

Radiofrequency heat-enhanced direct intratumoral chemotherapy for prostate cancer

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Abstract. A novel, minimally invasive interventional technique, radiofrequency heat (RFH), has been suggested to improve the efficacy of chemotherapy for solid organ tumors. However, the treatment for prostate cancer has not been completely characterized. The aim of the present study was to investigate the *in vitro* and *in vivo* efficiency of chemotherapy in combination with RFH for the treatment of prostate cancer. The following four treatment groups were included: i) No treatment (control); ii) RFH-only; iii) chemotherapy (docetaxel)-only; and iv) combination therapy of docetaxel and RFH in human prostate cancer (HPC) cell lines and mice with HPC xenografts. In the *in vitro* experiments, a heating guidewire was attached under the bottom of the last chamber of the four-chamber cell culture slide, and was then connected to a radiofrequency (RF) generator. In the *in vivo* experiments, a tumor model was generated by subcutaneously injecting human prostate cancer cells into 24 male nu/nu mice. RFH was conducted by inserting the 0.022-inch heating-guidewire into the tumor. The follow-up magnetic resonance imaging demonstrated a significant reduction in the average tumor size in animals treated with combination therapy compared with those receiving RFH-only and chemotherapy-only. The number of apoptotic cells and the average apoptotic index of the combination therapy group were significantly higher

compared with those of the other three treatment groups. In conclusion, the results of the present study suggested that RFH is able to increase the therapeutic efficiency of docetaxel in prostate cancer, and this study serves as a foundation for the future development of an interventional molecular image-guided local treatment strategy for prostate cancer that integrates RF technology, interventional oncology and direct intratumoral chemotherapy, as a replacement for systemic chemotherapy.

Introduction

Prostate cancer (PC) is the second most frequently diagnosed type of cancer and the sixth leading cause of cancer mortality worldwide (1,2). An estimated 160,000 new cases of prostate cancer were diagnosed annually in the United States (3). With the widespread use of prostate-specific antigen (PSA) screening and prostate biopsy, an increased number of men are being diagnosed with low-grade and localized prostate cancer, and a large portion of prostate cancer cases are being diagnosed as unifocal and unilateral lesions (4). Overtreatment has occurred in >30% of these patients (5). Traditional whole-gland radical therapies, including external beam radiotherapy and radical prostatectomy (RP), cannot achieve the local removal of lesions without damaging adjacent normal structures, such as the urethra and colorectum (5).

Docetaxel is one of the most potent chemotherapy drugs used for the treatment of prostate cancer (6). However, its clinical applications via systemic delivery are limited by its low chemotherapeutic distribution at the tumor site and its high toxicity to other vital organs (7). Furthermore, previous studies also suggested that an insufficient deposition of chemotherapeutic drugs at the target tumor via systemic administration contributes significantly to the development of cancer chemoresistance (8). Therefore, a local approach to administration to increase the accumulation of the chemotherapeutics at the target lesions and decrease the accumulation in other areas, such as by direct intratumoral administration

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of chemotherapeutics via interventional oncology techniques, may resolve this issue. In addition, hyperthermia with a temperature of $\sim 42^{\circ}\text{C}$ may increase the effectiveness of chemotherapy in a variety of malignancies (9-11). However, the lack of adequate devices for local heat delivery to the targets and appropriate temperature monitoring methods at the target sites, limits the application of hyperthermia-enhanced therapy for prostate malignancies (12).

Therefore, an image-guided, minimally invasive interventional approach that not only permits the local delivery of highly concentrated therapeutics to the target site, but also avoids therapeutic toxicity to other vital organs (which is often a consequence of systemic delivery approaches) should be investigated (13). Although previous advancements in interventional oncology technologies have improved the outcomes of patients with cancer (7), the present study hypothesized that the combination of interventional oncology and local hyperthermia may additionally improve the efficacy of chemotherapies in organ cancer. Therefore, a novel technique interventional radiofrequency heat (RFH), which combines these two aforementioned conditions, was considered. Previous studies by the authors have demonstrated an improvement in efficacy of chemotherapy in cholangiocarcinoma and breast cancer by RHF (10,14). Therefore, the aim of the present study was to identify whether the efficiency of direct intratumoral chemotherapy for prostate cancer may be enhanced using this novel method, RHF.

