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Original Article

Intratumoral radiofrequency hyperthermia-enhanced chemotherapy of liposomal doxorubicin on hepatocellular carcinoma

Jun Gao[1,2*], Jianfeng Wang[1,2*], Yin Jin[1], Feng Zhang[1], Xiaoming Yang[1]

[1]Image-Guided Bio-Molecular Intervention Research and Section of Vascular and Interventional Radiology, Department of Radiology, University of Washington School of Medicine, Seattle 98109, WA, USA; [2]Department of Hepatobiliary Surgery, Beijing Chao-Yang Hospital Affiliated with Capital Medical University, No. 5 Jingyuan Street, Beijing 100043, China. [*]Equal contributors.

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Abstract: Purpose: To investigate the possibility of using radiofrequency hyperthermia (RFH) to enhance intratumoral therapeutic effect of liposomal doxorubicin on hepatocellular carcinoma (HCC) via an interventional molecular imaging approach. Materials and methods: For both in-vitro confirmation and in-vivo validation, Luciferase/mCherry-labeled human HCC cells (HepG2) and mice subcutaneous hepatic cancer xenografts were treated by: (i) combination therapy of liposomal doxorubicin plus RFH at 42°C; (ii) liposomal doxorubicin alone; (iii) RFH at 42°C alone; (iv) phosphate buffered saline (PBS) as a control. For in-vitro confirmation, MTS assay, confocal microscopy, optical imaging and flow cytometry were used to evaluate and compare cell viabilities and apoptosis among different treatment groups. For in vivo validation, liposomal doxorubicin was directly injected into the tumor and RFH was performed subsequently under ultrasound imaging guidance. Changes of tumor sizes were quantified using ultrasound imaging and bioluminescence signal intensities after treatments were measured by optical imaging over 14 days, which were correlated with subsequent histology analysis. Results: Of in vitro experiments, MTS assay demonstrated the lowest cell proliferation in combination therapy group compared with the other three groups (25.0 ± 5.6% vs 49.7 ± 5.2% vs 94.2 ± 3.9% vs 100%, respectively, $P < 0.001$). Flow cytometry showed the highest percentage of early apoptotic HepG2 cells in combination therapy compared to the other three groups (37.9 ± 3.2% vs 32.2 ± 1.7% vs 2.9 ± 1.7% vs 1.8 ± 0.7%, respectively, $P < 0.001$). Of in vivo experiments, optical imaging demonstrated a significantly decreased bioluminescence signal intensities in the combination therapy group, compared with the other three groups (0.53 ± 0.10 VS 1.4 ± 0.5 VS 2.8 ± 0.8 VS 3.0 ± 0.3, $P < 0.05$). Ultrasound images showed the smallest tumor volumes of the combination therapy group, in comparison to other control groups (0.7 ± 0.1 VS 1.8 ± 0.4 VS 3.0 ± 0.8 VS 3.3 ± 0.3, $P < 0.05$). Both histologic correlation confirmed imaging findings. Conclusion: RFH can enhance intratumoral therapy with liposomal doxorubicin for HCC, which is effectively monitored by ultrasound imaging and optical imaging techniques. This concept may provide new avenues for eradicating the residual tumor cells when combining RFA with interventional molecular imaging guided direct intratumoral chemotherapy of HCC.

Keywords: Radiofrequency hyperthermia, liposomal doxorubicin, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and the third most common cause of cancer-related death worldwide, with an annual incidence of more than 1 million worldwide [1-3]. With the fast advancement of imaging techniques, increasing number of HCC cases are diagnosed at an early stage, which offered a great opportunity for completely eradicating the tumors using radical treatment approaches [1, 4, 5].

Liver transplantation has been considered as the most curative option for HCCs that meet the Milan criteria (one HCC 5 cm or as many as three nodules, 3 cm without vascular invasion or extrahepatic metastasis), but limited availability of liver donors precludes the wide application of this approach for treating HCC [2, 4, 6]. Surgical resection to remove the tumor or potion of tumor-involved liver used to be the best option for curing HCC patients [2, 7]. However, many patients may not be suitable for surgical resection because of the associated

high risks of further injury to the normal liver tissue, blood loss after the operation, and insufficient liver function in cirrhotic patients [4, 8, 9]. Highly effective local reginal ablation therapies such as radiofrequency ablation (RFA) have been accepted as the standard of care for early stage HCC [10-12]. Whether RFA can replace surgical resection as the first-line treatment for early HCC remains controversial due to its higher local recurrence rate than surgical resection [13]. Tumor recurrence post RFA is widely considered to stem from residual tumor cells in the peritumoral area, where microvascular invasion and satellite micronodules cannot be substantially ablated if the ablative thermal energy is not homogeneously distributed in the entire tumor area and a tumor-free margin of 0.5 cm is not warranted [14, 15].

