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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 5×10^6 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 μ l/kg) and low frequency 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 8/4MHz in SHI mode) followed by high frequency imaging with a Vevo 2100 scanner (Visualsonics, Toronto, ON, Canada) using a MS250 linear array transmitting and receiving at 24MHz. The radiofrequency data was filtered using a 4th order IIR Butterworth bandpass filter (11–13MHz) 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

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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 ± 0.017 vs. 0.39 ± 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.

Keywords

ultrasound contrast agent; subharmonic imaging; breast cancer; murine xenografts; signal processing

1. Introduction

Angiogenesis is an essential step in the growth of malignant tumors beyond $1\text{--}2\text{ mm}^3$ 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 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 $100\text{ }\mu\text{m}$ 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 neovasculature associated with cancers [11–13].

At higher acoustic pressure ($> 0.5\text{ MPa}$) 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% CO₂. 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 10⁶.

After culturing, 5×10⁶ 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 5×5×5 mm³) 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 to 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 3 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 based on our previous experiences [24]. All filters were implemented using MATLAB (R2013a, The Mathworks Inc., Natick, MA).

From each cine loop (i.e., each injection), an image corresponding to the time point of maximum contrast mean power (identified from RF spectra calculated using the VevoCQ software package supplied with the Vevo 2100) was selected. These images were then processed with every filter to produce a collection of HF SHI, which were assessed

qualitatively for image noise and contrast visualization. Images were scored by one reader on a visual-analogue-scale of 1 to 5 (worst to best, respectively) and compared to select the best of the bandpass filters across all images.

2.4. Pathology Data Analysis

Following the imaging studies, tumors were surgically removed and scanned *ex vivo* to identify and mark the imaging plane that was studied *in vivo*. The tumor specimens were placed in 10% neutral buffered formalin (Fisher Scientific, Houston, TX) for 12–24 hours to fix all the angiogenic markers, dehydrated in graded alcohols, cleaned in xylene and embedded in paraffin using standard methods. Each specimen was dissected corresponding to the imaging plane with a slice thickness of 15 μm and stained immunohistochemically against VEGF, CD31 and COX-2. A monoclonal antibody against PECAM (anti CD31; Dako Corporation, Carpinteria, CA), a monoclonal antibody against VEGF (Oncogene Research Products, San Diego, CA) and a polyclonal antibody against COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA) were used for staining. Finally, the stained sections were mounted onto glass slides for further analysis.

A semi-automated histomorphometry system based on a DXC-970MD color CCD camera (Sony Corporation, Tokyo, Japan) connected to a Labophot-2 microscope (Nikon, Melville, NJ, USA) was used to analyze the tumor specimens [29,30]. The histomorphometry system used a total magnification 100 $\times$ to provide digital images of the histologic slides. A motorized stage was used to move the specimen until red-green-blue (RGB) images of the entire tumor area were obtained. The blue image, which showed tissue enhancement, was extracted from the RGB color model. Then the RGB images were converted to hue saturation intensity (HSI) images and the saturation image, which indicated vessel enhancement, was extracted from HSI color model. The blue image was subtracted from the saturation image and the total area of the tumor was obtained from this image. The RGB channels were used to segment and count the number of colored or stained pixels as well as total number of pixels in the tumor. Relative expression (RE) was calculated for all specimens and each marker (i.e., VEGF, CD31 and COX-2) as the number of stained pixels (si) relative to the total number of pixels (si+xi) [29,30]:

$$RE = \frac{\sum si}{(\sum si + xi)} \quad (1)$$

To account for the heterogeneous nature of tumor vasculature [3,4], each tumor was divided into small regions of interest (ROIs) of 3–4 mm in diameter and the RE for each individual ROI was compared to the results from HF and LF SHI.

2.5. Ultrasound Data Analysis

The tortuous morphology of tumor angiogenesis was visualized in detail using the maximum intensity projection (MIP) technique. This technique retains the maximum contrast-enhanced pixels over several image frames in order to trace the path traversed by individual microbubbles within the microvasculature and more completely demonstrate the vascular architecture of the tumor [31,32]. The SHI fractional vascularity (FV) was determined by

classifying the pixels in the image into tumor vascularity pixels and tumor background pixels based on an optimum threshold value separating these two types of pixels using Otsu's segmentation technique [33] and then calculating the FV as their relative ratio (as in eqn. 1). The FVs corresponding to each of the ROIs marked on the tumor specimens were obtained. This process was performed for HF as well as LF SHI images.

