

## Original Article

# Cholangiocarcinoma: molecular imaging-guided radiofrequency hyperthermia-enhanced intratumoral herpes simplex virus thymidine kinase gene therapy

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**Abstract:** We investigated the feasibility of using radiofrequency hyperthermia (RFH) to enhance green fluorescent protein (GFP)/herpes simplex virus thymidine kinase (HSV-TK)/ganciclovir (GCV) gene therapy of cholangiocarcinoma. Cholangiocarcinoma cells and mice with cholangiocarcinoma were treated by (i) GFP/HSV-TK/plasmid combined with RFH at 42 °C, followed by ganciclovir administration; (ii) HSV-TK alone; (iii) RFH alone; and (iv) saline. The therapeutic effects among different treatments were evaluated by bioluminescent optical imaging and ultrasound imaging. For the technical validation, GFP/HSV-TK/plasmid was intrabiliarly injected into pig common bile duct (CBD) walls using a needle-integrated balloon catheter with or without RFH enhancement. GFP gene expression was evaluated by optical imaging, which was correlated with histology. The results show that combination therapy of HSV-TK plus RFH significantly induced lower cell viabilities and decreased bioluminescence signals compared the other three groups, which were further confirmed by the tumor volume decrease with combination therapy, as measured by ultrasound imaging. Optical imaging of CBD tissues demonstrated an increased GFP expression in the group with RFH enhancement, compared that with non-RFH treatment. We concluded that intratumoral RFH can enhance the therapeutic effect of GFP/HSV-TK/plasmid on cholangiocarcinoma, which may open new avenues for effective treatment of this deadly disease.

**Keywords:** Cholangiocarcinoma, molecular imaging, radiofrequency hyperthermia, suicide gene therapy

## Introduction

Cholangiocarcinoma is a malignant disease characterized by rapid invasiveness, marked resistance to chemo- and radiotherapies, and very poor prognosis [1, 2]. Despite continuous efforts in the past decades, the morbidity and mortality of cholangiocarcinoma has not been significantly improved, with the five-year survival rate still less than 10% [3]. Although surgical resection offers a potential for cure in early stage disease, patients are often diagnosed at advanced stages when surgical therapy is less effective or contraindicated [1, 2]. Systemic chemotherapy is the mainstay of treatment for advanced cholangiocarcinoma. However, the median survival in these patients receiving systemic chemotherapy remains extremely poor, due to rapidly evolving primary resistance of

tumors to chemotherapeutics [4, 5]. Because of these limited surgical and medical treatment options for advanced cholangiocarcinoma, it is imperative to explore treatment alternatives.

Gene therapy is one of the frontiers of modern medicine. To date, more than 1,800 clinical trials of gene therapy have been completed worldwide, with most of these trials focusing on cancer therapy (64.4% of all gene therapy trials) [6]. Among different gene therapeutic approaches, tumor suicide gene therapy using the herpes simplex virus thymidine kinase gene/ganciclovir prodrug (HSV-TK/GCV) system has been recognized as a more effective method for treating various malignancies [7-11]. The HSV-TK gene can not only convert GCV, a harmless prodrug, into its toxic metabolite to kill tumor cells, but also generate “bystander effect” to induce the

death of neighboring non-gene-transfected cells [12, 13]. However, the success of HSV-TK/GCV-mediated gene therapy completely relies on efficient HSV-TK gene transfection into the target tumors and subsequent sufficient gene expression in targeted tumor cells. While gene-carrying viral vectors, such as adenoviruses and lentiviruses, offer the greatest effectiveness for gene transduction, they often cause undesired immunogenicity or negative immune-reaction that can harm treated patients [14, 15]. Non-viral vectors, such as plasmids and various nanoparticles, are safe and relatively easy to be constructed for carrying genes. However, the efficiency of non-viral vector-mediated gene transfection is very low [16, 17]. With current technology, systemic delivery of any therapeutic genes usually cannot achieve sufficient therapeutic dose at the targets without causing toxicity to other vital organs [14, 18].

Our recent investigations have confirmed that radiofrequency hyperthermia (RFH, i.e., mild hyperthermia around 42°C) can significantly enhance gene transfection and expression in different diseased tissues such as atherosclerosis and various malignancies [19-21]. The possible mechanisms of hyperthermia-enhanced gene transfection include the effect of heating-induced tissue fracture, increased permeability of the plasma membrane, as well as increased cell metabolism and activity of the heat shock protein pathway [22, 23]. We have also established the methods for local therapeutic delivery by image-guided interventional approaches, via either percutaneous routes or naturally-existing anatomic lumens, which enable us to locally deliver high-dose therapeutics only into the targets and thereby solve the problems of systemic therapeutic delivery [24, 25]. These results underpin this current work, using molecular image-guided interventional RFH to enhance plasmid-mediated local HSV-TK/GCV gene therapy of cholangiocarcinoma by combining the advantages of three rapidly developing scientific fields-image-guided interventional oncology, RF technology, and direct intratumoral gene therapy.

## Materials and methods

### Study design

This study was conducted in three phases: (a) in-vitro experiments to establish the “proof-of-

principle” that RFH could enhance HSV-TK gene killing effects on human cholangiocarcinoma cells; (b) in-vivo experiments to further confirm the concept of RFH-enhanced HSV-TK gene therapy of cholangiocarcinoma in a mouse model, which could be assessed by molecular imaging; and (c) preclinical experiment of this new technique in near-human-sized pigs, to technically validate the feasibility of using image-guided intrabiliary RFH to enhance local HSV-TK gene delivery and expression in bile duct walls.

