of 5-LOX gene expression in SW1990 cells before and 24 h after shRNA vector transfection. As shown in Fig. 1a, 5-LOX expression was significantly inhibited in cells transfected with shRNA2, shRNA3 and shRNA4. Next, shRNA-induced gene suppression was confirmed by Western blotting, and the results showed that all of the four vectors are effective in suppressing endogenous 5-LOX expression (Fig. 1b). The effects of shRNA in cell growth were evaluated after 24 and 48 h treatments. As shown in Fig. 2a, the growth of SW1990 cells was suppressed by shRNA2, shRNA3 and shRNA4, and shRNA2 is more effective than the others, which is consistent with its strong activity to suppress 5-LOX gene expression. Next, significant increases in apoptosis of shRNA-treated cells were observed by analyzing DNA content with flow cytometry (Fig. 2b). Although shRNA-1 treatment resulted in an increased number of apoptotic cells compared to the negative control, but it is much lower than that of the other three groups. The results indicated that apoptosis may be responsible for the 5-LOX shRNA-mediated cell growth inhibition.

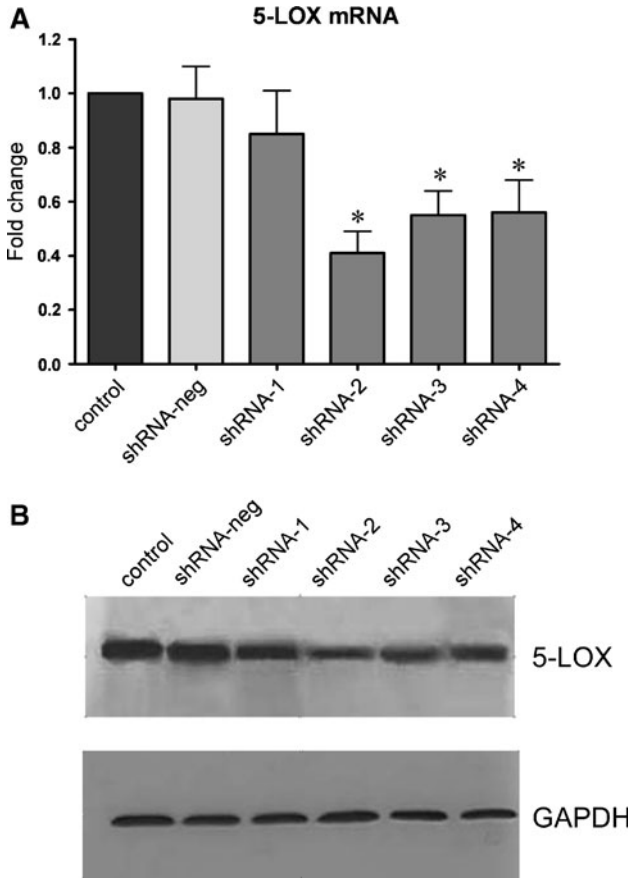

Fig. 1 5-LOX shRNA vectors efficiently inhibited gene expression in SW1990 cells. **a** SW1990 cells were transfected with 5-LOX shRNA vectors or a negative shRNA vector (shRNA-neg), and cells treated with transfection reagents only were used as control. Real-time PCR results demonstrated that among the four shRNA vectors, shRNA-1 was not functional, while shRNA-2 seems to be the most effective one, decreasing the 5-LOX gene expression to 40% of the control cells ($*p < 0.05$, $n = 4$). **b** The results of Western blotting confirmed that 5-LOX expression levels in SW1990 cells was decreased significantly in cells treated with shRNA-2, shRNA-3 and shRNA-4 vectors. The experiment was repeated at least 4 times, and representative result was shown in the figure

