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High intensity focused ultrasound-induced gene activation in solid tumors

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Abstract

In this work, the activation of heat-sensitive trans-gene by high-intensity focused ultrasound (HIFU) in a tumor model was investigated. 4T1 cancer cells (2×10^6) were inoculated subcutaneously in the hind limbs of Balb/C mice. The tumors were subsequently transduced on day 10 by intratumoral injection of a heat-sensitive adenovirus vector (Adeno-hsp70B-Luc at 2×10^8 pfu/tumor). On day 11, the tumors were heated to a peak temperature of 55, 65, 75, or 85 °C within 10–30 s at multiple sites around the center of the tumor by a 1.1- or 3.3-MHz HIFU transducer. Inducible luciferase gene expression was increased from 15-fold to 120-fold of the control group following 1.1-MHz HIFU exposure. Maximum gene activation (120-fold) was produced at a peak temperature of 65–75 °C one day following HIFU exposure and decayed to baseline within 7 days. HIFU-induced gene activation (75 °C-10 s) could be further improved by using a 3.3-MHz transducer and a dense scan strategy to 170-fold. Thermal stress, rather than nonthermal mechanical stress, was identified as the primary physical mechanism for HIFU-induced gene activation *in vivo*. Overall, these observations open up the possibility for combining HIFU thermal ablation with heat-regulated gene therapy for cancer treatment.

1. Introduction

High-intensity focused ultrasound (HIFU) has emerged as a viable noninvasive therapeutic modality for the treatment of a variety of solid tumors, including liver (Kennedy *et al.*, 2004), prostate (Chapelon *et al.*, 1999; Chaussy and Thuroff, 2003), breast (Hynynen *et al.*, 2001), and soft-tissue sarcoma (Wu *et al.*, 2004). New biomedical applications, such as hemostasis and gene delivery, have also been explored (Vaezy *et al.*, 1999; Miller and Song, 2003). Pioneering studies have demonstrated that HIFU, with spatial-peak temporal-average intensity (I_{SPTA}) between 10^3 and 10^4 W/cm², can produce well-defined thermal lesions in deep-sited tissue (ter Haar, 1995). A large volume of tumor tissue can be treated by scanning the HIFU focus in a matrix of positions. The fundamental physical mechanisms of HIFU ablation are coagulative thermal necrosis (>65 °C) and cavitation damage (Bailey *et al.*, 2003).

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In addition to thermal ablation, preliminary yet encouraging evidence suggest that HIFU may induce a distinct stress response in sublethally injured tumor cells surrounding the focal lesion. For example, significant up-regulation of heat shock proteins (hsp) has been observed at the border of HIFU-induced necrosis region in patients with benign prostatic hyperplasia (BPH) (Kramer *et al.*, 2004). It has been further postulated that the up-regulation of hsp may play a critical role in eliciting an antitumor immunity (Kramer *et al.*, 2004; Kennedy, 2005). Known as “molecular chaperones,” intracellular heat shock (or stress) proteins controlled by a specific family of hsp genes will be dramatically synthesized when cells are exposed to stressful or harmful environments (Morimoto, 1993). In particular, one member of the hsp70 gene family, hsp70B, is strictly stress inducible and absent in unstressed cells (Hildebrandt *et al.*, 2002).

In light of this, ultrasound-induced hyperthermia has been utilized as a noninvasive physical method to achieve spatial and temporal regulation of trans-gene expression under the control of hsp70B promoter both *in vitro* and *in vivo* (Vekris *et al.*, 2000; Guilhon *et al.*, 2003; Zhong *et al.*, 2004). Using ultrasound to regulate transgene expression could be particularly beneficial for site-specific gene therapy when systematic gene dissemination and expression in nontargeted areas are concerned. For example, following exposure to 2 W/cm² ultrasound for 20 min, Smith *et al.* (2002) demonstrated a locally induced luciferase or FasL gene expression after systemic delivery of adenoviral constructs under the control of hsp70B promoter. Furthermore, using a MRI-guided focused ultrasound system, trans-gene induction within internal organs was successfully achieved under well-controlled thermal dosage (42 °C, >30 min) in rat livers and in canine prostates (Plathow *et al.*, 2005; Silcox *et al.*, 2005). It is worth noting that heat shock gene expression has also been detected following ultrasound physiotherapy, which is presumably induced by cavitation-associated mechanical stresses (Barnett *et al.*, 1994; Angles *et al.*, 1990).