2.6. Statistical Analysis

The average HF bandpass filter scores were compared using a nonparametric Wilcoxon's signed rank test, since the values in a visual-analogue-scale are not continuous and a standard t-test can therefore not be applied. Least squares linear regression was used to compare the measures of FV obtained from HF and LF SHI to the corresponding FVs obtained from the tumor specimens stained for VEGF, CD31 and COX-2, respectively, on a per ROI basis. A p-value of less than 0.05 was used to define significance. All the statistical analysis was performed using IBM SPSS Statistics 20 (IBM Corporation, Endicott, NY)

3. Results

Sixteen out of 19 rats showed marked tumor growth with diameters ranging from 5–13 mm. The first 8 tumors that grew beyond $5 \times 5 \times 5 \text{ mm}^3$ were selected for the 21st day of scanning. On the 23rd day tumor growth was again monitored and the 4 tumors that exceeded $5 \times 5 \times 5 \text{ mm}^3$ were selected for scanning on the 24th day. Amongst the remaining 7 animals, only the 4 rats that developed tumors ($> 5 \times 5 \times 5 \text{ mm}^3$) were scanned on the 28th day. The remaining three rats that did not show any tumor growth were eliminated from the study.

All the rats were scanned in LF SHI mode using the Sonix RP and received 3 contrast injections per animal. However, for the HF ultrasound studies, just 1 successful Definity injection was achieved for 5 out of the 16 rats. Hence, those 5 were not included in the final data analysis and the ultrasound and pathological comparisons were carried out for the 11 rats each with 3 contrast injections per tumor. Thus, this study analyzed 33 pathological slides (3 markers $\times$ 11 tumors) and 66 ultrasound images (2 imaging modes $\times$ 3 injections $\times$ 11 tumors) with the 11 tumors split over 31 ROIs.

Mean scores for all the bandpass filters ranged from 1.0 ± 0.0 to 3.4 ± 0.8 (data not shown for the sake of brevity). A Wilcoxon's signed rank test was performed to compare the mean scores and filter BP9's scores were significantly higher than those of the other filters ($p < 0.002$). This filter (a 4th order IIR Butterworth bandpass filter with a -3 dB bandwidth from 11–13 MHz) showed less image noise and provided better contrast visualization as compared to other filters. Hence, filter BP9 was selected for filtering the subharmonic component from all the HF ultrasound scans in order to create the final HF SHI images.

The 11 tumors scanned had an average diameter of $8.69 \pm 2.23 \text{ mm}$. Figure 1 shows examples of LF and HF SHI of the same tumor. Both the LF and HF SHI tumor images demonstrated contrast within the microvasculature. Not surprisingly, the HF SHI scans had better resolution compared to LF SHI enabling better visualization of the tumor morphology, but also weaker average signal intensities (HF vs. LF SHI: 0.06 ± 0.017 vs. 0.39 ± 0.059 ; $p < 0.001$). The HF and LF SHI signals did not correlate ($r = 0.003$; $p = 0.98$). The

corresponding immunohistochemical stains for a small part of the tumor specimen can be seen in Figure 2. Marked staining is seen for VEGF and COX-2, while a more heterogeneous stain occurred with the expression of CD31.

An example of the segmentation performed to determine the fractional vascularity is presented in Figure 3. Table 1 presents the correlation coefficients and p values for the linear regression analysis performed (on a per ROI basis) between the SHI FV measures and the immunohistochemical markers. The strongest (and only significant) correlation was between tumor vascularity assessed with HF SHI and the percent area stained with VEGF ($r = -0.38$; $p = 0.03$); as depicted in the scatterplot of Figure 4. Hence, the FVs obtained from HF SHI and the relative areas stained for 3 immunohistochemical markers were analyzed by time point (Table 2). The only significant correlations were obtained for the expression of VEGF in 21 day rats ($r = -0.77$; $p = 0.02$) and the expression of CD31 ($r = 0.61$; $p = 0.04$) in 24 day rats. There were no significant results obtained when the expression of these markers was compared to LF SHI at the 3 different time points ($p > 0.1$).