Materials and methods

The present study was divided into two phases: i) *In vitro* 'proof-of-principle' experiments which demonstrate that RFH enhances chemotherapeutic efficiency in human prostate cancer cells; and ii) *in vivo* validation that interventional RFH enhances direct intratumoral chemotherapy for prostate cancer in a xenograft animal model.

Cell lines. The human prostate cancer PC-3 cell line (American Type Culture Collection, Manassas, VA, USA) was used. The PC-3 cell line was initiated from a bone metastasis of a grade IV prostatic adenocarcinoma from a 62-year-old male Caucasian. The PC-3 cell line was cultured in RPMI-1640 media (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified incubator with a 5% CO_2 atmosphere.

***In vitro* experimental model.** Each chamber of a Lab-Tek® 4-chamber cell culture slide™ (Thermo Fisher Scientific, Inc.) was seeded with 2×10^4 PC-3 cells. The slide was placed in a water bath at 37°C . A 0.032 inch heating guidewire was attached under the bottom of chamber 4 of the four-chamber cell culture slide and was then connected to a radiofrequency (RF) generator (MPG-4; OPTHOS Instruments, Inc., Rockville, MD, USA) When the RF generator was operated at 2-3 W through the heating guidewire, the temperature in chamber 4 increased from $37-42^{\circ}\text{C}$, creating a heat gradient along the four chambers (Fig. 1). The temperature of each chamber was recorded using a micro-thermometer (Photon Control Inc., Richmond, Canada).

To compare the therapeutic effects of different RFH temperatures, the same amount of docetaxel (52 nM) (Hopira, Lake forest, IL, USA) was placed into each of the four chambers as the bottom of chamber 4 was heated to 42°C for 20 min, and the temperature of chamber 1 was maintained at 37°C . To compare the therapeutic effects of different treatments, the prostate cancer cells were divided into four groups: No treatment (control), RFH only (42°C for 20 min), chemotherapy only (chemo-only, 52 nM docetaxel) and combination therapy (chemotherapy plus RFH). A docetaxel concentration of 52 nM was selected according to previous studies (15,16). Following this, the cells were then cultured at 37°C for 72 h, and subjected to different laboratory evaluations, including cell proliferation and apoptosis assays, to examine and compare the effects of the different treatments on prostate cancer cells.

The treatments were initiated when the cell confluency reached 80%. RFH-enhanced chemotherapy was performed by adding docetaxel into the medium and heating the cells to $\sim 42^{\circ}\text{C}$ with the RF power set to 12-14 W for 20 min. Docetaxel was maintained in the chamber for 24 h following RFH. The cells in the chemotherapy-only group were treated with docetaxel for the same duration (24 h).

Cell proliferation assay. Cell proliferation was evaluated by the MTS assay (Promega Corp., Madison, WI, USA). Briefly, 120 μl MTS reagent was added to 0.6 ml of medium in each chamber, and the cells were incubated at 37°C for 3 h. Then, 120 μl medium was transferred to a 96-well plate, and the absorbance was detected at 490 nm with a Microplate Reader (VersaMax; Molecular Devices, LLC, Sunnyvale, CA, USA). The relative cell proliferation rates of the different cell groups were evaluated using the following equation: $A_{\text{treated}}/A_{\text{control}}$, where A is the absorbance. All of the experiments for all cell groups were repeated six times.

***In vivo* experimental model.** The animal protocol was approved by Sir Run Run Shaw Hospital Institutional Animal Care and Use Committee (Hangzhou, China). A total of 24 male mice (athymic nu/nu; 4-6 weeks; weight, ~ 20 g; Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China) were used to generate a tumor model. The mice were maintained on Alpha-Dri bedding in temperature ($22 \pm 2^{\circ}\text{C}$) and humidity (30-50%) in controlled rooms with a 12/12 h light/dark cycle. The feeding method for mice was *ad libitum*. A suspension of 1×10^7 PC-3 cells in 100 μl PBS was injected subcutaneously and unilaterally into the back of each mouse to seed a prostate cancer mass. Within three weeks, the tumor masses had grown to 5-10 mm in diameter (Fig. 2).

