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Image-guided doxorubicin delivery with ultrasound and microbubbles in a mouse model of hepatocellular carcinoma using a diagnostic ultrasound system

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***Abstract*—Despite advances in chemotherapeutic drug development, hepatocellular carcinoma (HCC) is still the third leading cause of cancer-related deaths worldwide with a <30% 5-year survival rate. This poor prognosis can be attributed to the fact that HCC most commonly occurs in patients with pre-existing liver conditions, rendering many systemic options too aggressive. Patient survival rates could be improved by a more targeted approach. Ultrasound and microbubbles can provide a means for overcoming traditional barriers defining drug uptake. The goal of this work was to evaluate preclinical efficacy of image-guided drug delivery of doxorubicin with ultrasound and microbubbles. To this end, therapy settings were created on a Philips EpiQ and S5-1 phased array probe to provide focused sound for treatment. Sonovue was chosen as a clinically approved microbubble. A genetically engineered mouse model was bred and used as a physiologically relevant preclinical analog to human HCC. It was observed that ultrasound and microbubble therapy resulted in enhanced doxorubicin distribution as seen in fluorescent microscopy. Further, immediate vascular shutdown was observed in treated animals. The combination of these effects may be exploited to treat a challenging malignancy.**

Keywords—microbubble therapy, hepatocellular carcinoma, vascular disruption, doxorubicin

I. Introduction

Hepatocellular Carcinoma (HCC), the predominant form of primary liver cancer, is the third leading cause of cancer-related deaths worldwide [1]. This number is even higher in developing countries where liver cancer precursors such as Hepatitis B and cirrhosis are more prevalent [1]. The most successful treatment for HCC is through surgical techniques, including tumor removal or complete liver transplant. When removed in an early stage, both resection and liver transplants can yield greater than 50% 5-year survival rates. However, less than 30% of patients have tumor pathologies that can be removed with surgery [2].

When surgical resection is not an option, HCC is most commonly treated with systemic chemotherapy, radiation therapy or trans-arterial chemoembolization (TACE), a technique that introduces chemotherapeutics directly into the hepatic artery**,** limiting off-target side effects to healthy tissue [3]. These treatment options remain minimally effective, extending median survival rates by only a couple months [2]. This poor prognosis can be partly attributed to the fact that HCC most commonly occurs in patients with pre-existing liver conditions, rendering many systemic options too aggressive. HCC is also known as a relatively chemo- and radio-resistant cancer, so emerging research has used a more combinatorial approach to make the tumor more vulnerable to harsher chemotherapeutics [4].

Recent work in the field has focused on developing techniques for ultrasound-mediated drug delivery, in which sound pulses in conjunction with ultrasound contrast agents, gas-filled microbubbles, can be used as a method to enhance local delivery of drugs [5]-[8]. However, the lack of understanding of the mechanism of ultrasound and microbubble-mediated therapies have hindered the ability to move this technique to clinical practice. Moreover, most of the prior work in the field has been done using simplistic single-element ultrasound sources, which would not be available in hospitals [9], [10]. To truly make a clinical impact in this field, a strong knowledge of how to translate single element focused source conditions (or a close variety thereof) to a diagnostic system using FDA approved contrast agents is needed. A full validation of this system in a relevant preclinical model is similarly critical.

The objective of the present work is to evaluate preclinical efficacy of ultrasound-mediated drug delivery using a clinical ultrasound scanner in a mouse model of hepatocellular carcinoma. Our hypothesis is that using a diagnostic probe as a focused ultrasound source and clinical microbubbles will yield greater doxorubicin delivery in treated versus control animals. If successful, this would be a simple, inexpensive, and easily implementable technique for treating a notoriously difficult cancer pathology.

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II. Materials and Methods

A. Breeding of Pten-null mouse model

All animal work was conducted in accordance with national guidelines and was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Washington. *Pten*$^{flox/flox}$ and *Alb-Cre* mice (Jackson Laboratories, Bar Harbor, ME) were crossed, whose progeny have hepatocyte-specific *Pten* deletion. At 40 weeks of age, *Pten*-null mice develop tumors that are physiologically similar to human HCC [11], [12]. Moreover, the mice have hepatic steatosis, resulting in livers that are abnormally large even before they begin to develop tumors. Tumor progression was monitored through weekly ultrasound scans with an L15-7io imaging probe on a Philips iU22 (Philips Medical Systems, Bothell, WA) starting at 36 weeks of age. Mice (20 male and 18 female) were treated once tumors reached 1 cm in diameter.

