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Original Article

Synergistic effect of TTF and 5-FU combination treatment on pancreatic cancer cells

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Abstract: The present study investigated the therapeutic potential of combining tumor-treating fields (TTF), a novel cancer treatment modality that employs low-intensity, alternating electric fields, with 5-fluorouracil (5-FU), a standard chemotherapy drug used for treating pancreatic cancer. The HPAF-II and Mia-Paca II pancreatic cancer cell lines were treated with TTF, 5-FU, or their combination. Combination treatment produced a significantly greater inhibitory effect on cancer cell proliferation than each single modality. Furthermore, combination therapy induced a substantially higher rate of pancreatic cancer cell apoptosis and exhibited a synergistic effect in clonogenic assays. Additionally, combination treatment showed a greater inhibition of cancer cell migration and invasion than either TTF or 5-FU alone. In conclusion, these findings suggest that the synergistic properties of TTF and 5-FU result in greater therapeutic efficacy against pancreatic cancer cells than either modality alone and may improve survival rates in patients with pancreatic cancer.

Keywords: Tumor treating fields, 5-fluorouracil, combined therapy, pancreatic cancer

Introduction

Pancreatic cancer is a highly aggressive malignant tumor with a mortality rate of over 90% within 5 years after diagnosis, resulting in an increasing number of deaths [1, 2]. Because pancreatic cancer is often detected in advanced stages, it is frequently unresectable and has already metastasized to other parts of the body at the time of diagnosis [3]. Early-stage pancreatic cancer is treated with surgical resection followed by adjuvant chemotherapy, whereas advanced stages are treated with chemotherapy regimens, consisting of 5-fluorouracil (5-FU)/leucovorin plus irinotecan and oxaliplatin (FOLFIRINOX) or gemcitabine plus nab-paclitaxel [4, 5]. Despite the development and use of various types of anticancer drugs, chemoresistance remains a major obstacle, contributing to the poor prognosis of patients with pancreatic cancer. Therefore, there is an urgent need for new treatment strategies that can overcome chemoresistance and improve patient outcomes.

5-FU, a commonly used chemotherapeutic agent in the treatment of pancreatic cancer, inhibits the enzyme thymidylate synthase (TS), which is responsible for the synthesis of thymidine, a nucleotide necessary for DNA replication [6]. Incorporation of 5-FU into DNA disrupts its normal structure, inhibiting DNA replication and ultimately leading to cell death. In addition, 5-FU can be metabolized into active compounds that contribute to its anticancer effects. One of these metabolites, 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), binds to the enzyme dihydrofolate reductase (DHFR), depleting tetrahydrofolate (THF) and inhibiting DNA synthesis, resulting in cell death. Another metabolite, 5-fluorouridine triphosphate (FUTP), can be incorporated into RNA and interfere with protein synthesis, leading to cell death. Thus, 5-FU is an important component of pancreatic cancer treatment. However, pancreatic cancer cells can develop resistance to 5-FU through various mechanisms, making effective treatment of pancreatic cancer challenging. Resistance to 5-FU may be overcome and its ther-

apeutic efficacy enhanced by combining 5-FU with other chemotherapeutic agents and/or alternative treatment modalities.

Tumor Treating Fields (TTF) is a novel cancer treatment that uses low-intensity, intermediate-frequency electric fields to disrupt cell division and promote cancer cell death [7-9]. These electric fields are delivered via electrodes placed on the skin over the tumor region. By interfering with the proper alignment of microtubules during cell division, TTF leads to the formation of abnormal mitotic spindles and ultimately cell death. Additionally, TTF induces other effects in cancer cells, such as the formation of reactive oxygen species (ROS), alterations in signaling pathways, and changes in gene expression, all of which can further contribute to cancer cell death.

Preclinical and clinical studies of several types of cancer, including glioblastoma, mesothelioma, pancreatic cancer, ovarian cancer, and liver cancer, have shown that various cancer drugs can significantly enhance the therapeutic effects of TTF [10-13]. The United States Food and Drug Administration (FDA) has approved TTF for the treatment of glioblastoma and mesothelioma [14], and TTF has been reported to improve patients survival rates without negatively affecting their health-related quality of life [15]. One of the great advantages of TTF is that patients feel little pain during treatment and no serious side effects have been reported to date. Although more research is needed to fully understand the benefits of TTF therapy, its potential to overcome chemoresistance and improve patient outcomes suggests that it may have applications in cancer treatment.

