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Use of Ultrasound to Assess Drug Efficacy in Orthotopic Rat Models of HCC

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1. Introduction

1.1 Imaging in drug development

The development of novel therapeutics follows a typical path from chemical and biological activities in the laboratory through extensive clinical testing and if successful, to the commercialization of a drug for a given labeled indication. The attrition, the failure of a new product to successfully complete all stages of drug testing, is a key metric for defining productivity in the pharmaceutical industry. The lack of therapeutic efficacy and poor safety profiles are the leading causes of attrition. Several solutions to better address attrition have been proposed in order to enhance our knowledge regarding efficacy, safety and mechanism of action in pre-clinical and early clinical setups, all in order to minimize late-stage attrition and make informed decisions regarding the likelihood of the novel compounds to successfully complete all stages of drug development and become a product.
Clinical imaging has the potential to provide key biomarkers and to enable decision-making in drug development. Although, imaging is a complementary technology to biofluid-derived biomarkers, its non-invasive nature provides unique information regarding quantitative measurement of function at particular anatomical localization and is highly desirable in order to strengthen our confidence in positive clinical outcome. Imaging, therefore, has considerable potential to build upon well-established serum and urine biomarkers in order to better validate predictive values of biofluid-derived biomarkers in both the pre-clinical and clinical environments. In oncology, imaging techniques are complementary to methods that use biomarker techniques to detect presence of tumor tissue, tumor progression and response to therapy because imaging modalities provide precise anatomical localization of the tumor tissue(s) that generates biomarkers measured in fluids. Ultrasonography (US) is one of the emerging technologies that possess several key advantages over other molecular imaging modalities. These include frequency-dependent high spatial resolution, real-time imaging, both anatomical and molecular information in the single imaging session, freedom from ionizing radiation, inexpensive implementation, affordability worldwide, and finally, well-published preclinical and clinical use of US technology. Therefore, research teams can easily access information regarding adequate use and predictive value of ultrasound technology to address the specific project needs [1,2].

1.2 HCC and tumor models

Hepatocelluar carcinoma (HCC) is the fifth most common cancer and the third cause of cancer-related death globally that resists conventional chemotherapy and radiotherapy [3-5]. Also, the

liver is, in addition to lungs and bones, one of the most common sites of metastases of other tumors, in particular colorectal, pancreatic and ovarian cancers. Conventional cytotoxic chemotherapy does not significantly prolong survival of patients with primary liver tumors or liver metastases, therefore new therapeutic approaches are needed in order to curb the local growth of solid tumors as well as micrometastases of HCC. Given the complexity of the interactions between tumor cells and surrounding stroma and uniqueness of microvascularization of the liver, there is a strong rationale to combine agents with different mechanisms of action when treating HCC, but also to simultaneously target tumor cells and surrounding stroma in order to make the tumor microenvironment less friendly (fertile) for growth of solid tumors or development of micro-metastases. In this chapter we describe the difference in vascularization between xenograft and orthotopic preclinical models of HCC and use of ultrasonogrophy to assess tumor and organ vasculature. Additionally, we emphasize the unique value of contrast enhanced ultrasonography to monitor tumor growth and change in tumor vasculature over time in order to assess effect of applied therapies.

1.3 Angiogenesis in tumors

Angiogenesis is a critical process in local tumor growth and in the invasion and development of distant metastases [6,7]. Research investigating molecular pathways of tumor angiogenesis has led to the identification of a number of key molecules involved in the stimulation of new vessel growth from existing host vasculature. Several of these molecules, such as vascular endothelial growth factor and its main receptor 2 (VEGFR2) have become targets for antiangiogenic drugs [8,9]. However, successful application of novel therapies that target tumor vasculature will require accurate selection of susceptible tumors and precise evaluation of early treatment response using adequate preclinical models. Previous work has shown that angiogenesis can be successfully characterized *in vivo* by using ultrasonography with microbubble contrast agents bearing anti-integrin antibodies adhered to fibroblast growth factor-stimulated vessels [10-12].