4. Discussion and Conclusion

Both HF and LF SHI successfully imaged the tumor vasculature in a murine xenograft breast cancer model at their respective transmit and receive frequencies. SHI obtained using HF ultrasound (transmitting/receiving at 24/12 MHz) not only showed better resolution and less image noise than LF SHI (transmitting/receiving at 8/4 MHz), but also resulted in the only significant correlation relative to the angiogenic markers (VEGF; Table 1; Fig. 4) in this murine breast cancer model. Thus, contrast enhanced HF SHI appears to provide a noninvasive marker for angiogenesis; particularly for the expression of VEGF.

There has been a multitude of *in vivo* research studies conducted to assess vascularity and, in particular, the process of tumor angiogenesis in animal models using SHI [19–24,28] as well as other contrast enhanced ultrasound imaging modes [29,30,34–38]. Goertz and colleagues introduced HF IVUS SHI (transmitting at 20 and 30 MHz) using a prototype IVUS catheter [19,23] (subsequently implemented on a commercial IVUS system by Sridharan and co-workers [24]), while our group produced the initial *in vivo* SHI in animals at transmit frequencies below 10 MHz [14,18] and, more recently, in humans [39–41]. A study into HF SHI from 18–24 MHz on the Vevo 2100 by Needles et al concluded that SHI is most advantageous at 24 MHz [21]. This pilot study directly compared HF and LF SHI (transmitting at 24 and 8 MHz, respectively) and found that in this MDA-MB-231 murine model the resolution of HF SHI outweighed the weaker subharmonic signals obtainable further away from microbubble resonance. That notwithstanding, in human breast studies, where lesions at depths of 4–6 cm can be encountered, LF SHI will be the imaging mode of choice [41]. Interestingly, in this study the HF and LF SHI signals did not correlate, which is most likely due to the strong nonlinearity and frequency dependent, threshold phenomenon associated with subharmonic microbubble signals [12].

Moreover, while the correlation obtained in this project between contrast enhanced ultrasound imaging (specifically HF SHI) and tumor specimens stained for VEGF have been confirmed by other studies [29,34], there are also studies that did not report such a

relationship [30,35]. This includes the largest study to date comparing contrast ultrasound and angiogenic markers in rat tumor models (involving 144 rats) where our group reported correlations with CD31 and COX-2 [30]. Conversely, a number of studies have shown that the effect of anti-VEGF therapies in murine xenografts can be monitored with contrast ultrasound imaging [36–38]. Most likely, these differences are tumor model–, drug context– and tumor stage–dependent and additional studies will be needed to provide clarification [37].

There are also several limitations to this study. The number of rats studied (11) was small, which limits the statistical power of the study and hence, the associated conclusions. Also while the entire tumor area was taken into consideration for the vascularity measurements (unlike hot-spot assessments), the entire tumor volume (i.e., vascularity in 3D) was not evaluated. The elevation thickness of the ultrasound beam (in mm) is larger than the thickness of the specimens (in μm) by orders of magnitude, which means an exact match between SHI and tumor specimens cannot be obtained. Another limitation was the use of pulse inversion for LF SHI studies (which were optimized for pressure measurements rather than purely for imaging [28]) as opposed to the use of amplitude modulation for HF SHI and the use of two different transducers, which may also impact comparisons. Moreover, xenograft tumor models use cell lines, which do not exactly replicate the genetics and histology of human tumors. These cells lack the architectural and cellular complexity of *in vivo* tumors, which include inflammatory cells, vasculature, and other stromal components [25,42]. However, they also have high degree of predictability and rapid tumor formation, which makes them suitable for diagnostic studies.

Finally, a statistical limitation of this study is that multiple comparisons are being conducted, which increases the risk that a significant relationship will be found by pure chance. To compensate for this, a Bonferroni correction may be applied, which assigns the traditional 0.05 p-value divided by the number of comparisons to be the p-value of significance [43]. However, by controlling the group-wise error rate (i.e., preventing results that are not significant from being designated significant), each individual test is held to an unreasonably high standard, which makes it more likely that legitimately significant results will be discarded [44]. Hence, we chose not to adopt Bonferroni correction in this study.

In conclusion, quantifiable measures of tumor neovascularity derived from contrast-enhanced HF SHI appear to be a better method than LF SHI for estimating angiogenic marker expression in a murine xenograft model of breast cancer (corresponding in particular to the expression of VEGF); albeit based on a limited sample size.