The aim of this study was to investigate whether the combination of TTF and 5-FU can overcome chemoresistance in pancreatic cancer cells and provide scientific evidence of its potential to improve the prognosis of pancreatic cancer patients. Two pancreatic cancer cell lines were treated with TTF, 5-FU or their combination, and the effects of these agents on rates of cell growth, cell death, and cell invasion and migration were compared.

Materials and methods

Experimental setup for TTF

TTF was generated with a pair of insulated wires (Seoil Electric Wire Co., Ltd.; outer diameter, 0.4 mm; polyvinyl chloride insulation thickness, 0.17 mm; dielectric breakdown, 25 kV/mm) connected to a function generator (AFG-2112, Good Will Instrument Co., Ltd., Taiwan) and a high-voltage amplifier (A303, A. A. Lab Systems Ltd., Israel), which generated sine-wave signals ranging from 0-800 V [16]. TTF was applied to cell lines by attaching the pair of insulated wires to the bottom of each cell dish, 3 cm from each other. The electric field was applied for 72 h at an intensity of 1.1 V/cm and a frequency of 150 kHz.

Antibodies

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

Three-dimensional (3D) culture system

HPAF-II and Mia-Paca II pancreatic cancer cells were seeded in 96-well plates at 1×10^4 cells/well. In the 3D culture model, the 96-well plates were precoated with Matrigel as a basement membrane by adding 40 µl of Matrigel to each well, followed by incubation at 37°C for 30 min. Cells in appropriate medium were plated onto the gel, and the wells were photographed 10 d later.

Colony formation assay

Cells were treated with TTF and/or 5-FU and incubated for 14-20 d. The resulting colonies were stained with 0.4% Crystal Violet (Sigma, St. Louis, MO, USA). The plating efficiency (PE) was defined as the percentage of seeded cells that formed colonies under specific culture conditions. The survival fraction, expressed as a function of irradiation, was calculated using the equation: survival fraction = colonies counted/(cells seeded × PE/100).

Flow cytometry

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

Western blotting

After treatment, pancreatic cancer cells were lysed with RIPA buffer; and the resulting proteins were separated by sodium dodecyl sul-

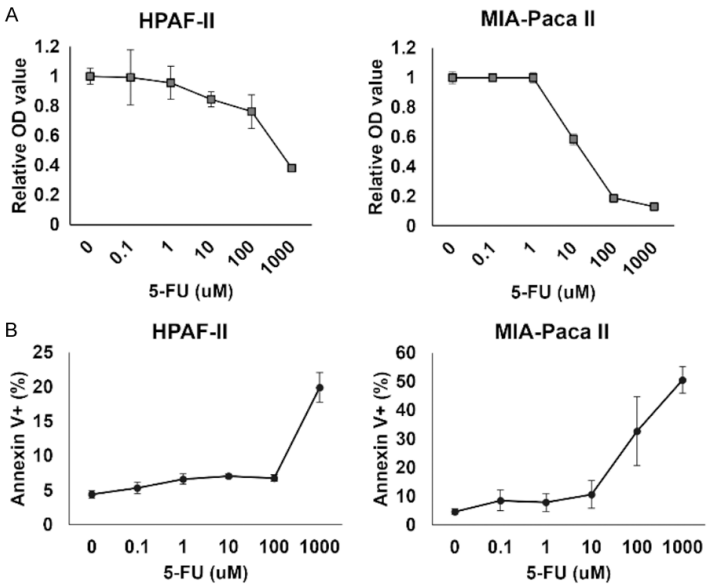

Figure 1. A. Concentration-dependent effects of 5-FU on the viability of the HPAF-II and Mia-Paca II pancreatic cancer cell lines. Following the addition of 5-FU, the cells were incubated for 72 hours, and cell viability determined by MTT assays. B. Concentration-dependent effects of 5-FU on pancreatic cancer cell apoptosis, as determined by flow cytometry. The values represent the means ± standard deviations (SDs) of three independent experiments. *, P < 0.05; **, P < 0.01.

fate-polyacrylamide gel electrophoresis and electro-transferred to nitrocellulose membranes. The membranes were blocked with 1% (v/v) nonfat dried milk in Tris-buffered saline with 0.05% Tween 20 and incubated with the appropriate primary antibodies diluted 1:1000; followed by incubation with secondary antibodies diluted 1:5000. Immunoreactive protein bands were visualized using a Chemiluminescent imaging system.