B. Therapuetic acoustic parameters: design and measurement

Focused beams suitable for ultrasound and microbubble-mediated drug delivery were created using a Philips EpiQ and S5-1 transducer in PW Doppler mode, where a longer focal length was preferentially chosen to provide a large uniform nearfield insonation area. The scanner was modified to produce ultrasound pulses 200 cycles long at acoustic pressures of 2-3 MPa and with a PRF of 50 Hz. The spatial extent of the sound field was measured in a water tank with a 0.4 mm membrane hydrophone (Precision Acoustics Ltd., Dorchester, UK), acquired using a DPO7054C Oscilloscope (Tektronix, Inc.) and analyzed in MATLAB (The MathWorks, Inc., Natick, MA, USA) (Fig. 1).

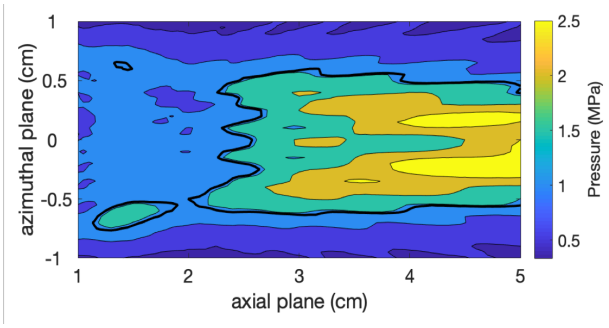

Fig. 1: Measured PW Doppler field of the Philips S5-1 in water. The black contour shows the 6 dB down from the maximum region.

C. Therapy protocol

Mice were anesthetized under 1-3% isoflurane and placed supine. After removing fur from the abdomen of the animal, pre-therapy b-mode images were taken to orient the imaging plane in the center of the tumor. A pre-therapy CEUS scan was then performed with a Philips iU22 and L12-5 linear array probe. 75 µL Sonovue (Bracco, Suisse SA, Geneva, Switzerland) was injected retro-orbitally and 60 s loops were recorded. The S5-1 probe of the EpiQ with programmed long pulses was then used for application of therapy. Therapy consisted of 4 injections of MB + DOX or DOX alone (30 mg/kg). In the treatment cohort, each treatment started 30 s after injection and alternated between "on" for 5 s and "off" for 5 s, for a total "on" time of 30 s. The start time of 30 s was chosen based on preliminary contrast experiments, where time intensity curve analysis was used to determine the optimal time for treatment (Fig. 2). After treatment, mice were euthanized via transcardial perfusion and samples of tumor and healthy liver were frozen in OCT compound for sectioning.

III. Results

A. Fluorescent imaging of doxorubicin distribution

5 µm slices of frozen tissue were evaluated using fluorescent microscopy for doxorubicin distribution. As seen in Fig. 3, doxorubicin intercalates into the nucleus of cells, hence the punctate areas of fluorescence shown. It can be seen in both treated and untreated animals that doxorubicin is present in the outer edges of the tumors. However, the penetration into the tumor is greater in the treated animal than the untreated animal.

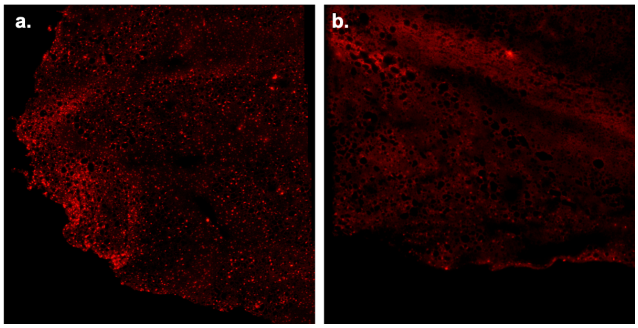

Fig. 3: Tissue slices from treated (a) and control (b) animals. Fluorescence intensity indicates doxorubicin presence. While both treated and untreated samples have high doxorubicin accumulation on the outer edge of the tumor, there is more penetration further in the tumor in the treated animal.