Various strategies have been developed to overcome the inherent limitations of high tumor recurrence rate using RFA alone to treat HCC [16, 17], such as RFA combined with chemoembolization using drug eluting beads or an emulsion of chemotherapeutic drugs in Lipidol [4, 18-20]. Some retrospective studies show that combination therapy can offer some benefits to the patients in term of decreasing the rate of tumor recurrence and prolonging the overall survival time, but a variety of factors affect the synergistic effect of combination therapy, such as the status of the tumor blood supply and tumor stages. Intravenous administration of liposomal Doxorubicin had been combined with RFA to treat liver or neck and head cancers in rodent models [16, 17]. The results of the studies suggest that RF-induced thermal energy could enhance the therapeutic effect by promoting the rapid release of Doxorubicin from the long-circulating drug/liposome complex and increasing the dose of drugs in the tumor tissue [21-23]. However, the majority of patients with HCC cannot undergo systemic chemotherapy because of hepatic insufficiency or medical comorbidity. On the other hand, barriers still exist in the systemic chemotherapy that limited drugs can be delivered to the tumor targets. The development of cluster ablation electrodes with multiple infusion needles provides the possibility that alongside with RFA, we can inject high dose of chemotherapeutic drugs in the target tumor area, especially at the margin of the tumors. Recent studies have confirmed that non-ablative hyperthermia at around 42°C can significantly enhance the chemotherapeutic effect through the mechanism of increasing chemosensitivity and reversing chemoresistance [24, 25]. The aim of this study was to determine whether RF hyperthermia (RFH) could enhance the therapeutic effect of liposomal doxorubicin in a HCC cell line (HepG2 cells) and intratumoral chemotherapy of liposomal Doxorubicin on mice HepG2 tumor xenografts.

Materials and methods

Study design

This study was divided into two stages: (a) in vitro experiments to confirm if RFH can enhance the efficacy of liposomal doxorubicin on HCC cells; and (b) in vivo experiments validate the synergistic effect of the combination therapy of local liposomal doxorubicin with RFH.

In vitro experiments

Cell culture and RFH-enhanced cell killing effect of liposomal doxorubicin

Cellular viability assay: HepG2 cells (JCRB Cell Bank, Osaka, Japan) were transfected with luciferase (Luc)/m-Cherry/lentivirus gene, to create Luc/RFP$^+$ HCC cells per the manufactural protocol (GeneCopoeia Inc., Rockville, MD). Luc/RFP-positive cells were sorted out using fluorescence-activated cell sorting technique (Aria II, Becton Dickinson, Franklin Lakes, NJ). Cells were then seeded in four-chamber cell culture slides (NalgeNunc International, Rochester, NY) and maintained in Delbecco's modified Eagle's medium/F12 (1:1) supplemented with 10% fetal bovine serum (Gibco, Grand island, NY). RFH was performed as described in the literature [24, 25]. Cells in different groups were treated by (a) liposomal doxorubicin (650 μM) (Doxil; ALZA Pharmaceuticals, Palo Alto, CA) plus 30-min RFH at approximately 42°C; (b) liposomal doxorubicin (650 μM) alone; (c) 30-min RFH alone; and (d) phosphate buffer saline (PBS) to serve as a control group. We used the 50-percent inhibitory concentration (IC50) doses of liposomal doxorubicin for cell treatments. Cells viability was evaluated by MTS assay 24 h after the treatments. Relative cell viability of different cell groups was calculated using the equa-