### In-vitro experiments

RFH-enhanced HSV-TK gene killing effect on cholangiocarcinoma cells: Human cholangiocarcinoma cells (Mz-Cha-1) were first transfected with luciferase/m-cherry lentiviral particles, to create luciferase/m-cherry-positive cells according to the protocol provided by the manufacturer (Gene Copoeia Inc., Rockville, MD). Luciferase/m-Cherry-positive cells were sorted out using fluorescence-activated cell sorting technique (Aria II, Becton Dickinson, Franklin Lakes, NJ).

$1 \times 10^5$  cells were seeded in 24-well cell culture plates (Becton Dickinson and Company, Franklin Lakes, NJ) and maintained in Opti-MEM (Gibco) on the day of transfection. 0.5- $\mu$ g GFP/HSV-TK/plasmid (ABM, Richmond, Canada), 1.5- $\mu$ l lipofectamine 3000 and 1- $\mu$ l p3000 (Invitrogen, Carlsbad, CA) with 50- $\mu$ l Opti-MEM were added to each well for transfection per manufacturer's protocol (Invitrogen, Carlsbad, CA). The GFP/HSV-TK positive cells were then quantified using flow cytometry to confirm the gene expression rate.

The same  $1 \times 10^5$  cells were then seeded in four-chamber cell culture slides (Nalge Nunc International, Rochester, NY) and maintained in Delbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco). A mixture of 0.5- $\mu$ g GFP/HSV-TK/plasmid, 1.5- $\mu$ l lipofectamine 3000 and 1- $\mu$ l p3000 was added to each of the four chambers. RFH was performed by attaching a 0.022-inch RF heating wire under the bottom of chamber 4 connected to a radiofrequency (RF) generator for heating. A sterilized 1.1-mm fiber optic temperature probe was placed in the bottom of each chamber and connected to a thermometer (Photon-Control, Burnaby BC, Canada) for monitoring of

the temperature change during RF heating. GFP/HSV-TK gene expression in cells was quantified by western blotting.

Cells in different groups were treated by (a) 30-min RFH at approximately 42°C with GFP/HSV-TK/plasmid, followed by 72 hours of exposure to 100-μg/mL GCV one day after gene transfection; (b) 30-min RFH-only; (c) GFP/HSV-TK/plasmid+GCV therapy only; and (d) no treatment to serve as a control.

## Cell viability assay

Cell viability was evaluated by MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega Corporation, Madison, WI) 72 hours after GCV treatment. Briefly, MTS agent was added to the cell chambers and incubated for 1 hour. The absorbance was measured using a microplate reader at 490 nm (SpectraMax, Molecular Devices, Orleans Drive, Sunnyvale). Relative cell proliferations of different cell groups were evaluated using the equation of  $A_{\text{treated}} - A_{\text{blank}} / A_{\text{control}} - A_{\text{blank}}$ , where A is absorbance. Cells on slides were subsequently washed twice with phosphate-buffered saline (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 bioluminescence assay

In-vitro bioluminescent optical imaging was performed for cells in each group 72 hours after GCV treatment. Cells were digested by 0.25% trypsin and washed with PBS, and then 5-μL Pierce D-Luciferin (ThermoFisher Scientific, Rockford, IL) was added into the solution and incubated for additional 20 minutes. Cell medium was mixed with 100-μL 1% agarose (Invitrogen, Carlsbad, CA) in a cylindrical glass tube for optical imaging using an in vivo optical imaging system (Bruker Corp., Billerica, MA). Bioluminescent signal intensity was quantified as the mean of all detected photon counts 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 treat-

ment group, and C represents the control group.

## Western blotting

Expression of HSV-TK/GFP in cells transfected by GFP/HSV-TK/plasmid with and without RFH was quantified by analyzing the GFP gene expression with western blotting two days after gene transfection. Western blotting was performed according to the protocol provided by life technologies. All the antibodies were obtained from Thermo Fisher Scientific and Santa Cruz Biotechnology (Santa Cruz, USA). The gene expression was quantified by measuring the chemiluminescence areas and subsequently normalized to relative areas (RA) by using the following equation:  $RA = A_{\text{RFH+gene}} / A_{\text{gene}}$ , where  $A_{\text{RFH+gene}}$  is the chemiluminescence area of the group of gene transfection plus RFH, and  $A_{\text{gene}}$  is the group with gene transfection alone.

## In vivo experiments on mice

### Animal model

The Institutional Animal Care and Use Committee approved the animal protocol. Thirty-six nu/nu mice (Charles River Laboratories, Wilmington, MA), aged 4-6 weeks, were used for creating subcutaneous cholangiocarcinoma.  $5 \times 10^6 - 1 \times 10^7$  luciferase-positive cells in 100-μL Matrigel were inoculated in the right back of each mouse. After the size of tumor had reached 5-10 mm in diameter, the animals were anesthetized with 1-3% isoflurane (Piramal Healthcare, Andhra Pradesh, India) in 100% oxygen, and placed on a warm pad for experiments during the treatment.

### GFP-based optical imaging to confirm and quantify GFP gene expression in tumors

Optical imaging was performed using the in-vivo x-ray/optical imaging systems (Bruker). The first group of 12 mice was set to investigate the GFP/HSV-TK/plasmid expression levels over a period of 5 days. Three mice in 4 groups were euthanized at different time points of 1, 2, 3 and 5 days after the intratumoral gene injection. The mean fluorescence signal intensities of tumors were measured using the Bruker MI software.

