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Original Article

Combined treatment of cholangiocarcinoma with interventional radiofrequency hyperthermia and heat shock protein promoter-mediated HSV-TK gene therapy

Jingfeng Luo[1], Jiali Zhou[3], Fengnan Xie[4], Yali Zhu[1], Fei Zhou[1], Shuanglin Zhang[1], Shaojie Jiang[1], Jie He[1], Jiaxin Liu[1], Xia Wu[1], Yanhua Zhang[2], Jihong Sun[1], Xiaoming Yang[1,5]

Departments of [1]Radiology, [2]Pathology, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang, China; [3]No. 1 Clinical Medical School, Zhejiang Chinese Medicine University, Hangzhou, Zhejiang, China; [4]Medical Imaging School, Hangzhou Medical College, Hangzhou, Zhejiang, China; [5]Image-Guided Bio-Molecular Intervention Research, Department of Radiology, University of Washington School of Medicine, Seattle, WA, USA

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Abstract: Cholangiocarcinoma is a most lethal malignancy frequently resistant to chemotherapy. Herpes simplex virus thymidine kinase/Ganciclovir (HSV-TK/GCV) suicide gene therapy is a promising approach to treat different cancers, including cholangiocarcinoma. However drawbacks including low therapeutic gene expression and lack of precise targeted gene delivery limit the wide clinical utilization of the suicide gene therapy. We attempted to overcome these obstacles. We established the "proof-of-principle" of this concept via serial in-vitro experiments using human cholangiocarcinoma cells and then validated the new interventional oncology technique in vivo using mice harboring the same patient derived cholangiocarcinomas. Curative effects were evaluated by magnetic resonance imaging and confirmed by pathology and laboratory examinations. Intratumoral radiofrequency hyperthermia (RFH) significantly elevated the targeted expression of HSV-TK gene and further enhanced the therapeutic effects of direct intratumoral HSV-TK/GCV gene therapy, evident as the least number of survival tumor cells, smallest tumor size, and the highest apoptosis index in the combination treatment of HSV-TK plus RFH, compared to other control treatments. The novel combination of image-guided interventional oncology, RFH technology, and direct gene therapy may be valuable for the effective treatment of cholangiocarcinoma.

Keywords: Radiofrequency hyperthermia, HSV-TK, gene therapy, cholangiocarcinoma

Introduction

Cholangiocarcinoma is one of the deadest diseases worldwide with poor outcomes and an increasing mortality rate [1, 2]. Surgery and direct curative liver transplant are the best options for patients with this difficult to cure malignancy. However, these treatments are unsuitable for patients suffering from metastatic cholangiocarcinoma [3]. Although chemotherapy is a beneficial aid for inoperable patients, the use of chemotherapy is still limited in many cases of cholangiocarcinoma, because of the low chemotherapeutic dose attained at the target using systemic delivery and the rapid development of drug resistance by cholangiocarcinomas [4]. Hence, it is essential to explore alternative treatment options.

Enzyme prodrug therapy is an alternative approach for patients with different neoplastic diseases, wherein the enzyme can convert the non-toxic prodrug to a toxic anti-tumor drug in transfected tumors. In addition, in the "bystander effect", toxic anti-tumor drugs can enter and kill surrounding tumor cells. Activating the systemic immune response after enzyme and prodrug treatment might further enhance the therapeutic effects [5].

The herpes simplex virus thymidine kinase/Ganciclovir (HSV-TK/GCV) suicide gene system is a promising enzyme/prodrug system for various malignancies [6-8]. The main barrier inhibiting the wide clinical application of HSV-TK/GCV system is the low transfection and expression efficiency of systemically administered

HSV-TK genes [5]. Image-guided interventional procedures for the direct delivery of high dose anti-tumor therapeutics to the target tumors could solve this problem.

Utilization of tissue- or cell-specific promoters is an ideal option to elevate the therapeutic effects of HSV-TK genes. Heat shock proteins (HSPs) are a set of chaperone proteins that function in the cytoprotection from denaturation or misfolding of cellular proteins corresponding to heat stress or temperature stimuli. HSPs are generally classified as small HSPs (such as HSP60, HSP70, and HSP90) and large HSPs according to their molecular weights [9]. HSPA6 (also known as HSP70B') belongs to the HSP70 family. HSPA6 is a heat shock- and stress-inducible protein. The expression of HSPA6 is low under normal physiological conditions, with increased induction of protein synthesis in the presence of extreme temperatures [9, 10]. The HSPA6 promoter plays a crucial role in this process. Thus, transferring this promoter upstream of an HSV-TK gene is beneficial, as it can elevate the HSV-TK gene expression level when the treated cells experience heat shock.