Invasion/migration assay

Invasiveness was measured *in vitro* using Transwell chamber assays (Chemicon, Millipore, GA, USA), in accordance with the manufacturer's protocol. Briefly, cells, at a concentration of 4×10^5 cells/mL in 150 μL of DMEM, were seeded onto the membrane of each well of the Transwell upper chamber. These cells were left untreated or treated with TTF, HT, or both. The medium in the upper chamber was serum-free, whereas the medium in the lower chamber contained 10% FBS as a source of chemoattractants. Cells that passed through the Matrigel or gelatin-coated membrane were incubated for 24 h with Cell Stain solution containing Crystal Violet, (Chemicon, Millipore, GA, USA) and photographed.

Wound-healing scratch assay

HPAF-II and Mia-Paca II pancreatic cancer cells were seeded onto 6-well plates (Corning) at 2.5×10^4 cells/well in 3 ml medium supplemented with 10% FBS. After 2 days, monolayers were disrupted mechanically using a sterile 200-μl pipette tip. All assays were performed in duplicate. Cells were untreated or treated with TTF, 5-FU or both for 24 h, and the wells were photographed. Cells were then stained with 0.2% crystal violet. Cell migration was monitored using a Nikon Eclipse Ti microscope with a DS-Fi1 camera, and cells were counted using ImageJ software (United States National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Means were compared using Student's t-tests. *P*-values < 0.05 were defined as statistically significant.

Results

Effects of 5-FU in pancreatic cancer cells

The sensitivity of the pancreatic cancer cell lines HPAF-II and Mia paca-II to 5-FU was tested by analyzing the dose-dependent effects of 5-FU on cell viability. As the concentration of 5-FU increased, the viability of these cancer cells decreased, with HPAF-II exhibiting stronger drug resistance (**Figure 1A**). Additionally, annexin V, a marker of cell apoptosis, increased with increasing concentrations of 5-FU (**Figure 1B**).

Effects of combined treatment

To evaluate the effects of the combination of 5-FU and TTF on these pancreatic cancer cell lines, cells were treated with 5-FU, TTF, or their combination (5-FU+TTF), and cell viability was measured. Cell density after treatment with 5-FU, TTF or their combination was lower than that of untreated cells, with the difference