2. Preclinical models of hepatocellular carcinoma

Preclinical experimentation allows for simultaneous longitudinal implementation of various technologies and biomarkers to monitor the tumor take rate, growth and response to treatment as well as to confirm and correlate histological and histochemical results at various time points with serum or imaging biomarkers, which cannot always be determined in HCC patients.

2.1 Tumor vasculature in xenograft HCC model

The vast majority of *in vivo* oncology studies are performed in xenograft models, subcutaneously placed tumor cells in immunodeficient mice or rats. Xenograft models are relatively easy to perform since tumor cells are injected in subcutaneous tissue of mice or rats and simple caliper measurement of tumor size provides insight regarding tumor take rate, growth and the compound efficacy (Figure 1). Since human malignancies never start or metastasize to subcutaneous regions of the body, the xenograft models lack many critical characteristics of human cancers including lack of preexisting organ vasculature and interaction between the tumor cells and cells of the organ where tumor initiated or metastasized. Therefore, the main goal of studies using xenograft models is to confirm that the "targeted" therapy under investigation hits the intended target that should be present in the tumor cell line used in the particular study. In this model there are no pre-existing blood

vessels and the newly formed tumor vasculature is fairly simple and consists of nutritional arteries designed to provide oxygen and nutrients to the tumor (Figure 1). Consequently, antiangiogenic therapy in the HCC xenograft models seems to be very effective against the tumor growth [13]. In general, the xenograft models are very informative and allow for several types of measurements to be performed simultaneously including caliper measurements, IVIS imaging, ultrasonography with and without the contrast agents, PET and MRI imaging, measurements of serum or urine biomarkers and finally deployment of many histological and histochemical methods. Recently, several groups have demonstrated that quantification of intra-tumoral flow of an ultrasound contrast agent with gray-scale imaging can be used for monitoring tumor vascular response to anti-angiogenic therapy in animal models, a technique that can also be used in the clinic [13-16]. In those studies data obtained with ultrasonography paralleled tumor volume data obtained by caliper measurement and showed good correlation with tumor histology, allowing assessment of necrotic and perfused tumor areas *in vivo*.

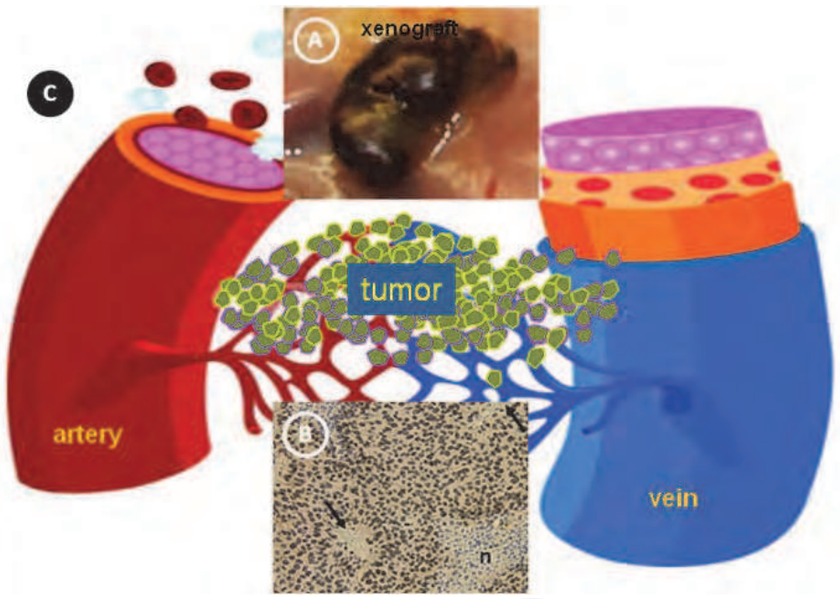

Fig. 1. Image of a xenograft of HCC tumor (A), histological appearance of the tumor (B) with arrows indicating a blood vessel, and schematic representation of the nutritional blood supply to the tumor (C).