Recent studies have confirmed that image-guided interventional radiofrequency hyperthermia (RFH at approximately 42-45°C) can facilitate the effects of different anti-tumor treatments for a variety of carcinomas [11-14]. However, hyperthermia-enhanced therapy for cancer still has many drawbacks that include inadequate delivery systems for local heat administration and lack of efficient temperature monitoring.

In this study, we investigated the capability of a combination treatment by constructing an HSP promoter-mediated HSV-TK gene therapy system. We used an image-guided interventional procedure to deliver a high dose of HSP promoter-mediated HSV-TK genes to the tumors. Simultaneously, intratumoral RFH further enhance the HSV-TK gene therapeutic effects on cholangiocarcinomas.

Materials and methods

The present study consisted of two parts: (1) *in vitro* establishment of the "proof-of-principle" of RFH-enhanced lethality of the HSV-TK gene on human cholangiocarcinoma cells, and (2) *in vivo* feasibility of image-guided interventional RFH-enhanced direct intratumoral gene therapy of cholangiocarcinoma in mice.

In vitro experiments

Construction of the P_{HSP}-TK/GFP-lentivirus: We successfully constructed the co-expressed lentiviral vectors of HSP-TK and green fluorescent protein (GFP) genes as published previously [15]. Since the same HSPA6 promoter simultaneously regulated the co-expression of P_{HSP}-TK and GFP genes, the expression efficiency of P_{HSP}-TK in transfected cholangiocarcinoma cells was detectable by measuring GFP signals using fluorescent microscopy.

Cell culture: Informed consent was provided from the human subjects to collect cells for experimentation. The experiment was approved by the institutional human ethics committee. Cholangiocarcinoma tissues were harvested from patients suffering from cholangiocarcinoma. The primary human cholangiocarcinoma cell line, ZJU-1125, was established using a single clone culture. The patient derived cholangiocarcinoma cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 1 × penicillin-streptomycin solution (Thermo Fisher Scientific, Waltham, MA, USA). The cells were cultured at 37°C in a thermostat incubator in an atmosphere containing 5% CO_2. The ATCC provided the STR identification for our cholangiocarcinoma cells line ZJU-1125.

Detection of P_{HSP}-TK gene transfection and expression: After cholangiocarcinoma cells were seeded into a 30-cm culture vessel for 24 h, the cells were incubated with P_{HSP}-TK/lentivirus [multiplicity of infection (MOI) = 20] for 12 h. The culture medium was then replenished. HSV-TK gene expression at 72 h post-transfection was evaluated using flow cytometry analysis (BD Biosciences, Franklin Lakes, NJ, USA).

RFH: Cholangiocarcinoma cells were cultivated in each chamber of a four-chamber cell culture slide (Nalge Nunc International, Rochester, NY, USA) at a density of 5×10^4 cells per well and incubated in a 37°C water bath. These cells were divided into four groups: gene transduction of P_{HSP}-TK/GFP lentivirus plus RFH; RFH-only; P_{HSP}-TK/GFP gene transduction-only; and untreated control. For gene transduction, P_{HSP}-TK/lentivirus (MOI = 20 per chamber) was added to the cell-containing chambers for 48 h, followed by heating at 45°C for 20 min using the RFH system. For the RFH, a 0.032-inch radiofrequency heating wire was placed under the