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References

1. Li WW. Tumor angiogenesis: molecular pathology, therapeutic targeting, and imaging. *Acad Radiol*. 2000; 7:800–811. [PubMed: 11048878]

2. Bamias A, Dimopoulos MA. Angiogenesis in human cancer: implications in cancer therapy. *Eur J Intern Med.* 2003; 14:459–469. [PubMed: 14962697]
3. Makrilia N, Lappa T, Xyla V, Nikolaidis I, Syrigos K. The role of angiogenesis in solid tumours: an overview. *Eur J Intern Med.* 2009; 20:663–671. [PubMed: 19818284]
4. Folkman J. What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst.* 1990; 82:4–6. [PubMed: 1688381]
5. Holmgren L, O'Reilly MS, Folkman J. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nature Med.* 1995; 1:149–153. [PubMed: 7585012]
6. Patel N, Harris AL, Gleeson FV, Vallis KA. Clinical imaging of tumor angiogenesis. *Future Oncol.* 2012; 8:1443–1459. [PubMed: 23148617]
7. Laking GR, West C, Buckley DL, Matthews J, Price PM. Imaging vascular physiology to monitor cancer treatment. *Critical Rev Oncol Hematol.* 2006; 58:95–113. [PubMed: 16387510]
8. Delisser HM, Baldwin HS, Albelda SM. Platelet endothelial cell adhesion molecule 1 (PECAM-1/CD31): a multifunctional vascular cell adhesion molecule. *Trends Cardiovasc Med.* 1997; 7:203–210. [PubMed: 21235886]
9. Mohammed SI, Dhawan D, Abraham S, Snyder PW, Waters DJ, Craig BA, et al. Cyclooxygenase inhibitors in urinary bladder cancer: in vitro and in vivo effects. *Mol Cancer Ther.* 2006; 5:329–336. [PubMed: 16505106]
10. Denkert C, Köbel M, Pest S, Koch I, Berger S, Schwabe M, et al. Expression of cyclooxygenase 2 is an independent prognostic factor in human ovarian carcinoma. *Am J Pathol.* 2002; 160:893–903. [PubMed: 11891188]
11. Lamuraglia M, Bridal SL, Santin M, Izzi G, Rixe O, Paradiso A, et al. Clinical relevance of contrast-enhanced ultrasound in monitoring anti-angiogenic therapy of cancer: current status and perspectives. *Crt Rev Oncol Hematol.* 2010; 73:202–212.
12. Goldberg, BB.; Raichlen, JS.; Forsberg, F. *Ultrasound contrast agents: basic principles and clinical applications.* 2nd ed.. London: Martin Dunitz; 2001. p. 25-57.
13. Eisenbrey JR, Forsberg F. Contrast-enhanced ultrasound for molecular imaging of angiogenesis. *Eur J Nucl Med Mol Imaging.* 2010; 37:S138–S146. [PubMed: 20461376]
14. Forsberg F, Shi WT, Goldberg BB. Subharmonic imaging of contrast agents. *Ultrasonics.* 2000; 38:93–98. [PubMed: 10829636]
15. Shankar PM, Krishna PD, Newhouse VL. Advantage of subharmonic over second harmonic backscatter for contrast-to-tissue echo enhancement. *Ultrasound Med Biol.* 1998; 24:395–399. [PubMed: 9587994]
16. Chomas J, Dayton P, May D, Ferrara K. Nondestructive subharmonic imaging. *IEEE Trans Ultrason Ferroelec Freq Contr.* 2002; 49:883–892.
17. Faez T, Emmer M, Docter M, Sijl J, Versluis M, de Jong N. Characterizing the subharmonic response of phospholipid-coated microbubbles for carotid imaging. *Ultrasound Med Biol.* 2011; 37:958–970. [PubMed: 21531498]
18. Shi WT, Forsberg F, Hall AL, Chiao RY, Liu JB, Miller S, et al. Subharmonic imaging with microbubble contrast agents: initial results. *Ultrason Imaging.* 1999; 21:79–94. [PubMed: 10485563]
19. Goertz DE, Needles A, Burns PN, Foster FS. High-frequency, nonlinear flow imaging of microbubble contrast agents. *IEEE Trans Ultrason Ferroelectr Freq Control.* 2005; 52:495–502. [PubMed: 15857059]
20. Faez T, Skachkov I, Versluis M, Kooiman K, de Jong N. In vivo characterization of ultrasound contrast agents: microbubble spectroscopy in a chicken embryo. *Ultrasound Med Biol.* 2012; 38:1608–1617. [PubMed: 22766113]
21. Needles A, Arditi M, Rognin NG, Mehi J, Coulthard T, Bilan-Tracey C, et al. Nonlinear contrast imaging with an array-based microultrasound system. *Ultrasound Med Biol.* 2010; 36:2097–2106. [PubMed: 21092832]
22. Pollard RE, Broumas AR, Wisner ER, Vekich SV, Ferrara KW. Quantitative contrast enhanced ultrasound and CT assessment of tumor response to antiangiogenic therapy in rats. *Ultrasound Med Biol.* 2007; 33:235–245. [PubMed: 17306694]