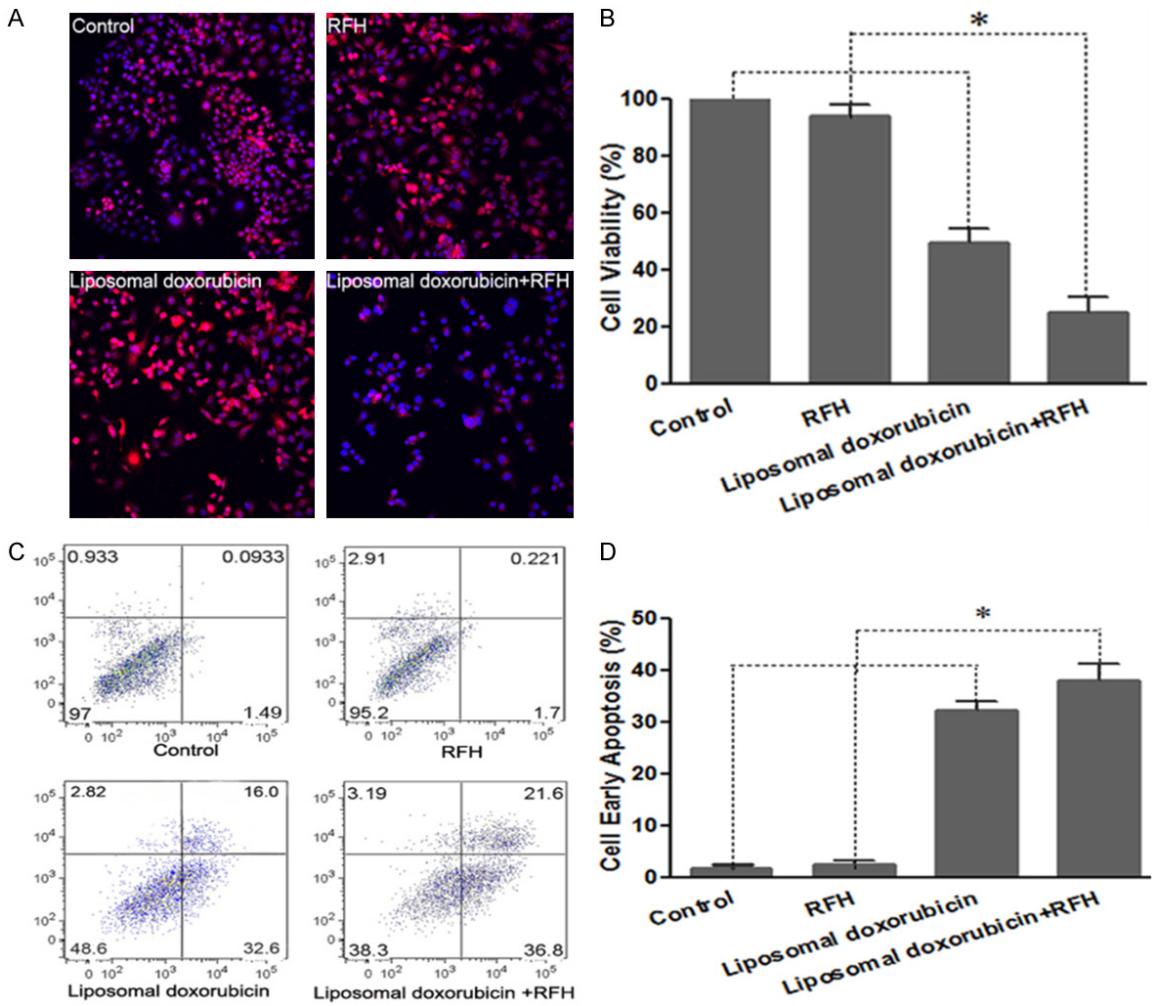

Figure 1. A. Confocal microscopy showed the lowest number of viable cells in the group treated with combination therapy (liposomal doxorubicin + RFH), compared with the other three control groups. B. MTS assay further demonstrated the lowest cell viability in combination therapy group (*P < 0.001). RFH = Radiofrequency hyperthermia. C and D. Flow cytometry showed the highest percentage of early apoptotic HepG2 cells in combination therapy group (liposomal doxorubicin + RFH) compared with those in other three groups (*P < 0.001). RFH = Radiofrequency hyperthermia.

tion of $A_{treated}$-A_{blank}/$A_{control}$-A_{blank}, where A is absorbance.

Cells on cell culture slides were subsequently washed twice with PBS, fixed in 4% paraformaldehyde, counterstained with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA), and then imaged with a fluorescent microscope. All experiments for each of cell groups were repeated six times.