RFH-enhanced chemotherapy. A total of 24 mice bearing human prostate cancer xenografts were randomly stratified into four study groups (6 mice per group) for receipt of different intratumoral treatments: i) PBS (control); ii) RFH-only (42°C for 20 min via the heating guidewire); iii) chemo-only (intratumoral injection of 75 mg/m^2 docetaxel); and iv) combination therapy (chemo plus RFH). RFH was conducted by inserting the 0.022 inch heating-guidewire into the tumor such that its hot spot was at the center of the tumor. A 400 μm micro-optical temperature fiber (Photon Control Inc.) was placed subcutaneously parallel to the heating guidewire, such that the real

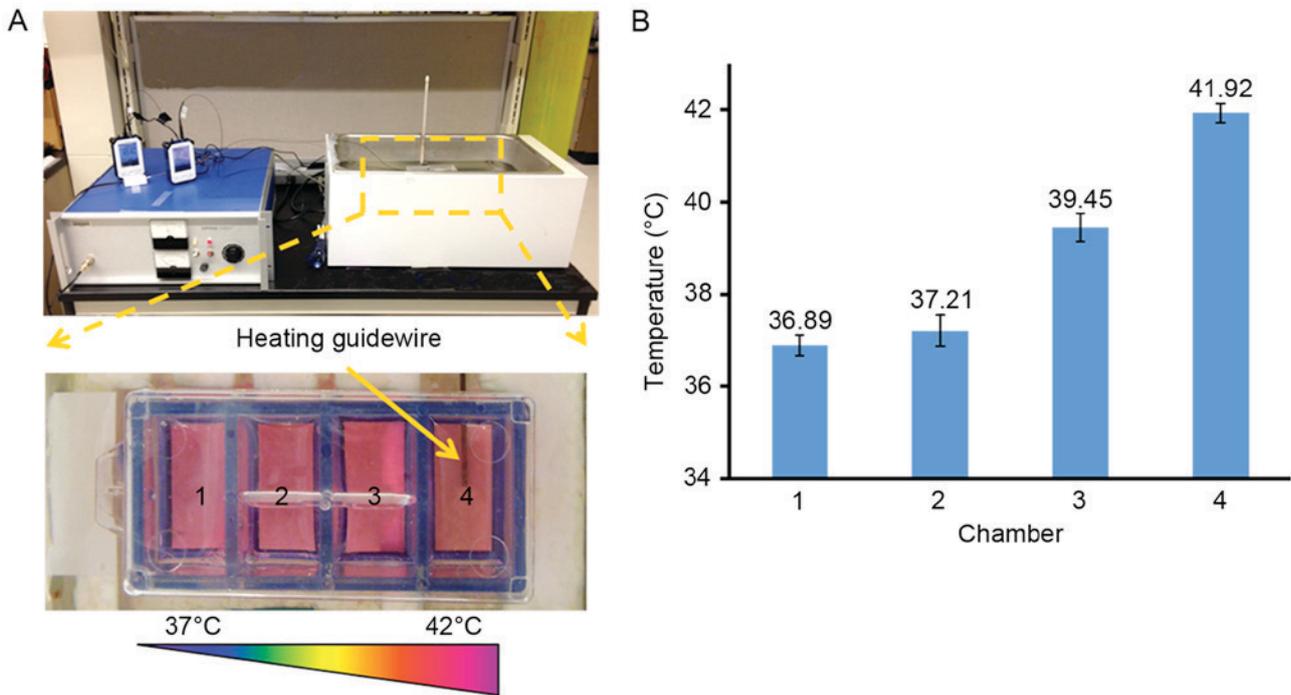


Figure 1. (A) *In vitro* experimental set-up for radiofrequency heating of human prostate cancer cells. The cells were seeded in a four-chamber cell culture slide, which was then placed in a water bath at 37°C. A 0.032 inch heating guidewire (arrow) was positioned under the bottom of chamber 4. (B) When the temperature reached 42°C in chamber 4, a stable heat gradient was created along the four chambers.

