



The underlying molecular mechanism of intratumoral radiofrequency hyperthermia-enhanced chemotherapy of pancreatic cancer



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ABSTRACT

Background: To investigate the underlying molecular mechanisms of radiofrequency hyperthermia (RFH)-enhanced direct chemotherapy of pancreatic cancers.

Method: Rat ductal PaCa cell line DSL-6A/C1 and orthotopic pancreatic cancers of Lewis rats were divided into four study groups with various treatments: i) phosphate-buffered saline (PBS) as a control; ii) RFH alone; iii) intratumoral chemotherapy alone (gemcitabine); and (iv) combination therapy of gemcitabine plus intratumoral RFH at 42 °C for 30 min. In the in-vitro confirmation experiments, the viability and apoptosis of DSL-6A/C1 cells in each treatment group were evaluated using cell live/dead staining, flow cytometry, and Western blot. In the in vivo validation experiments, related proteins were evaluated by immunohistochemistry (IHC) staining of tumors.

Results: Of the in-vitro experiments, the lowest cell viability and more apoptotic cells were shown in the group with combination therapy compared to other treatments. Western blot data showed elevated Bax/Bcl-2, Caspase-3, and HSP70 expressions in DSL cells with combination therapy, compared to other treatments. Of the in vivo experiments, IHC staining detected the significantly increased expressions of HSP70, IL-1 β , TNF- α , Bax, and Caspase-3 in pancreatic cancer tissues of the animal group treated by combination therapy of gemcitabine with RFH.

Conclusion: Molecular imaging-guided interventional RFH can significantly enhance the chemotherapeutic effect on pancreatic cancers via potential molecular mechanisms of up-regulating Bax/caspase-3-dependent apoptosis pathways.

1. Introduction

Pancreatic cancer remains one of the most lethal malignancies in western countries.¹ In the United States, it is the third leading cause of cancer-related deaths with an estimated mortality of 40,560 in 2015.² Pancreatic cancer is a highly aggressive and lethal cancer characterized by high invasiveness and resistance to chemotherapy and radiation therapy.³ The poor prognosis for patients with pancreatic cancer could be mainly attributed to the limited efficacy of available systemic treatments⁴ or the development of cellular chemotherapeutic resistance, which can lead to therapy failure.⁵

Radiofrequency ablation (RFA), which has been widely used in the palliative treatment of a variety of solid parenchymal tumors,^{6–10} appears to be an attractive option in patients with unresectable, locally advanced,

and non-metastatic pancreatic cancer^{11–14} as well. However, RFA may cause inadvertent thermal injuries to peripancreatic structures, including the portal vein, duodenum, and common intra-pancreatic bile duct.⁸ To minimize the risk of inadvertent thermal damage to the portal vein with possible thrombosis, duodenal injury, bleeding and late duodenal stenosis, or necrotizing pancreatitis,¹⁵ RF-induced ablation must be confined at a safe distance from nearby important structures to reduce the effect of unwanted thermal damages to adjacent organs,⁸ which will induce the consequence that complete eradication of the pancreatic mass cannot be achieved.^{8,16} Combination therapy with other treatment modalities is needed to eradicate the untreated tumor cells at the peripheral areas that are spared by RF-induced thermal energies. Our studies confirmed that RFH can facilitate the delivery of chemotherapeutic drugs in tumors and enhance the sensitivity of cancer cells to certain

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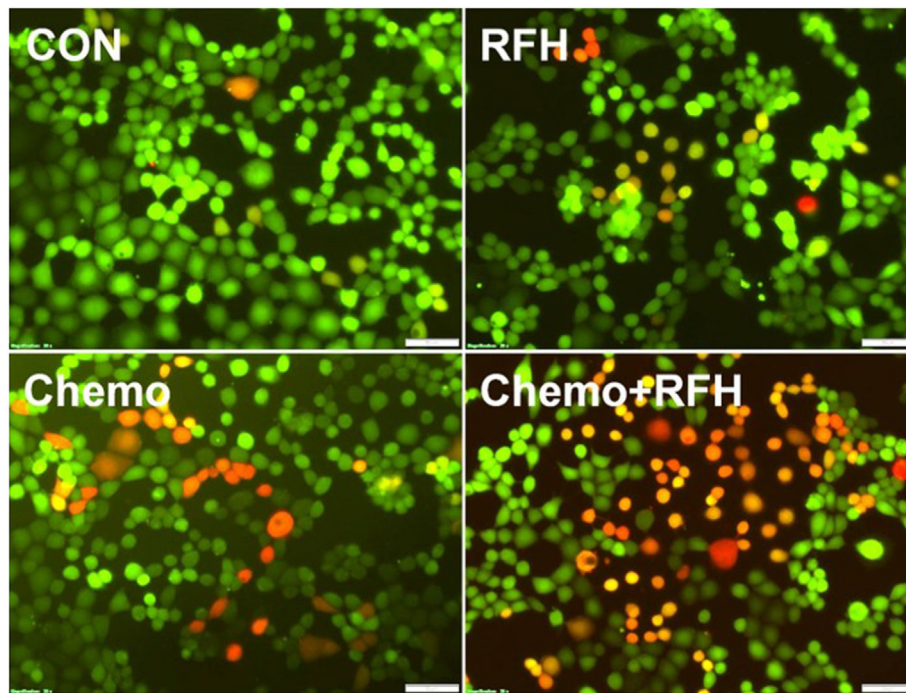