2.2 Tumor vasculature and liver vasculature in orthotopic HCC model

Orthotopic HCC models of liver tumors are labor-intensive to perform as they require surgical inoculation of tumor cells into the liver (or spleen) and the use of sophisticated imaging technologies and/or biofluid based biomarkers to monitor the tumor take rate, tumor growth and effects of therapy on tumor progression [17]. Because in orthotopic HCC models the tumor is placed in the organ of origin, studies performed in those models provide more realistic and clinically relevant data regarding compound systemic and tumor exposure, efficacy and safety.

The local vasculature of the liver is extremely complex and is composed of nutritional and functional blood supplies that in addition to neo-vasculature created by the tumor itself, collectively support tumor growth within the liver (Figure 2). The hepatic artery accounts

for only about 65% of the oxygen supply to the liver [18,19]. Slow circulation in the liver and an intense nutritional (hepatic artery) and functional (portal vein) vascular network allows the tumor to establish, survive and eventually metastasize [20,21]. The blood flow to the liver tumor is carried almost exclusively by systemic arterial vessels [22,23]. However, oxygen and nutrient supply through the portal vein is respectable, and it has been hypothesized that outside edges of the tumor use this alternate route to survive and spread to surrounding tissues [24] causing these tumors to be very resistant to antiangiogenic therapies. Finally, vascularization in the liver of cancer patients can be further complicated by liver cirrhosis [25-27]. Due to technical and other difficulties to reliably describe the exact anatomical location of the tumor within the liver, tumor size and growth propensity, presence or lack of secondary tumors, as well as tumor response to therapy, scientists that utilize orthotopic HCC models in their research as well as clinicians almost entirely depend on imaging modalities, including various ultrasound methods.