## *RFH-enhanced HSV-TK gene transfection*

The remaining 24 mice were divided into four groups (six mice per group) and treated with: (a) intratumoral injections of 25- $\mu$ g GFP/HSV-TK/plasmid, 75- $\mu$ l lipofectamine 3000 and 50- $\mu$ l p3000 in 25- $\mu$ L Opti-MEM (Gibco), immediately followed by intratumoral RFH at approximately 42°C for 30 mins; (b) 30-min intratumoral RFH alone; (c) intratumoral injection of gene and transfection reagents with no RFH; and (d) injection of 200- $\mu$ L PBS to serve as controls. RFH was carried out by inserting a 0.022-inch RFH wire into the tumor with its heating spot located at the center of each tumor mass, which was precisely guided under real-time ultrasound imaging. At the same time, a 400- $\mu$ m fiber optical thermal probe was subcutaneously placed at the margin of each tumor to instantaneously monitor the temperature. For the mice with intratumoral gene injection, GCV at 5 mg/kg/day was intraperitoneally administered for 7 days.

## *Bioluminescence-based optical imaging to follow up the tumor growth*

Animals in the treatment group were imaged at day 0 before treatment and days 7 and 14 after the treatment. Bioluminescence optical images were acquired 20 minutes after an intraperitoneal injection of 150 mg/kg d-luciferin. Bioluminescence signal intensity of the tumor was quantified as the sum of detected photon counts using the Bruker MI software. The recorded signal intensities were subsequently 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 to follow up the tumor growth*

Immediately after optical imaging, ultrasound imaging (Sonosite Inc, WA, USA) was performed to measure the tumor size. Two longest perpendicular axes at X and Y planes, as well as the depth axes defined as Z of each tumor were measured by using a 6-13 MHz ultrasound transducer. The volume of each tumor was then calculated according to the equation of volume =  $X \times Y \times Z \times \pi / 6$ . Data were normalized to relative tumor volume (RTV) by using the following

equation:  $RTV = V_{Dn} / V_{D0}$ , where V is tumor volume, Dn represents days after treatment, and D0 is the day before treatment.

## *Histologic correlation/confirmation*

Upon achieving satisfactory images, the animals were euthanized and tumors were harvested at day 14 after treatments. Tumor tissues were cryosectioned at 10- $\mu$ m slices for apoptosis staining. Level of apoptosis was determined with a terminal deoxynucleotidyl transferase dUTP nick end labeling assay (TUNEL). On one slide, six fields were randomly photographed using a digital camera (Olympus DP72, Tokyo, Japan). Apoptosis results were analyzed as the apoptotic index, defined as the number of apoptotic cells/total number of cells in each field. Expression of GFP/HSV-TK in tumor tissues with and without RFH was detected and quantified by analyzing the GFP gene expression with western blotting two days after intratumoral gene injection. Tissue samples were homogenated and digested, and then processed for western blotting, as performed in the in-vitro experiments with cells.

## **In vivo experiments on pigs**

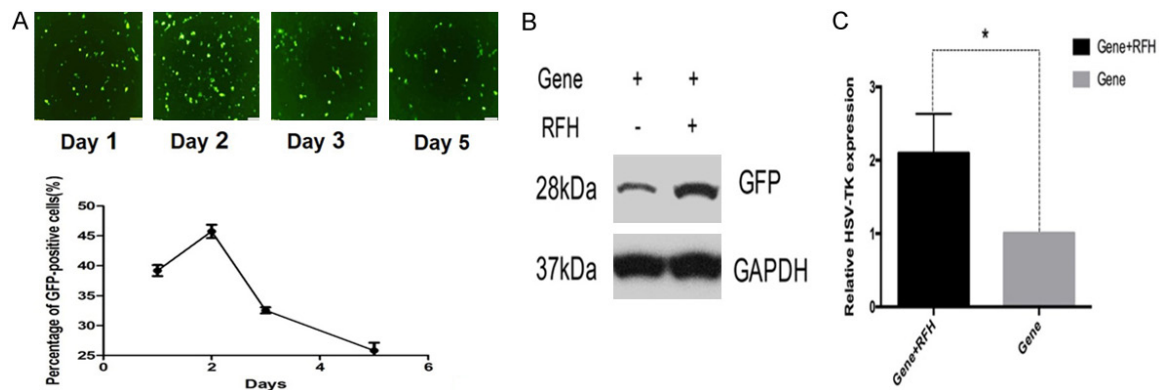
### *Animals*

Twelve domestic pigs, weighing 40-60 kg, were sedated by intramuscular injection of Telazol at 4.4 mg/kg and xylazine at 1 mg/kg (Fort Dodge Animal Health, Fort Dodge, Iowa) and were then mechanically ventilated with 1-3% isoflurane delivered in 100% oxygen.