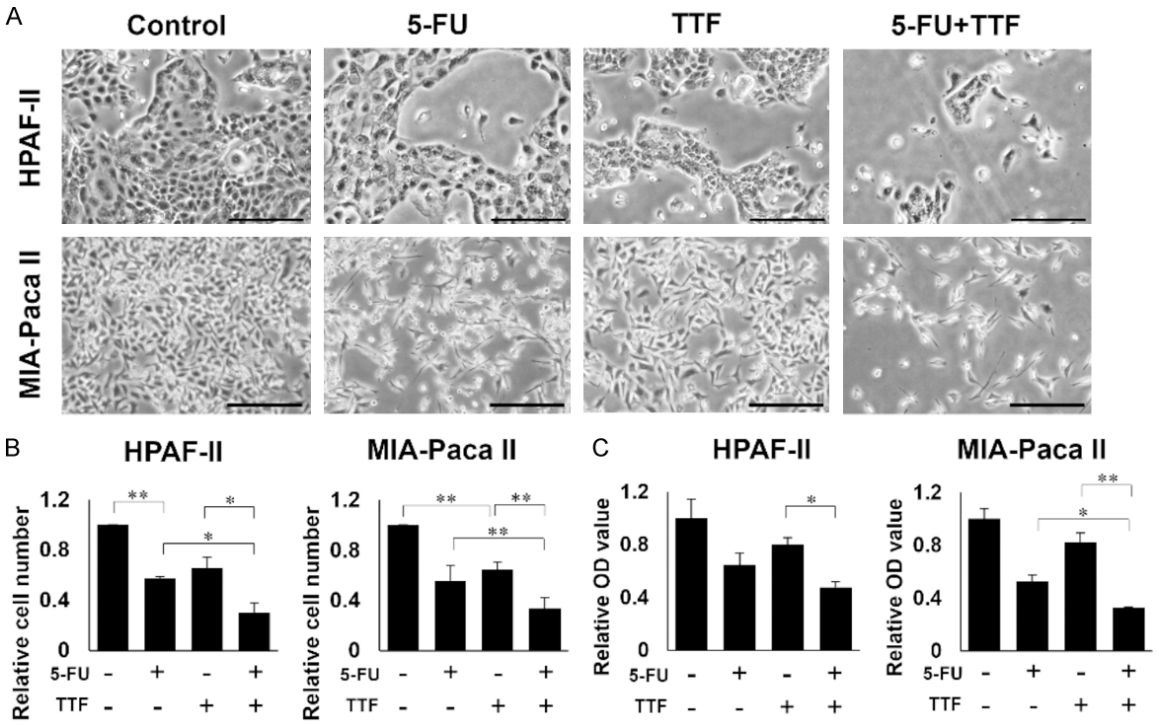

Figure 2. Effects of 5-FU, TTF, and 5-FU+TTF on the viability of the HPAF-II and Mia-Paca II pancreatic cancer cell lines, as determined by (A) microscopic imaging, (B) cell counts, and (C) MTT assays. Scale bar; 100 μm. The values represent the means ± SDs of three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

being particularly noticeable in cells treated with 5-FU+TTF (**Figure 2A**). Cell counting (**Figure 2B**) and MTT assays (**Figure 2C**) also showed that the numbers of viable cells were significantly lower after treatment with 5-FU+ TTF than after treatment with either agent alone.

The effects of 5-FU and TTF treatment on the reproduction of these pancreatic cell lines were evaluated by clonogenic assays (**Figure 3A**, **3B**). The numbers of colonies were lowest in HPAF-II and MIA-Paca II cells treated with 5-FU+TTF. Based on the fraction of surviving cells, the synergistic effects of 5-FU and TTF treatment were evaluated using the Valeriote and Carpentier equations [17, 18]. These equations showed that 5-FU and TTF had a synergistic effect on both pancreatic cancer cell lines. In addition, 3D culture assays showed that the formation of cell spheres in pancreatic cancer was inhibited in cells treated with 5-FU, TTF and 5-FU+TTF compared with untreated control cells (**Figure 3C**), with sphere diameter being smallest in cells treated with 5-FU+TTF.

Effects of 5-FU and TTF on apoptosis

TTF treatment has been reported to induce apoptosis in several types of cancer cells, including glioma, hepatocellular carcinoma, and pancreatic cancer cells [19-21]. To evaluate whether 5-FU increases TTF-induced apoptosis in pancreatic cancer cells, the levels of expression of the apoptosis markers annexin V and cleaved-poly (ADP-ribose) polymerase (PARP) were measured. Flow cytometry showed that treatment of these cells with 5-FU, TTF, and 5-FU+TTF increased the numbers of annexin V-positive cells (**Figure 4A**). The increase in apoptosis was more pronounced in cells treated with TTF than with 5-FU, and even more pronounced in cells treated with 5-FU+TTF. The combination of 5-FU and TTF markedly increased the level of expression of cleaved-PARP compared with either agent alone (**Figure 4B**).

Effects of 5-FU and TTF on metastasis

Treatment with 5-FU [19] and TTF [20] alone has been reported to inhibit tumor metastasis.