Most recently, we have explored the physical conditions for HIFU-induced gene activation in sublethally injured cancer cells *in vitro* (Liu *et al.*, 2005). Following exposure to peak temperatures of 50 to 70 °C for 1 to 10 s, the heat shock responses of two cancer cell lines (HeLa and R3230Ac) were investigated. Consistent gene expression in the surviving cell population was detected under various HIFU thermal doses and the maximum gene activation was produced by 60 °C in 5-s heat shock exposure. These exposure conditions are similar to those experienced by the sublethally injured tumor cells surrounding HIFU-induced necrosis lesion. The logical extension of this preliminary *in vitro* study is to confirm *in vivo* that HIFU treatment can indeed induce trans-gene expression under the control of hsp70B promoter in tumor models. If such a hypothesis is confirmed, one could potentially take advantage of this unique biological response to explore a synergistic combination of *in situ* gene therapy with HIFU-produced thermal ablation to improve the overall efficacy and quality of cancer therapy.

The present study is therefore aimed to investigate the feasibility of simultaneous tumor ablation and activation of a transgene under the control of hsp70B promoter using a tumor-bearing mouse model. Specifically, after an intratumoral injection of adenovirus vector (Ad-hsp70B-Luc) for gene transduction, target tumors were scanned by either a 1.1- or 3.3-MHz HIFU transducer under the guidance of B-mode ultrasound imaging. *In vivo* gene expression activities following HIFU exposures using different combinations of peak temperature, treatment duration, and scanning strategy were evaluated. The results were compared with conventional hyperthermia treatment. Furthermore, the roles of two potential physical mechanisms, i.e., thermal stress and cavitation-associated nonthermal mechanical stress, in HIFU-induced gene activation *in vivo* were investigated.

II. Materials and Methods

A. Tumor inoculation and gene transduction

All *in vivo* experimental procedures were carried out in accordance with the protocol approved by the Duke University Committee on the Use and Care of Animals. Female Balb/C mice (Charles River Laboratory, Wilmington, MA), 6–8 weeks old, were selected with a compatible mouse mammary carcinoma cancer cell line (4T1) to construct the murine tumor model. 4T1 cells were maintained routinely in DMEM culture medium with 10% heat-inactivated fetal bovine serum and 5% antibiotics in a humidified incubator at 37 °C containing 5% CO₂. For tumor inoculation, 2×10^6 4T1 cells suspended in 50 μ L PBS were injected subcutaneously (s.c.) into the shaved right hind limb of the mouse. When the tumor reached a size of ~ 8 mm in maximum diameter in about 10 days, 30 μ L adenoviral luciferase vectors with hsp70B promoters (Ad-hsp70B-Luc) were injected into the center of the tumor using a 30-gauge needle at a dosage of 2×10^8 pfu/tumor, following an established protocol (Wang *et al.*, 2005). After virus injection, the anesthetized animals were sent back to vivarium for virus dissemination and gene transduction inside the tumor for 24 h before HIFU treatment.