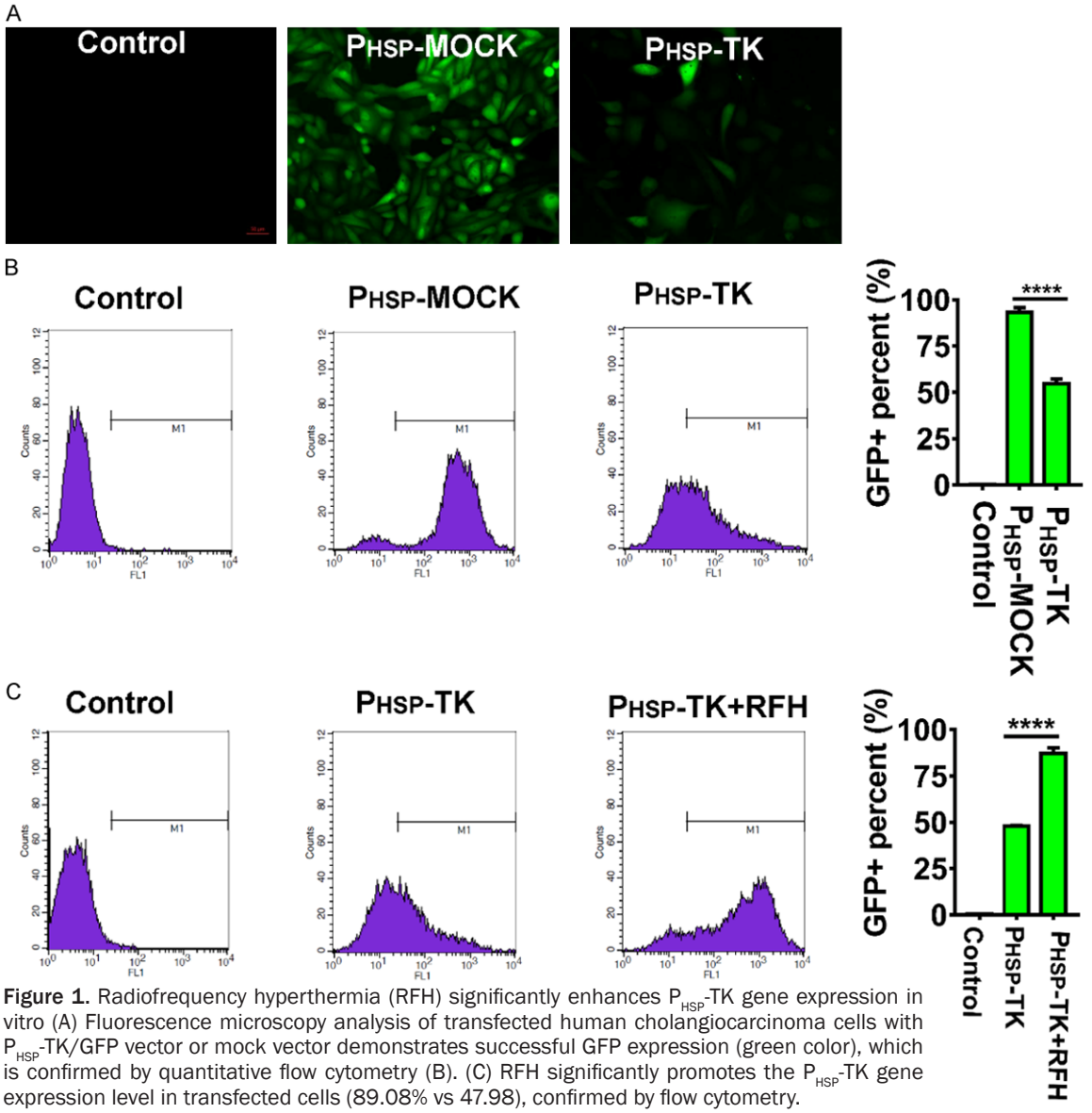

Figure 1. Radiofrequency hyperthermia (RFH) significantly enhances P_{HSP}-TK gene expression in vitro (A) Fluorescence microscopy analysis of transfected human cholangiocarcinoma cells with P_{HSP}-TK/GFP vector or mock vector demonstrates successful GFP expression (green color), which is confirmed by quantitative flow cytometry (B). (C) RFH significantly promotes the P_{HSP}-TK gene expression level in transfected cells (89.08% vs 47.98), confirmed by flow cytometry.

bottom of chamber number one of the four-chamber cell culture slide and then connected to a 2450 MHz RF generator (GMP150; OPTH-OS, Rockville, MD, USA) to heat the slide at 45°C for 20 min. The temperature of the chambers was constantly monitored using a digital thermometer (Photon Control, Burnaby, British Columbia, Canada). Forty-eight hours later, GCV was added to each cell culture chamber (10 μg/ml) for 48 h.