Fig. 1. Murine pancreatic cancer DSL cells were stained with calcein and propidium iodide to identify surviving (green) and dead (red) cells, demonstrating more dead cells with combination treatment (Chemo + RFH) (200× magnification, bar = 50 μ m). (Chemo = chemotherapy, RFH = radiofrequency hyperthermia, CON = control).

therapeutic agents. The major mechanisms may include the loss of membrane integrity,¹⁷ impairing DNA repair system accompanying with apoptosis,¹⁸ altering pH value,¹⁹ and enhancing immunostimulatory effects,²⁰ which eventually lead to tumor cell necrosis or apoptosis.

In the last decade, we have developed a radiofrequency heating guidewire, which functions as an intraluminal thermal energy source to enhance the treatment effect of lentivirus-mediated tumor suicide gene therapy/chemotherapies in cancers.^{21,22} Our group has already reported the technical feasibility of using ultrasound-guided intratumoral RFH to enhance local gemcitabine treatment of pancreatic cancer in animal models.^{23,24} However, the mechanism for increasing the efficacy of RFH for antitumor agents remains unclear. In the present study, we attempted to investigate the associated molecular mechanisms involved in enhancing therapeutic effects by the combined treatment.

2. Methods

2.1. Cell culture and RFH-enhanced chemotherapy

Rat ductal PaCa cell line DSL-6A/C1 was maintained in Waymouth's MB 752/1 medium (Gibco, Grand Island, NY) at 37 °C in humidified air with 5% CO₂. RF hyperthermia was performed as described in our previous literature.²⁵ Cells in different groups were treated with (a) gemcitabine (5.0 μ M) plus 30-min RFH at approximately 42 °C; (b) gemcitabine alone; (c) 30-min RFH alone, and (d) no treatment to serve as a control. We used the 50-percentage inhibitory concentration (IC₅₀) dose of gemcitabine for cell treatment, which was determined by Cell-Titer 96 Aqueous One Solution Cell Proliferation-assay (Promega Corporation, Madison, WI).

2.2. The live/dead cell assay

Viability/Cytotoxicity Kit for Mammalian cells (Invitrogen, Eugene, OR) was used to determine the cell viability.²⁶ Briefly, the cells were seeded at 1×10^5 cells per well in 2 mL Waymouth's medium in 6-well plates. 24 h after the treatments as described above, cells were dually

stained with calcein-AM or EthD-1, which enables the simultaneous determination of live and dead cells, according to the manufacturer's protocol. The polyanionic dye calcein-AM is converted to the intensely fluorescent calcein by intracellular esterase activity within live cells (495–515 nm). EthD-1 was used to identify dead or dying cells as it exclusively enters cells through the disrupted cell membrane and binds to nucleic acids (495–635 nm). Fluorescent images of cells were acquired with a 20 × magnification by an Olympus DP72 digital camera.

2.3. Apoptosis assay by flow cytometry

Flow cytometry was performed using propidium iodide (PI) and fluorescein isothiocyanate (FITC)-labeled annexin V (BD Pharmingen, San Diego, CA) to detect phosphatidylserine externalization as an endpoint indicator of early apoptosis. 24 h after the treatments, the cells were washed twice with cold PBS at 4 °C and centrifuged at 1000×g for 5 min. 5- μ l FITC-labeled Annexin V, 10- μ l PI, and 5- μ l Hanks' balanced salt solution were added to the cell suspension and mixed gently. After 30-min incubation in the dark, the cells were analyzed by flow cytometry (BD Bioscience, USA).