Cellular apoptosis assay: The percentages of apoptotic cells in each group were quantified by flow cytometry using Annexin V-FITC/PI staining kit (BD Biosciences, San Diego, CA). Cells were stained with Annexin-V/FITC and PI in binding buffer along with the appropriate control. Total number of Annexin V- and PI-positive cells were counted using a FACScan flow cytometer (BD Biosciences). The data was analyzed using the FlowJo software version 10.

Cellular bioluminescence assay: In vitro bioluminescence optical imaging of cells of each group was performed 24 hours after the treatments. 5 μL Pierce D-Luciferin (ThermoFisher Scientific, Rockford, IL) was added into the cell culture medium and incubated for additional 20 minutes. 100 μl cell medium was mixed with 100 μl 1% agarose in a cylindrical glass tube. Optical imaging was performed using an in vivo

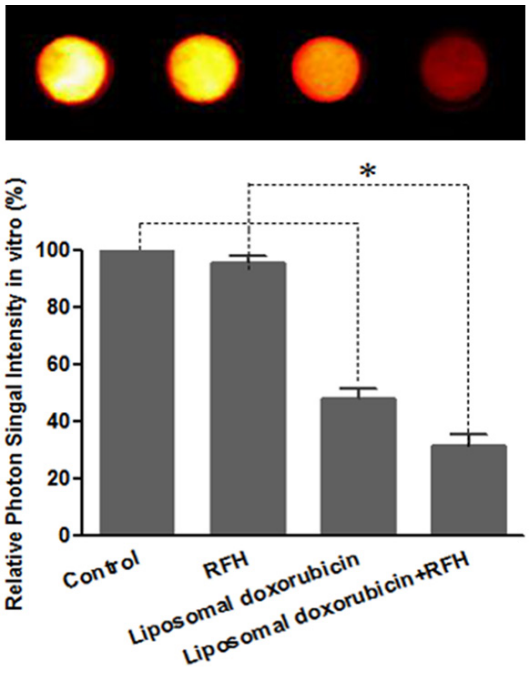

Figure 2. Optical imaging in vitro confirmed that relative photon signal intensity with combination therapy was significantly lower than that in the groups treated with chemotherapy alone, RFH alone and PBS (*$P < 0.001$).

optical imaging system (Bruker Corp., Billerica, MA). Bioluminescent signal intensity was quantified as the sum of all detected photon counts, which was subtracted by the background photon flux within a manually derived region of interest (ROI) using the Bruker MI software. Data were normalized to relative signal intensity (RSI) by using the following equation: RSI = SI_T/SI_C, where SI is signal intensity, T represents the treatment group, and C represents the control group.

In vivo experiments

Animal model

The animal protocol was approved by our Institutional Animal Care and Use Committee. The animals were anesthetized with 1-3% isoflurane (Piramal Healthcare, Andhra Pradesh, India) in 100% oxygen. Mice models with subcutaneous HCC cancer xenografts were created by inoculating 5 × 10^6-1 × 10^7 Luc/RFP-positive HepG2 cells in 100-μl Matrigel into the backs of 24 nu/nu mice. Once the size of tumor reached 8-10 mm in diameter, we started the treatments.

RFH-enhanced intratumoral chemotherapy on mouse models

Six mice in each of four groups were treated by (a) direct intratumoral injections of 10 mg/kg liposomal doxorubicin, immediately followed by local RF heating at approximately 42°C for 30 minutes; (b) intratumoral injection of 10 mg/kg liposomal doxorubicin; (c) 30 min RFH alone; and (d) intratumoral injection of 100 μL PBS to serve as the control.

RFH was carried out by inserting a 0.022 inch RF heating wire into the tumor with its hot spot at the center of each tumor mass, which was precisely guided by ultrasound imaging. A 400 μm micro-fiber optical thermal probe was subcutaneously placed at the margin of the tumor, so that the temperature at the RF-heated tumor mass could be measured instantly. By setting the RF output power at 10-15 watts, the temperature could be controlled at 42±1°C.

Post-treatment follow-up with optical and ultrasound imaging

Optical imaging was performed on an Optical In-Vivo Imaging Systems (Bruker Corp., Billerica, MA). Each animal was imaged at day 0 before the treatment and days of 7 and 14 after the treatment. The animals were anaesthetized with 2% isoflurane delivered in 100% oxygen. Optical images were acquired 20 minutes after intraperitoneal injection of Pierce D-Luciferin at 150 mg/Kg.