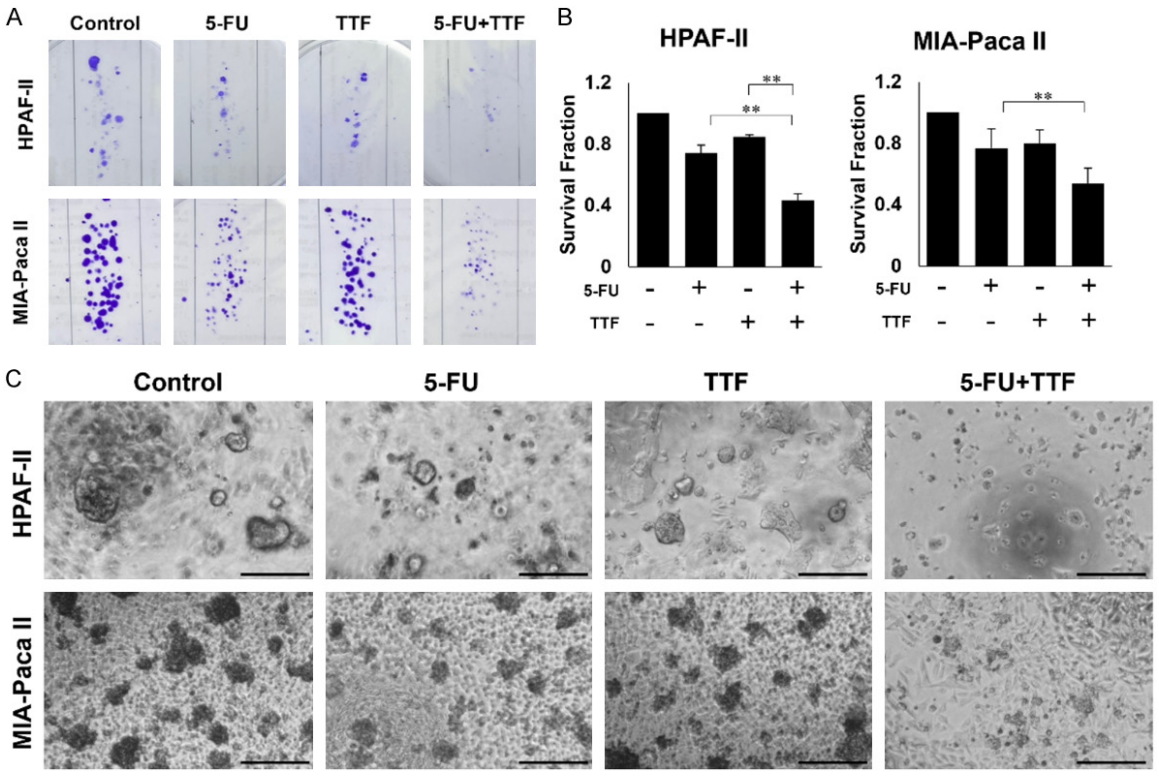

Figure 3. A, B. Effects of 5-FU, TTF, and 5-FU+TTF on the survival of the HPAF-II and Mia-Paca II pancreatic cancer cell lines, as determined by clonogenic assays. Cells were treated with TTF (1.1 V/cm and 150 kHz), 5-FU (5 μM), or both for 72 hours. The values represent the means ± SDs of three independent experiments. *, $P < 0.05$; **, $P < 0.01$. C. Effects of TTF, 5-FU, and 5-FU+TTF on sphere diameters of 3D cultures of pancreatic cancer cells. Scale bar; 100 μm.