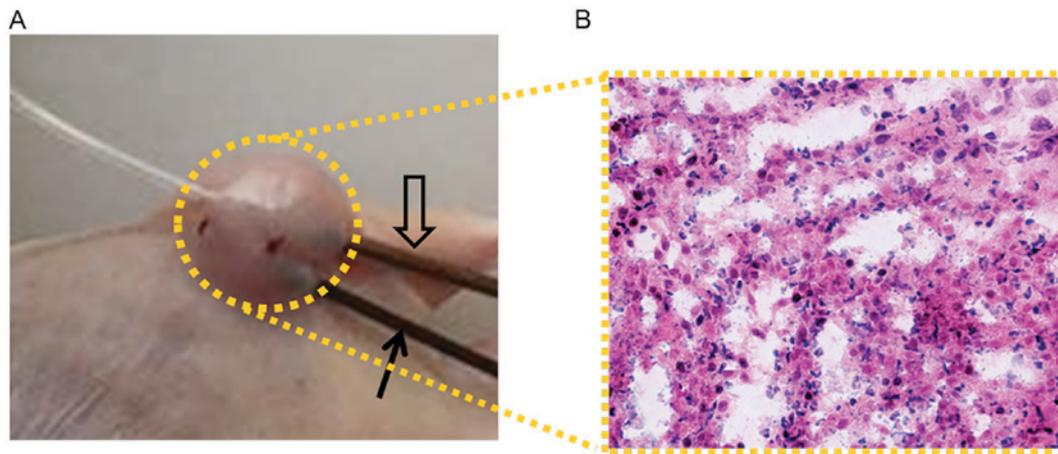


Figure 2. (A) *In vivo* experimental set-up for radiofrequency heat-enhanced direct intratumoral chemotherapy of a mouse with a prostate cancer xenograft (circle). Trans-tumor insertion of a 0.032 inch heating guidewire was performed (open arrow). The radiofrequency-heated tumor was maintained at 42°C by continuously measuring the temperature with a micro-thermometry fiber (solid arrow) placed parallel to the heating guidewire within the tumor. (B) A pathological study with hematoxylin and eosin staining confirmed the successful generation of a prostate cancer xenograft (magnification, x400).

temperature in the tumor mass was measured continuously (Fig. 2A). By adjusting the RF output power to ~10 W, the temperature of the tumor mass was maintained at ~42°C. Throughout the entire procedure, the animals were anesthetized with 1-3% isoflurane in 100% oxygen.

Magnetic resonance imaging (MRI) follow-up. The animals were placed in the supine position in a 3.0 Tesla MR scanner with a 100 mm micro-imaging coil (GE Healthcare, Chicago, IL, USA). MRI scans were acquired prior, and 7 and 14 days subsequent to the treatment. Axial and sagittal T2-weighted imaging was performed using a Fast Spin Echo sequence

with the following parameters: TR/TE=3,100 ms/80 ms, field of view=8 cm, matrix=256x256, section thickness=1.5 mm, intersection gap=0.5 mm, and Number of Excitation (NEX)=3. Axial T1-weighted imaging was then achieved using a spin echo (SE) sequence with the following parameters: TR/TE=550 ms/15 ms, field of view=8 cm, matrix=256x256, section thickness=1.5 mm, intersection gap=0.5 mm, and NEX=2.

Imaging analysis. For the tumor size measurements, the tumor volume was expressed in cubic millimeters (mm³) and was calculated based on the axial T2-weighted images. The tumor

border was delineated manually, and the area was automatically calculated using ImageJ 1.46r software (Wayne Rasband, National Institutes of Health, USA). Given the irregular contours of the tumors, the tumor volume was calculated according to the following equation: $V = s \cdot (a_1 + a_2 + a_3 + \dots + a_n)$, where s is the section interval and $a_1 - a_n$ are the areas of various sections.

The relative tumor volume (RTV) at different time points was then calculated for each tumor using the following equation: $RTV = TV_{Dn} / TV_{D0}$, where TV is the tumor volume, Dn is day 1, 7, or 14 following treatment and $D0$ is the day prior to treatment (8).

As the present study primarily focused on proving the principle of this novel concept, a longitudinal follow-up of the animals to time points at which the treated tumors had completely disappeared was not performed.