Fluorescent signal intensity was quantified as the sum of all detected photon counts, which was subtracted by the background photon flux within a manually derived region of interest (ROI) using the Bruker MI software. Data were normalized to relative signal intensity (RSI) by using the following equation: RSI = SI_{Dn}/SI_{D0}, where SI is signal intensity, Dn represents days after treatment, and D0 is the day before treatment.

Ultrasound imaging was performed simultaneously to assess tumor growth (Sonosite Inc, Bothel, WA) at days 0, and days of 7 and 14 after treatment. The axial (X) and longitudinal (Y) diameters of tumors, as well as tumor depths (Z) were measured on the ultrasound images at their largest profiles. The volume of

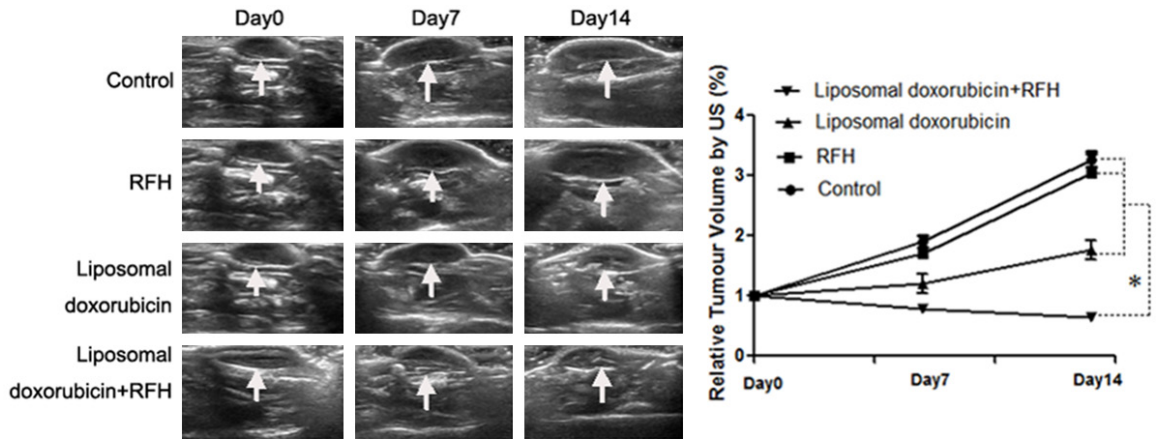

Figure 3. Ultrasound imaging is used to follow tumor growth (white arrows) and response to the treatments at days 0, 7 and 14. A significant decrease of relative tumor volume with combination therapy (liposomal doxorubicin + RFH) was seen compared with other three treatment groups (*$P < 0.05$). RFH = Radiofrequency hyperthermia.

each tumor was then calculated according to the equation: tumor volume = X * Y * Z * π/6. Data was expressed as relative tumor volume (RTV) by using the following equation: RTV = V_{Dn}/V_{D0}, where V is tumor volume, Dn represents days after the treatment and D0 is the day before the treatment.

Histologic correlation/confirmation

Mice Tumors were harvested at day 14 after the treatment. The xenograft tissues were fixed in 10% formalin for 4 h and then embedded in paraffin. Hematoxylin-eosin (HE) staining was performed to estimate the pathological change and TUNEL was performed for apoptosis analysis. The TUNEL assay was carried out according to the manufacturer's protocol (Roche, Indianapolis, IN). A positive result was brown staining in the nucleus. Apoptosis results were analyzed as the apoptotic index, defined as the number of apoptotic cells/total number of cells × 100%. At least 1000 cells from at least 10 scopes were counted using the Olympus BX51 system (Olympus, Tokyo, Japan).

Statistical analysis

Statistical software (SPSS, Version 19.0; Chicago, Ill) was used for all data analyses. The non-parametric Mann-Whitney U test was used to compare (i) relative proliferation rates among different cell groups; (ii) relative signal intensity as well as (iii) relative tumor volumes at different time points among different animal groups. *P* value of less than 0.05 indicates the significant difference.