5-LOX shRNA inhibited growth of transplanted pancreatic tumor

Pancreatic tumor xenograft mouse model was established by injecting SW1990 cells into nude mice. The transplanted tumor is visible usually after 5 days injection, and it grows to around 100 mm^3 on day 10. From day 11, two most effective 5-LOX shRNA vectors (shRNA-2 and shRNA-3) and a negative control shRNA vector were injected into the tumors every 3 days until day 30. The dose of shRNA vector was determined by preliminary experiments, and no obvious toxic effects of intra-tumoral injection were observed. The volumes of tumor were measured just before administration of shRNA during the course of treatment. The results showed that both 5-LOX shRNA significantly inhibited tumor growth compared with the control mice (transfection reagent only) or the negative shRNA-treated mice (Fig. 3a–c). Since preliminary results indicated that administration of the transfection reagent had no effects on tumor growth in vivo, it was a bit surprising that the negative shRNA treatment, although slightly but significantly, suppressed the tumor growth. It could be due to unwanted non-specific effects because the negative shRNA did not change endogenous 5-LOX gene expression in the cell lines in vitro (Fig. 1). Nevertheless, two 5-LOX shRNA vectors consistently showed abilities to suppress tumor growth, suggesting that the inhibitory effects of the shRNA in vivo are associated with 5-LOX gene suppression. In order to confirm this, we examined the levels of 5-LOX expression in the tumor tissues with immunohistology and real-time PCR. 5-LOX is highly expressed in tumor cells and is mostly located in the plasma, but its expression was significantly suppressed in shRNA-2- and shRNA-3-treated mice compared to the control and the negative shRNA-treated mice (Fig. 3d). Real-time PCR showed similar results, indicating successful knocking down of 5-LOX gene expression in vivo (Fig. 3e). No differences were observed between the two groups of 5-LOX shRNA-treated mice (Fig. 3d, e). We did not find changes in 5-LOX expression in negative shRNA-treated mice compared to the control mice, suggesting that the negative shRNA-medicated inhibitory effects probably were resulted from its unrecognized activity (Fig. 3d, e).

TL treatment induced tumor suppression and enhanced antitumor effects of 5-LOX shRNA

In vitro experiment indicated that TL is able to suppress pancreatic tumor cell growth [5]. We questioned whether it can suppress tumor growth in vivo as well, and whether additive effects exist when TL is used in combination with 5-LOX shRNA. According to the results of above experiments, we used shRNA-2 for all of the following experiments. Mice carrying pancreatic tumor were treated with either 5-LOX shRNA, TL or both shRNA and TL. As shown in Fig. 4a, both shRNA and TL were effective to suppress the tumor growth when they were used alone, and combined use of the two drugs produced more striking antitumor activities. Compared to the control mice, the weight of tumor from shRNA- or TL-treated mice was significantly lower than the control mice, and a threefold decrease in tumor mass was observed in the mice treated with both drugs (Fig. 4b), suggesting that there are additive effects between the two treatments.