B. High-intensity focused ultrasound (HIFU) exposure system

The experiments were carried out utilizing a B-mode ultrasound imaging-guided HIFU exposure system shown in Fig. 1(a). Briefly, a HIFU transducer (H-102, Sonic Concepts, Seattle, WA) with a focal length of 63 mm, operated at either 1.1 MHz (fundamental) or 3.3 MHz (third harmonic), was mounted at the bottom of a heated (37 °C) chamber ($40 \times 30 \times 15$ cm³, L $\times$ W $\times$ H) filled with degassed water. The transducer was driven by continuous sinusoidal signals produced by a function generator (33120A, Agilent, Palo Alto, CA), connected in series with a 55-dB power amplifier (A150, Electronic Navigation Industries, Rochester, NY). The operation and exposure parameters of the HIFU system were controlled by LabView programs via a GPIB board installed in a PC. The animal was placed in a holder specially designed to facilitate HIFU exposure to the tumor-bearing hind limb while protecting the internal organs of the mouse. Using a 5/7 MHz imaging probe (Terason 2000, Terason Inc., NJ), the target tumor area could be outlined to guide the HIFU treatment [Fig. 1(a)]. The pressure waveform and distribution in the focal plane of the HIFU transducer were measured by using a fiber optical probe hydrophone (FOPH-500, RF Acoustics, Leutenbach, Germany). When the function generator was operated at 0.1 V_{pp} , a peak positive (p^+)/peak negative (p^-) pressure of 3.1/–2.7 MPa and 7.1/–4.5 MPa were measured at the beam focus of the HIFU transducer at 1.1 and 3.3 MHz, respectively [Fig. 1(b)]. The corresponding –6-dB beam sizes at the focus along and transverse to the transducer axis were determined to be 11 mm $\times$ 1.64 mm at 1.1 MHz and 5 mm $\times$ 0.6 mm at 3.3 MHz, respectively.

C. Temperature measurement and thermal dose evaluation

A 0.2-mm bare-wire thermocouple (Customer designed IT-23, Physitemp Inc., Clifton, NJ) was used to measure the temperature rise inside the tumor tissue. Temperature output was conditioned by an electronically compensated isothermal terminal block (TC-2190, National Instrument, Austin, TX) and registered at a 6-Hz sampling rate using a Data Acquisition Board (NI4351, National Instrument, Austin, TX) controlled by a LabView program. For alignment, the mouse tumor embedded with the thermocouple was scanned across the acoustical field of the HIFU transducer operated at low intensity using a computer-controlled 3-D step motor (Velmex Inc., Bloomfield, NY) until the maximum temperature rise (<42 °C) was detected at the beam focus. Using this approach, temperature elevations and distribution inside the tumor tissue under the designated HIFU exposures were recorded. In this study, all the HIFU exposures are in continuous wave (CW) mode with an I_{SATA} between 605 and 2657 W/cm² calculated based on an established protocol (Harris, 1985). Figure 2 shows the temperature profiles produced at 1.1-MHz HIFU focus inside the tumor. By adjusting the exposure pressure,

the temperature at the transducer focus could reach a peak value of 55, 65, 75, and 85 °C within a 10-s exposure duration [$p^- = -4.9$ to -9.1 MPa, Fig. 2(a)] or the same temperature of 75 °C in 5, 10, 20, and 30 s, respectively [$p^- = -9.1$ to -5.3 MPa, Fig. 2(b)]. Moreover, the lateral temperature distributions (perpendicular to the HIFU beam axis) under the 75 °C-10 s exposure condition, i.e., under the transducer output conditions for which a temperature rise to 75 °C was reached at the focus in 10 s, were also measured under 1.1- and 3.3-MHz exposure, which revealed a -6-dB lateral temperature beam width of 6 and 1.5 mm, respectively.

Sapareto and Dewey (1984) proposed the concept of equivalent thermal dose in order to elucidate the relationship between thermal dosimetry and biological response. The following equation is defined for single point equivalent thermal dose, also known as equivalent minutes at 43 °C (EM_{43}), as a function of exposure temperature, $T(x,y,z,t)$, and the total treatment duration D . When the temperature history of a point within the tissue is known, the thermal dose at that specific position can be calculated by

$$EM_{43}(x, y, z, T) = \int_0^D R^{43-T(x,y,z,t)} dt,$$

$$R = \begin{cases} 0, & T < 37^\circ \text{C}, \\ 0.25, & 37^\circ \text{C} \leq T < 43^\circ \text{C}, \\ 0.5, & T \geq 43^\circ \text{C}. \end{cases}$$

Based on the temperature profile measured during HIFU exposure, the spatial distributions of the equivalent thermal dose (represented by EM_{43}) in the focal plane (transverse to the beam axis) and in a longitudinal plane across the beam axis of the transducer were evaluated in order to better understand the role of thermal dose in HIFU-induced gene activation and lesion formation.