Cytotoxic and cell apoptosis assays: Cytotoxic and cell apoptosis assays were performed to evaluate the cell killing effects of P_{HSP}-TK/GCV on cholangiocarcinoma cells. Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was used to assess cell viability. Briefly, CCK-8 solution (100 μl) was dispensed in each chamber and incubated at 37°C for 2 h followed by measurement of the absorbance of each chamber at 450 nm using a Universal Microplate Reader (BIO-TEK Instruments, Minneapolis, MN, USA). Apoptosis was detected by staining with Annexin V and APC solution (Thermo Fisher Scientific) followed by flow cytometry analysis (BD Biosciences).

In vivo experiments

RFH-mediated gene therapy: The animal experiments were approved by the Institutional Animal Care and Use Committee. Twenty-four 8-

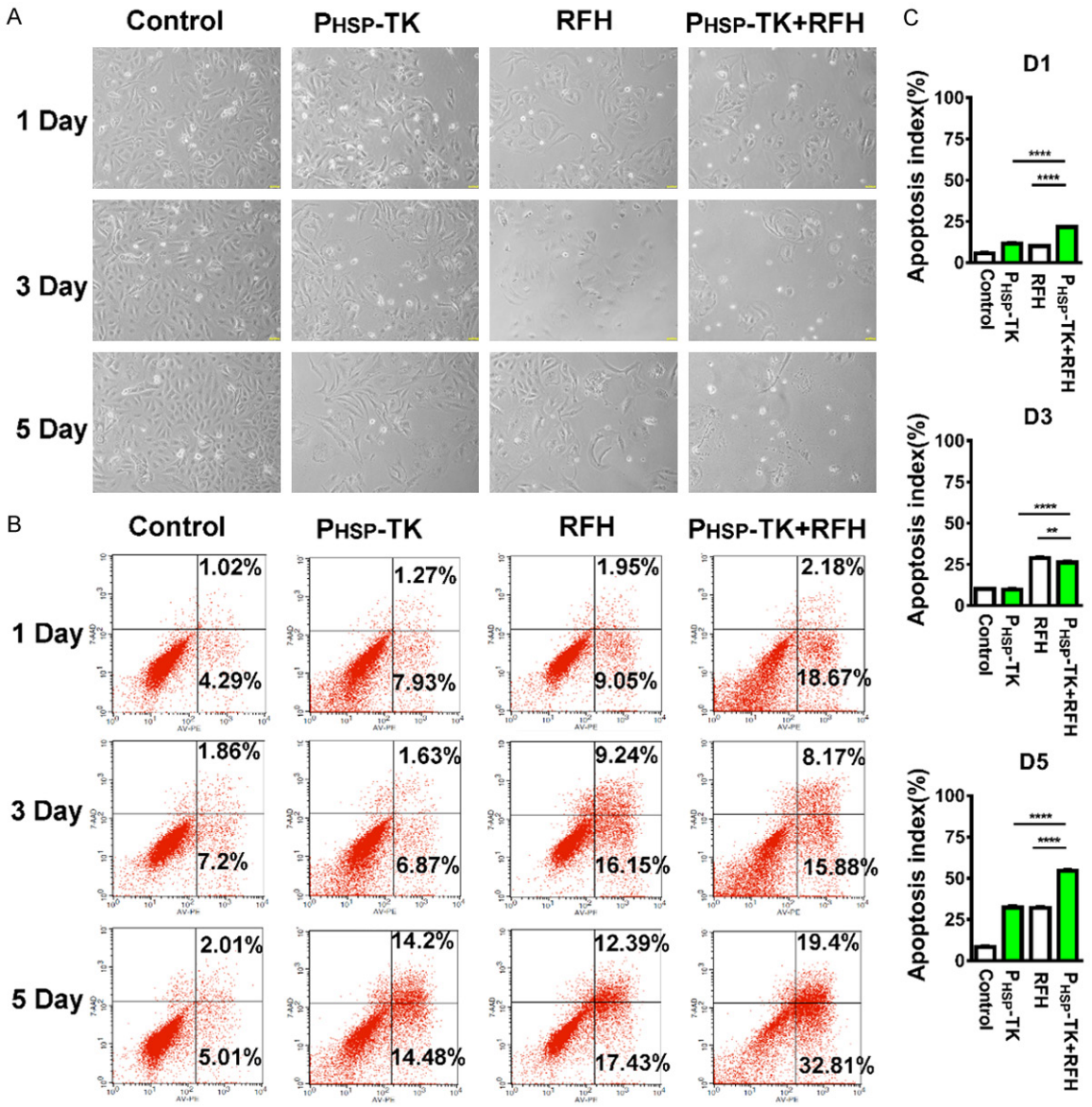

Figure 2. Combined treatment using the P_{HSP}-TK/GCV system plus RFH causes significant apoptosis in human cholangiocarcinoma cells *in vitro* (A) Light microscope assays in four different cell groups at day 5 after the treatments reveal decreased survival of cells by the combination treatment compared to other control cell groups, which is confirmed by subsequent quantitative flow cytometry analysis (B). (C) Apoptosis index of combinational treatment groups were significantly higher than those of other control groups. (** < 0.01, *** < 0.0001, **** < 0.0001).

week-old BALB/c nude female mice (Slac laboratory animal center, Shanghai, China) were divided into four treatment groups (n = 6 per group): RFH+P_{HSP}-TK/GFP; P_{HSP}-TK/GFP-only; RFH-only; and phosphate-buffered saline (PBS) as a control. After human cholangiocarcinoma cells were transfected with P_{HSP}-TK/GFP-lentivirus (MOI = 20) for 12 h, the residual medium was replaced with fresh culture medium, followed by analysis of HSV-TK-positive expression. HSV-TK-positive cells (5×10^6) were subcutaneously