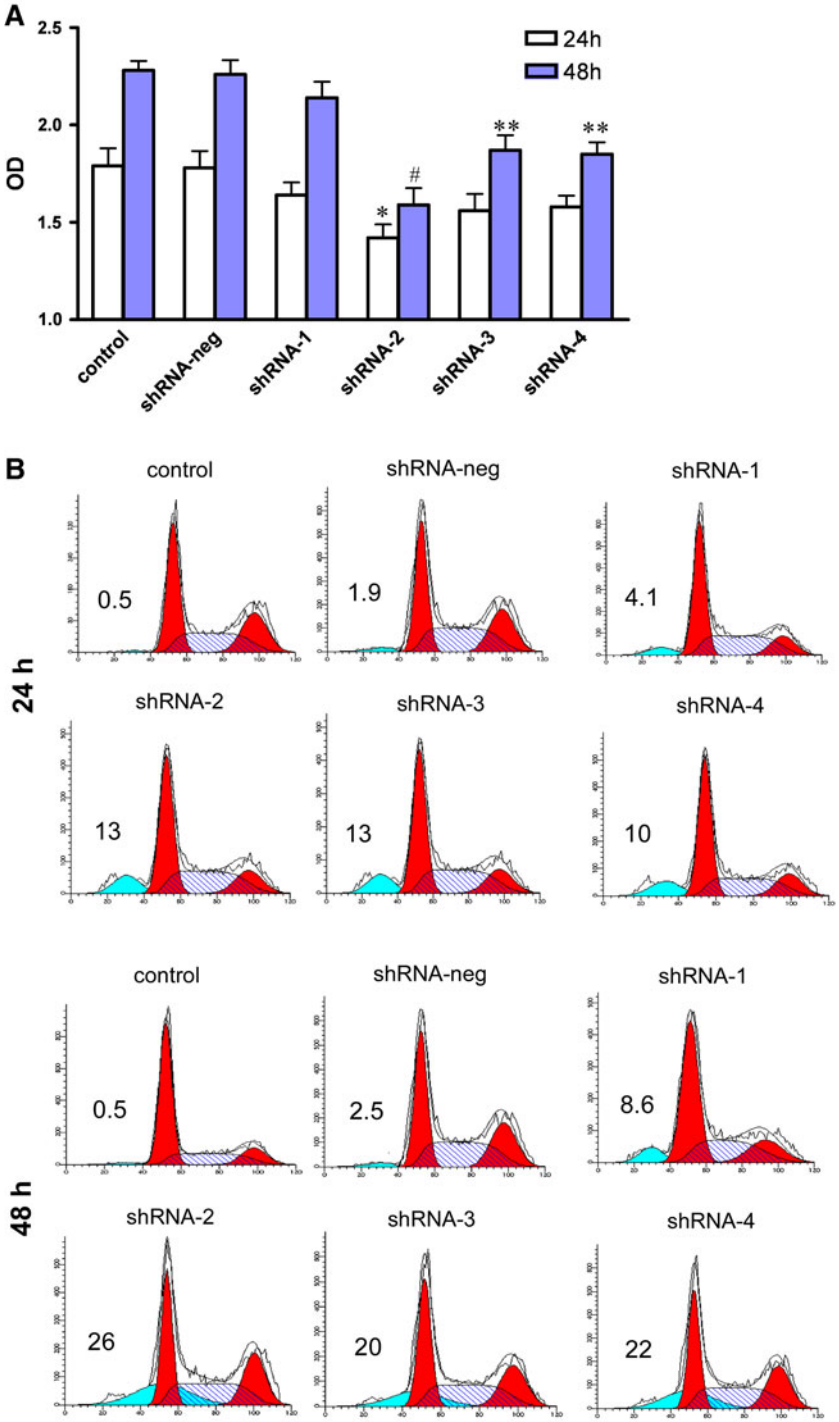

Fig. 2 Transfection with 5-LOX shRNA suppressed the growth of SW1990 cells and induced apoptosis. **a** 1×10^4 cells were seeded in 96-well cell culture plate (200 μl/well) and allowed to attach overnight. The cells were then transfected with 5-LOX vectors or a negative control. After 24 and 48 h of transfection, cell proliferation was measured by Cell Counting Kit-8 (CCK8) reagents and displayed as optical densities (OD) at a wavelength of 450 nm. The results showed that knocking down 5-LOX gene suppressed cell growth, with shRNA-2 showing most striking effects ($*p < 0.05$, $**p < 0.01$, $\#p < 0.001$, $n = 6$; compared to negative shRNA-treated cells,). **b** The levels of apoptosis were measured by flow cytometry. Apoptotic cells were defined according to their DNA content (sub-G1, *green*), and the percentages of apoptosis were displayed in the figures. The results showed that the 5-LOX RNAi leads to increased levels of apoptosis in SW1990 cells after 24 and 48 h transfection compared with the negative shRNA-transfected group ($*p < 0.05$). The experiment was repeated 3 times, and representative result was shown in the figure

5-LOX and VEGF suppression is involved in the enhanced antitumor effects of combined use of 5-LOX shRNA and TL

