An in-vivo investigation of the therapeutic effect of pulsed focused ultrasound on tumor growth

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Purpose: High-intensity focused ultrasound (HIFU) has been investigated for ablative therapy and drug enhancement for gene therapy and chemotherapy. The aim of this work is to explore the feasibility of pulsed focused ultrasound (pFUS) for cancer therapy using an in vivo animal model.

Methods: A clinical HIFU system (InSightec ExAblate 2000) integrated with a 1.5 T GE MR scanner was used in this study. Suitable ultrasound parameters were investigated to perform nonthermal sonications, keeping the temperature elevation below 4 °C as measured in real time by MR thermometry. LNCaP cells (10^6) were injected into the prostates of male mice (n = 20). When tumors reached a diameter of about 5 mm in 3D as measured on magnetic resonance imaging (MRI), the tumor-bearing mice (n = 8) were treated with pFUS (1 MHz frequency; 25 W acoustic power; 0.1 duty cycle; 60 s duration). A total of 4–6 sonications were used to cover the entire tumor volume under MR image guidance. The animals were allowed to survive for 4 weeks after the treatment. The tumor growth was monitored on high-resolution (0.2 mm) MRI weekly post treatment and was compared with that of the control group (n = 12).

Results: Significant tumor growth delay was observed in the tumor-bearing mice treated with pFUS. The mean tumor volume for the pFUS treated mice remained the same 1 week after the treatment while the mean tumor volume of the control mice grew 42% over the same time. Two weeks after the pFUS treatment, the control group had a mean tumor volume 40% greater than that of the treated group. There was a greater variation in tumor volume at 4 weeks post treatment for both treated and control mice and a slightly faster tumor growth for the pFUS treated mice.

Conclusions: The authors’ results demonstrated that pFUS may have a great potential for cancer therapy. Further experiments are warranted to understand the predominantly nonthermal cell killing mechanisms of pFUS and to derive optimal ultrasound parameters and fractionation schemes to maximize the therapeutic effect of pFUS. © 2014 American Association of Physicists in Medicine. [http://dx.doi.org/10.1118/1.4901352]

Key words: pulsed focused ultrasound (pFUS), nonthermal effect, cancer therapy, prostate cancer

1. INTRODUCTION

High-intensity focused ultrasound (HIFU) has a great potential for cancer therapy because of its qualities of noninvasiveness, high spatial precision, and certain penetration in tissues. HIFU has been used clinically for thermal tissue ablation in the treatment of uterine fibroids and various solid tumors. Recent in vivo animal studies have also suggested that pulsed focused ultrasound (pFUS) exposures may temporarily alter the tissue properties such as the vascular or cell membrane permeability to enhance drug delivery for chemotherapy and gene therapy. Enhancement of drug delivery to the tumor target by pFUS exposures and its effect on tumor growth inhibition in vivo have been reported by several investigators.

Over the past decades, there have been encouraging findings from in vitro studies on the predominantly nonthermal (＜42 °C) biological and cell killing effects of HIFU, which resemble those of high linear energy transfer (LET) radiation cell damage that is less affected by the local biochemical environment and shows less radiation resistance. Despite the positive findings from many in vitro studies, there have been no in vivo animal studies on pulsed HIFU to demonstrate its nonthermal therapeutic potential. This was most likely due to the lack of image-guided HIFU treatment systems for small animal models. Advanced imaging systems would be required to determine the gross tumor volume, to plan the HIFU treatment, to place the ultrasound beam precisely, to monitor the treatment process, and to assess the treatment outcome in order to maximize the therapeutic effect of pulsed HIFU on tumor cells and to minimize unnecessary damage to surrounding normal tissues.

