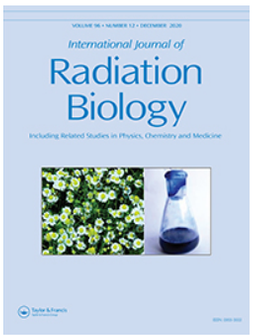

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RAPID COMMUNICATION

Tumor treating fields (TTF) treatment enhances radiation-induced apoptosis in pancreatic cancer cells

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ABSTRACT

Purpose: Tumor treating fields (TTF) therapy is a noninvasive method that uses alternating electric fields to treat various types of cancer. This study demonstrates the combined effect of TTF and radiotherapy (RT) in vitro on pancreatic cancer, which is known to be difficult to treat.

Materials and methods: In CFPAC-I and HPAF-II pancreatic cancer cell lines, the combined in vitro effect of TTF and RT was evaluated by measuring cell counts, markers of apoptosis, and clonogenic cell survival. The synergy effects were verified using the Valeriote and Carpentier equations.

Results: TTF and RT inhibited cancer cell growth more effectively than did monotherapy with TTF or RT. The combined treatment also enhanced apoptosis more than monotherapy, as shown by assays for cleaved poly (ADP-ribose) polymerase (PARP) and annexin V. In addition, on the survival curve, this treatment method has been shown to work synergistically.

Conclusion: These results suggest that combined treatment with TTF and RT may be a good alternative treatment for patients with pancreatic cancer.

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KEYWORDS
Tumor treating fields; radiotherapy; pancreatic cancer; combined therapy; cancer therapy

Introduction

The incidence of pancreatic cancer, one of the most aggressive and deadly forms of cancer, continues to increase yearly (Siegel et al. 2019). The incidence of pancreatic cancer almost parallels its mortality, as the 5-year survival rate of patients with pancreatic cancer is only 6% (Kamisawa et al. 2016). These results indicate that traditional therapies, such as surgery, chemotherapy and radiotherapy, that have been used to treat patients with pancreatic cancer, are ineffective, suggesting the need for new therapeutic modalities.

Tumor treating fields (TTF) is a method in which alternating electric fields of low-intensity (<3 V/cm) and intermediate-frequency (100–300 kHz) are applied to tumors. TTF selectively damages cancer cells, eventually causing their death (Kirson et al. 2004, 2007). TTF treatment has been approved by the U.S. FDA and EU regulatory agencies for patients with treatment-refractory forms of cancer, including glioblastoma (GBM) and mesothelioma (Fennell 2019). In addition, TTF treatment is also undergoing phase III clinical trials for patients with pancreatic cancer (Weinberg et al. 2019), as phase II trials in pancreatic cancer have yielded promising results, including the possibility of better prognosis (Rivera et al. 2019).

Clinical studies in different types of cancer have shown that the addition of TTF to chemotherapy was more effective than chemotherapy alone (Pless et al. 2013; Stupp et al. 2015; Rivera et al. 2019). As such, the synergistic effect of TTFs therapy in combination with chemotherapy has been demonstrated in clinical trials, and studies on nonclinical trials have also been conducted (Kirson et al. 2009; Stupp et al. 2015). However, there is a lack of research on TTF treatment in combination with radiation therapy that has strong anticancer effects. Although several studies have shown that combinations of radiotherapy (RT) and TTF have synergistic effects, these findings are insufficient to support future clinical trials (Giladi et al. 2015; Kim et al. 2016; Karanam et al. 2017). Radiation is a type of electromagnetic wave with physical properties similar to those of TTF (Kim et al. 2016). The advantages of both RT and TTF include their noninvasive nature and the absence of severe pain. To assess the applicability of combined TTF and RT in patients with pancreatic cancer, the present study tested the synergistic effects of TTF and RT on pancreatic cancer cells in vitro.

Methods and materials

Experimental setup for TTF

The experimental setup for TTF has been described previously (Jo et al. 2019).

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Supplemental data for this article can be accessed here.

Antibodies and chemicals

Anti-cleaved poly (ADP-ribose) polymerase (PARP) and anti-beta actin antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA).

Cell culture

Human pancreatic adenocarcinoma CFPAC-I and HPAF-II cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in accordance with the information supplied by the ATCC.

Cell viability assay

Cell viability was assayed according to the manufacturer's protocol (EZ3000, Daeillab Service). Absorbance at 450 nm was measured using a microplate reader (PHOmo, autobio labtec instruments).