23. Goertz DE, Frijlink ME, Tempel D, Bhagwandas V, Gisolf A, Krams R, et al. Subharmonic contrast intravascular ultrasound for vasa vasorum imaging. *Ultrasound Med Biol*. 2007; 33:1859–1872. [PubMed: 17683850]
24. Sridharan A, Eisenbrey JR, Machado P, deMuinck ED, Doyley MM, Forsberg F. Delineation of atherosclerotic plaque using subharmonic imaging filtering techniques and a commercial intravascular ultrasound system. *Ultrason Imaging*. 2013; 35:30–44. [PubMed: 23287505]
25. Kerbel RS, Guerin E, Francia G, Xu P, Lee CR, Ebos JM, et al. Preclinical recapitulation of antiangiogenic drug clinical efficacies using models of early or late stage breast cancer metastasis. *Breast*. 2013; 22:S57–S65. [PubMed: 24074794]
26. Lewis MT, Landua JD, Adams HC 3rd, Medina D. A mystery wrapped in an enigma: Matrigel enhancement of mammary cell growth and morphogenesis. *J Mammary Gland Biol Neoplasia*. 2012; 17:99–101. [PubMed: 22581302]
27. Eckersley RJ, Chin CT, Burns PN. Optimising phase and amplitude modulation schemes for imaging microbubble contrast agents at low acoustic power. *Ultrasound Med Biol*. 2005; 31:213–219. [PubMed: 15708461]
28. Halldorsdottir VG, Dave JK, Eisenbrey JR, Machado P, Zhao H, Liu JB, et al. Subharmonic aided pressure estimation for monitoring interstitial fluid pressure in tumours—in vitro and in vivo proof of concept. *Ultrasonics*. 2014; 54:1938–1944. [PubMed: 24856899]
29. Forsberg F, Ro RJ, Liu JB, Lipcan KJ, Potoczek M, Nazarian LN. Monitoring angiogenesis in human melanoma xenograft model using contrast-enhanced ultrasound imaging. *Ultrason Imaging*. 2008; 30:237–246. [PubMed: 19507677]
30. Forsberg F, Ro RJ, Fox TB, Liu JB, Chiou SY, Potoczek M, et al. Contrast enhanced maximum intensity projection ultrasound imaging for assessing angiogenesis in murine glioma and breast tumor models: A comparative study. *Ultrasonics*. 2011; 51:382–389. [PubMed: 21144542]
31. Linden RA, Trabulsi EJ, Forsberg F, Gittens PR, Gomella LG, Halpern EJ. Contrast enhanced ultrasound flash replenishment method for directed prostate biopsies. *J Urol*. 2007; 178:2354–2358. [PubMed: 17936814]
32. Sugimoto K, Moriyasu F, Kamiyama N, Metoki R, Yamada M, Ymai I, et al. Analysis of morphological vascular changes of hepatocellular carcinoma by microflow imaging using contrast-enhanced sonography. *Hepatol Res*. 2008; 38:790–799. [PubMed: 18507694]
33. Otsu N. A threshold selection method from gray-level histograms. *IEEE Trans Cybern*. 1979; 9:62–66.
34. Chen ZK, Cai MX, Yang J, Lin LW, Xue ES, Huang J, et al. Chemotherapy with PLGA microspheres containing docetaxel decreases angiogenesis in human hepatoma xenograft. *Med Oncol*. 2012; 29:62–69. [PubMed: 21136211]
35. Lee HJ, Hwang SI, Chung JH, Jeon JJ, Choi JH, Jung HS. Evaluation of tumor angiogenesis in a mouse PC-3 prostate cancer model using dynamic contrast-enhanced sonography. *J Ultrasound Med*. 2012; 31:1223–1231. [PubMed: 22837286]
36. Forsberg F, Ro RJ, Marshall A, Liu JB, Chiou SY, Merton DA, et al. The antiangiogenic effects of a vascular endothelial growth factor decoy receptor can be monitored in vivo using contrast-enhanced ultrasound imaging. *Mol Imaging*. 2014; 13:1–9. [PubMed: 24622811]
37. Eichten A, Adler AP, Cooper B, Griffith J, Wei Y, Yancopoulos GD, et al. Rapid decrease in tumor perfusion following VEGF blockade predicts long-term tumor growth inhibition in preclinical tumor models. *Angiogenesis*. 2013; 16:429–441. [PubMed: 23238831]
38. Guibal A1, Taillade L, Mulé S, Comperat E, Badachi Y, Golmard JL, et al. Noninvasive contrast-enhanced US quantitative assessment of tumor microcirculation in a murine model: effect of discontinuing anti-VEGF therapy. *Radiology*. 2010; 254:420–429. [PubMed: 20093514]
39. Forsberg F, Piccoli CW, Merton DA, Palazzo JJ, Hall AL. Breast lesions: imaging with contrast-enhanced subharmonic US—initial experience1. *Radiology*. 2007; 244:718–726. [PubMed: 17690324]
40. Eisenbrey JR, Dave JK, Halldorsdottir VG, Merton DA, Machado P, Liu JB, et al. Simultaneous grayscale and subharmonic ultrasound imaging on a modified commercial scanner. *Ultrasonics*. 2011; 51:890–897. [PubMed: 21621239]