In our laboratory, we have developed techniques for prostate tumor implantation orthotopically. In a previous study, we developed MR guided high-intensity focused ultrasound (MRgHIFU) treatment techniques for a small animal model (nude mouse) using a clinical patient treatment device integrated with a 1.5 T MR scanner for MR guidance during treatment. We performed in vivo experiments to investigate the use of pFUS for the enhancement of chemotherapy drug delivery in prostate tumors grown in nude mice using [3H]-docetaxel. Our experimental data showed that the [3H]-docetaxel concentration in tumors treated with pHIFU was significantly increased (by two folds) compared with those without the pFUS treatment.
The purpose of this study was to investigate the feasibility of pFUS for cancer therapy using an *in vivo* mouse model with implanted prostate tumors. We will describe the MRgHIFU system and the pFUS treatment procedures for our study. We will quantify the therapeutic effect of pFUS by measuring the tumor growth delay as a result of the pFUS treatment in comparison with the control group. The therapeutic effect of pFUS will also be compared with that of radiation therapy using the same animal model.

2. MATERIALS AND METHODS

2.A. Experimental setup

A clinical HIFU system (ExAblate 2000, InSightec-Tx-Sonics, Haifa, Israel) integrated with a 1.5 T MR scanner (Signa Excite HD, GE Healthcare, Milwaukee, WI) was used for the pFUS treatment (Fig. 1).

This treatment system was approved by the Food and Drug Administration (FDA) for treating uterine fibroids clinically and, recently, for palliative treatment of painful bone metastases. It has also been used for clinical investigations of prostate and breast cancer ablation under local IRB approval. Quality control (QC) for the clinical HIFU treatment unit was performed according to the procedures provided by the vendor to check the transducer output, the focal spot location, and the mechanical motion system before animal treatments as described previously.

Figure 1 shows the animal setup for pFUS treatments. A gel pad (InSightec-Tx-Sonics, Haifa, Israel) was warmed in a water bath to approximately 37 °C and a shallow hole measuring about 2 × 3 cm with 8 mm in depth was created in the center of the gel (facing up). The hole was used to accommodate degassed water in contact with the animal for ultrasonic coupling. The gel pad was placed on the HIFU table above the transducer with degassed water in the interface between the table and the gel pad. Mice were anesthetized by the intraperitoneal injection of a mixture of Ketamine (60 mg/kg) and Ace-promazine (2.5 mg/kg). The animal was carefully placed in the hole of the gel pad with degassed water. A 3-in. surface coil (GE Healthcare, Milwaukee, WI) was placed around the animal to receive the MR signals. A small (4 × 2 × 2 cm) tissue-mimicking phantom (InSightec-Tx-Sonics, Haifa, Israel) was placed beside the mouse within the MR coil for verification of the focal spot location prior to animal sonication. A surgical glove filled with warm (37 °C) water was placed on top of the mouse to protect the animal from hypothermia and to increase the acoustic depth (to reduce the effect of ultrasound backscatter). The glove temperature decreased by 5–7 °C during the imaging and treatment duration. Ideally, a heater could be used to keep the water temperature constant but it was unavailable for this work. The mouse body temperature ranged typically from 35 to 32 °C during the sonication period. The room was air-conditioned keeping the temperature at 22 °C.

2.B. Tumor model

In order to investigate tumor growth delay with nonthermal pFUS treatment, a good *in vivo* tumor model is needed, which is well confined locally and less likely to metastasize to other organs. Human prostate cancer (LNCaP) cells were obtained from the American Type Culture Collection and cultured in Dulbecco’s modified Eagle’s medium (DMEM)-F12 medium, containing 10% fetal bovine serum (FBS), 1% l-glutamine, and 1% penicillin-streptomycin as described previously. Male athymic Balb/c nude mice (6 weeks old) were purchased from Harlan (Indianapolis, IN). All of animal studies were carried out in compliance with the approval of the institutional animal care and use committee (IACUC). Aseptic techniques were used for injection and implantation of LNCaP cells in the prostates of nude mice as described previously. Nude mice were anesthetized using methoxyflurane. A lower midline incision was made approximately 1.5 cm above the presumed location of the bladder. The seminal vesicles were gently brought out through the incision and LNCaP cells (1 × 10⁶) in 25 µl of total volume were injected into the dorsal prostate lobes using a 30-gauge 1-in. needle. The incision was sealed by suturing the muscle layer (suture size-4.0 silk) and 2–3 wound clips for the skin layer.