2.4. Western blot analysis of proteins

The cells were collected and lysed in lysis buffer (Pierce RIPA, Thermo Scientific, MA) for 20 min. After brief sonication, the lysates were centrifuged at 16,000 g for 30 min at 4 °C, and the protein content in the supernatant was measured using a Pierce BCA Protein Assay kit (Thermo Scientific). After mixing with 4 fold SDS-loading buffer (Life Technologies, Carlsbad, CA), the protein lysates were denatured at 96 °C for 5 minutes, applied on an SDS polyacrylamide gel (Invitrogen, Life Technologies, Carlsbad, CA) for electrophoresis, and transferred to nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK). Western blot analysis was performed using primary antibodies (1:1000) to caspase-3 (1:1000, Cell Signaling Technology), Bcl-2 (1:1000, Abcam), Bax (1:1000, Abcam), HSP70 (1:1000, Abcam), GADPH (1:2000, ThermoFisher Scientific). The secondary horseradish

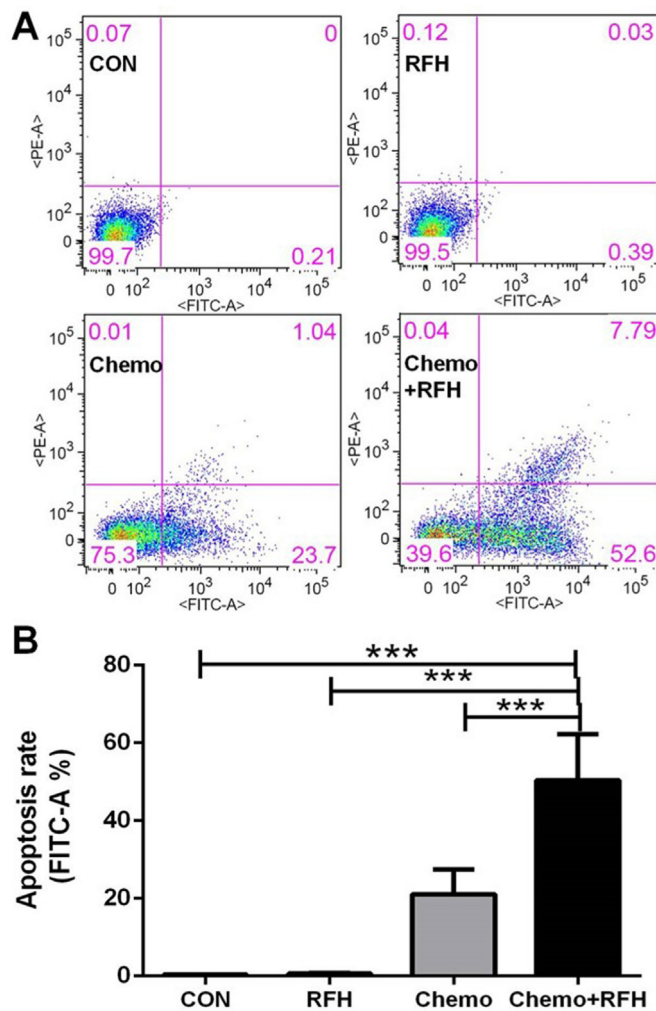


Fig. 2. A combination treatment (Chemo + RFH) induces DSL cell apoptosis. (A) Apoptotic cells were measured by flow cytometry 24 h after treatment. (B) The histogram shows the highest percentage of apoptotic cells in the cell group treated with Chemo + RFH (** $P < 0.001$). (Chemo = chemotherapy, RFH = radiofrequency hyperthermia, CON = control).

peroxidase (HRP)-conjugated antibodies (1:2000) were purchased from Cell Signaling Technology. The level of protein expression was quantified by measuring the density of the immunoreactive bands and subsequently normalizing to GADPH.

2.5. In vivo experiments

The animal protocol was approved by our Institutional Animal Care and Use Committee (Protocol number:4120-02). Rat models with orthotopic pancreatic cancer were created in Lewis rats weighted 150–200 g and RF hyperthermia was performed as described in our previous study.²³ When the orthotopic pancreatic tumors had grown to 5–10 mm in diameter, six rats in each of four groups were treated by (a) intratumoral injections of gemcitabine (100-mg/kg for rats), followed by RFH at approximately 42 °C for 30 minutes; (b) intratumoral injection of gemcitabine alone; (c) 30-min RFH alone; and (d) injection of 100 μ L PBS to serve as a control. All groups of rats were sacrificed 24 h after the treatments for tumor collection. We used a multi-functional perfusion-thermal RF electrode and an RF generator (Welfare Electronics Co., Beijing), which consists of multiple prongs to deliver hyperthermia and chemotherapeutic drugs to the tumors.