3D Culture system

Human pancreatic adenocarcinoma CFPAC-I and HPAF-II cells were seeded and 3D culture assay was performed as described previously (Jo, Kim, et al. 2018).

Colony formation assay

The surviving number of cells, expressed as a function of irradiation, was calculated using the formula: surviving fraction = colonies counted/(cells seeded × plating efficiency/100).

Western blotting

After TTF treatment, pancreatic cancer cells were exposed to radiation and then incubated for 48 h. Then, Western blotting was performed as described (Jo, Kim, et al. 2018).

Flow cytometry

Cells were stained for propidium iodide (PI) and annexin V in accordance with the manufacturer's protocol and fractionated on a FACSCalibur flow cytometer (BD). A minimum of 10,000 cells were counted for each sample, and data were analyzed using CellQuest pro software (BD Biosciences).

Enzyme-Linked immunosorbent assay

After treatment, human pancreatic adenocarcinoma CFPAC-I and HPAF-II cells were seeded and Enzyme-Linked Immunosorbent Assay was assayed according to the manufacturer's protocol (ab223863, Abcam).

Statistical analysis

Means were compared using Student's *t*-tests. Differences were considered significant if the *p*-value was $<.05$ or $<.01$.

Results

Effect on TTF or radiation alone

To optimize the in vitro effects of TTF on pancreatic cancer cells, electric field conditions, such as voltage and frequency, were varied. First, in order to evaluate the voltage dependence of TTF, the cell number and viability of each group were checked after treatment with increasing voltage. Increasing the voltage from 0.9 to 1.5 V/cm for 48 h resulted in a gradual reduction in the number of cells compared with control, untreated cells. When cell viability was checked under the same conditions, the same tendency was observed (Figure 1(A)). These results therefore indicate that the therapeutic effects of TTF on cancer cells depend on the voltage and clearly showed that the decrease in cell number and viability compared to the control group was due to TTF. Because TTF requires optimization for different types of cancer cells (Kirson et al. 2007), its voltage was fixed at 1.2 V/cm and its frequency adjusted from 100 to 250 kHz for 48 h. Immediately after, the cell number and viability were checked. The lowest number of cells and the lowest cell viability were observed at 150 kHz in both cell lines (Figure 1(B)).

Next, the effects of radiation alone treatment on two pancreatic cancer cell lines were verified. Cells were treated with increasing doses of radiation, up to 30 Gy, and their viability assessed after incubation for an additional 48, 72 and 96 h. Cell viability decreased with increasing radiation dose (Figure 1(C)). Combined treatment with TTF was performed at 5 Gy, a dose of about 70% viability compared to the control group.

Effect on combined therapy

To confirm the effects of combined treatment with TTF and RT, cells were treated with TTF, RT, both (TTF + RT) and neither, and the numbers of cells were counted. In the combined therapy group, cells were treated with TTF at 0.9 V/cm and 150 kHz for 48 h, irradiated with 5 Gy immediately afterward, and incubated for an additional 48 h. Both cell imaging and cell counting showed that the number of cells following combined treatment was significantly lower than the numbers of cells following treatment with TTF or RT alone (Figure 2(A,B)).

To find out if there are four group interactions, a generalized estimating equation (GEE) was used to reduce Type I error (significant but rejected error) and not to be affected by normality and missing values. In the two cell lines, the interaction between group and time was statistically significant (Suppl. Table 1).

A 3D culture assay showed that these treatments, especially TTF plus RT, prevented the growth of cell spheres. The diameter of the spheres compared to the control was also evaluated (Figure 2(C)).

Apoptosis in combination therapy

Because other studies have assessed the effects of TTF and/or RT on apoptosis (Haimovitz-Friedman et al. 1994; Kim