2.C. MR imaging

Image guidance plays an important role in the target delineation for planning, target localization during treatment, ultrasound beam placement, and treatment assessment during and after treatment for accurate pHIFU therapy using small animals. In this study, the tumor volume was directly monitored...
by magnetic resonance imaging (MRI) after the orthotopic implantation of LNCaP cells using a 1.5 T GE MR scanner following an imaging protocol established in a previous study. The protocol provides MR image of both high resolution and SNR to identify the prostate tumor in a nude mouse within approximately 1 h anesthesia time for the entire imaging and treatment procedure for both control and treated mice. The scans were first performed for the 3D localization of the tumor using a fast SE echo planar imaging (EPI) sequence with a 8 s scan time and then for the coronal and axial image sequences with a ~4 min scan time following the imaging protocol. The MR parameters were $T2$-weighted MR images acquired using fast-recovery fast-spin-echo (FRFSE) sequence, repetition time (TR)/echo time (TE) = 2200/85 ms, number of excitations (NEX) = 3, matrix = 288×288 for image acquisition and reconstruction, field of view (FOV) = 7×7 cm² (resolution = 0.243×0.243 mm²), and slice thickness = 2 mm for coronal and = 1 mm for axial scans, respectively. The acquired MR images were loaded immediately into the pFUS treatment planning system for the treatment planning process. Figure 2 shows an example of the MR image used for tumor delineation and treatment planning in real time for this study.

2.D. Pulsed FUS treatment

A total of 50 mice were implanted with tumor cells. MR scans were performed weekly beginning at 3 weeks after tumor implantation. To minimize the statistical effect of the tumor growth rate variation, only 20 mice with similar tumor sizes (average diameter of about 5 mm in 3D) at weeks 3 and 4 after the tumor implantation were selected and randomized into the control group ($n = 12$) and pFUS treatment group ($n = 8$) for this study. Because of the limited anesthesia time (1 h) precise tumor contouring and tumor volume calculation was performed for both treated and control mice immediately after the imaging/treatment procedure.

Pulsed FUS treatment was performed using a previously described method, which is briefly described below. For mice randomized for treatment, both coronal and axial MR images were loaded on the focused ultrasound treatment work station after image scanning. The tumor was outlined and a treatment plan was generated. Prior to focused ultrasound treatment, the effective focal spot location was verified on a small tissue-mimicking phantom beside the animal using a machine built-in MR thermometry software function, which was based on the MR proton resonance frequency shift sequence with approximately ±3 °C uncertainties. MR thermometry scans were acquired using a fast spoiled gradient echo (FSGR) sequence with parameters: TR/TE = 25.9/12.8 ms, flip angle = 30°, NEX = 1, number of echo = 1, matrix = 256×128 for image requisition and reconstruction, FOV = 22×22 cm² (resolution = 0.86 × 1.72 mm²), and slice thickness = 3 mm. Animals were treated using 1 MHz ultrasound, 25 W acoustic power with a pulse of 0.1 s power on/0.9 s power off (10% duty cycle) for a 60 s duration per sonication depending on the experimental design (see below). The focal length was 98–102 mm and the aperture of the pFUS transducer was 12 cm. The ultrasound focal zone (full width at half maxima, FWHM) is an elongated ellipsoid with a longitudinal length of 6.8 mm and a radial diameter of 1.25 mm. The estimated peak-negative pressure in the focal zone was 7.8 MPa and the average acoustic intensity was 20.4 W/mm². The treatment parameters were derived from tissue-mimicking