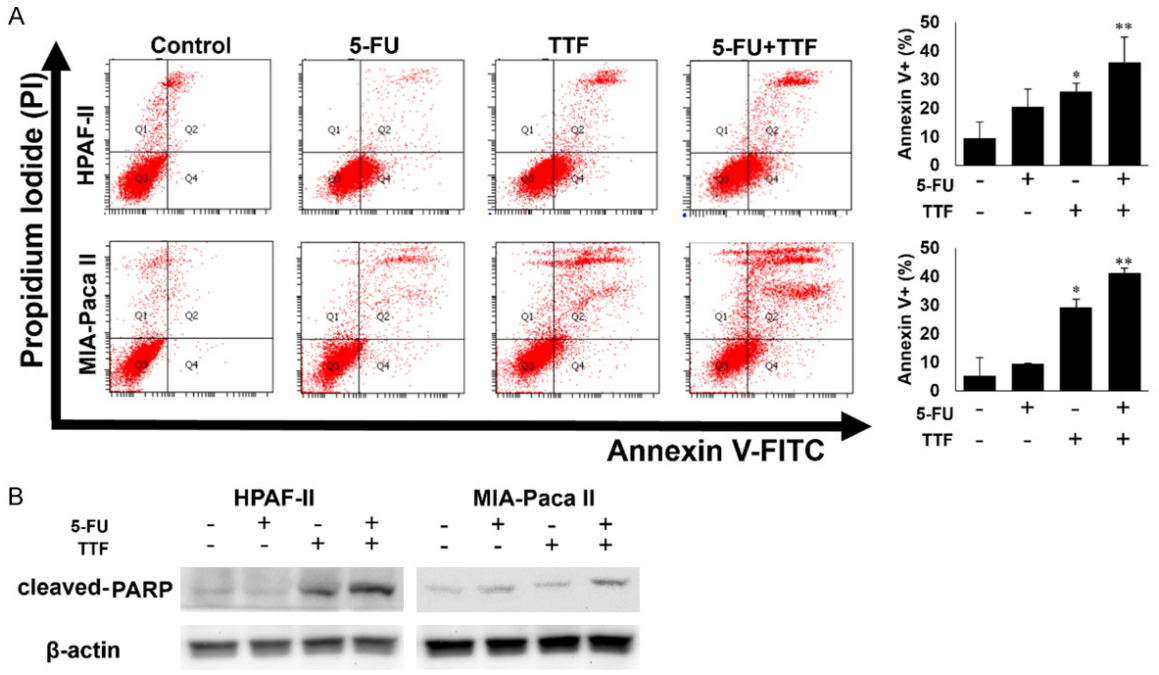

Figure 4. Effects of 5-FU, TTF, and 5-FU+TTF on the expression of annexin V and cleaved PARP by pancreatic cancer cells. A. Apoptotic cell rates were determined by flow cytometry. The values represent the means ± SDs of three independent experiments. *, $P < 0.05$; **, $P < 0.01$. B. Equal amounts of cell lysates were separated by SDS-PAGE electrophoresis, transferred to nitrocellulose membranes, and analyzed by western blotting with anti-cleaved PARP antibodies.

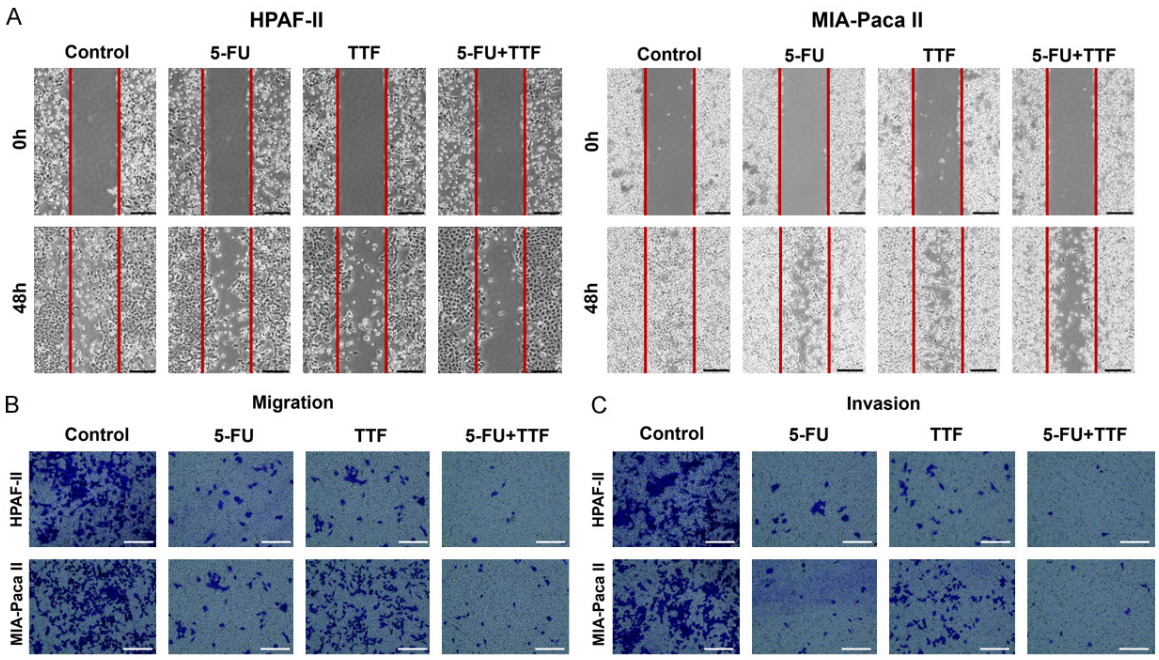

Figure 5. Effects of 5-FU, TTF, and 5-FU+TTF on pancreatic cancer cell migration, as assessed using (A) scratch assays and (B) Transwell chamber and on (C) pancreatic cancer cell invasion, as determined by Matrigel invasion assays. Scale bar; 100 μm (A) and 200 μm (B, C).