We asked whether TL further downregulated 5-LOX gene expression in tumors, thus contributed to the enhanced effects of combined treatment. Real-time PCR was used to examine levels of 5-LOX gene expression in tumor tissues. As shown in Fig. 4c, 5-LOX mRNA levels were suppressed by shRNA or TL treatment alone and further reduced by the combined treatment. We also found that TL, but not 5-LOX shRNA, is able to suppress VEGF gene expression in tumor tissues. Interestingly, the VEGF expression in the mice treated with two drugs was significantly lower than the TL-treated mice (Fig. 4d), suggesting a synergistic effect between the two drugs in VEGF expression. In addition, the

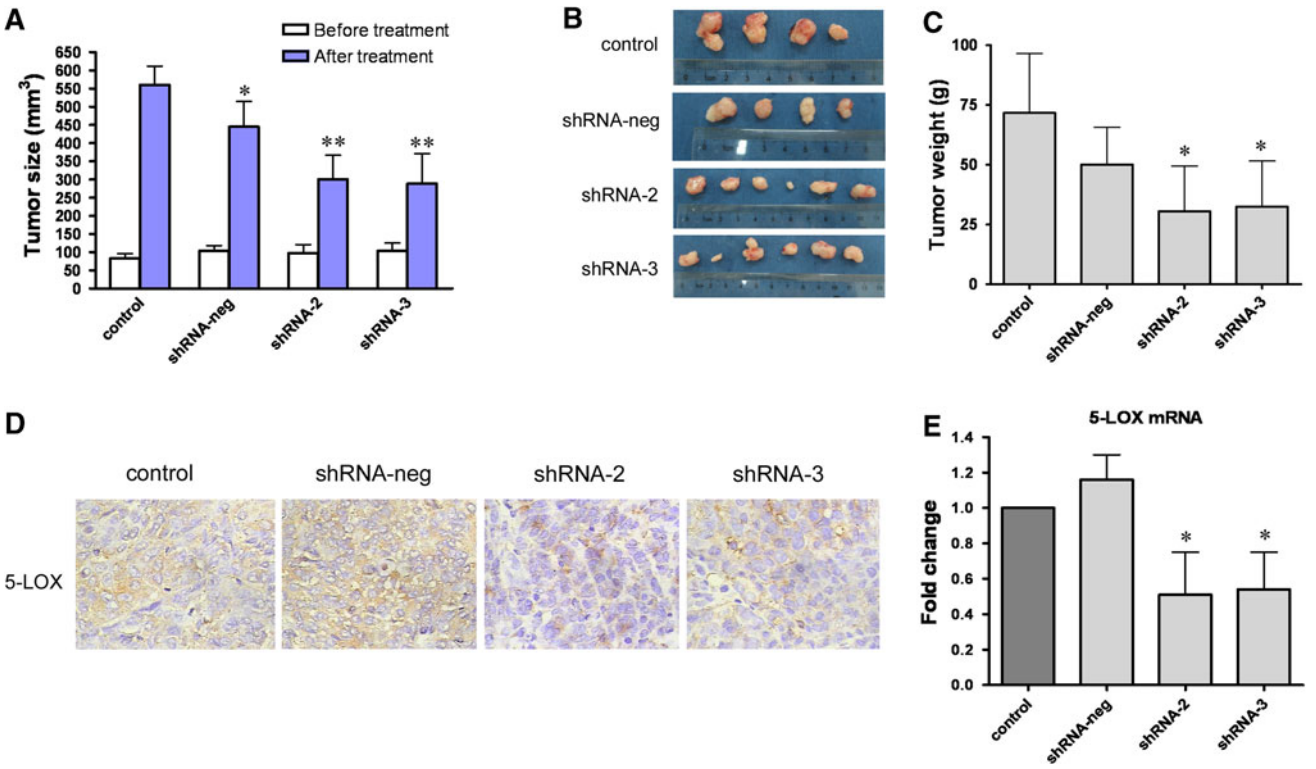

Fig. 3 Treatment with shRNA vectors inhibited pancreatic tumor growth in vivo. **a** After the tumor xenografts grew for 10 days (around 100 mm^3, before treatment), shRNA vectors (shRNA-2, -3 or a negative control, 50 μg per mouse) were administrated by intratumoral injection every 3 days for 7 times. Control mice were treated with transfection reagents only. Tumor volumes were measured every 3 days during the course of treatment. The tumor volumes at the end of treatment (after treatment) were shown in the figure. Compared to the control group, tumors grew more slowly in the mice that were treated with shRNA-2, shRNA-3 or a negative shRNA (*$p < 0.05$, **$p < 0.01$, $n = 6$). After 3 weeks treatment, the mice were euthanized. Tumor burden at the end of the experiment was calculated as tumor weight. The results showed that the tumors from 5-LOX shRNA-2 and shRNA-3-treated mice were smaller than the control group **b**, and the tumor weight **c** was also significantly less than the control group (*$p < 0.05$, $n = 6$). **d** The levels of 5-LOX expression in tumor tissues were evaluated with immunohistology. The results indicated that shRNA-2 and shRNA-3 treatments decreased 5-LOX protein levels in the tumor tissues, while such effects were not seen in the