![Fig. 2. Window capture of real-time treatment planning for the pFUS treatment: (a) a prostate tumor, (b) the tumor target delineated on the MR image, and (c) seven sonication spots covering the target volume on both coronal and axial images.](image-url)
phantom measurements as described by Chen et al.\textsuperscript{14} Planned sonication spots had a cylindrical shape with a diameter of 3.8 mm and a length of 10.3 mm. A total of 4–6 sonication spots were used to cover the entire treatment volume under MR image guidance. Figure 2 is the window capture from the HIFU workstation showing real-time treatment planning for the MR guided pFUS treatment. The tumor target was outlined and covered by multiple sonication spots (see arrows) on both the coronal and axial images. The real-time temperature elevation was monitored by MR thermometry (with a 3 s delay).

2.G. Morphologic and immunohistochemistry evaluation

Additional experiments were conducted to compare the tissue morphology and the expression of apoptotic (caspase-3) and DNA damage (\(\gamma\)H2AX and Chk2) biomarkers between pFUS-treated prostate tumors and control. Tumor-bearing mice were given the same pFUS sonifications as described above and euthanized at 24 h \((n = 2)\) or 48 h \((n = 2)\) after treatment. Additional mice were used for control \((n = 2)\) and 2 Gy radiation treatment \((n = 2)\). Following animal sacrifice, prostate tumors were harvested, fixed in 10% neutral buffered formalin, and embedded in paraffin. Tissue sections (5 µm) were cut from paraffin blocks, deparaffinized, and hydrated through xylenes and graded alcohol series. Hematoxylin and eosin (H&E) staining was performed to evaluate the histopathology of prostate tumors. For immunohistochemistry, tissue sections were subjected to antigen retrieval (0.01 M sodium citrate buffer, pH 6.0). After blocking endogenous peroxidase activity with 3% hydrogen peroxide and nonspecific binding with 10% normal goat serum, sections were incubated overnight with the following primary antibodies (1:200 dilution) at 4 °C in a humidified chamber: rabbit anticleaved caspase-3 (Cell Signaling Technology, Inc., Danvers, MA), mouse anti-\(\gamma\)H2AX (Abcam, Inc., Cambridge, MA), and rabbit anti-phospho-Chk2 (Cell Signaling Technology, Inc., Danvers, MA). The appropriate secondary biotinylated antibodies (goat anti-rabbit or anti-mouse IgG) were incubated with the sections for 30 min at room temperature. The antibody complex was detected by the LSAB system (Dako, Caripenteria, CA) and was visualized with the chromogen 3,3’-diaminobenzidine. Sections were lightly counterstained with hematoxylin. Slides were examined by light microscopy (Nikon, Melville, NY) to observe the difference in these markers between treated and untreated groups.

2.H. Data analysis and statistics

The absolute tumor volume for each animal was calculated based on the axial MR images and the relative volume was expressed as a ratio of the tumor volume measured weekly after treatment to the tumor volume on the day of treatment. Statistical analysis was performed for the measured tumor volumes. The mean tumor volume and standard deviation of the mean (SEM) were calculated and the results were expressed as mean ± SEM. To determine if there was a significant difference between different groups, Student’s \(t\)-test was used and a \(p\)-value, \(p \leq 0.05\), was considered to be statistically significant.

3. RESULTS

3.A. Temperature variation

Pulsed HIFU exposures with an acoustic power of 25 W and a 10% duty cycle were used in this study, which generated a temperature elevation of \(<5^\circ\text{C}\) in our tissue-mimicking phantom measurements\textsuperscript{16} and in targeted tumor tissues in mice as measured by MR thermometry (Fig. 3).