41. Sridharan A, Eisenbrey JR, Machado P, Ojeda-Fournier H, Wilkes A, Sevrakov A, et al. Quantitative analysis of vascular heterogeneity in breast lesions using contrast-enhanced three-dimensional harmonic and subharmonic ultrasound imaging. *IEEE Trans Ultrason, Ferroelectr Freq Control*. 2015; 62:502–510. [PubMed: 25935933]
42. Becher OJ, Holland EC. Genetically engineered models have advantages over xenografts for preclinical studies. *Cancer Res*. 2006; 66:3355–3358. [PubMed: 16585152]
43. Bland JM, Altman DG. Multiple significance tests: the Bonferroni method. *Br Med J*. 1995; 310:170. [PubMed: 7833759]
44. Perneger TV. What's wrong with Bonferroni adjustments. *Br Med J*. 1998; 316:1236–1238. [PubMed: 9553006]

- High and low frequency subharmonic imaging were compared to markers of angiogenesis in a murine breast cancer model
- A 4th order IIR Butterworth bandpass filter (11–13MHz) was optimal to isolate high frequency subharmonic signals (24MHz)
- HF SHI demonstrated better resolution, but weaker signals than LF SHI (0.06 ± 0.017 vs. 0.39 ± 0.059 ; $p < 0.001$)
- The strongest overall correlation in this breast cancer model was between HF SHI and VEGF ($r = -0.38$; $p = 0.03$)

Figure 1.

An 8×7 mm tumor (big arrows) depicted in pre contrast (a) LF SHI and (b) HF SHI modes as well as post contrast with (c) LF SHI and (d) HF SHI showing areas of flow (small arrows).

Figure 2.
Immunohistochemical staining (arrows) of tumor for (a) VEGF, (b) CD31 and (c) COX-2.

Figure 3.
Segmentation of enhanced (i.e., vascular) pixels in red within a tumor (outlined in green) depicted with HF SHI for calculating the corresponding FV.

Figure 4.

Scatterplot of FVs obtained from HF SHI and percent area stained for VEGF. Notice the plot is somewhat dominated by one large data point.

Table 1

Regression analysis (r- and p-values) between SHI FV (LF as well as HF) and histology (N = 31). Significant p-values are in bold.

		VEGF	CD31	COX-2
LF SHI	r	-0.04	-0.18	-0.31
	p	0.80	0.32	0.09
HF SHI	r	-0.38	0.32	-0.14
	p	0.03	0.08	0.47

Table 2

Regression analysis (r- and p-values) between HF SHI FV and histology by time point. Significant p-values are in bold.

HF SHI		VEGF	CD-31	Cox-2
21 days (N = 9)	r	-0.77	0.34	-0.44
	p	0.02	0.39	0.26
24 days (N = 11)	r	-0.15	0.61	-0.22
	p	0.67	0.04	0.51
28 days (N = 11)	r	-0.56	0.22	-0.38
	p	0.09	0.49	0.22