transplanted into the unilateral back of each mouse to create a human cholangiocarcinoma-bearing animal model. Two days after the transplantation of HSV-TK-positive cells, which was a sufficient time to allow HSV-TK expression, the 0.032-inch radiofrequency heating wire was inserted into the targeted tumors for local heating at 45°C for 20 min. A 2.7 mm micro-thermometry fiber was inserted into tumor parallel to the RF heating wire to instantly measure the temperature changes caused by RFH. Intraperitoneal GCV (25 mg/kg) was administered every 2 days for 14 days.

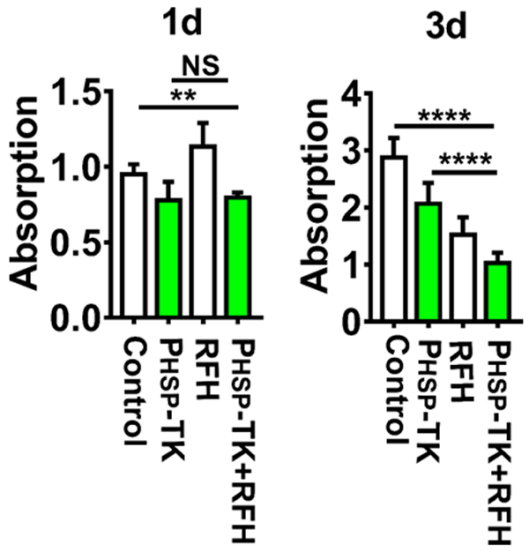

Figure 3. In vitro cytotoxicity. The CCK8 assay at day 3 after the treatments demonstrates that the combined treatment using the P_{HSP}-TK/GCV system plus RFH significantly inhibits the growth of human cholangiocarcinoma cells. ** < 0.01, **** < 0.0001.

In-vivo magnetic resonance imaging (MRI) follow-up: The treated animals were mechanically ventilated with 3% isoflurane mixed with 0.5 l/min oxygen during MRI follow-up. MRI was done using a 3.0-Tesla MR scanner (GE Healthcare Corporation, Chicago, IL, USA) by placing the mouse into a 100 mm-diameter micro-imaging coil. T2-weighted images (T2WI) were acquired by rapid acquisition with the following OAx T2 FSE spin echo sequence: TR/TE = 2660/80 ms, field of view = 8 cm, matrix = 256 × 256, section thickness = 1.5 mm, intersection gap = 0.5 mm, NEX = 2, and total scan time = 1 min, 51 sec. MRI was performed on days 0, 7, and 14 after gene therapy.

MRI measurement of tumor volume: The tumor volume was measured using the Volume Rendering software on the GE workstation. Measurements were performed independently by two radiologists with 5 years-experience in the MRI diagnosis of cholangiocarcinoma. A region of interest (ROI) was manually drawn around the entire visible tumor. Once the ROIs were defined, the tumor volume was calculated automatically.