D. Experimental design

A total of three series of experiments were conducted to investigate the trans-gene activation in the mouse tumor model during HIFU treatment. The first series were designed to demonstrate the feasibility of HIFU-elicited gene activation *in vivo*. A total of five spatially distributed 1.1-MHz/10-s HIFU exposures with a peak temperature of 55, 65, 75, and 85 °C were delivered to the center of the tumor at 2-mm spatial interval in order to identify the appropriate HIFU dosimetry for gene activation. Water-bath hyperthermia treatment (42 °C-30 min) was used as positive control throughout this study.

Following the feasibility study, the second series of experiments was conducted to determine the optimal HIFU conditions for gene activation *in vivo*. Specifically, the 1.1-MHz HIFU exposures used were for increasing durations of 5, 10, 20, and 30 s with transducer outputs chosen so that, for each exposure, the peak temperature reached was 65 °C or 75 °C. In addition, the time course of hyperthermia- or HIFU-induced gene activation in a week was monitored and compared. The corresponding tumor growth curve was also monitored for 20 days after HIFU treatment. Once the proper thermal dose was identified, the effect of the scanning strategy on HIFU-induced gene activation (75 °C-10 s) was further investigated, using the 3.3-MHz HIFU transducer and a dense-scan pattern across a $6 \times 6 \text{ mm}^2$ area with 1-mm step size.

In the third series of experiments, the contributions of two potential physical mechanisms, i.e., thermal stress and nonthermal mechanical stress, to HIFU-induced gene activation *in vivo* were compared. The thermal HIFU (75 °C-10 s) was evaluated by scanning the tumor within the focal plane ($6 \times 6 \text{ mm}^2$ at 1-mm step size) of the 3.3-MHz HIFU, which enhances thermal absorption while suppressing cavitation in the targeted tissue compared to the 1.1-MHz transducer. Under the same scanning protocol, the mechanical HIFU was evaluated by

increasing the acoustic output pressure of the transducer by fourfold while reducing its duty cycle concomitantly from 100% (thermal HIFU) to 6.3% so that the same total acoustic power could be delivered. Using this approach, the accumulated thermal effect could be eliminated because the peak temperature inside the tumor during the 10-s mechanical HIFU exposure was kept below 40 °C when the ambient temperature in the water bath was maintained at 23 °C. Moreover, cavitation activities inside the tumor tissue during both the thermal and mechanical HIFU treatments were monitored by B-mode ultrasound imaging and passive cavitation detection (PCD) technique. For PCD measurements, a 3.5-MHz focused transducer (V380, Panametrics Inc., Waltham, MA) was positioned confocally with the 3.3-MHz HIFU transducer [Fig. 1(a)]. The data acquisition and signal processing protocols used are similar to previously reported procedures (Chen *et al.*, 2004, Rabkin *et al.*, 2005). Briefly, fast Fourier transform (FFT) spectrums of the acoustic emission signals emanated from HIFU-induced cavitation bubbles inside the tumor were averaged and compared at different exposure settings. The rms amplitude of the broadband noise signals for each FFT spectrum between 4.5 and 5.5 MHz was further calculated and presented in time sequence. The inertial cavitation (IC) activity was quantified by computing the cumulated IC dosage (ICD), which is defined by the integration of the rms amplitude (in dBm) of the power spectrum between 4.5 and 5.5 MHz over the entire 10-s exposure period (Chen *et al.*, 2003).

E. Gene expression assay

On the day for gene expression assay, 100 µL of aqueous D-luciferin solution was injected intraperitoneally into the anesthetized animal 20 min before *in vivo* gene expression analysis using a Xenogen *in vivo* bioluminescence imaging system (Xenogen Inc., Alameda, CA). The expressed luciferase protein catalyzes the oxidation of injected D-luciferin (enzyme substrate), resulting in the emission of bioluminescence (Wang *et al.*, 2005). Bioluminescence images were generated by integrating photon emission from the mice tumor during an exposure time of 60 s at a fixed sensitivity. The results were shown in pseudo-colors indicated by the color bar. The final images were represented by superimposing the pseudo-color bioluminescence images on conventional gray scale images taken separately (Wang *et al.*, 2005).