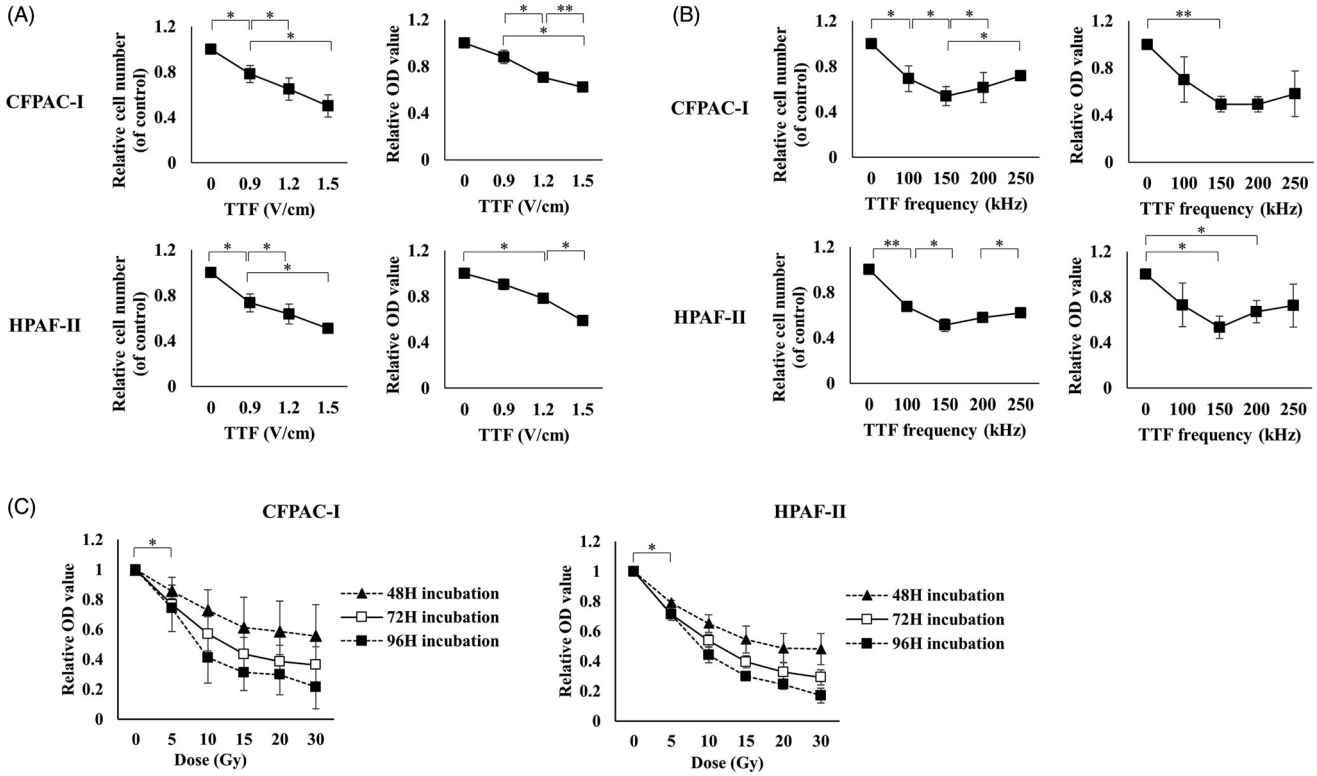

Figure 1. (A) TTF inhibited pancreatic cancer cell viability in an intensity-dependent manner. Cell counts using 0.4% Trypan Blue stain (left) and MTT assay (right) confirmed the voltage dependence of TTF effects at 0, 0.9, 1.2, and 1.5 V/cm for 48 h. (B) Cell counts using 0.4% Trypan Blue stain (left) and MTT assay (right) confirmed the frequency dependence of TTF effects at 0, 100, 150, 200, and 250 kHz. (C) Radiotherapy inhibited pancreatic cancer cell viability in a dose-dependent manner. Cells were incubated for 48, 72 and 92 h after 0, 5, 10, 15, 20 and 30 Gy irradiation, respectively, and the effects were confirmed by MTT assay. The values represent the means ± SD for 3 experiments. $^{*}p < .05$; $^{**}p < .01$.