Histology confirmation

After achieving the satisfactory MR images, all tumor-bearing animals were sacrificed under anesthesia. The subcutaneous tumors were harvested. The volume (V) of each tumor was calculated using the formula: $V = A \times B^2/2$ (where A is the longer diameter and B is the shorter one). Due to the variation in tumor size, we used the relative tumor volume (RTV) to compare the tumor size changes, which was calculated as $RTV = V_n/V_0$ (where V_n = tumor volume on day 7 or 14 post-treatment and V_0 = tumor volume pre-treatment). Hematoxylin and eosin (H&E) staining was performed to confirm the formation of cholangiocarcinoma. Terminal dUTP nick end labeling (TUNEL) was used to examine cell apoptosis. The number of apoptotic cells was counted using Image J software (Media Cybernetics, Rockville, MD, USA).

Statistical analyses

The significance of differences was evaluated using one-way ANOVA to compare *in vitro* gene expression levels, the efficacy of cytotoxicity, and apoptosis indices. Two-way ANOVA was performed to compare *in vivo* tumor sizes among different subject groups at three time points. A *p-value* < 0.05 was considered statistically significant.

Results

In vitro radiofrequency heating enhances P_{HSP}-TK gene expression

After human cholangiocarcinoma cells were transfected with the P_{HSP}-TK/GFP lentivirus, the percentage of GFP+ cells corresponded to the percentage of HSP-TK positive cells (**Figure 1A**). As compared to 94% GFP+ mock cells, only 55% of the cells were GFP+ after the cells were transfected with lentivirus containing the P_{HSP}-TK gene (**Figure 1B**). However, the percentage of GFP+ cells rapidly increased from 47% to 89% after the cells were heated with RFH for 20 min at 45°C (**Figure 1C**). It indicated that HSP promoter was significantly activated due to the heat shock of RFH, more effective P_{HSP}-TK gene expression were achieved.

In vitro RFH significantly promotes cytotoxic effects of P_{HSP}-TK gene therapy

RFH resulted in a significant increase of apoptosis ratio combined with P_{HSP}-TK gene therapy after the addition of GCV (10 μg/ml) (**Figure 2A**). The apoptosis index increased from 10 ±

