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# High and low frequency subharmonic imaging of angiogenesis in a murine breast cancer model

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ABSTRACT

This project compared quantifiable measures of tumor vascularity obtained from contrast-enhanced high frequency (HF) and low frequency (LF) subharmonic ultrasound imaging (SHI) to 3 immunohistochemical markers of angiogenesis in a murine breast cancer model (since angiogenesis is an important marker of malignancy and the target of many novel cancer treatments). Nineteen athymic, nude, female rats were implanted with $5 \times 10^6$ breast cancer cells (MDA-MB-231) in the mammary fat pad. The contrast agent Definity (Lantheus Medical Imaging, N Billerica, MA) was injected in a tail vein (dose: 180 μl/kg) and LF pulse-inversion SHI was performed with a modified Sonix RP scanner (Analogic Ultrasound, Richmond, BC, Canada) using a L9–4 linear array (transmitting/receiving at 8/4 MHz in SHI mode) followed by HF imaging with a Vevo 2100 scanner (Visualsonics, Toronto, ON, Canada) using a MS250 linear array transmitting and receiving at 24 MHz. The radiofrequency data was filtered using a 4th order IIR Butterworth bandpass filter (11–13 MHz) to isolate the subharmonic signal. After the experiments, specimens were stained for endothelial cells (CD31), vascular endothelial growth factor (VEGF) and cyclooxygenase-2 (COX-2). Fractional tumor vascularity was calculated as contrast-enhanced pixels over all tumor pixels for SHI, while the relative area stained over total tumor area was calculated from specimens. Results were compared using linear regression analysis. Out of 19 rats, 16 showed tumor growth (84%) and 11 of them were successfully imaged. HF SHI demonstrated better resolution, but weaker signals than LF SHI ($0.06 \pm 0.017$ vs. $0.39 \pm 0.059$; $p < 0.001$). The strongest overall correlation in this breast cancer model was between HF SHI and VEGF ($r = -0.38$; $p = 0.03$). In conclusion, quantifiable measures of tumor neovascularity derived from contrast-enhanced HF SHI appear to be a better method than LF SHI for monitoring angiogenesis in a murine xenograft model of breast cancer (corresponding in particular to the expression of VEGF); albeit based on a limited sample size.



## 1. Introduction

Angiogenesis is an essential step in the growth of malignant tumors beyond 1–2 $mm^3$ and for the development of metastases [1–3]. This process is a cascade of several events in which host endothelial cells are stimulated to obtain oxygen and blood supply for vascular – ingrowth [3]. It thus, provides a pathway for cancer cells to spread via the vascular and lymphatic systems [3–7].

Tumors are able to stimulate angiogenesis by directly secreting angiogenic substances or activating and releasing angiogenic compounds in the extracellular matrix [6,7]. This process involves activation, migration and proliferation of endothelial cells and is regulated by specific growth factors [6,7]. Vascular endothelial growth factor (VEGF) is an important angiogenic factor that promotes the growth of tumor by forming immature, tortuous and leaky blood vessels [3]. Platelet endothelial cell adhesion molecule (PECAM-1 or CD31), a monocyte found on the surface of

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endothelial cell junctions is also a potential endothelial cell marker for angiogenesis [8]. Cyclooxygenase-2 (COX-2) is another molecule that may be involved in expansion and proliferation of tumor and hence is also a regulator of angiogenesis [9,10].

There is an increased interest in noninvasive imaging of tumors to monitor the process of angiogenesis to evaluate the response of anti-angiogenic agents and therapies [6,7]. Ultrasound is one such imaging modality, which can provide real-time information related to angiogenesis by measuring tumor flow and vascular volume [11–13]. Conventional Doppler imaging cannot visualize vessels smaller than 100 μm and hence, only limited conventional ultrasound data is available on the early stages of angiogenesis [12,13]. However, the use of ultrasound contrast agents (i.e., gas-filled and shell stabilized microbubbles) improves the signal to noise ratio up to 25 dB and allows imaging of the neovasculature associated with cancers [11–13].

At higher acoustic pressure (>0.5 MPa) ultrasound contrast agents show nonlinear behavior by producing harmonic frequency components (ranging from sub- to ultra-harmonic) in the received echoes. These nonlinear components can be used to produce contrast-specific imaging modes with improved contrast relative to the surrounding tissue (by up to 25 dB) [12,13]. Harmonic imaging is one such commercially available nonlinear imaging technique, which utilizes the second harmonic frequency component from the backscattered echoes to improve contrast visualization [12,13]. However, this technique suffers from accumulation of tissue harmonic signals, which reduces blood to tissue contrast [12,13]. Subharmonic imaging (SHI), where echoes are received at half the fundamental frequency, can be used as a substitute for harmonic imaging, since subharmonic signal components are not generated in the tissue [14]. There have been many *in vitro* and *in vivo* studies demonstrating the feasibility of SHI [14–24] but, to the best of our knowledge, no one has directly compared high frequency (HF; >15 MHz transmission) and low frequency (LF; <10 MHz transmission) implementations of SHI *in vivo*.