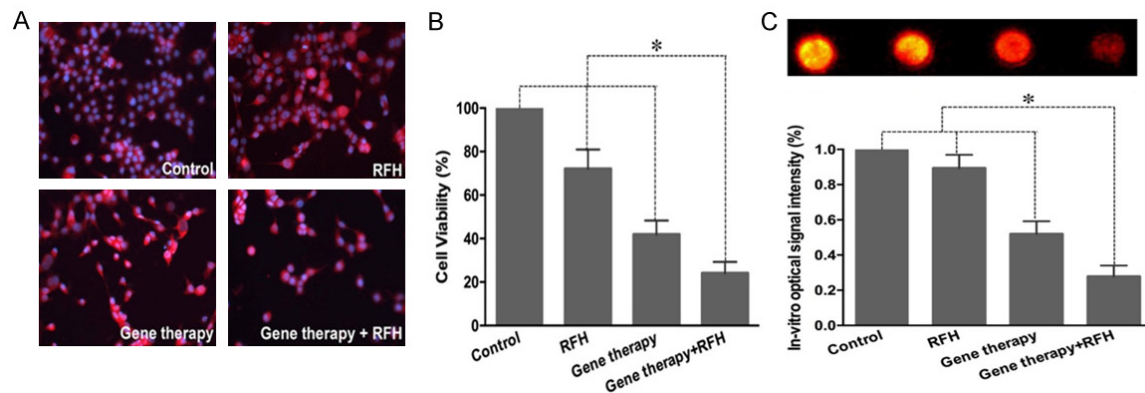
### *Intrabiliary GFP/HSV-TK/plasmid gene delivery and RF heating*

Six pigs in group 1 were treated by intrabiliary injection of a mixture of 0.1-mg GFP/HSV-TK/plasmid, 0.3-ml lipofectamine 3000 and 0.2-ml p3000 in 50  $\mu$ L Opti-MEM with 100- $\mu$ L X-ray contrast agent (Visipaque, GE healthcare, Milwaukee, WI) and 70- $\mu$ L motexafin gadolinium (MGd, Pharmacyclics, Sunnyvale, CA) (a multifunctional agent emitting red fluorescence). Six pigs in group 2 received the same mixture of agents as the pigs in group 1, followed by intrabiliary RFH at approximately 42°C for 30 minutes. For each pig, a laparotomy via a right subcostal incision was made to expose the gallbladder. The cystic duct was





**Figure 1.** In-vitro experiments. A. Fluorescent microscopy with quantitative analysis of GFP-positive cells shows the highest GFP gene expression at day 2 after the GFP/HSV-TK/plasmid transfection. B, C. Western blot analysis of GFP gene expression demonstrates a two-fold increase of gene expression in cells treated with RFH in comparison to the cells without RFH (\*= $p < 0.001$ ).



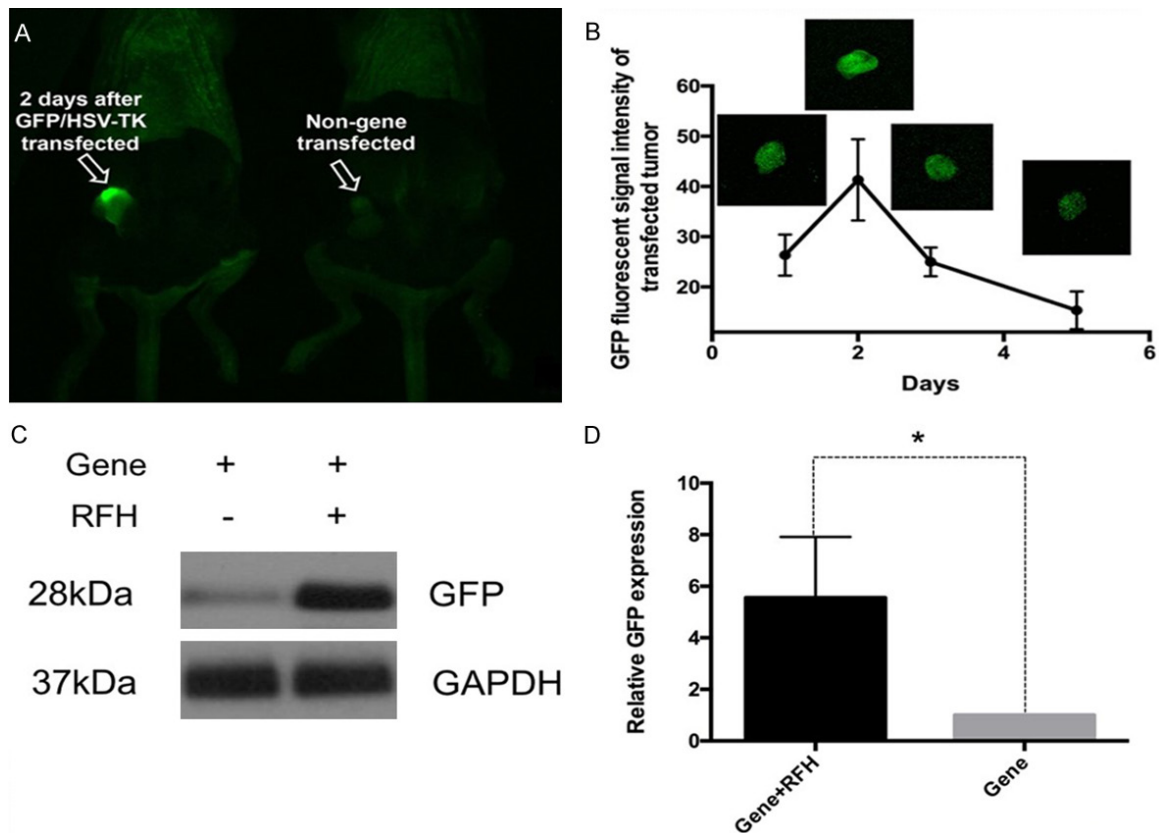
**Figure 2.** In-vitro experiments. (A) m-Cherry fluorescent microscopy shows a decreased number of cells with combination therapy (Gene therapy+RFH), which is significantly lower than those of other three groups (B) (\*= $p < 0.001$ ). (C) In-vitro bioluminescence optical imaging further confirms the lowest signal in the cells treated with combination therapy of GFP/HSV-TK/GCV plus RFH (\*= $p < 0.001$ ).