Fig. 3. Real-time temperature measurement with MR thermometry during animal sonication (top red line: maximum temperature measured at the focal spot in a single voxel of 0.86 × 1.72 × 3.00 mm; bottom green line: average temperature measured over 3 voxels). The ambient temperature was set to 35 °C, which was the typical mouse body temperature in the beginning of the sonication period.
This was to ensure a temperature of \(<42\,^\circ C\) so that the observed therapeutic effect was predominantly nonthermal (the mouse temperature measured by an anal thermometer under anesthesia before and after the imaging/treatment procedure was \(37\,^\circ C\) and \(30\,^\circ C\), respectively). These results were consistent with our previous measurements using an acoustic power of 5 W and a 50% duty cycle for pFUS drug delivery studies.\(^{14,16}\)

### 3.B. Post-treatment tumor growth on MRI

Figure 4 shows prostate tumor growth for a control mouse and a pFUS treated mouse as observed on MRI. The tumor volume for the pFUS treated mouse was 141, 70, 33, 18, and 18 mm\(^3\) on the treatment day and 1, 2, 3, and 4 weeks after the pFUS treatment, respectively, while for the control mouse it was 139, 222, 282, 445, and 595 mm\(^3\), respectively. Tumor boundaries were clearly identified in MRI and the difference in the tumor growth trend was well demonstrated between the control and treated groups. Tumor in the control group shows a continuous, nearly exponential increase in size during the follow-up weeks, while a tumor growth delay (or even regression for one) was clearly observed in the pFUS treated group with similar variations among individual animals.

### 3.C. Tumor growth delay

There was a large variation in tumor growth rate among the 50 mice implanted with the same number of tumor cells. Only 20 mice of similar tumor sizes (average diameter of 5 mm in all 3D) at 3 or 4 weeks after the tumor implantation were selected and randomized into the control and pFUS treatment groups. The mean tumor volume for the 20 mice was 120 mm\(^3\) (control tumor volume: mean 121 mm\(^3\), range 97–139 mm\(^3\); pFUS treated tumor volume: mean 116 mm\(^3\), range 94–141 mm\(^3\)). Figure 5 shows the mean tumor volumes measured on the treatment day and weekly after the randomization for the 12 control mice and 8 FUS-treated mice.

A continuous growth was observed for the control mice. The largest variation in tumor volume among the control mice was found at week 4 (Fig. 6); some tumors developed separate lobes and necrotic zones were formed as tumor volumes became large. In the pFUS treated group, tumor growth delay was observed for all mice. This delay was clearly demonstrated by the tumor volumes one week after treatment; most tumors showed slower growth compared to the control group while one even regressed (mouse number 5 in Fig. 6).

Statistically, tumors in the control group showed an exponential increase and relative tumor volumes changed to 142\% ± 6\%, 205\% ± 12\%, 286\% ± 22\%, and 410\% ± 33\% at 1, 2, 3, and 4 weeks after the randomization, respectively, comparing to the initial tumor volume on the randomization day (Fig. 5). In contrast, relative tumor volumes in the pFUS treated group changed to 101\% ± 9\%, 156\% ± 22\%, 262\% ± 40\%, and 372\% ± 64\% at 1, 2, 3, and 4 weeks after the randomization (treatment), respectively. Tumors treated by pFUS showed an early response. Significant tumor growth delay was observed in the first week with a 29\% smaller mean tumor volume in the pFUS treated group than in the control group (\(p < 0.05\)). The mean tumor volume differences were reduced to 9\% starting at week 3 comparing to the control group (\(p = 0.29\), statistically not significant), indicating an accelerated tumor growth for the pFUS treated tumors compared to the control group.

### 3.D. Histology and immunohistochemistry analysis

All pFUS treated mice tolerated the treatment well with no damage to their skin and no changes in their body weight.
both immediately after the treatment and in follow-ups of up to 6 weeks. There were no signs of tissue damage in any healthy organs in the autopsy of sacrificed animals from one day to six weeks post treatment. On the H&E slides, the treated areas showed normal morphological appearances without typical signs of “direct” ultrasound-mediated thermal damage, which is generally marked by areas of cell disruption or destruction, together with the presence of “implosion cysts.” Other histological changes such as extracellular matrix disintegration or increased intracellular spacing were not clearly evident.