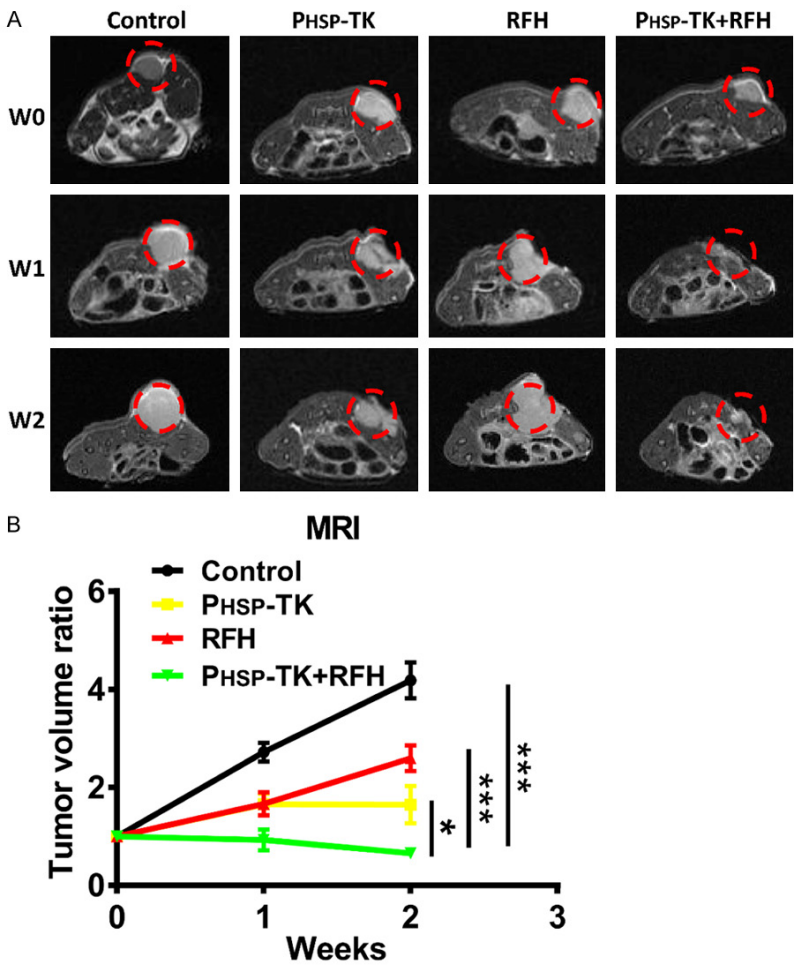

Figure 4. *In vivo* follow-up MRI to monitor tumor size. Interventional RFH treatment significantly promotes the inhibitory effects of P_{HSP}-TK/GCV therapy in cholangiocarcinoma bearing mice. A. The combined treatment with P_{HSP}-TK gene therapy plus RFH reduces tumor volume compared to those with other control treatments within 2 consecutive weeks (w). B. Statistical analysis of MRI evaluation for the four different groups of mice, showing significantly decreased size with the combination therapy using P_{HSP}-TK plus RFH at week 2 after the treatment, in comparison to other control treatments. * < 0.05, *** < 0.001.

0.14% in the HSP-TK treated group to 21.62 ± 0.17% in the P_{HSP}-TK plus RFH group at day 1 (**Figure 2B**). As the time past, the apoptosis index of tumor cells increased. At day 5 after treatment with GCV, the tendency of cell death was more pronounced in the combination treatment compared to other control treatments (54.42 ± 0.65% vs 8.39 ± 0.23% for control, 32.34 ± 0.61% for P_{HSP}-TK, and 31.98 ± 0.77% for RFH; **Figure 2B**, **2C**). The CCK8 assay also indicated the stronger cytotoxic effects of the P_{HSP}-TK plus RFH treatment in comparison with the P_{HSP}-TK-only treatment. After GCV treatment for 3 consecutive days, the growth of the cholangiocarcinoma in P_{HSP}-TK plus RFH group was more prominently inhibited compared to the cholangiocarcinoma in the P_{HSP}-TK treatment group (**Figure 3**). It suggested that RFH significantly promoted the cell killing effects of P_{HSP}-TK/GCV system on cholangiocarcinoma.

In vivo treatment of P_{HSP}-TK plus RFH leads to significant reduction in the volume of cholangiocarcinoma

One week after the treatment, we found the combined therapy of P_{HSP}-TK plus RFH significantly inhibited the tumor growth in the cholangiocarcinoma, as compared to other controlled treatments of RFH-only, P_{HSP}-TK-only, or PBS treatment by using MRI evaluation. As the therapy continued, the difference between the P_{HSP}-TK plus RFH group and other groups further increased. Two weeks after the treatments, the combinational treatment lead to the significant reduction of tumor volume, however the tumor volume increased in the other groups. It indicated that the only P_{HSP}-TK plus RFH treatment had more powerful inhibitory effect on the growth of cholangiocarcinoma in vivo (**Figure 4**). These results showed that combinational treatment of P_{HSP}-TK plus RFH might be the superior option compared to the RFH-only treatment, or P_{HSP}-TK-only treatment in cholangiocarcinoma.

In vivo combinational approach of P_{HSP}-TK plus RFH offered an effective treatment via promoting apoptosis of cholangiocarcinoma

Reversed fluorescence microscopy examination (**Figure 5A**) confirmed that the number of apoptosis cells of the P_{HSP}-TK plus RFH group

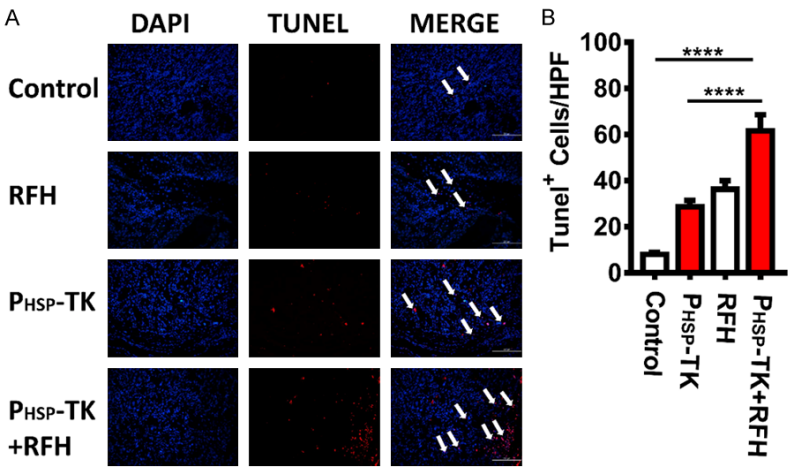

Figure 5. *In vivo* detection of apoptosis in cholangiocarcinomas using the TUNEL assay in the four different treatment groups. (A) Reverse fluorescence microscopy reveals a significant increase of apoptosis cells in P_{HSP}-TK+RFH group compared to the other three control groups, (red dots), which is further confirmed by the TUNEL assay, **** < 0.0001 (B).