F. Tumor growth regression assay

Following HIFU treatment, tumor sizes were measured using an electronic digital caliper every other day and the tumor volume (V) was calculated using the formula for an ellipsoid, $V = (\pi/6)W^2L$, where L is the longest dimension and W is the shortest dimension of the tumor. The tumor growth curves for 20 days were determined and compared with the control group.

G. Statistical analysis

Each experiment data point was averaged from six samples unless otherwise indicated. Student's t test was used to determine the statistical significance and $p < 0.05$ was considered to indicate a statistically significant difference between two experimental configurations. All statistical analyses were computed using the Office Excel program (Microsoft Inc., Seattle, WA).

III. Results

A. Feasibility of HIFU-induced gene activation *in vivo*

Enhanced luciferase expression in the tumor was clearly detected in a wide range of temperatures at day 1 following 1.1-MHz/10-s HIFU treatment [Fig. 3(a)]. It is important to note that no transgene expression was observed in other vital organs, such as lung and liver. Compared to the control group, luciferase expression intensity within the tumor volume was found to increase by 15-, 95-, 120-, and 55-fold with a peak HIFU temperature of 55, 65, 75,

and 85 °C, respectively [Fig. 3(b)]. Maximum luciferase expression of 120-fold was detected in the 75 °C HIFU treatment group although it is not statistically different from the 65 °C group. Interestingly, higher (85 °C) or lower (55 °C) temperature levels induced less gene expression activity in the solid tumor. These observations of temperature-dependent gene activation during *in vivo* study are consistent with our *in vitro* experiments using HeLa and R3230Ac cell lines (Liu *et al.*, 2005), as well as 4T1 cells (data not shown). Altogether, these results support the notion that an optimal window of peak temperature (65 °C–75 °C) exists for eliciting maximum gene expression following a 10-s HIFU exposure.

B. Effect of treatment duration on HIFU-induced gene activation

As illustrated in Fig. 4(a), gene expression increased initially with the exposure duration and saturated after 10–20-s exposure at a peak temperature of 65 °C or 75 °C. Further increase of exposure duration to 30 s was found to inhibit the overall gene expression intensity at both temperature levels. Compared to the control group, gene expression following 1.1-MHz HIFU at the peak temperature of 75 °C was found to increase by 40-, 120-, 115-, and 60-fold for exposure durations of 5, 10, 20, and 30 s, respectively. Overall, 10–20-s HIFU exposure with a peak temperature between 65 °C and 75 °C led to a maximum gene activation in the target tumor tissue. Furthermore, the time course of gene activation elicited by hyperthermia (42 °C-30 min) and 1.1-MHz HIFU (75 °C-10 s) treatment were compared. As shown in Fig. 4(b), peak luciferase expression level was observed at day 1 following both treatment regimens and decayed gradually to the background level within 7 days. Specifically, luciferase intensity rose to 160- and 58-fold on day 1 and day 3 following water-bath hyperthermia (42 °C-30 min) compared to 120- and 33-fold increase induced by the 1.1-MHz HIFU treatment (75 °C-10 s). The discrepancy might be caused in part by the heterogeneous temperature distribution inside the solid tumor produced by the 1.1-MHz HIFU exposure protocol, in contrast to the relatively uniform temperature field in water-bath hyperthermia.

C. Tumor growth regression

Following the HIFU treatment (75 °C-10 s, 1.1 MHz), the tumor volume shrunk to approximately 33% in 12 days while the tumor in the control group increased to 300% of the original size, at which point the animal was euthanized (Fig. 5). The volume of HIFU-treated tumor, however, rebound gradually to 65% of the original size at the end of a 20-day observation period.

D. Effect of scanning strategy on HIFU-induced gene activation

Under the same thermal exposure (75 °C-10 s), the efficiency of HIFU-induced gene activation could be further improved by proper selection of transducer frequency (1.1 vs. 3.3-MHz) and scanning density [5 vs. 36 spots as shown in Fig. 6(a)]. Figure 6(b) shows that HIFU-induced gene activation was elevated from 120-fold at 1.1 MHz with a sparse scan to 170-fold at 3.3 MHz with a dense scan ($p < 0.05$). The latter is statistically comparable to the 168-fold increase induced by hyperthermia ($p > 0.3$). It should be noted, however, because both frequency and scanning density were changed, the current results could not pinpoint which variable is more important in HIFU-induced gene activation.