Activation of caspase-3 is commonly used as a biomarker for the assessment of apoptosis and for understanding the mechanisms of the programmed cell death induction. The immunohistochemical staining results of prostate tumors for cleaved caspase-3 at 24 h after treatment are shown in Fig. 7. Compared to the control areas, pFUS treated areas showed qualitatively increased apoptotic cell death, which was similar to that in areas treated by 2 Gy irradiation. Both the γH2AX focus assay and Chk2 staining represent a fast and sensitive approach for the detection of one of the critical types of DNA damage—double-strand breaks (DSB) induced by various cytotoxic agents including ionizing radiation. Figure 8 shows the immunohistochemical staining results with increased levels of γH2AX and Chk2 at 48 h after the pFUS treatment.

4. DISCUSSION

In this study, we have investigated the feasibility of pFUS for cancer treatment using tumor-bearing mice and a clinical HIFU treatment system with MR image guidance. To the best of our knowledge, this was the first in vivo experimental study to quantify the predominantly nonthermal effect of pFUS (alone) on cancer cell killing using an animal tumor model. Our results indicated significant tumor growth delays in pFUS treated mice as a result of a single treatment. As can be seen in Fig. 5, the greatest difference in the mean tumor volume was observed at one week after the pFUS treatment (142% ± 6% for control vs 101% ± 9% for pFUS treated mice, relative to the mean tumor volumes on the treatment day), indicating an early response of cancer cells to the pFUS treatment. This mean volume difference was statistically significant (P < 0.05). It was found that when the tumors were relatively small, they grew more consistently following an exponential growth. As the tumors grew larger, the variations of the tumor growth rate increased for both control and pFUS treated mice partially because some tumors became necrotic and some developed separate lobes, which also caused greater statistical uncertainties in the mean tumor volume values.

Although individual tumors exhibited large variations in the tumor growth rate, detailed analysis showed significant tumor growth delays for all pFUS treated mice. In this study, mice were MR scanned weekly starting from 3 weeks after the tumor implantation. If the tumors reached a certain volume (average diameter of 5 mm in 3D as measured on MRI), pFUS treatment was performed immediately after the MR scan so that the same images could be used for pFUS treatment planning. The mice were randomly assigned into the control and pFUS group once their tumors reached the target size. The accurate tumor volume for each mouse was calculated based on precise contours on saved MR images after the pFUS treatment. The uncertainty of the tumor volume calculated using this technique was estimated to be <3% by direct comparisons of the same tumor volumes on a Bruker Biospec 7 T MicroMRI scanner with sub 0.1 mm resolution. The mean tumor volumes for the 20 mice were 120 ± 7 mm³ on the day of treatment. Figure 6 shows the mean tumor volumes one week before treatment, on the day of treatment, and one week after treatment for the control mice. The average tumor growth rate was 54% for the week before treatment and 57% for the week after treatment, respectively.
For the eight pFUS treated mice, the tumor volumes one week before treatment differed significantly between each other, indicating a large variation of the tumor growth rate among individual mice, which was similar to that of the control mice. If we assume that these tumors would grow at the same rate for the week before and after treatment as we have observed for the control mice, we could project the tumor volume one week after treatment as if they had not received the pFUS treatment. As can be seen in Fig. 6, despite the large variations of tumor growth rate among these mice, the measured tumor volumes one week after the pFUS treatment were consistently smaller than the projected tumor volumes had they not been treated. This exercise was intended to help understand the likely tumor growth rate and the potential ability of the pFUS treatment to prohibit tumor growth for individual pFUS treated mice, not to predict their actual growth characteristics.