negative shRNA-treated mice. The experiment was repeated three times, and representative result was shown in the figure. **e** The levels of 5-LOX mRNA in tumor tissues were evaluated with real-time PCR, and the results showed that an in vivo suppression of 5-LOX gene was obtained by both shRNA-2 and -3, but not the negative shRNA ($p < 0.05$, $n = 6$)

expression levels of VEGF and 5-LOX were examined by immunohistological staining, and the results consistently indicated that the dramatic downregulation of VEGF and 5-LOX is involved in the enhanced antitumor effects of the combined treatment (Fig. 4e).

Discussion

The poor prognosis of pancreatic cancer, as well as the limited treatment options, emphasizes the need for novel therapeutic strategies. A series of studies suggested that abnormal AA metabolism is involved in pathogenesis of pancreatic cancer [18]. Both COX-2 [19] and 5-LOX [15] were reported to be overexpressed in pancreatic tumor tissues. Therefore, COX-2 and 5-LOX inhibitors may become effective reagents in pancreatic cancer therapy. However, recent study showed that COX-2 specific inhibitors suppressed tumor growth through a COX-independent way [20–22], calling for the further study to elucidate the underlying molecular mechanisms of their antitumor effects. In addition, clinical research showed that celecoxib, a widely used COX-2 inhibitor, did not have significant antitumor, or antiangiogenic effects in a pancreatic tumor xenograft model, although its usage dramatically decreased PGE2 synthesis in those mice [23].

Antitumor effects of a 5-LOX inhibitor zileuton have been seen in a variety of tumor cells [24–26], suggesting that blockage of 5-LOX pathway may be an effective way to treat pancreatic cancer [27]. Our preliminary data do find that high dose of zileuton (125 μm) suppresses the growth of pancreatic cell line SW1990 (unpublished data). However, recent study showed that 100 μm zileuton did not reduce proliferation of a pancreatic cancer cell