Therefore, the present study evaluated the effects of combined treatment with 5-FU and TTF on the migration and invasion of the pancreatic cancer cell lines HPAF-II and Mia-Paca II cell lines using the wound healing scratch assay and chamber analysis method. Wound healing scratch assays showed that migration over 48 hours was lowest in cells treated with the combination of 5-FU and TTF (**Figure 5A**). Furthermore, treatment with 5-FU or TTF alone reduced the migration and invasion of these cancer cells, with particularly significant results observed in cells treated with 5-FU+TTF (**Figure 5B**, **5C**).

Discussion

Although overall survival rates of cancer patients have been increasing, pancreatic cancer remains difficult to treat, with 5-year survival rates < 10% [1, 2]. At present, pancreatic cancer ranks as the fourth leading cause of cancer-related deaths worldwide [22], indicating the need for new treatment methods.

A phase 2 clinical trial targeting pancreatic cancer patients showed that the addition of TTF to chemotherapy (gemcitabine or gemcitabine plus nab-paclitaxel) resulted in a > 2-fold increase in 1-year survival rates, as well as increases in OS and progression-free survival (PFS), compared with chemotherapy alone [10]. These findings indicated that the addition of TTF significantly improved the effectiveness of chemotherapy. A prospective, randomized phase 3 trial comparing TTF plus chemotherapy with chemotherapy alone in patients with pancreatic cancer is currently underway [23].

5-FU is an anti-cancer agent that acts by inhibiting the activity of thymidylate synthase (TS), thereby disrupting essential biosynthetic activity or incorporating metabolites into RNA and DNA [6]. Despite these activities, however, the clinical application of 5-FU is limited due to drug resistance. Efforts to overcome this resistance have included the development of treatment modalities that synergize with 5-FU. Combinations of 5-FU with other anticancer drugs, including oxaliplatin, liposomal irinotecan, and cisplatin, have been found to significantly increase patient survival rates [24, 25]. Furthermore, in colon cancer, 5-FU has been reported to act as a sensitizer for TTF, and combination therapy has been shown to downregulate mechanisms associated with cell proliferation, invasion, and migration, thereby enhancing anticancer effects [26].

TTF has been reported to inhibit DNA repair in cancer cells. For example, TTF was found to downregulate the expression of genes in the BRCA and Fanconi anemia (FA) pathways, thereby increasing replication stress [27]. Because 5-FU has been shown to induce double-strand DNA breaks (DSB) in cancer cells, TTF therapy inhibiting the BRCA pathway can sensitize cancer cells to 5-FU treatment [28]. Therefore, the synergistic effect of TTF and 5-FU can be attributed to the TTF associated interference with DNA repair of damage caused by 5-FU.

To our knowledge, the present study is the first to evaluate the in vitro effects of TTF plus 5-FU in pancreatic cancer cell lines. Administration of TTF plus 5-FU to pancreatic cancer cell lines resistant to 5-FU resulted in significant increases in annexin-V staining, indicating increased apoptosis, and cleaved-PARP. Additionally, combination treatment reduced cell invasion and migration, which are crucial steps in metastasis. These results suggest that combination treatment with TTF and 5-FU inhibits the proliferation and metastasis of pancreatic cancer cells while increasing cell death. Because these findings were based solely on in vitro results, the effects of TTF plus 5-FU should be evaluated in animal tumor models.

In conclusion, this study provides evidence that the combination of 5-FU and TTF exhibits synergistic effects on pancreatic cancer cell lines. These findings suggest that combination treatment may improve the prognosis of patients with pancreatic cancer.

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Disclosure of conflict of interest

None.

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