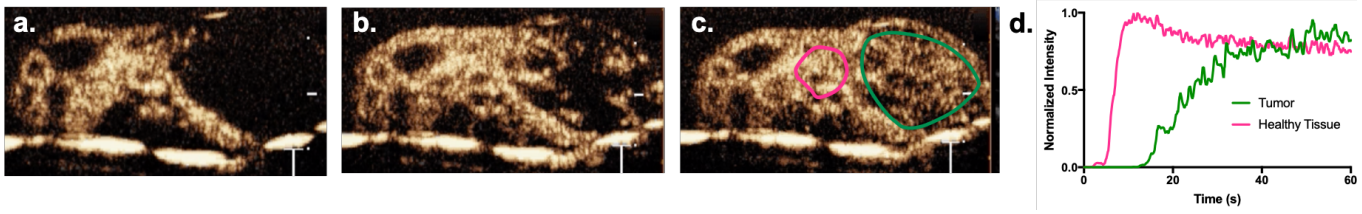

Fig. 2: (a)-(c) CEUS scan used to inform treatment protocol. The wash-in of microbubbles is slower in the tumor than the surrounding tissue. Time-intensity curves of the HCC and the normal parenchyma are shown in (d). Ultrasound treatment was chosen to start at 30 s, when the tumor had sufficient microbubble accumulation.

B. *Immediate post-therapy perfusion changes*

Pre- and post-therapy CEUS exams were used to show immediate changes in vascularity following treatment. As shown in the time-matched pre- and post- therapy contrast images in Fig. 4, treated animals show significant reduction in contrast agent present in the tumor region following treatment. This effect is not seen in control animals. Additionally, it was seen that healthy tissue was not as susceptible to these perfusion changes, implying that the effect is inherently specific to the tumor.

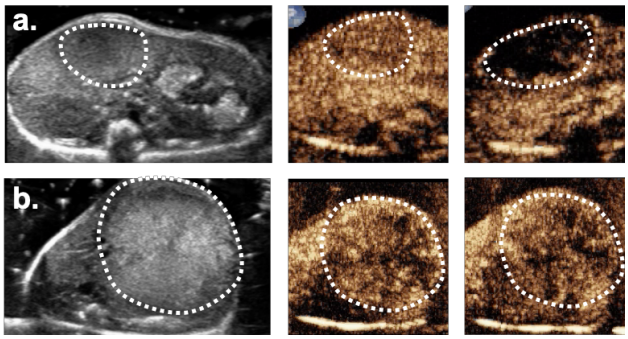

Fig. 4: Anatomical b-mode images and time-matched perfusion images of pre- and post- treatment CEUS scans of a treated mouse (a), and a control mouse (b). Ultrasound treatment resulted in immediate vascular disruption in tumor areas, but no change in surrounding areas. Control mice show no appreciable change in perfusion.

IV. Discussion

Through this work, it was shown that therapy with ultrasound and microbubbles can cause both enhanced doxorubicin penetration and immediate perfusion changes. While the mechanism of action of ultrasound and microbubble-based therapies is widely thought to be microscale forces from the cavitation of microbubbles causing enhanced permeability of endothelial cells to macroscale therapeutics [13], results from this study have shown that microbubble cavitation on its own may cause acute vascular shutdown preferentially in tumors. The concept of ultrasound and microbubbles acting as a vascular disrupting agent is not new [9], [14]; however, the specificity of this effect in the tumor microenvironment and not in healthy tissue has not been studied. One possible explanation for this is due to the differences in vascular morphology in tumors versus healthy liver; hepatocellular carcinomas tend to result in tortuous vessel structures that are perhaps more prone to damage or occlusion than normal tissue [15].

While it has been reported previously that these vascular shutdown effects do not persist long term due to the highly proliferative outer edge of the tumor [9], when used in concert with chemotherapeutics, the dual action of vascular disruption and cytotoxicity may be an effective therapeutic strategy. Furthermore, if the biology of the tumor makes it more susceptible to these effects than healthy tissue, then the precise ultrasound focusing that is usually required for ablative ultrasound procedures may not be necessary. More work still needs to be done to optimize the vascular collapse and to evaluate the extent of damage that occurs following treatment through histological analysis, but if proven safe, this would be a simple, inexpensive, and easily implementable technique in the clinic.

V. Conclusion

We have demonstrated preclinical efficacy and clinical relevance of ultrasound-mediated drug delivery with microbubbles using a "modified" diagnostic ultrasound scanner, clinical microbubbles, and a genetically engineered mouse model of hepatocellular carcinoma. It was shown that diagnostic probes can be modified to produce acoustic fields sufficient to implement ultrasound and microbubble-mediated drug delivery. Through fluorescent microscopy, we observed increased doxorubicin penetration in treated versus control animals. Finally, we observed that therapy with ultrasound and microbubbles causes significant perfusion changes in tumors. Interestingly, these changes seem to be specific to tumor tissue. Future work will involve further understanding the specificity of these vascular changes through histology and longitudinal studies.

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