E. Physical mechanism: Thermal stress versus mechanical stress

Figure 7 shows the equivalent thermal dose (represented by EM_{43}) in tumor tissues, calculated based on temperature measurements in both the focal and beam planes during a single 3.3-MHz HIFU exposure (75 °C-10 s). The calculated iso-exposure contours for 55 °C-10 s, 65 °C-10 s, and 75 °C-10 s were outlined explicitly, together with the empirical EM_{43} (=240 min) contour for thermal necrosis (Damianou *et al.*, 1995). In the focal plane, the 55, 65, and 75 °C iso-exposure contours are concentric circles with diameters of 1.6, 0.8, and 0.3 mm,

respectively [Fig. 7(a)]. In comparison, the corresponding contours in the beam plane are close to “cigar” shapes. For instance, the axial and lateral beam diameters for the 65 °C-10 s contour were calculated to be 2.2 and 1.2 mm [Fig. 7(b)]. The total areas within the 55 °C-10 s contour, which is the primary gene activation zone based on preliminary *in vitro* studies, were calculated to be 2.5 and 7.0 mm² in the focal and beam planes, respectively.

Compared to thermal HIFU, mechanical HIFU exposure often generates a bright hyperechoic spot at the beam focus, which is presumably associated with the induction of cavitation bubbles in the target tumor tissue [Fig. 7(c)]. A significant increase in the broadband noise of the acoustic emission signal spectrum was also detected during mechanical HIFU exposure [Fig. 7(d)]. In particular, generation of subharmonic and second harmonic components could be clearly detected, which might be produced by the nonlinear scattering of cavitation bubbles inside the tumor tissue (Miller and Bao, 1998). Figure 7(e) shows the average rms amplitude of the broadband noise in the frequency range of 4.5–5.5 MHz during a 10-s treatment period. The inertial cavitation dosage (ICD) of 498.2±42.5 dBm s produced by the mechanical HIFU exposure is significantly higher than the corresponding value of 245.67±27.3 dBm s generated by the thermal HIFU exposure ($p<0.05$). Altogether these results suggest that significantly higher level of cavitation activities and associated mechanical stresses were generated in the targeted tumor tissue by the mechanical HIFU exposure than its counterpart of thermal HIFU exposure.

As shown in Fig. 8, thermal HIFU was found to stimulate a gene activation of 170-fold in the target tumor tissue compared to the control group, while the mechanical HIFU only produced a fourfold increase. Together with the results shown in Fig. 7, it is concluded that thermal stress is the primary physical mechanism for HIFU-induced gene activation *in vivo*. The mechanical stresses produced during HIFU exposure (primarily associated with cavitation), although strong enough to cause cell lysis and tissue damage, may not be sufficient to elicit a measurable heat shock response (i.e., activation of hsp70B gene promoter). It is worth noting, however, that mechanical and thermal stresses may interact synergistically to enhance the thermal deposition during HIFU treatment (Bailey *et al.*, 2003).

IV. Discussion

In clinical HIFU therapy, the peak temperature at the beam focus could exceed 70 °C within several seconds, leading to coagulative necrosis and lesion formation surrounded by sub-lethally injured tumor tissues (ter Haar, 1995). Up-regulated heat shock response has been detected in the border zone of necrosis lesion during HIFU treatment of BPH, raising the possibility of hsp-mediated immune response following HIFU (Kramer *et al.*, 2004; Kennedy, 2005). The present study demonstrates that HIFU can indeed elicit strong trans-gene activation under the control of hsp70B promoter *in vivo*, presumably in sublethally injured tumor tissues. It is observed that HIFU-induced gene activation depends considerably on exposure conditions, such as peak temperature, duration, frequency, and scan strategy. Furthermore, thermal stress, instead of cavitation-associated mechanical stress, was found to be the primary physical mechanism for HIFU activation of hsp70B promoter *in vivo*, which is consistent with the results of our previous *in vitro* study (Liu *et al.*, 2005).