Hence, the objective of this pilot study was to compare HF and LF SHI in a murine breast cancer xenograft model and investigate their correlations with the expression of three immunohistochemical markers. Initially, the tumor model and the imaging strategies employed for *in vivo* HF and LF SHI data acquisition are described. The filter design for HF SHI is established next and then the two SHI imaging modes are compared using histopathology as the reference standard.

## 2. Materials and methods

### 2.1. Tumor model

Human breast adenocarcinoma cells (MDA-MB-231) were purchased from ATTC (Manassas, VA), since this is a well-established model of human breast cancer with a predictable growth pattern making it well suited for pre-clinical investigations [25]. The MDA-MB-231 cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM; Mediatech Inc., Manassas, VA) at 37 °C in 5% $CO_2$. After the cells reached approximately 80% of confluence, they were sub cultured using 0.25% trypsin (to detach cells adhered to the walls of petri dishes) and growth medium (to stop the enzymatic action of trypsin) and then split into even volumes and incubated as before. The sub culturing was repeated until the number of cells was on the order of $10^6$.

After culturing, $5 \times 10^6$ cells were mixed with matrigel [26] and injected subcutaneously into the right mammary fat pads of 19 athymic, nude rats (RNU rats; Charles River Laboratories, Fredrick, MD). The growth of the tumors was monitored over 3 weeks before animals (with tumors greater than $5 \times 5 \times 5$ mm$^3$) were selected for ultrasound imaging conducted 21, 24 or 28 days after tumor implantation. After the ultrasound scans were completed, the animals were euthanized using standard techniques. All the animal studies were carried out in an ethical fashion under the supervision of a veterinarian and were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University.

### 2.2. Ultrasound data acquisition

For the imaging studies, rats were intubated and anesthetized with 0.5–2% Isoflurane (Iso-thesia; Abbott Laboratories, Chicago, IL). A warming blanket was used to maintain normal body temperature. A Sonix RP scanner (Analogic Ultrasound, Richmond, BC, Canada) was configured in the Research Setting to operate in pulse-inversion mode (i.e., two pulses with a 180° phase difference are transmitted and the received echoes summed to cancel out the fundamental and odd nonlinear signals while enhancing the even nonlinear signals) [21,27]. Grayscale LF SHI was performed at a depth of 4 cm using a L9–4 linear array (bandwidth: 9–4 MHz) transmitting at 8 MHz and receiving at 4 MHz (selected based on our previous experiences and with the subharmonic amplitude extracted over a 1 MHz bandwidth around the subharmonic peak [28]) with an acoustic power setting of −10 dB (approximately 760 kPa peak-to-peak *in situ* pressure measured using a 0.2 mm needle hydrophone (Precision Acoustics, UK) with a sensitivity of 47.0 mV/MPa at 8 MHz). A digital LF SHI clip was acquired for each contrast injection and transferred to a PC for off-line analysis.

A Vevo 2100 system (Visualsonics, Toronto, ON, Canada) was used to obtain HF ultrasound scans with an MS250 linear array (approximately 14–30 MHz bandwidth [21]) operating in nonlinear contrast imaging mode at 24 MHz. In the nonlinear contrast mode, the Vevo scanner uses amplitude modulation where two consecutive, differently scaled ultrasound pulses are transmitted followed by subtraction of their (appropriately scaled) echo signals to cancel linear tissue signals and retain nonlinear contrast agent echo signals thus, improving sensitivity [21,27]. Image acquisition was performed at a depth of 14 mm (to cover the tumor) with 4% output power (approximately 780 kPa peak-to-peak *in situ* pressure obtained using the 0.2 mm needle hydrophone; sensitivity: 51.0 mV/MPa). Digital cine loops of the HF ultrasound radiofrequency (RF) data were captured for all contrast injections.

To ensure reproducibility, imaging (LF as well as HF) was performed in the largest cross-sectional plane of each tumor. The contrast agent Definity (Lantheus Medical Imaging, N Billerica, MA) was selected because of its marked nonlinear properties [24] and injected (dose: 180 μl/kg) into a tail vein using a 24 gauge needle followed by a 0.2 ml saline flush. Three contrast injections were administered in each imaging mode (i.e., LF SHI as well as HF ultrasound imaging) with around 5 min between injections; to be sure the contrast agent could not be seen with imaging and had time to clear the blood pool.

### 2.3. HF SHI filter design and selection

To convert the HF ultrasound data obtained at 24 MHz to HF SHI (at 12 MHz; around 12 dB below the signal peak [21]), the RF data was filtered using digital IIR Butterworth bandpass filters (since such filters have a maximally flat frequency response and have previously been used successfully in intravascular (IVUS) SHI applications [23,24]). Filters were designed and tested for their ability to extract the subharmonic signal component from the contrast microbubbles and suppress the background tissue signals. Twenty IIR Butterworth bandpass filters with 4 different orders (2, 4, 6 and 8) and 5 different bandwidths (11.5–12.5 MHz, 11–13 MHz, 10–14 MHz, 9–15 MHz and 8–16 MHz) were evaluated