then accessed by using an 8 French introducer, via which a 0.035-inch guidewire was placed. Over the guidewire, a needle-integrated-balloon catheter (Mercator MedSystems, Emeryville, CA) was advanced into the common bile duct (CBD). Under the guidance of fluoroscopy, the needle-integrated balloon was inflated with 50% X-ray contrast agent, which pushed the needle to puncture through the bile duct wall. Subsequently, 100- $\mu$ g GFP/HSV-TK/plasmid mixed with transfection reagents was injected into the CBD wall. For the group of pigs treated with intrabiliary RFH, a 10  $\times$  20 mm balloon attached with a 1.1 mm fiber optic thermal sensor was immediately advanced into the gene-treated segment of CBD, where local RFH at 42°C was applied via the RFH wire. After intrabiliary gene delivery and RFH, the gallblad-

der was removed with suture ligation and the abdominal incision was closed with suture in layers.

#### Ex vivo optical imaging of the excised CBD

Two days after gene delivery, animals were euthanized and the CBDs were harvested, weighed and applied on optical imaging system to acquire the fluorescence images. Gene delivery segment of CBD was confirmed according to the MGD-based optical imaging, and then the signal to noise ratio (SNR) of gene delivered CBD tissues were calculated,  $SNR = SI_{\text{gene}} / Sd_{\text{background}}$ , where  $SI_{\text{gene}}$  represents mean fluorescence signal intensities of gene-delivered CBDs, and  $Sd_{\text{background}}$  is the standard deviation of the background.



**Figure 3.** A. GFP optical imaging detects a high green fluorescent signal at the day 2 after intratumoral GFP/HSV-TK/plasmid gene transfection (left arrow), which is not seen in non-gene transfection (right arrow). B. Optical imaging detects the highest green fluorescent signal at the day 2 after intratumoral GFP/HSV-TK/plasmid gene injection. C, D. Western blot analysis of GFP gene expression in tumor tissues showed an approximately six-fold increase of gene expression in tumor treated with RFH (n=6) than tumors with gene transfection alone (n=6, \*p=0.001).

#### Statistical analysis

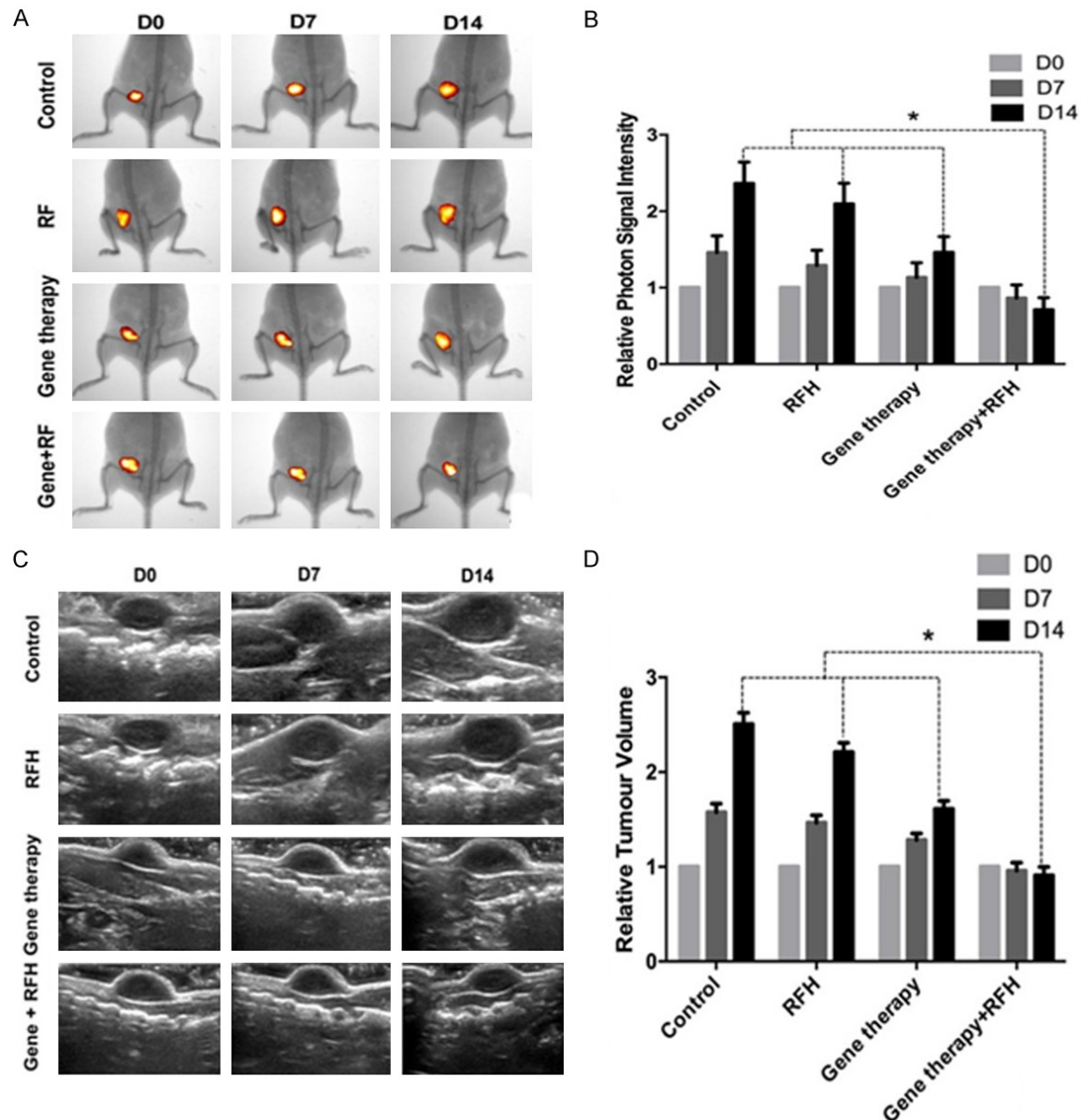
Statistical software (SPSS, Chicago) was used for all data analyses. A non-parametric Mann-Whitney U test was used to compare (i) relative proliferation rates and relative bioluminescent signal intensity among different cell groups of in-vitro experiments; (ii) relative signal and bioluminescent signal intensities, as well as (iii) relative tumor volumes at different time points among various animal groups of in-vivo experiments on mice and pigs. *P* value of less than 0.05 was considered significant.