The therapeutic effect of pFUS observed in this work was considered to be predominantly nonthermal since the tissue temperature was kept well below 42 °C (the mouse body temperature was about 35 °C in the beginning of the sonication period, and the tissue temperature at the focal zone increased gradually and was close to 40 °C for only a few seconds). Attempts were made to understand the cell killing mechanisms of pFUS by immunohistochemical staining of caspase-3 at 24 h after treatment and γH2AX and Chk2 at 48 h after treatment. Elevated levels of γH2AX and Chk2 expressions from our preliminary results indicated that the induction of DNA damage could be an important mechanism for the nonthermal pFUS therapeutic effect, resulting in mitotic cell death or clonogenic cell death. Similar to the effects of 2 Gy irradiation, pFUS also induced apoptotic cell death in prostate tumors, which may not be as significant as mitotic cell death, but one could not preclude the contribution of spontaneous and induced apoptosis in pFUS treated tumors to the early treatment response (i.e., the largest tumor growth delay occurred at one week after treatment). In contrast, clinical regression of solid tumors after completion of radiotherapy is generally observed over weeks to months; whereas, our previous experimental experiences with this prostate tumor model indicated that the largest tumor growth delay by 2 Gy irradiation occurred at 3 weeks after RT treatment. The early response of pFUS treated tumors

![Figure 7. Immunohistochemical staining of prostate tumors for cleaved caspase-3 to detect apoptotic cells at 24 h after treatment (×20): (a) control, (b) treated with pFUS, and (c) treated by 2 Gy irradiation. The apoptosis induced by pFUS is comparable to that by 2 Gy irradiation.](image7)

![Figure 8. Immunohistochemical staining of γH2AX (top) and Chk2 (bottom) at 48 h after the pFUS treatment (×10) indicating increased levels of DNA damage: (a) control and (b) treated with pFUS.](image8)
suggested other mechanisms that might cause cell damage resulting in necrosis besides mitosis and apoptosis. The effect of pFUS alone or the interaction of pFUS with some contrast agents (CA) may lead to violent oscillation and/or collapse of CA and, in vivo, the hypothetical formation of shear stress forces or thermal changes (heat or microjets), denaturation and coagulation of cell membrane and cytoplasmic proteins and cell necrosis.

In this work, we have used an acoustic power of 25 W and a 10% duty cycle for the pFUS sonication, which kept the temperature elevation below 5 °C in our phantom measurements and in targeted tumor tissues in mice as measured by MR thermometry (Fig. 3). These results were consistent with our previous measurements using an acoustic power of 5 W and a 50% duty cycle for pFUS drug delivery studies,\textsuperscript{14,16} which delivered the same acoustic energy for the same sonication duration, leading to an identical temperature elevation. The 10% duty cycle was the lowest duty cycle available on this clinical HIFU system, and; therefore, the 25 W acoustic power would provide the highest focal acoustic intensity for our experiments, keeping the total energy input unchanged. The maximal tumor growth delay with this acoustic power was observed at about a week after the pFUS treatment. Individually, prostate tumors responded differently to the same sonication conditions. For example, one tumor almost completely regressed after one sonication treatment (Fig. 4). It should be mentioned that the sonication spot size (3.8 mm in diameter) used in treatment planning was larger than the focal spot size (1.25 mm in diameter) of the pFUS system, resulting in heterogeneous acoustic intensity within the treatment volume that might have led to compromised cell killing. To improve this, we may need to use smaller sonication spots in treatment planning to achieve more uniform acoustic intensity distribution inside the treatment target. This will require more knowledge about the threshold acoustic intensity for pFUS-induced tumor cell killing. To further increase the therapeutic effect of pFUS, one may either increase the acoustic intensity, which will require a lower duty cycle in order to keep the total energy input the same, or increase the sonication duration. Alternatively, one can carry out multiple sonication fractions similar to those used in radiation therapy with different fractionation and dose schemes. Based on the treatment parameters of this work, 25–30 pFUS fractions may be required to achieve a 10^-9 control rate for LNCaP tumor cells. Optimal pFUS parameters may be derived through further studies (e.g., using higher and more uniform acoustic intensity) to reduce the fraction numbers required. It may also be combined with other treatment modalities such as radiotherapy, chemotherapy, gene therapy, and immunotherapy. These feasibilities may be explored clinically since the MRgFUS system has been installed in a number of oncology centers similar to our center. It is important to point out that keeping the temperature below 42 °C in this study was only an experimental condition to evaluate the predominantly nonthermal effect of pFUS treatment. In an ideal situation, thermal ablation, hyperthermia, and nonthermal pFUS may be combined to achieve the best tumor control clinically.