Pathological confirmation. Following satisfactory MRI, the mice were sacrificed with 5% CO_2 , and the tumor masses were harvested. The tumor tissues were embedded in Tissue Freezing Medium (O.C.T compound; Thermo Fisher Scientific, Inc.), frozen in liquid nitrogen, maintained in a freezer at $-80^\circ C$ and then sectioned into 5 μm thick slices. The tissue slices were stained with the In Situ Cell Death Detection kit and fluorescein (Roche Diagnostics, Basel, Switzerland), and were then exposed to freshly prepared proteinase K working solution for 15-30 min at $37^\circ C$ (10-20 $\mu g/ml$ in 10 mM Tris/HCl;

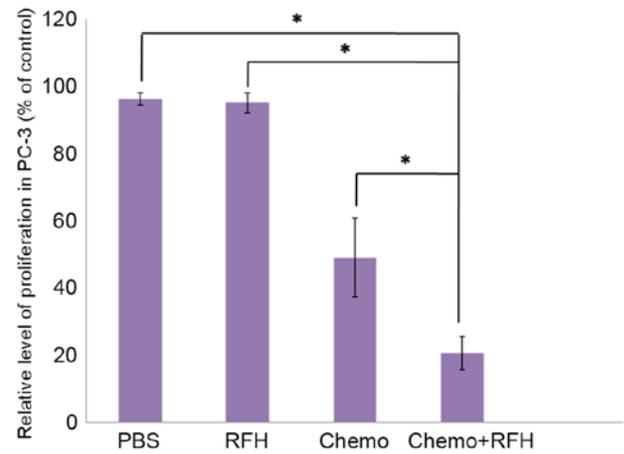


Figure 3. MTS assay demonstrating that the combination therapy (chemo + RFH) exhibited a greater inhibitory effect on growth compared with RFH-only, chemotherapy-only and control group. * $P < 0.01$. RFH, radiofrequency heat.

pH 7.4-8). Subsequent to washing with PBS, the slices were incubated in 50 ml TUNEL reaction mixture for 60 min in a dark and humidified environment. The slides were washed twice with PBS, stained overnight with DAPI (Invitrogen; Thermo Fisher Scientific, Inc.) and sealed with Prolong Gold Antifade reagent (Thermo Fisher Scientific, Inc.). All of the slides were examined under a fluorescence microscope (Leica