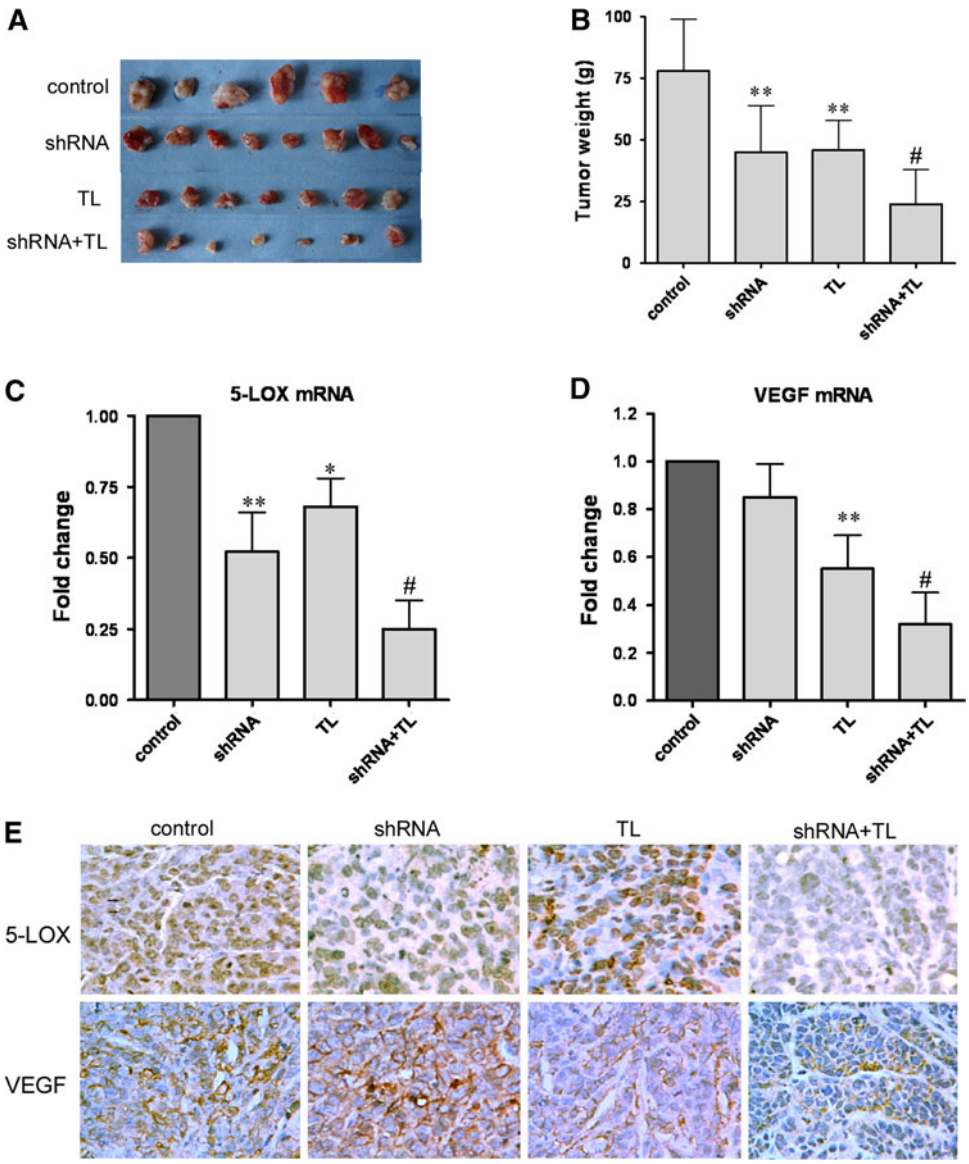

Fig. 4 TL treatment suppressed pancreatic tumor growth in vivo and augmented the antitumor effects of 5-LOX shRNA**.** The tumor size (**a**) and weight (**b**) in the mice that were treated with TL, 5-LOX shRNA or both (shRNA + TL) were shown, indicating that 0.25 mg/kg TL effectively suppressed tumor growth in vivo. Stronger antitumor effects were observed in the mice that were treated with two reagents simultaneously, and was indicated by markedly *decreased* tumor size and weight by the end of the 3 weeks treatment (**$p < 0.01$, $n = 8$, compared to the control group; #$p < 0.05$, compared to the shRNA group or the TL group). **c** The expression levels of 5-LOX in tumors were measured by real-time PCR, and the results showed a synergistic inhibitory effect between TL and 5-LOX shRNA (*$p < 0.5$, **$p < 0.01$, $n = 6$, compared to the control group; #$p < 0.05$, $n = 6$, compared to shRNA group or TL group). **d** VEGF gene expression levels were lower in the TL-treated mice than the control mice, and were further reduced by the combined treatment. (**$p < 0.01$, $n = 6$, compared to the control group; #$p < 0.05$, $n = 6$, compared to TL group). **e** Immunohistological staining confirmed that both 5-LOX and VEGF in the tumors were dramatically suppressed by the combined treatment. The experiment was repeated 3 times, and representative result was shown in the figure