(61.6 ± 7.3) per high power field was significantly higher than that of the other treatment groups of control, RFH-only, or P_{HSP}-TK-only (8 ± 0.9, 36.2 ± 3.9, 28.6 ± 3.0, respectively; $P < 0.0001$, $P < 0.01$, and $P < 0.0001$, respectively; **Figure 5B**).

Discussion

The HSV-TK/GCV system is the most broadly utilized enzyme/prodrug in studies of suicide gene therapy [6, 16, 17]. Despite the abundant use of HSV-TK/GCV, low gene expression remains the main obstacle that limits the curative effects for suicide gene therapy [5]. To address this problem, tissue-specific promoters were used to elevate suicide gene expression levels [7, 18, 19]. HSP70B' is a heat shock inducible protein. The heat shock element (HSE) in the HSP70B' promoter plays an important role in response to heat shock [9, 20]. We cloned the promoter of HSP70B' upstream of the HSV-TK gene to construct a new suicide gene delivery system. The construct was activated by heat shock, resulting in significantly elevated HSV-TK gene expression levels, which produced sufficient toxic GCV triphosphate at the target tumors.

To further enhance HSV-TK/GCV efficacy, we also specifically developed a method using image-guided interventional RFH to create local and precise heating at the targeted tumors only [12, 21], which allowed targeted heat shock in cholangiocarcinoma cells. The innovative integration of image-guided interventional RFH with the HSP promoter-mediated HSV-TK gene therapeutic system permitted the confirmation that RFH at approximately 45°C significantly enhanced HSV-TK gene expression and cytotoxic effects on cholangiocarcinoma cells.

Concurrent with the experimental results *in vitro*, the tumor volume of the combinational therapy group shrank more significantly as compared to those of RFH-only group, P_{HSP}-TK-only group or saline group *in vivo* in cholangiocarcinoma bearing mice. The synergistic effects of HSV-TK gene therapy and RFH indicate that the HSP promoter-mediated suicide gene therapy combined with RFH is a promising approach to the treatment of cholangiocarcinoma.

Image-guided anti-tumor therapies avoid various drawbacks of systemic therapeutic administration, which include lethal effects on normal cells, low therapeutic dose at the targets, and toxic damages to other vital organs due to systemic administration [22]. The interventional procedure enables the precise transport of therapeutic genes to the targets, while interventional RFH further contributes to the targeted heating of selected areas. These potent synergistic effects improved the therapeutic efficacy of cholangiocarcinoma in the current study.

The measurement of tumor volume is an important assessor of the clinical stage and disease outcomes of cancer [23, 24]. We evaluated tumor volumes using MRI and demonstrated that intratumoral RFH significantly promoted the direct therapeutic effects of HSV-TK gene therapy throughout the therapeutic process.

To conclude, image-guided interventional RFH significantly enhanced the HSP promoter-mediated HSV-TK gene therapeutic efficacy for cholangiocarcinoma. The new combinational treatment can eliminate multiple obstacles of sys-

temic HSV-TK gene therapy, which include low gene transfection and expression, and killing of normal cells due to less precise gene administration. The novel technique may open new avenues for image-guided combinational therapy of cholangiocarcinoma via the innovative integration of image-guided delivery of high-dose therapeutic genes with the simultaneous generation of local RFH at the targets, which further enhances HSP promoter-mediated direct HSV-TK gene therapy.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiaoming Yang, Image-Guided Bio-Molecular Intervention Research, Department of Radiology, University of Washington School of Medicine, 815 Mercer Street, Room S470, Seattle, WA 98109, USA. Tel: 206-685-6967; E-mail: xmyang@zju.edu.cn; xmyang@uw.edu; Dr. Jihong Sun, Department of Radiology, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, 3 East Qingchun Road, Hangzhou 310016, Zhejiang, China. Tel: (0571)86006764; E-mail: sunjihong@zju.edu.cn

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