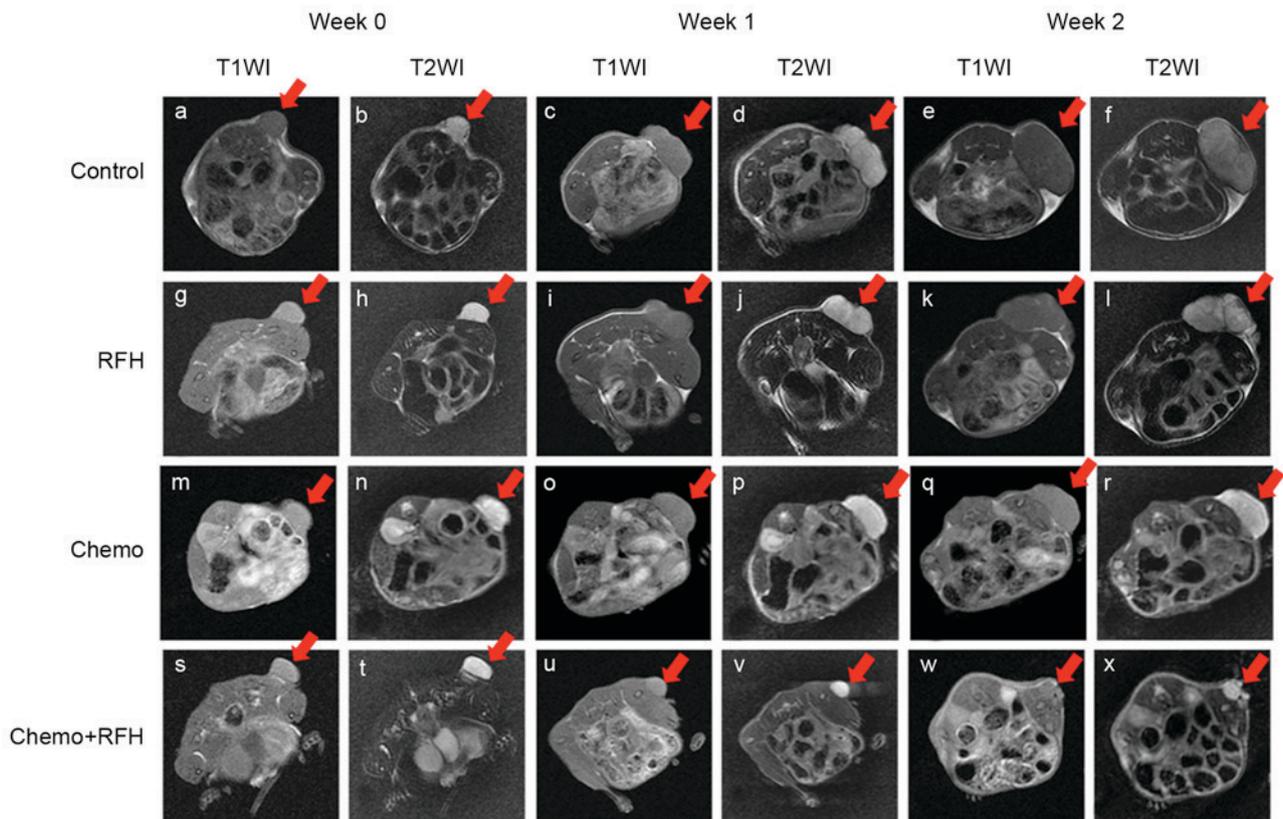


Figure 4. Representative T1-weighted images and T2-weighted images of mice bearing prostate cancer xenografts in the four treatment groups demonstrating homogeneous hypointense (T1WI) and hyperintense (T2WI) tumors (arrows) on the unilateral backs of the mice. The follow-up images of the tumors at different time points demonstrate that the tumor size in the chemo plus radiofrequency heat (RFH) group (S-X) was clearly decreased at week 2 following treatment (arrow on x) compared within the control (a-f), RFH-only (g-l), and chemo-only (m-r) groups. RFH, radiofrequency heat; T1WI, T1-weighted image; T2WI, T2-weighted image.

Microsystems GmbH, Wetzlar, Germany), and a total of 200 cells were counted in 7 fields of view to calculate the mean percentage of apoptotic cells as the apoptotic index. Apoptotic cells were counted at $\times 400$ magnification using Image-Pro Plus 6 software (Media Cybernetics, Inc., Rockville, MD, USA).

The tissue slices ($5\ \mu\text{m}$ in thickness) were stained with Hematoxylin and Eosin Staining kit (Nanjing SenBeiJia Biological Technology, Nanjing, China). The slides were immersed for 30 sec and agitated by hand in H_2O . Slide were submerged into a Coplin jar containing Mayer's hematoxylin (Nanjing SenBeiJia Biological Technology, Nanjing, China) and agitated for 30 sec prior to being washed in H_2O for 1 min. Slides were stained with 1% eosin Y solution (Nanjing SenBeiJia Biological Technology, Nanjing, China) for 30 sec with agitation. Sections were then dehydrated with two changes of 95% alcohol and two changes of 100% alcohol for 30 sec each. The alcohol was then extracted with two changes of xylene. All of the slides were examined at $\times 400$ magnification under a light microscope (Leica, Wetzlar, Germany).

Statistical analysis. Data are presented as the means \pm standard deviation. The statistical analyses were performed using SPSS 13.0 (IBM Corp., Armonk, NY, USA). One-way analysis of variance was performed to compare the mean cell proliferation rate, cell apoptotic index, tumor volume and RTV, and Tukey's post hoc test was used for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

RFH enhanced chemotherapeutic efficiency in prostate cancer cells. In the *in vitro* experiments, the combination therapy significantly reduced the proliferation of prostate cancer cells compared with the control, RFH-only and chemo-only groups (Fig. 3; 20.50 ± 4.99 vs. $96.18 \pm 1.82\%$, $P = 0.005$; $95.06 \pm 2.95\%$, $P = 0.003$; $48.98 \pm 11.74\%$, $P = 0.007$, respectively).