#### Results

Of in-vitro experiments, flow cytometry showed that GFP/HSV-TK gene expression peaked at day 2 after the gene transfection, followed by a marked decrease in number of GFP cells over 5 days of observation (Figure 1A). Western blotting assay demonstrated that GFP/HSV-TK gene expression level was significantly higher

in the cell group with combination therapy (gene therapy plus RFH) than gene transfection alone ( $2.10 \pm 0.54$  vs  $1.0$ ,  $p < 0.001$ ) (Figure 1B, 1C). Fluorescent microscopy showed that more cells were killed in the group with combination therapy than three control groups, which was further confirmed by quantitative MTS assay. This demonstrated the lowest cell proliferation with combination therapy, compared to those of three control group of gene therapy-only, RFH-only, and the negative control ( $24.0 \pm 5.3\%$  vs  $41.9 \pm 6.4\%$  vs  $72.0 \pm 9.0\%$  vs  $100\%$ ,  $p < 0.001$ ) (Figure 2A, 2B). Bioluminescence optical imaging of cells further confirmed a significantly lower relative photon signal intensity in the cell group with combination therapy, compared to three control groups ( $27.8 \pm 6.2\%$  vs  $51.9 \pm 7.3\%$  vs  $89.4 \pm 7.6\%$  vs  $100\%$ , respectively,  $P < 0.001$ ) (Figure 2C).

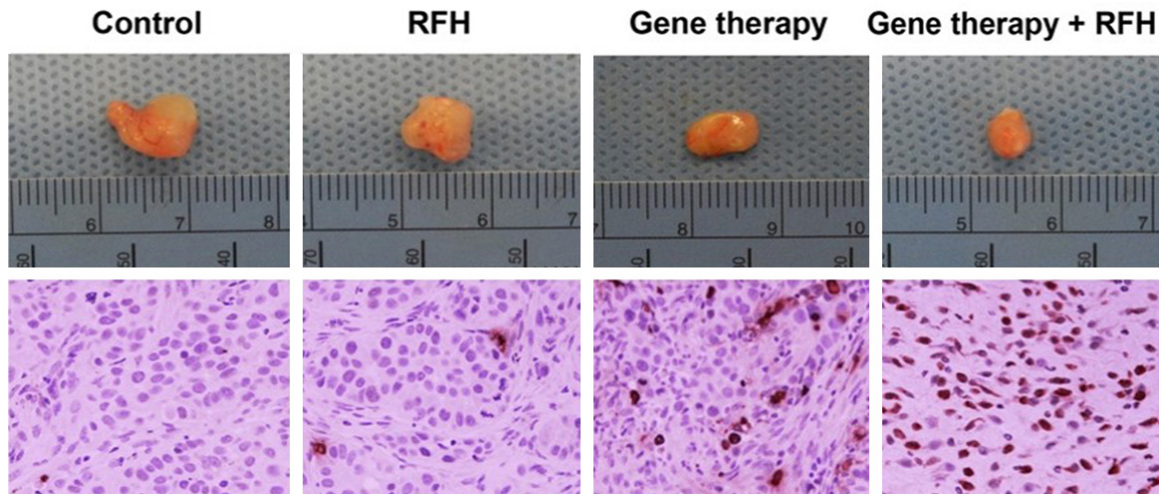
Of the in-vivo experiments on mice, optical imaging demonstrated the highest green fluorescent signal at day 2 after intratumoral GFP/



**Figure 4.** A, B. Optical/x-ray imaging to follow up the tumor growth and response to the treatments at days 0, 7 and 14. There is a significant decrease in both bioluminescence signal intensity (red-yellow color) and tumor size with combination therapy, compared to the other three groups. C, D. Ultrasound imaging further showed the smallest relative tumor volumes of the combination therapy group, in comparison to those of other control groups.

HSV-TK/plasmid gene injection, which was significantly greater than non-gene transfected tumors ( $41.33 \pm 14.01$  VS  $9.33 \pm 5.51$ ,  $p < 0.05$ ) (Figure 3A, 3B). Western blot analysis of GFP gene expression in tumor tissues showed an approximately six-fold increase of gene expression in tumor treated with RFH than tumors with gene transfection alone ( $5.55 \pm 1.06$  vs 1,  $p = 0.001$ ) (Figure 3C, 3D). Optical imaging demonstrated decreased bioluminescent signals of

tumors in the combination therapy group compared with those of three other groups ( $0.71 \pm 0.16$  VS  $1.46 \pm 0.21$  VS  $2.10 \pm 0.27$  VS  $2.36 \pm 0.28$ ,  $p < 0.001$ ) (Figure 4A, 4B). Follow-up ultrasound imaging showed the smallest relative tumor volumes of the mouse group with combination therapy compared to the three control groups ( $0.90 \pm 0.21$  VS  $1.61 \pm 0.21$  VS  $2.22 \pm 0.25$  VS  $2.50 \pm 0.30$ ,  $p < 0.001$ ) (Figure 4C, 4D).