line Capan-2, but another 5-LOX inhibitor MK886 greatly inhibited the tumor cell growth with much lower doses [28]. The author suggested that certain 5-LOX inhibitors may have 5-LOX-independent anti-proliferative effects [28]. In the present study, we examined the direct effects of 5-LOX inhibition by RNA interference instead of blocking its enzymatic activity. We successfully screened out several 5-LOX shRNA vectors that not only inhibited 5-LOX gene expression in SW1990 cells, but also suppressed cell proliferation in vitro and the tumor growth in vivo. The results demonstrated that 5-LOX pathway may play a crucial role in carcinogenesis of pancreatic cancer.

It has been acknowledged that combined treatment may produce more benefit for cancer therapy [10, 29, 30].

TL has been shown great value when used in combination with other antitumor treatments, inducing higher levels of cell death by increasing tumor cell sensitivity to chemotherapy or radiation [31, 32]. Although a body of evidence suggests that TL has antitumor effects in a wide range of tumors, the effects of TL in pancreatic cancer has not been tested in vivo. We therefore speculated that TL might be a good candidate to treat pancreatic cancer individually or in combination with 5-LOX RNAi. Our results clearly indicated that TL effectively suppressed the growth of pancreatic cancer xenografts in mice, and the effects were even stronger when it was used in combination with 5-LOX shRNA. It has been reported that TL suppresses 5-LOX gene expression in SW1990 cells [33], and it functions as small molecule inhibitor of tumor angiogenesis [34]. Consistently, we found that the levels of VEGF and 5-LOX in the TL-treated mice were reduced, and that combined use of 5-LOX shRNA and TL further suppressed their expression. We proposed that the synergism between 5-LOX shRNA and TL in 5-LOX and VEGF suppression is an underlying mechanism of the enhanced antitumor effects.

A major concern about using TL for clinical antitumor applications is its toxicity. Shamon et al. [35] reported that TL exerted a modest antitumor activity when administered at a dose of 25 μg/mouse 3 times per week intravenously to nude mice carrying human breast tumors, but higher doses (>50 μg/mouse) were lethal, suggesting a narrow therapeutic window of TL treatment. Severe side effects happened in a recent phase I clinical study using F60008, which is a semi-synthetic derivate of TL, in patients with solid tumors [36]. In preliminary study, we found that i.p. administration of 0.25 mg/kg or higher doses of TL exerted significant antitumor effects in a dose-dependent way, and that the mice treated with 0.25 mg/kg TL did not show any obvious side effects. However, weight loss, skin inflammation and vessel inflammation were observed in the mice treated with 0.5 mg/kg TL, and higher doses of TL have stronger effects but the side effects were more severe (data not shown).

In addition to cytostatic drugs and small molecule inhibitors, RNAi extended the portfolio toward specific antitumor treatment and so far has not shown unmanageable side effects [37, 38]. Here, 5-LOX shRNA was used to suppress the tumor growth in vivo by intra-tumoral injection individually or in combination with TL. Efficient gene delivery has been obtained, and the endogenous 5-LOX was successfully suppressed by introducing shRNA vectors but not the negative control. Compared to 5-LOX inhibitors, which may have untoward side effects and unpredictable targets, suppressing 5-LOX gene expression with RNAi provide a reliable way to delineate the role of 5-LOX in pancreatic cancer. Our results clearly demonstrated that suppression of 5-LOX gene is an effective way to inhibit pancreatic tumor growth in vivo. Moreover, its antitumor effects could be further enhanced by TL in a relative low dose, suggesting a promising way to treat the devastating disease.

However, it should be noted that at the moment, delivery of shRNA vectors directly into tumor is not feasible in most clinical settings. In the past recent years, breakthroughs in RNAi research are taking place with an unprecedented speed, and there are several RNAi-based human clinical trials in progress [39–41]. Further study is warrant to test the two effective approaches, 5-LOX RNAi and TL, individually or in combination for its safety and efficacy in clinical usage.

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Conflict of interest The authors indicated no potential conflict of interest.

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