RFH increased chemotherapeutic efficacy in a prostate cancer xenograft model. All of the animals survived throughout the *in vivo* experiments. A MRI analysis demonstrated significant reductions in tumor size in the mice treated with combination therapy (chemo plus RFH) compared with those that received the control, RFH-only and chemo-only treatments (0.28 ± 0.16 vs. 1.42 ± 0.27 ; 0.96 ± 0.23 ; $0.75 \pm 0.18\ \text{cm}^3$, respectively; Fig. 4; $P < 0.001$). These results were confirmed by subsequent pathological examination. The average RTV in the combination treatment group was significantly lower compared with the control, RFH-only and chemo-only groups (0.57 ± 0.17 vs. 3.95 ± 0.26 ; 2.68 ± 0.43 ; 2.08 ± 0.30 , respectively; $P < 0.001$; Fig. 5). The number of apoptotic cells and the average apoptotic index of the combination therapy group were significantly higher compared with the control, RFH-only and chemo-only groups (Fig. 6; 43.22 ± 10.26 vs. $8.00 \pm 2.87\%$, $P = 0.003$; $10.91 \pm 4.31\%$, $P = 0.032$; $23.20 \pm 6.00\%$, $P = 0.025$, respectively).

Discussion

Prostate cancer is a common malignancy in men, and the worldwide burden of this disease is increasing (2). Although

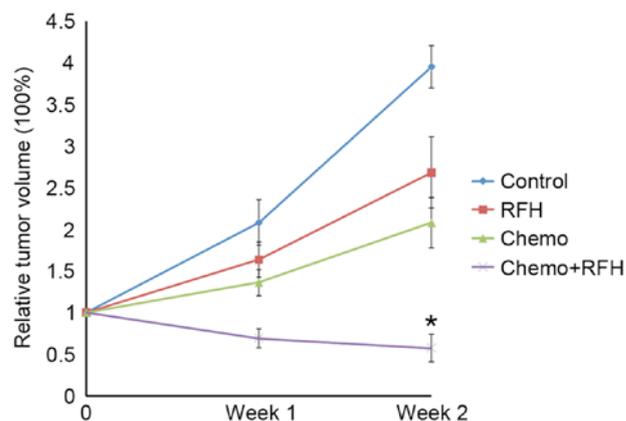


Figure 5. A comparison of the relative tumor volumes in the four treatment groups indicates that RFH-enhanced chemotherapy significantly inhibited tumor growth at week 2 post-treatment ($P < 0.001$). RFH, radiofrequency heat.