2.6. Immunohistochemistry analysis

Tumors were harvested 14 days after treatments and fixed in PBS-buffered 10% formalin for at least 24 h and embedded in paraffin. The samples were then sectioned using a microtome (Leica, Germany) and mounted in Poly-L-Lysine-coated slides for IHC examination. Slides were placed in 1:20 citrate buffer in a decloaking chamber under pressure for 5 minutes after being deparaffinized, dehydrated, washed, and then depressurized for 5 minutes and allowed to cool. Endogenous peroxidase was quenched by 15-min incubation in distilled water containing 0.3% hydrogen peroxide. The transverse paraffin sections were incubated in 3% H₂O₂ and 80% carbinol for 30 min and then in blocking solution at room temperature for 1 h. Subsequently, the sections were incubated with the following primary antibodies overnight at 4 °C, including caspase-3 (1:200, Cell Signaling Technology), Bcl-2 (1:200, Abcam), Bax (1:200, Abcam), HSP70 (1:200, Abcam), IL-1 β (1:200, Abcam), and TNF- α (1:200, Abcam). After triple washing in PBS, the sections were incubated with horseradish peroxidase-conjugated secondary antibodies at 37 °C for 1 h. Then, the reaction was stopped with 3,3'-diaminobenzidine, and the image was captured at a 400 \times magnification. Digital histological scans of a whole section were acquired with a 400 \times magnification on an Olympus DP72 digital camera and image processing software (Image-pro plus 6.0) and quantitative data were counted randomly from 10 fields of each sample slice.

2.7. Statistical analysis

Statistical analysis was carried out by one-way ANOVA followed by post-hoc Turkeys multiple comparisons. All p-values were two-sided, and a p-value less than 0.05 indicated statistical significance. Values are reported as mean \pm standard deviation (SD) unless otherwise indicated.

3. Results

3.1. RFH-enhanced chemotherapeutic effect on pancreatic cancer cells

Of the in-vitro experiments, compared to the other three treatments, the two-color fluorescence cell viability/cytotoxicity assay demonstrated the lower cell viability in the group with combination therapy (Fig. 1). To further examine the effects of combination therapy-induced cell death, PI/Annexin V-FITC assay was carried out following the treatment in DSL cells, demonstrating the highest percentage of early apoptotic cells in the cell group with combination therapy (combination therapy vs gemcitabine vs RFH vs saline: 0.51 ± 0.33 vs 0.21 ± 0.026 vs 0.062 ± 0.003 vs 0.050 ± 0.004 , $P < 0.001$, Fig. 2).

3.2. Determination of apoptosis-related biomarker levels by western blotting

To further investigate the molecular mechanism of RFH-enhanced chemotherapy, we also evaluated the expression levels of cell apoptosis-related proteins by Western blot analysis. The results showed that gemcitabine in combination with RFH markedly increased the expressions of cleaved caspase-3 and Bax, compared with RFH treatment alone (Fig. 3). Furthermore, suppression of Bcl-2 expression was observed in cells 24 h after combination treatment. Western blot analysis also showed that gemcitabine slightly increased RFH-induced HSP 70 expression.

3.3. Determination of apoptosis-related biomarker levels by IHC in tumors

Chemotherapy significantly elevated the expression of caspase 3 in the tumors. Furthermore, combination therapy of intratumoral gemcitabine with RFH significantly argument the upregulation of caspase-3 in tumors (IOD values in control vs. RFH vs gemcitabine vs. combination therapy: 2742 ± 374.2 vs. 2946 ± 44.1 vs. 6969 ± 675.8 vs. $11,289 \pm$