Results

In-vitro evaluation: RFH-enhanced chemotherapeutic effect on HepG2 cells

Confocal microscopy demonstrated diminished number of cells survived combination therapy compared with those in other three treatment groups (**Figure 1A**). Quantitative MTS assay further confirmed that cell proliferation in the cell group with combination therapy was significantly lower than those in the groups treated with chemotherapy alone, RFH alone and PBS (25.0 ± 5.6% vs 49.7 ± 5.2% vs 94.2 ± 3.9% vs 100%, respectively, $P < 0.001$) (**Figure 1B**). Flow cytometry showed that the highest percentage of early apoptotic HepG2 cells in combination therapy compared with those in other three groups (37.9 ± 3.2% vs 32.2 ± 1.7% vs 2.9 ± 1.7% vs 1.8 ± 0.7%, respectively, $P < 0.001$) (**Figure 1C**, **1D**). Bioluminescence optical imaging of cells confirmed that the relative photon signal intensity in the cell group with combination therapy was significantly lower than that in the groups treated with chemotherapy alone, RFH alone and PBS (31.5 ± 3.9% vs 48.0 ± 3.6% vs 95.3 ± 2.7% vs 100%, respectively, $P < 0.001$) (**Figure 2**).

In-vivo confirmation: RFH-enhanced chemotherapy of mouse HCC cancer xenografts

All the treatment in four groups was performed under ultrasound imaging guidance. All animals survived the experimental procedures without complications. Ultrasound imaging demonstrated the smallest relative tumor volu-

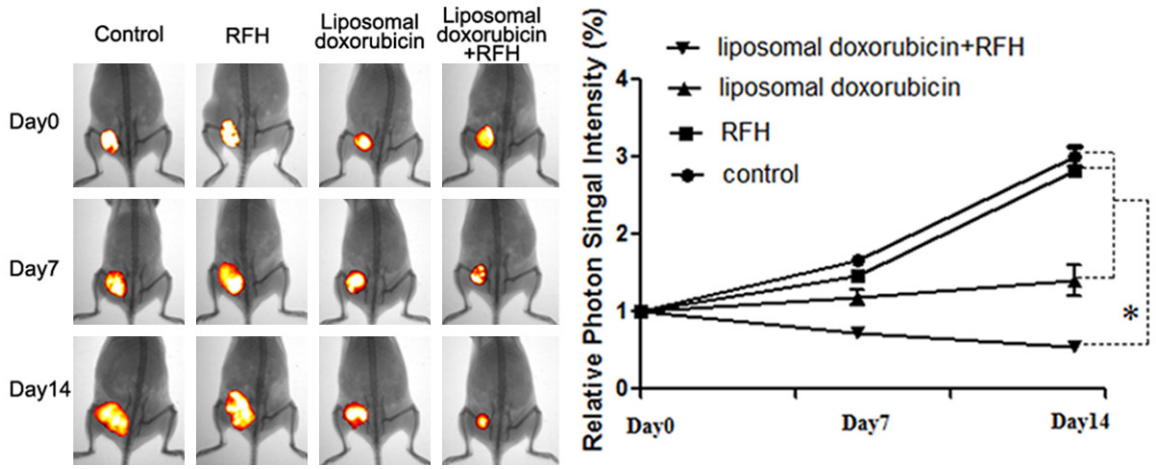

Figure 4. Optical/x-ray imaging follow-up of tumors after the treatments. A significant decrease of both relative bioluminescence signal (golden-yellow color) and tumor size was seen in the group with combination therapy (liposomal doxorubicin + RFH), compared to other three treatment groups (*$P < 0.05$). RFH = Radiofrequency hyperthermia.