**Figure 5.** (Upper row) Pathology confirms the smallest tumor size in the combination group. (Lower row) Apoptosis by TUNEL staining further shows more apoptotic cells (brown) in the combination therapy group than those in the other three groups.

Gross specimens obtained after the treatments revealed the smallest tumor size in the combination therapy group compared with other groups. Histological analysis of apoptosis by TUNEL staining further confirmed more apoptotic cells in the combination therapy group than in the groups treated with RFH only or gene therapy only ( $37.7\% \pm 10.2\%$  vs  $12.8\% \pm 4.5\%$  vs  $4.3 \pm 2.4\%$ ,  $P < 0.001$ ) (**Figure 5**).

For in-vivo validation experiments on pigs, optical imaging of the GFP/HSV-TK/plasmid-delivered CBD tissues demonstrated an increased SNR of green fluorescence in the group with intrabiliary gene delivery combined with RFH, compared with gene delivery alone group ( $1.28 \pm 0.61$  VS  $0.49 \pm 0.31$ ,  $P < 0.05$ ) (**Figure 6**). There was no pathological evidence of thermal damage to the normal bile duct tissues.

### Discussion

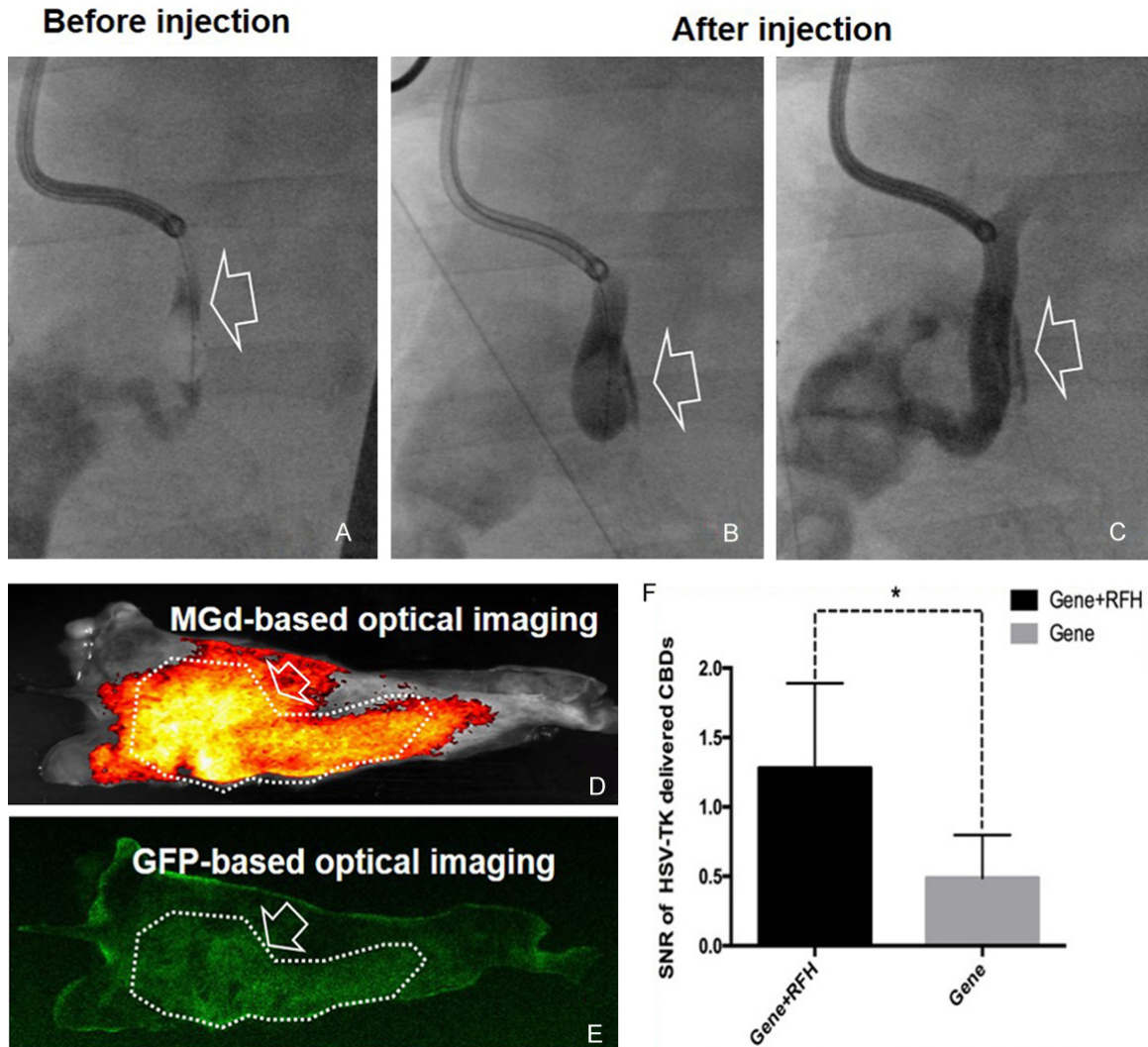
The findings of our study demonstrate that RF heating can significantly enhance the therapeutic effect of HSV-TK/GCV on human cholangiocarcinoma, manifested as greater cell death by the combination therapy of HSV-TK gene plus RFH in vitro, and shrunken tumor volumes and decreased bioluminescence signal intensities of treated cholangiocarcinoma in mice, in comparison to control treatments. The preclinical validation experiments on near-human-sized pigs have further confirmed that it is possible to adapt the above concept, “intratumoral RFH-

enhanced direct HSV-TK gene therapy,” to further clinical application in humans. Specifically, image-guided intrabiliary local gene delivery associated with local RF hyperthermia has the potential to effectively treat the unresectable cholangiocarcinoma in human patients.