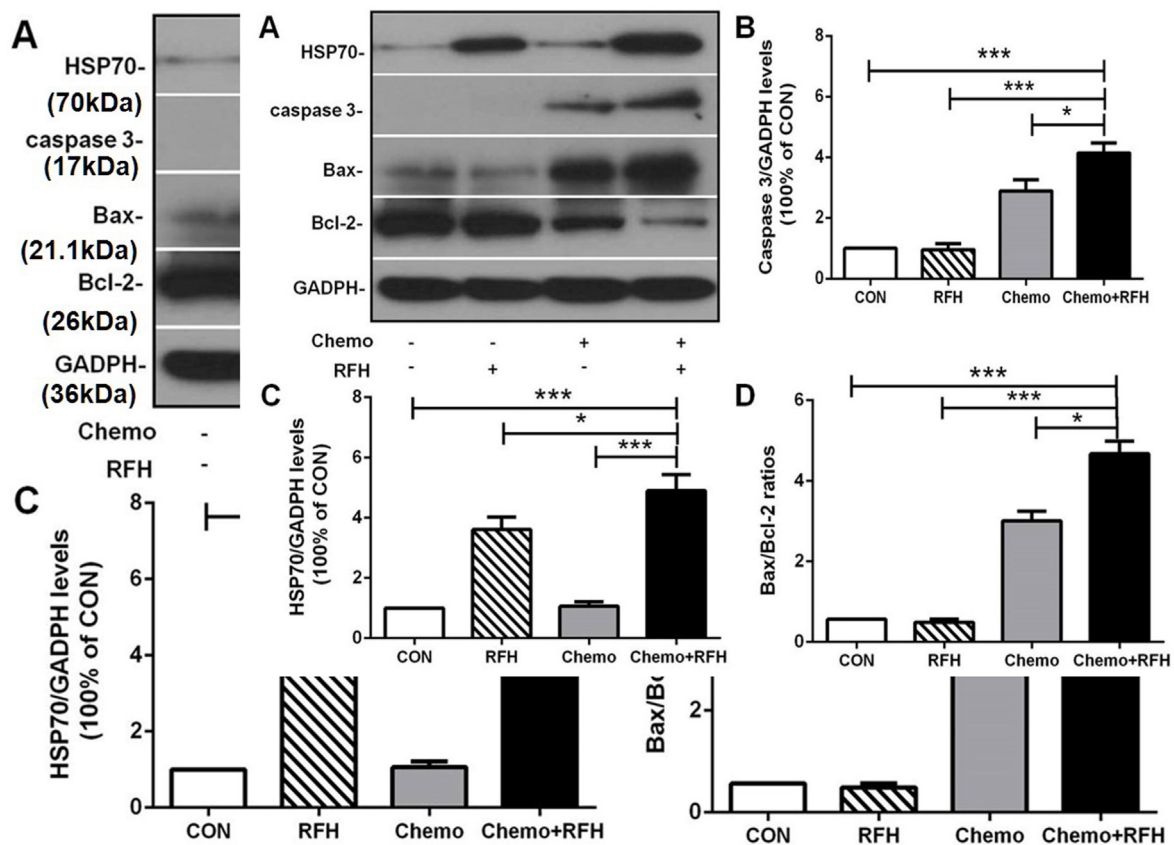


Fig. 3. Western blot analysis was performed to detect the expressions of cell apoptosis and immune response-related proteins. (A) Expressions of caspase-3, Bax, Bcl-2, and HSP70 were detected by Western blot analysis. The anti-NADPH antibody was used as an internal control for the Western blot analysis. (B–D) The caspase-3, Bax/Bcl-2, and HSP70 signals were normalized to the NADPH signals, and the relative ratios are shown below each band. (Chemo = chemotherapy, RFH = radiofrequency hyperthermia, CON = control).

1925, $P < 0.05$, Fig. 4a). Simultaneously, the highest expression level of Bax, the pro-apoptosis factor, was observed in the rat group with the treatment of chemotherapy plus RFH, as compared with the chemotherapy-only group, RFH-only group, and the control group (control vs. RFH-only vs. chemotherapy-only vs. chemotherapy plus RFH: 2929 ± 404.5 vs. 3242 ± 400.3 vs. 6343 ± 613.2 vs. $10,774 \pm 1416$, $P < 0.05$, Fig. 4b). However, the level of Bcl-2, the anti-apoptosis factor, was significantly decreased in the rat group with combination therapy ($187,875 \pm 28,195$ vs. $146,385 \pm 21,326$ vs. $50,517 \pm 6306$ vs. 9795 ± 2269 , $P < 0.05$, Fig. 4c).