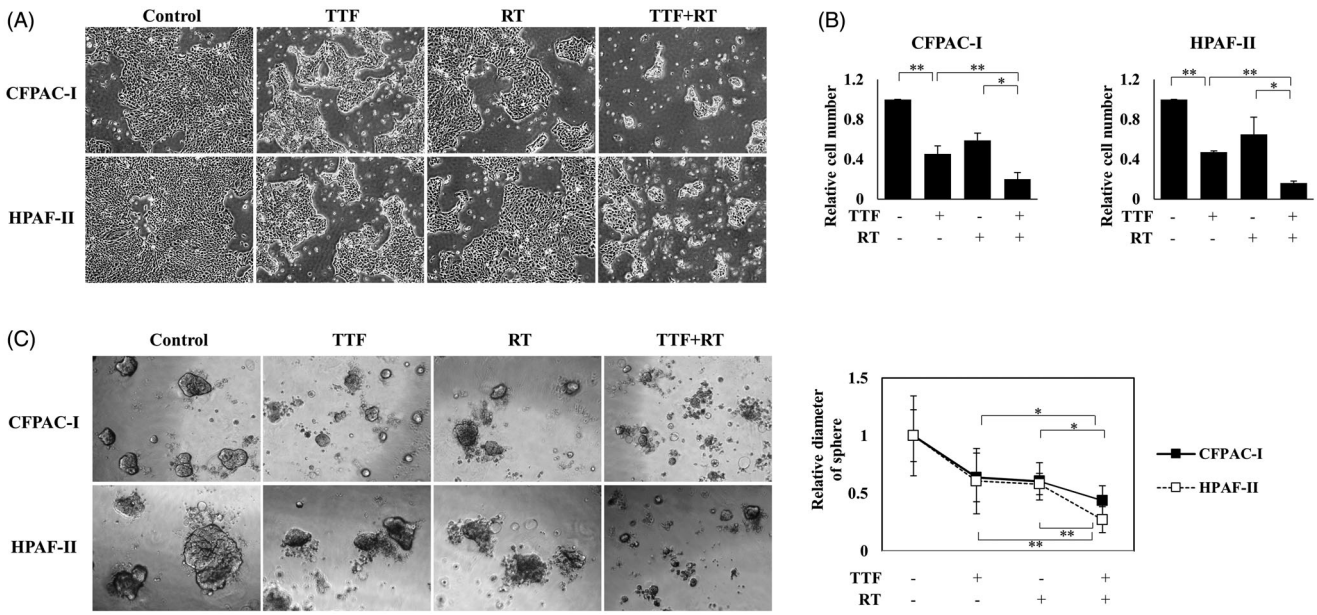

Figure 2. The viability of cells treated with a combination of TTF and RT was significantly lower than that of cells treated with TTF or RT alone. Effects of TTF, RT, or both on (A) microscopic images and (B) cell counts after post-treatment incubation for 48 h. (C) In each treatment condition, 3D culture assay was performed using Matrigel. The sphere diameter is graphed. The values represent the means ± SD for 3 experiments. $^{*}p < .05$; $^{**}p < .01$.

et al. 2016), their effects on apoptosis of pancreatic cancer cells were evaluated by measuring the levels of expression of the apoptosis markers cleaved-poly(ADP-ribose) polymerase (PARP) and annexin V. PARP was detected, and expression levels were upregulated in the order of TTF + RT, TTF and RT (Figure 3(A)). Annexin V, another marker of apoptotic cells, was detected to confirm the upregulation effect of apoptosis by TTF + RT treatment (Figure 3(B)). Along with this, necrosis marker PI was also analyzed from flow cytometry. After treatment, cells were incubated for 24, 48 and