Under the 1.1-MHz HIFU exposure protocol, maximum gene expression following a 10-s exposure was produced at peak temperature in the range of 65 °C to 75 °C both *in vitro* and *in vivo*. Lower peak temperature (<55 °C) cannot stimulate sufficient heat shock response while higher peak temperature (>85 °C) may activate extensively cellular apoptosis pathways, leading to programmatic cell death and thus hindering the overall gene expression (Barry *et al.*, 1990). This interpretation is further supported by the fact that higher gene expression was achieved at 10–20-s exposure rather than 5 or 30 s with a fixed peak temperature of 65 °C or

75 °C [Fig. 4(a)]. Heat-sensitive gene expression triggered by HIFU and hyperthermia revealed a similar time course, which was found to peak at day 1 post-treatment and decay gradually within a week [see Fig. 4(b)]. Biologically, the same molecular pathway of heat shock response may be activated by these two treatment regimens through a thermal dose threshold mechanism, despite the dramatically different rate at which the thermal energy is delivered to the target tissue. Our results also suggest that optimization of HIFU transducer frequency and scan strategy is important for achieving maximum gene activation *in vivo* (see Fig. 6).

Because of the nonuniform thermal field produced by a HIFU transducer, it is likely that the gene expression pattern within the targeted tumor volume will be heterogeneous. However, such spatial variation in gene expression cannot be resolved by the Xenogen *in vivo* bioluminescence imaging system used in this study. Future investigations are warranted to determine the spatial distribution of HIFU-induced gene activation in tumor tissues, which may provide critical insight for optimizing HIFU treatment strategies to maximize gene activation. Based on pilot *in vitro* cell studies, we have observed that luciferase positive cells surviving the HIFU treatment were primarily produced between the 55 °C and 75 °C thermal dose contours as outlined in Fig. 7(a) (data not shown). In this range, escalating HIFU thermal exposure levels will increase progressively the resultant cell death while elevating concomitantly the heat shock response (Liu *et al.*, 2005). A combination of cell survival rate and the intensity of heat shock response per cell determines the total accumulated gene expression in the tumor tissue, as shown in Figs. 3 and 8(a).

It is interesting to note that the thermal necrosis criteria (i.e., $EM_{43}=240$ min) widely quoted in HIFU literature also falls within the thermal exposure range for gene activation [see Fig. 7 (a)]. This empirical thermal necrosis criteria were derived based on the histological analysis of muscle tissues in 4–21 days following 44 °C-60 min hyperthermia treatment (Damianou *et al.*, 1995; Jansen and Haveman, 1990). Although majority of the cancer cells or tissue exposed to HIFU at a thermal dose of $EM_{43}=240$ min will die acutely or gradually as a result of apoptosis or depletion of blood and nutrition supplies, a few percent of HIFU treated cells and/or tissue could survive (Liu *et al.*, 2005; Wu *et al.*, 2004). This observation probably reflects primarily the heterogeneous nature of the biological response of cells and tissues to stress. Therefore, it would be more accurate to interpret the thermal necrosis criteria ($EM_{43}=240$ min) as a threshold at which coagulative necrosis may result in biological cells and tissues, but not as a criterion to ensure total necrosis. It is also desirable in the future to carry out systematic investigations both *in vitro* and *in vivo* to better determine HIFU-induced stress responses at cellular and molecular levels and their correlation with sublethal heat shock response and tissue necrosis.

HIFU-induced gene activation may also be useful in regulating site-specific gene expression. Systemic dissemination of viral vectors following intratumoral delivery has been found to be a serious problem that may limit its application in cancer gene therapy (Wang *et al.*, 2005). While others have focused on development of novel carriers that can significantly reduce the systemic leakage of viral vectors from the injection site in the solid tumor (Wang *et al.*, 2005), the results of this study suggest that by employing a heat-sensitive hsp-70B promoter HIFU can be used as a noninvasive physical method to control trans-gene expression both spatially and temporally, thus eliminating the adverse effects due to systemic dissemination of the viral vectors.