IHC staining of the tumor tissues of rat pancreatic cancers showed that RFH rather than chemotherapy slightly increased HSP70 expression and combination therapy of RFH with gemcitabine significantly increased the expression levels of HSP 70 in tumors (control vs. RFH vs. chemotherapy vs. combination therapy: $116,874 \pm 18,362$ vs. $900,361 \pm 161,749$ vs. $150,274 \pm 19,932$ vs. $1,440,061 \pm 14,156$, $P < 0.001$, Fig. 5a). Compared with the other three groups, the combination therapy induced the highest levels of the inflammatory cytokines (TNF- α : 1790 ± 148.2 vs. 2934 ± 476 vs. $11,694 \pm 1371$ vs. $19,597 \pm 439.1$; IL-1 β : 6794 ± 858.7 vs. 7765 ± 739.1 vs. $10,444 \pm 797.4$ vs. $15,017 \pm 1841$, $P < 0.05$, Fig. 5b–c), which indicates that the local immune responses promoted by the upregulated HSP70 expression may play a role in the enhanced chemotherapeutic effect by RFH.

4. Discussion

In our study, RFH can increase cell apoptosis level in murine pancreatic cancer cell lines and pancreatic cancer tumors, as manifested by flow cytometry assay, which demonstrated the highest percentage of apoptotic

cells with combination therapy in in-vitro experiments.²³ Furthermore, in this study, we investigated the potential mechanisms of intratumoral RFH-enhanced chemotherapeutic effects in cancers. Our findings showed that: (a) some factors related to immune responses, such as HSP70, IL-1 β , and TNF- α in tumor tissues, were elevated significantly after combination therapy, indicating HSP70-dependent local immune responses may involve in the mechanisms of the therapy; (b) both in vitro and in vivo experiments showed significant changes in Bax, Bcl-2, and caspase-3 expressions in the pancreatic cancer cell groups with combination therapy, compared to other controls, suggesting caspase-3-dependent cell apoptosis pathways may also involve in the enhancement of RFH-induced apoptosis.

4.1. HSP70 dependent immune response

HSP70 is a highly conserved protein that plays a role in response to a variety of stresses, such as aging, metabolic challenge, oxidative stress, and hyperthermia.²⁷ In an organism, HSP70 plays a dual role in the pathogenic progression of cancers.²⁷ Firstly, it protects the cells from antitumor agents. It was reported that the majority of cancer cells contain a high amount of HSP70 and, therefore, are protected from environmental stressors and drugs.²⁷ However, when transported out of a cell, HSP70 can also regulate immune function, including antigen presentation, dendritic cell maturation, NK cell, and MDSC activities.^{28,29} Then this immunization resulted in a T cell-mediated immune response including a significant increase in CD4⁺ or CD8⁺ T cells,^{29,30} even induction of cytokines, such as IL-1 β and TNF- α .³¹

Our study also shows that the combination therapy of chemotherapy with RFH significantly enhanced the HSP 70 levels. In local tumor samples of the tumor group with the combination of RFH and