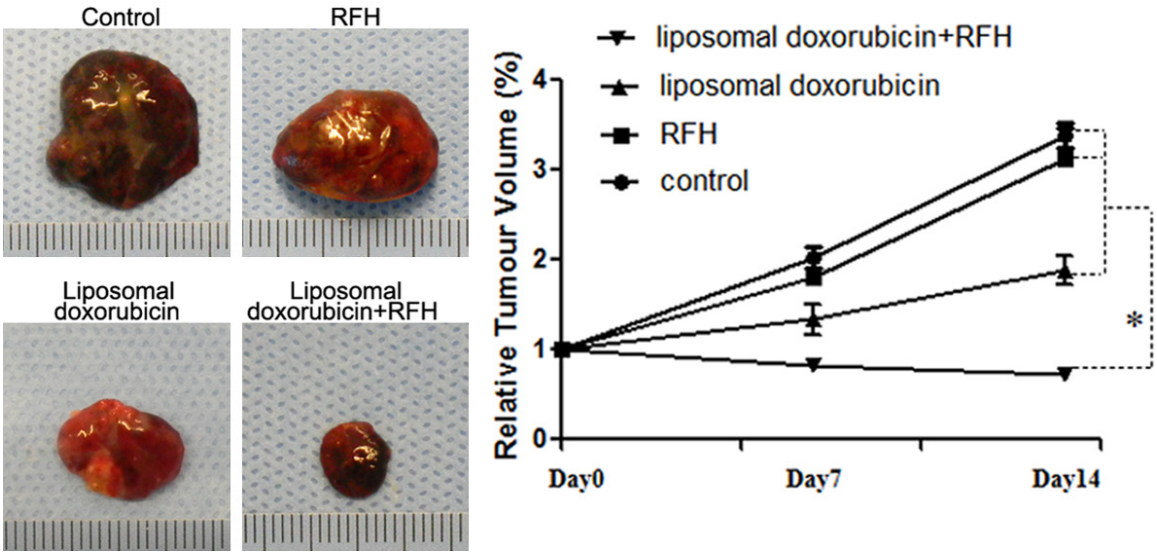

Figure 5. Representative tumors harvested from four different animal groups, demonstrated the smallest tumor size in the combination therapy group (liposomal doxorubicin + RFH), compared with three control treatment groups (*$P < 0.05$). RFH = Radiofrequency hyperthermia.

me in the combination therapy group, compared with the other three groups (0.7 ± 0.1 VS 1.8 ± 0.4 VS 3.0 ± 0.8 VS 3.3 ± 0.3, $P < 0.05$) (**Figure 3**). Optical imaging was used to follow the response of tumors to the treatments, demonstrating a significant decrease in relative photon signal intensity in the combination therapy group, compared with the other three groups (0.53 ± 0.10 VS 1.4 ± 0.5 vs 2.8 ± 0.8 vs 3.0 ± 0.3, $P < 0.05$) (**Figure 4**).

Gross specimens obtained at the end of the experiment revealed the smallest tumor size in combination therapy group compared with the other three groups (**Figure 5**). Histological analysis of apoptosis by TUNEL staining further confirmed more apoptotic cells in the combination therapy group than in the three other groups (51.3 ± 10.5% vs 17.0 ± 4.4% vs 2.9 ± 1.7% vs 1.8 ± 0.7%, respectively, $P < 0.001$) (**Figure 6**).

Discussion

Our study evaluated the feasibility and efficacy of using RFH to enhance intratumoral chemo-

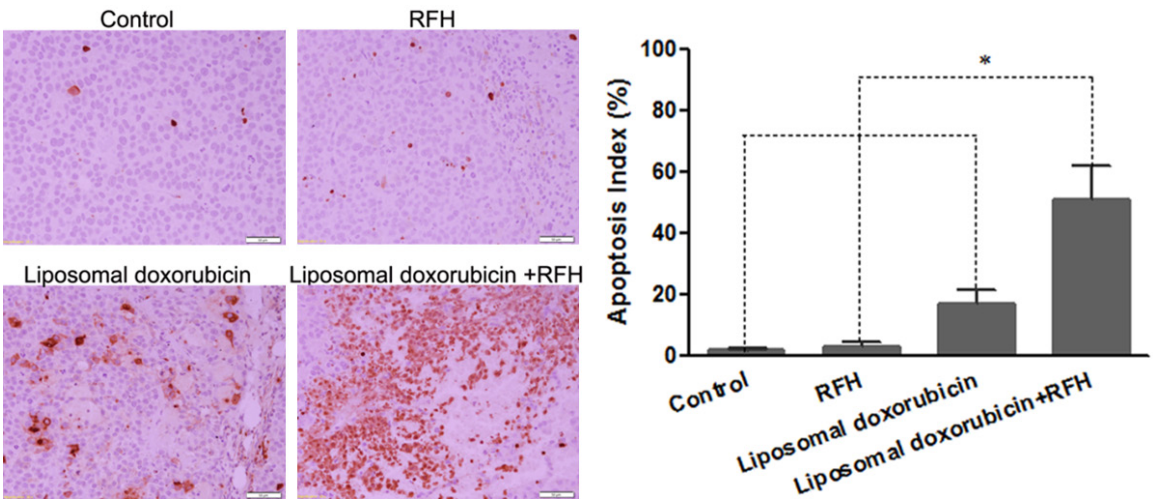

Figure 6. Apoptosis analysis using TUNEL staining showed more apoptotic cells (yellow brown dots) in combination therapy group than other three groups (20X). RFH = Radiofrequency hyperthermia.