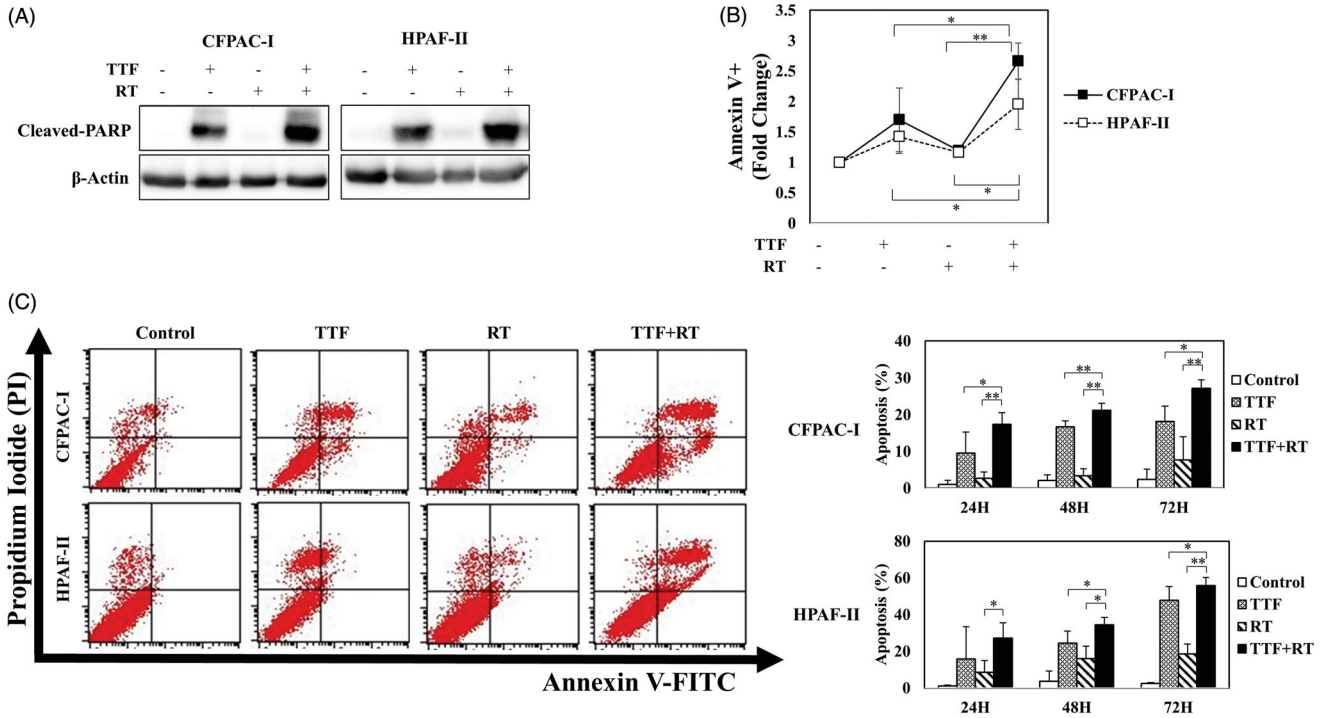

Figure 3. Effects of TTF, RT, or both on expression of cleaved PARP and annexin V by pancreatic cancer cells. (A) Equal amounts of cell lysates were separated by electrophoresis and analyzed by western blotting using anti-cleaved PARP antibodies. (B, C) Apoptotic cell rates, as determined by staining for annexin V and flow cytometry. The values represent the means ± SD for 3 experiments. $^{*}p < .05$; $^{**}p < .01$.

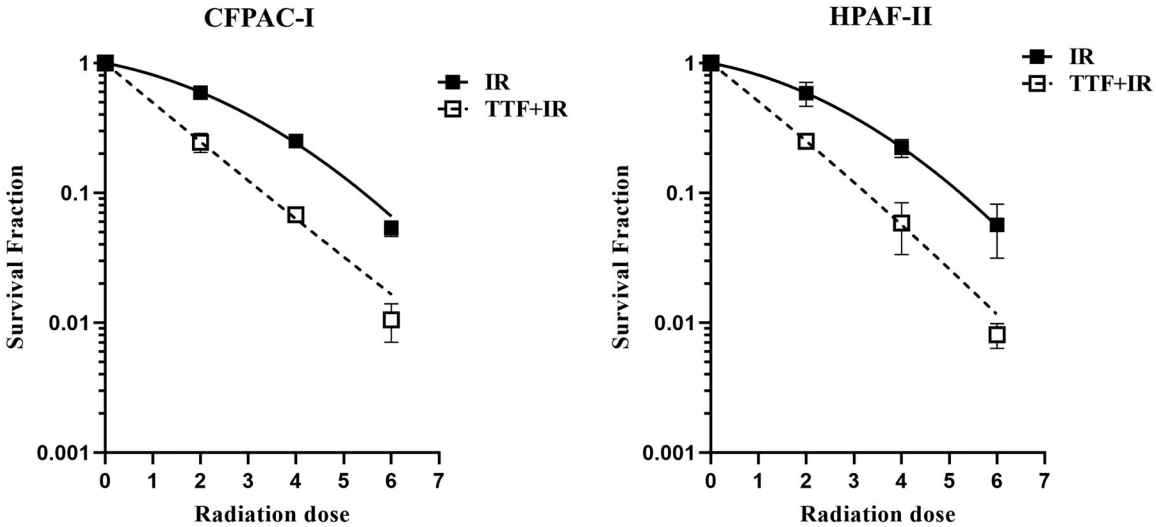

Figure 4. Effects of TTF on survival of CFPAC-I and HPAF-II pancreatic cancer cells in a clonogenic assay. Cells were treated with or without TTF (0.9 V/cm and 150 kHz) and immediately irradiated with 0, 2, 4, or 6 Gy RT. The surviving cell fraction from the clonogenic assay was expressed as survival curves. The values represent the means ± SD for three experiments.