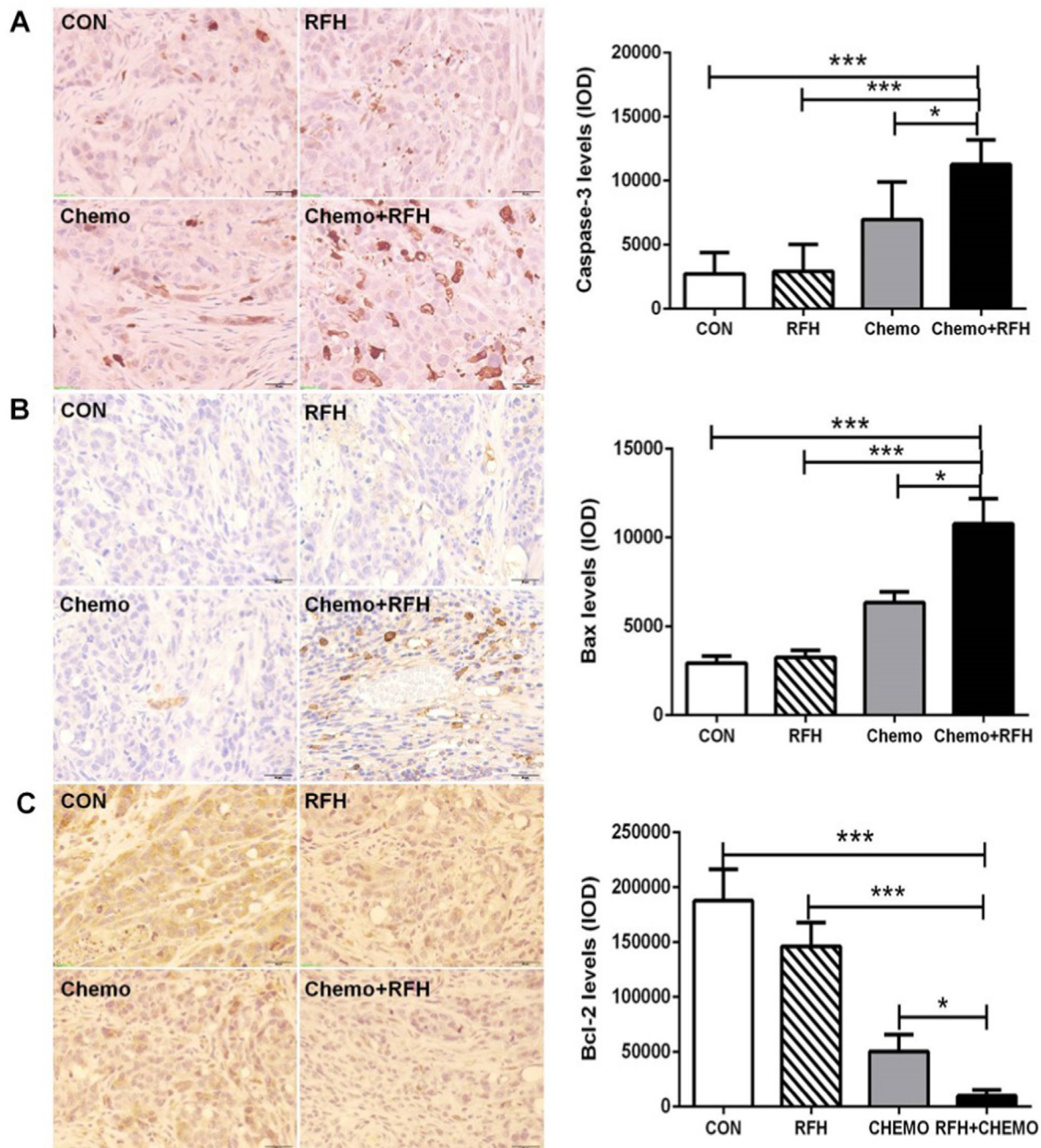


Fig. 4. Quantitative analysis of tumor tissue immunohistochemistry staining revealed the overexpression of caspase-3 (A), Bax (B), and reduced expression of Bcl-2 (C) proteins in rat orthotopic pancreatic cancers with combination therapy, compared to the other three treatment groups ($n = 6 \times$, $400 \times$ magnification, scale bar: $50 \mu\text{m}$, $*P < 0.05$, $***p < 0.001$). (Chemo = chemotherapy, RFH = radiofrequency hyperthermia, CON = control).

gemcitabine therapy, elevated IL-1 β and TNF- α levels were confirmed. Both results suggested elevated local immune responses after the combination therapy of RFH with different agents. Pancreatic cancer is a highly aggressive cancer characterized by low immune responses in the tumor microenvironment and resistance to treatment.^{3,32} HSP70 can contribute to anti-tumor immunity, such as regulating immune function, instead of for oncogenesis. Novel therapies of combining different therapeutic agents with RFH have been shown to target the tumor microenvironment to decrease this resistance, improve immune tolerance and increase the efficacy of the current treatment.³³ Additional clinical trials with effective combination therapy need to be further evaluated in the future.

4.2. Caspase-3 dependent cell apoptosis pathway

Caspases are the important executioners of apoptosis induced by various apoptotic stimuli.³⁴ In the present study, both IHC and Western blot analysis data showed the increased expression of caspase-3 protein in pancreatic cancer cells and rat cancers by the combined treatment of RHF plus Gemcitabine. Caspases play a critical role in the initiation and execution of apoptosis. They transduce the apoptotic cell death signals in a cascade manner, where the initiator caspases (caspase-8) cleave and activate the effectors (caspase-3), which then leads to cell apoptosis.^{18,35,36} As zymogen caspase-3 exists in the cytoplasm and is activated in the early stage of apoptosis to degrade the substrates in cells, eventually leading to apoptosis.³⁷