therapy of liposomal doxorubicin on HCC. Our data demonstrated that RFH can significantly enhance the direct intratumoral chemotherapy on human HCC cells, as manifested by decreased survival of HCC cells in in-vitro experiments, as well as shrunken tumor volumes and decreased optical signal intensities in both in-vivo confirmation and validation experiments. Our study also provided the evidence that (a) both chemotherapeutics and RFH can been locally delivered simultaneously to HCC tumors under ultrasound imaging guidance and (b) both optical imaging and ultrasound imaging are useful tools to assess the response of HCC to local therapy.

A few studies combined RFA with intravenous liposomal doxorubicin to treat tumors of different animal models, which demonstrated an increased interstitial accumulation of doxorubicin in target tumor tissue and widened coagulation necrosis area of the tumor [16, 17]. Preclinical studies further found that a combination of intravenous lyso-thermosensitive liposomal doxorubicin with local mild hyperthermia at temperatures ≥ 39.5°C induced up to 15-fold greater increase of doxorubicin in tumor tissue, compared with the same dose of free doxorubicin [27]. This regimen suggested the potential to increase the concentration of doxorubicin in a tumor while limiting systemic exposure. However, dose limiting toxicity (DLT) is still a critical clinical issue when liposomal doxorubicin is administered systemically in conjunction with thermal ablation. The observed DLT of neutropenia indicates the existence of systemic exposure to non-entrapped drug especially given the dose dependency of neutropenia, leukopenia, and alopecia. The overall toxicity aspects of liposomal doxorubicin appear to be similar to doxorubicin [28]. Furthermore, intravenous liposomal doxorubicin still poses a matter of potential risks of hepatic or kidney injury in HCC patients with impaired hepatic or renal function reserve [12, 29]. Interventional molecular imaging may offer an opportunity to address this problem [30]. Under imaging guidance, we can precisely place the cluster ablation electrode integrated with infusion needle in tumor targets and thereby deliver highly concentrated therapeutics and thermal energy simultaneously to the targets. Such an efficient local drug/hyperthermia delivery approach can circumvent the systemic administration of chemotherapeutic agents and thus minimize toxicity to other organs [30].

A combination of intratumoral RFA with local chemotherapy may offer an opportunity of destructing the residual tumor cells in the peritumoral zone of an ablated HCC tumor. At the margin of a RF ablating HCC tumor, the temperature may not be high enough at the tumoricidal level, but we can take the advantage of mild hyperthermia at the sublethal level to enhance the local chemotherapy, with a goal of achieving a complete tumor destruction of the entire tumor mass. Studies have suggested that mile hyperthermia at a temperature of approximately 42°C can enhance the chemotherapeutic

effect through a possible mechanism of fracturing tissue, increasing permeability of the cytoplasmic membrane, cellular metabolism and accelerating apoptosis of tumor cells [24, 26]. Our current study proved the principle that intratumoral RF-induced hyperthermia can play a promising role in boosting the efficacy of liposomal doxorubicin on human HCC cancer cells, which can be effectively monitored by in vitro and in vivo bioluminescence optical imaging and ultrasound imaging.

Our study has limitations. We only followed up the tumor growth for up to two weeks and didn't observe the effect of combination therapy on prolonging the overall survival after the treatments, because HCC tumor xenografts created with HepG2 cells grow very fast. This poses the risk that the tumor in the control group may grow a relatively large size of more than 10% body weight, which is not permitted by our IACUC. We need additional experiments to investigate the possibility of RFH to enhance local chemotherapy of animal models with orthotopic liver cancer, which may pave the way for clinical translation of this technique.

In conclusion, we validated the feasibility of using RFH to enhance intratumoral therapy with liposomal doxorubicin for HCC. This concept may provide new avenues for destructing the residual tumor when combining RFA with interventional molecular imaging guided direct intratumoral chemotherapy of HCC.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiaoming Yang, Image-Guided Bio-Molecular Intervention Research and Section of Vascular and Interventional Radiology, Department of Radiology, University of Washington School of Medicine, Seattle 98109, WA, USA. E-mail: xmyang@uw.edu

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