As shown in Fig. 5, following HIFU treatment the volume of tumor was initially reduced by 70% in 2 weeks and, subsequently, the tumor started to regrow. This recurrence, also frequently observed following clinical HIFU therapy (Wu *et al.*, 2004), may arise from sublethally injured tumor cells that survive the HIFU treatment. Such a limitation of current HIFU cancer therapy may be potentially overcome by a synergistic combination of HIFU thermal ablation with heat-inducible cytotoxic or immunostimulatory gene therapy. Conceptually, HIFU can be used to

activate therapeutic genes in sublethally injured tumor cells while thermally debulking the primary tumor mass. Using this strategy, heat-induced cytotoxic gene products or immunostimulatory factors may be produced simultaneously during HIFU therapy to improve the killing of residual or distal metastasis tumor cells via “bystander” effects or an enhanced antitumor immune response (Li and Dewhirst, 2002). Alternatively, a pretreatment of HIFU-induced gene activation at sublethal thermal dose in the target tumor may be applied to boost the host antitumor immune response before a full dose of regular HIFU for thermally ablating the tumor mass is executed. With the advance of transducer technology and real-time thermal dosimetry monitoring by MRI (Hynynen *et al.*, 2001), such new therapeutic strategies are feasible and should be explored in the future to improve the effectiveness of HIFU cancer therapy.

In conclusion, the present work opens up a new paradigm for HIFU-regulated trans-gene activation *in vivo*. Further animal studies are underway to explore the potential for a synergistic combination of HIFU-induced thermal ablation with heat-induced gene therapy to improve the overall quality and effectiveness of cancer therapy.

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FIG 1.

(a) A schematic diagram of the B-mode ultrasound imaging-guided HIFU system. (b) Pressure distribution across the focal plane of the 1.1/3.3-MHz HIFU transducer in free field. p^+ : peak positive pressure and p^- : peak negative pressure.

FIG 2.

Temperature profiles produced in mouse tumor tissues under (a) different peak temperatures (55 °C–85 °C) and (b) different exposure durations (5–30 s). (c) Lateral distribution of peak temperature in mouse tumor tissue produced by the 1.1-MHz/3.3-MHz HIFU transducer during 75 °C-10-s exposures.

FIG 3.

(a) Representative bioluminescence images of luciferase distribution in the mouse model 24 h after HIFU exposure. (b) Quantitative luciferase intensity after 1.1-MHz/10-s HIFU exposure at peak temperatures of 55 °C–85 °C. HT: hyperthermia (42 °C, 30 min).

FIG 4.

(a) Effect of HIFU treatment duration on transgene activation (5–30 s) at a peak temperature of 65 °C or 75 °C produced by the 1.1-MHz transducer. (b) Comparison of the time course of luciferase gene expression induced by HIFU and hyperthermia (HT) treatments.

FIG 5. The time course of tumor growth regression following 1.1-MHz/10 s HIFU treatment (75 °C-10 s).

FIG 6.

(a) Schematic illustrations of sparse (1.1 MHz) versus dense (3.3 MHz) HIFU scan strategies and (b) corresponding gene activation efficiency following 75 °C-10 s HIFU exposure.

FIG 7.

The distribution of equivalent thermal dose (represented by EM_{43} in logarithmic scale) within (a) focal plane and (b) beam plane of the 3.3-MHz transducer during 75 °C-10 s HIFU exposure. (c) B-mode images of cavitation activity in mouse tumor tissues induced by thermal and mechanical HIFU. (d) Representative frequency spectrum of PCD signals, and (e) time evolution of the averaged broadband noise (4.5–5.5 MHz) in the PCD spectrum during a 10-s HIFU exposure.

FIG 8.

(a) Representative bioluminescence images in the mouse model and (b) corresponding luciferase intensity induced by 3.3-MHz thermal and mechanical HIFU treatments. Output conditions are $I_{\text{SAPA}}=430 \text{ W/cm}^2$ at CW mode for 10 s for thermal HIFU (75 °C-10 s) and $I_{\text{SAPA}}=6849 \text{ W/cm}^2$ with a duty cycle of 6.3% (burst mode) for mechanical HIFU.