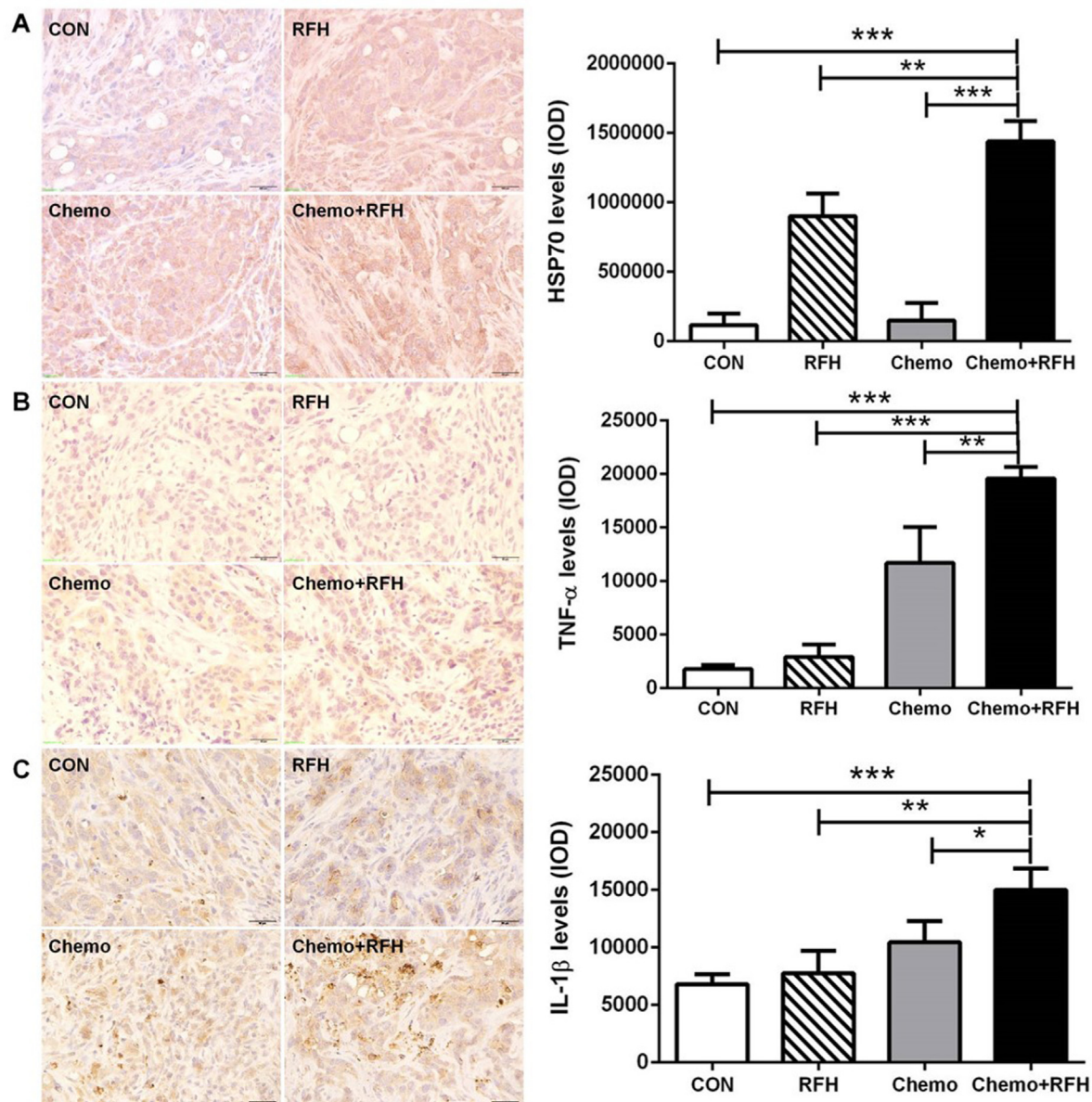


Fig. 5. Quantitative analysis of tumor tissue immunohistochemistry staining revealed the overexpression of caspase-3 (A), TNF- α (B), and IL-1 β (C) proteins in rat orthotopic pancreatic cancers with combination therapy, compared to the other three treatment groups ($n = 6 \times$, 400 \times magnification, scale bar: 50 μ m, $*P < 0.01$, $***p < 0.001$). (Chemo = chemotherapy, RFH = radiofrequency hyperthermia, CON = control).

4.3. Conclusions

Molecular imaging-guided interventional RFH can significantly enhance the therapeutic effect of gemcitabine in pancreatic cancers via the molecular mechanisms for upregulating the expressions of HSP70/IL-1 β /TNF- α -dependent immune regulation and Bax/Bcl-2/caspase-3-dependent apoptosis pathways. These mechanisms may provide new targets for further improving the efficacy of interventional molecular image-guided RFH-enhanced direct intratumoral therapy for pancreatic cancer, one of the deadliest malignancies worldwide.

Conflict of interest

We have no conflict of interest to declare.

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