Although we have quantified the tumor growth delay with the pFUS treatment using an animal model implanted with prostate cancers, it would be useful to compare its therapeutic efficacy to a well-established treatment modality. In comparison with the control, the mean tumor volume one week after the pFUS treatment (25 W acoustic power, 0.1 duty cycle, 60 s duration) was 29% smaller while the mean tumor volume three weeks after a 2 Gy RT treatment was 21% smaller,\textsuperscript{27} indicating an even higher therapeutic effect from the pFUS treatment. It was also observed that pFUS treated mice exhibited a slightly accelerated tumor growth rate compared to the control mice. This accelerated repopulation seemed to be consistent with those observed in previous investigations in which treatment with any cytotoxic agent could trigger surviving cells (clonogens) in a tumor to divide faster than before.\textsuperscript{29} There was evidence of similar phenomenon in RT treated patient population suggesting the need for adequate treatment fractionation.\textsuperscript{30} Further experiments are warranted to test various pFUS parameters and different tumor models to provide useful preclinical data for clinical trial designs utilizing different fractionation schemes aiming at complete local tumor control. It is also interesting to note that the sonication volume was usually larger than the tumor volume, especially in the longitudinal direction (ultrasound beam axis), and; therefore, the surrounding normal structures could have been exposed to relatively high acoustic intensities (e.g., bowel and bladder were immediately adjacent to the target volume). Although the pFUS treatment induced considerable cancer cell death, animals tolerated the pFUS treatment well and no noticeable normal tissue toxicities were observed in the autopsy of pFUS treated mice either immediately after treatment or sacrificed later up to 6 weeks.

5. CONCLUSIONS

In this study, the feasibility of pFUS for cancer therapy has been investigated using an in vivo mouse model with implanted prostate tumors. The therapeutic effect of pFUS has been quantified by measuring the tumor growth delay as a result of the pFUS treatment in comparison with the control group. The possible mechanisms of the pFUS effect, which were considered to be predominantly nonthermal, have been explored by histology and immunohistochemistry. A summary of our findings is given below:

(1) The therapeutic effect of pFUS may be clinically significant; the tumor growth delay by one sonication (25 W acoustic power, 0.1 duty cycle, 60 s duration) was comparable to that of 2 Gy irradiation for the prostate tumor model.

(2) There seemed to be an earlier treatment response to pFUS than to radiation, indicating different cell killing mechanisms between the two modalities. There was a tendency toward accelerated tumor growth in pFUS treated tumors compared to the control mice.

(3) The therapeutic effect of pFUS may be a combined result from mitotic, apoptotic, and necrotic cell death due to the biophysical and biochemical reactions mediated by the high-intensity ultrasound with different
components of the tumor cell including DNA, cell membrane, mitochondria, etc.

(4) No skin damage was observed immediately and up to 6 weeks after the pFUS treatment and no tissue damage in any normal organs was observed in sacrificed animals. This may allow repeated pFUS treatment to achieve tumor control.

(5) Further experiments were warranted to understand the cell killing mechanisms of pFUS and to derive optimal ultrasound parameters and fractionation schemes to maximize the therapeutic effect of pFUS.

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