72H, respectively, and apoptosis cells by TTF + RT were increased than by TTF or RT (Figure 3(C)).

Survival curve

Clonogenic assays were performed after irradiating pancreatic cancer cells with 0, 2, 4 and 6 Gy, respectively, with or without TTF (Figure 4). Survival curves of both cell lines were fitted to a linear quadratic (LQ) model. More curved graphs were observed in cells treated with TTF + RT than with either alone, suggesting that TTF sensitized both cell lines to RT. Radio-sensitizing effect in pancreatic cancer cells was evaluated by the Valeriote and Carpentier equations (Valeriote and Lin 1975; Carpentier et al. 1993). The results showed that TTF and RT had synergistic effects in both pancreatic cell lines (Suppl. Table 2). Moreover, these findings suggest that TTF may enhance the therapeutic efficacy of RT in patients with pancreatic cancer who are difficult to treat in the clinic.

Discussion

According to several reported studies, TTF induces DNA damage to cancer cells and causes DNA repair delays

(Giladi et al. 2017; Karanam et al. 2017; Jo, Hwang et al. 2018; Karanam et al. 2020). This DNA damage is known to cause apoptosis if not repaired properly. PARP is a protein involved in cell death due to DNA damage (Bouchard et al. 2003), and our studies have also shown that TTF induces apoptosis via upregulation of cleaved PARP.

The survival rate of patients with pancreatic cancer, known as one of the most difficult to treat than any cancer, is sadly very frustrating. Phase II clinical trials of TTF + gemcitabine and TTF + gemcitabine + nab-paclitaxel in patients with pancreatic cancer have recently been completed. Median progression-free survival (PFS) was 8.3 months and median overall survival (OS) was 14.9 months when gemcitabine treatment was performed with TTF, and 3.7 months and 6.7 months, respectively, for historical control without TTF (gemcitabine only). Median PFS was 12.7 months and median OS was not yet reached when gemcitabine + nab-paclitaxel chemotherapy with TTF, and the historical control without TTF (gemcitabine + nab-paclitaxel) was 5.5 months and 8.5 months, respectively (Von Hoff et al. 2013; Rivera et al. 2019). These findings suggested that combinations of TTF with existing therapies may increase the very low survival rate of patients with pancreatic cancer. Phase III clinical trials are currently underway (Weinberg et al. 2019).

Despite ongoing Phase III clinical trial of TTF + chemothearpy, few preclinical studies to date have tested the effects of TTF or TTF plus radiotherapy on pancreatic cancer cells. Radiation therapy is used to treat cancer by directly or indirectly damaging the DNA of cells and inducing apoptosis through various mechanisms. The pancreas is moderately sensitive to radiation, so a dose of 70 Gy or higher is recommended for treatment (Wilkowski et al. 2005). However, due to radiation-sensitive organs such as the liver, kidneys, and spinal cords, which are organs around the pancreas, side effects of radiation are inevitably concerned. Therefore, the use of radiosensitizers can optimize the therapeutic effect with lower doses of radiation, which means lower side effects. Although plenty of radiosensitizers exist, we previously confirmed that TTF in glioblastoma itself has cancer treatment as well as synergistic effect as a radiosensitizer when combined with radiotherapy (Kim et al. 2016).

The present study confirmed that TTF and TTF + RT induced apoptosis in pancreatic cancer cell lines, as shown by the upregulation of cleaved-PARP and annexin V. This result may be related to the synergistic effect of TTF with radiotherapy which also cause apoptosis. Further studies are needed to determine the pathways through which TTF induces cell apoptosis.

The addition of TTF to radiotherapy or chemotherapy is expected to enhance the therapeutic efficacy of these traditional treatments in pancreatic cancer patients. The present study confirmed this and showed that TTF and RT had synergistic effects in vitro. Studies are needed to show synergistic effects of these treatments in vivo. This study presents the possibility of improving the prognosis of patients with pancreatic cancer when combined with radiotherapy and TTF, and this combined therapy are promising treatments for future clinical use.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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