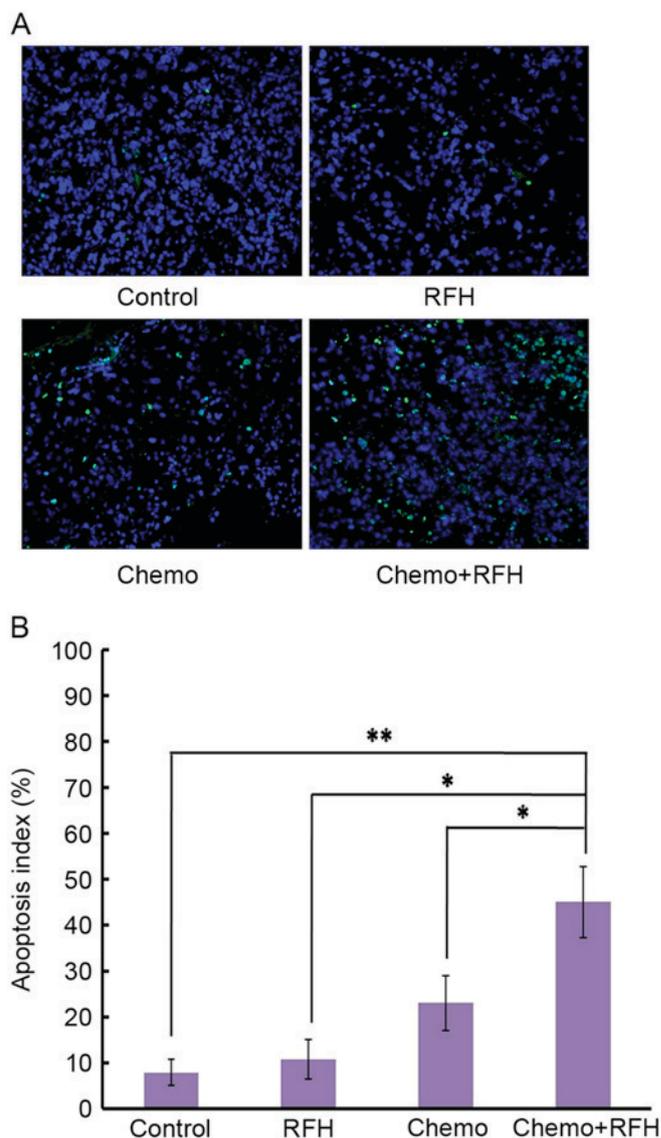


Figure 6. (A) Apoptosis assay demonstrating that there was a greater number of apoptotic cells (green) in the combination therapy group (chemo + RFH) compared with the other three groups. (B) This finding was confirmed by the analysis of the apoptotic index: The apoptotic index of the combination treatment groups was higher compared with the other treatment groups. $*P < 0.05$; $**P < 0.01$. RFH, radiofrequency heat.

external beam radiotherapy and whole-gland radical therapies have improved patient survival, the overtreatment of patients with low-grade and localized prostate cancer is of great concern (7). Although systemic administration of chemotherapy is a routine approach for the treatment of prostate cancer, the technology currently available does not guarantee a sufficient accumulation of chemotherapeutic agents at the target tumor site, and may cause toxicities to other vital organs (17).

Minimally invasive interventions have been demonstrated to improve the management of prostate cancer in previous studies (7). Under imaging guidance, interventional devices may be placed precisely at the target tumor site, allowing the delivery of highly concentrated therapeutics to the target cells (18). Such local approaches avoid the systemic administration of chemotherapeutic agents and therefore minimize toxicity to other organs (19,20).

The present study attempted to overcome the disadvantages of the currently used systemic chemotherapy for prostate cancer by developing a novel interventional therapeutic approach that integrates the benefits of multiple technologies, namely RF technology, interventional oncology and direct intratumoral chemotherapy. Proliferation of prostate cancer cells significantly decreased in the combination therapy group compared with the control, RFH-only and chemo-only groups. To the best of our knowledge, this provided the first demonstration that RFH may significantly improve the efficacy of the first-line chemotherapeutic agent docetaxel in human prostate cancer cells. These findings were consistent with previous studies by the authors (10). Zhang *et al* (10) demonstrated that combination therapy (chemotherapy plus RF hyperthermia) killed many more cancer cells compared with the chemotherapy-only and RF hyperthermia-only treatments in a preclinical study of cholangiocarcinoma.

Then, in the animal models with human prostate cancer xenograft in the present study, the reduction of the tumor size and RTV and the increment of the number of apoptosis cells demonstrated significant improvement using combination therapy in comparison of other groups following two weeks of treatment. Those results additionally confirmed that the direct intratumoral delivery of RFH significantly enhances the efficacy of local chemotherapy for prostate cancer. In a breast cancer study, Zhou *et al* (14) also reported similar efficacies of using RHF combined with doxorubicin compared to three other treatment groups: Controls, RFH-only, and chemo-only.

The present study also hypothesized that the mechanisms underlying RFH-enhanced chemotherapy may include heating to fracture the tissue, increasing the permeability of the cytoplasmic membrane, increasing cellular metabolism and increasing the activity of the heat shock protein pathway (21,22). In addition, RFH may also impair the drug efflux ability of cancer cells. All of these mechanisms facilitate the entry of therapeutics into targeted tumor cells for the effective destruction of tumor tissues, and thereby improve the therapeutic outcome of prostate cancer.

Additional efforts are required to determine whether this novel combination therapy approach may increase endpoint survival by optimizing the efficacy of therapeutic regimens. In the present study, the human prostate cancer PC-3 cell line was used. As PC-3 cells are relatively fast-growing prostate cancer

cells, the follow-up time was limited to two weeks following treatment. A longer follow-up period would have resulted in the xenograft tumor masses, particularly those in the control animal group, reaching >10% of the body weight of the mice, which was not approved by the Institutional Animal Care and Use Committee. Therefore, a limitation of the present study is that it did not evaluate the long-term therapeutic effects with follow-up MRI.

In conclusion, the present study has introduced the novel concept of using interventional RFH to increase the therapeutic efficacy of local chemotherapy for prostate cancer. The present study has successfully established a foundation for the future development of a novel interventional oncology technology, namely 'interventional RFH-enhanced local chemotherapy', for the effective management of prostate cancer through the integration of RF technology, interventional oncology and direct intratumoral chemotherapy.

Acknowledgements

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