Currently, either viral or non-viral vectors can be used to transduce or transfect cells with therapeutic genes. It is well known that although viral vectors can generate higher gene transduction than non-viral vectors, they can often cause some critical safety issues, such as viral immunogenicity and toxicity. Non-viral vectors, such as plasmid and nanoparticles, create lower immunogenicity and toxicity than viral vectors, but they cannot achieve high level gene transfection and expression in the target tumor cells [26-29]. In our study, we attempted to overcome these problems by developing an alternative approach of using intratumoral RFH to enhance non-virus-mediated gene transfection/expression. Our study has confirmed that RFH can boost HSV-TK gene transfection and expression in cholangiocarcinoma cells, and enhance HSV-TK gene therapy of mouse models with cholangiocarcinoma.

Molecular-genetic imaging allows the visualization of pathophysiologic events in living subjects at the molecular or genomic level rather than at the anatomic level [30, 31]. Because the therapeutic efficacy of gene-directed enzyme prodrug therapy is dependent on the level





**Figure 6.** In-vivo experiments on pigs. (A-C) Through a transcystic approach, a needle/balloon catheter is placed in the pig common bile duct. After inflating the balloon with contrast agent, the micro-needle is pushed to puncture the bile duct wall through, and then a mixture of gene with contrast agent is injected into the bile duct wall. Arrow indicates the penetration and distribution of gene/contrast mixture in the bile duct wall tissue. (D, E) Optical images confirm the penetration and distribution of MGD in the bile duct wall (red-orange signals on D). GFP-based fluorescence optical imaging displays the GFP signals in the bile duct tissue. (F) Optical imaging quantitative analysis of GFP expression further confirms stronger GFP signal in the RFH-treated bile duct (n=6) than that in gene delivery only (n=6,  $p < 0.05$ ).

of transgene expression in tumors, it is important to monitor the expression of the exogenous gene using a safe, repetitive and noninvasive method [32]. Molecular optical imaging can be exploited for its capability of detecting either endogenous contrast of tumors in organs, or exogenous labeling of tumor cells that are transplanted or implanted into laboratory animals [33-35]. When a fluorescent reporter gene, such as green fluorescence protein gene (GFP), is constructed with a therapeutic gene

under the same promoter, the therapeutic gene expression can be simultaneously monitored and quantified by optical imaging with a very low cost and in a real-time manner. Reporter genes with optical signatures (e.g., fluorescent and bioluminescent) are a low-cost alternative for real-time analysis of gene expression in animal models [36]. To establish the “proof-of-principle” of the new concept, in our study we used the dual gene-carrying vector, i.e. GFP and HSV-TK genes that are con-

structed in the same plasmid and simultaneously function under the same promoter of cytomegalovirus (CMV). Thus, we could use green fluorescence as a direct biomarker to indirectly detect and quantify the expression level of HSV-TK gene in target tumors. The response of tumors to treatment is usually determined by the morphologic change, for example, shrinkage of tumor size measured on computed tomography (CT), ultrasound imaging, or magnetic resonance images (MRI). However, these morphologic changes usually occur later than biological changes of treated tumors [37]. To overcome this limitation, we transfected the cholangiocarcinoma cells with luciferase gene, which enabled us to utilize bioluminescence optical imaging to monitor the response of cholangiocarcinoma to RFH-enhanced HSV-TK gene therapy. Our study demonstrates that optical imaging of both in-vitro cells and in vivo tumors displayed the marked responses to the combination therapy of RFH with HSV-TK gene therapy.

In this study, since we primarily focused on this new technical development, we only followed the tumor growth for up to 14 days and did not follow up to time points when the treated tumors completely disappeared. This was due to the consideration that a longer follow-up period would result in the tumor size exceeding ten percent of the body weight, which was not allowed by our Institutional Animal Care and Use Committee. In addition, since no large animal model with cholangiocarcinoma is currently available worldwide, in this study we could only use normal pigs to validate technical feasibility. We have confirmed the possibility of (i) using the needle-integrated balloon catheter to locally deliver highly-dose GFP/HSV-TK/plasmids into pig bile ducts; and (ii) using the intrabiliary RF hyperthermia to simultaneously enhance GFP/HSV-TK gene expression.

In conclusion, this study has confirmed the possibility of image-guided, intratumoral RFH-enhanced local HSV-TK/GCV gene therapy of cholangiocarcinoma, which can be monitored by molecular imaging. This concept may open new avenues for effective management of those patients with unresectable cholangiocarcinoma by simultaneous integration of image-guided interventional oncology, radiofrequency technology, and direct intratumoral gene therapy.

## Acknowledgements

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## Abbreviations

HSV-TK, Herpes Simplex Virus Thymidine Kinase; RFH, Radiofrequency Hyperthermia; GCV, ganciclovir; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; GFP, Green fluorescence protein; PBS, phosphate-buffered saline; RSI, relative signal intensity; SNR, signal to noise ratio; RTV, relative tumor volume; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling assay.

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