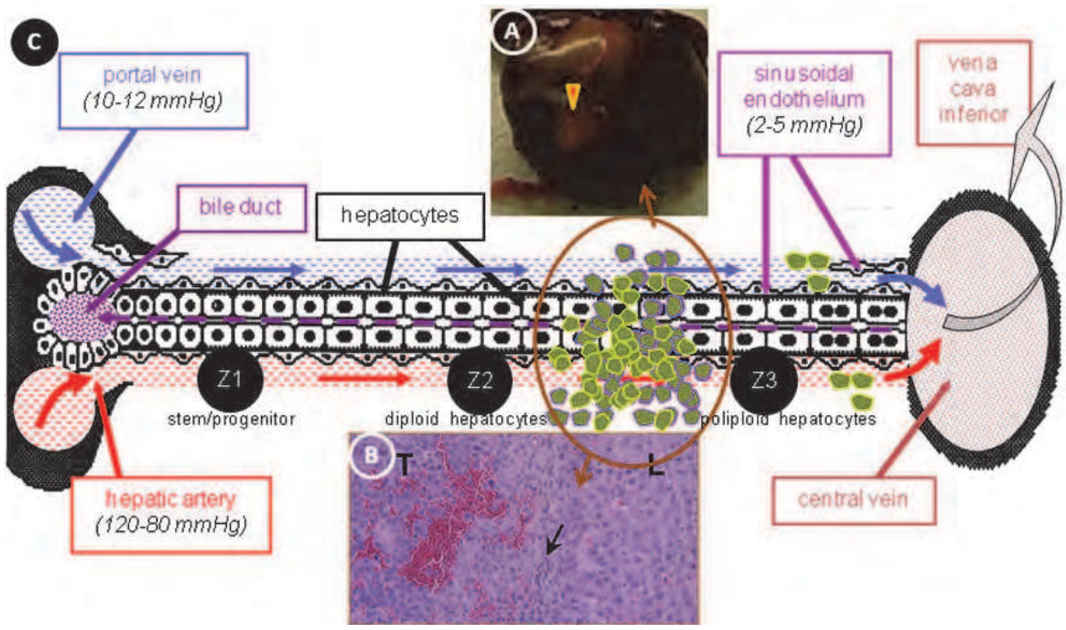

Fig. 2. Overview of a rat liver (A) with orthotopic HCC tumor (arrow head), histological appearance of intrahepatic tumor (B) depicting borderline area (arrow) between the tumor (T) and normal liver tissue (L) and schematic representation (C) of the nutritional (hepatic artery) and functional (portal vein) blood supply in the liver and the intrahepatic tumor, emphasizing differences in blood pressure between the two blood systems.

2.3 Delivery of contrast and/or drug in orthotopic HCC model

One of the most clinically effective ways to deliver drugs to the liver is through the hepatic artery because it allows continuous infusion directly into the arterial bed from which the tumor derives nearly all of its blood supply [27,28]. The use of orthotopic animal models allows for cannulation of the hepatic artery and portal vein to deliver drugs directly to the liver, thus mimicking the clinical arrangement. Due to technical difficulties associated with intra-arterial drug delivery, the most common way of dosing animals in preclinical studies is via tail-vein injections. Even though tail-vein injections have little similarity with the clinical

setup, this method is acceptable as long as the frequency of repeated dosing does not cause local tissue damage and necrosis. However, a single bolus injection of a drug or contrast agent through the hepatic artery or portal vain is more desirable since it mimics human setup and delivers much better results. The differences in the microbubble concentrations in the liver following tail-vain, portal vein and hepatic artery delivery are shown in Figures 3.

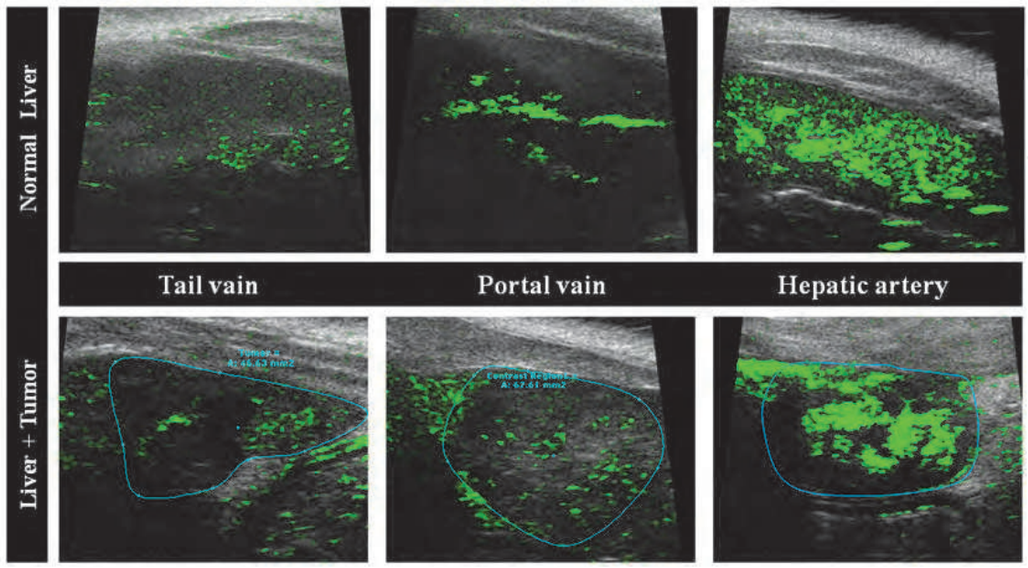

Fig. 3. Ultrasound images of normal rat livers and tumor bearing livers following bolus injection of microbubbles (50 μL) into the tail vein, portal vein, or hepatic artery. Take of the microbubble contrast (green) by the liver is very high if the injection is made through hepatic artery or portal vein, but poor if injection is made through the tail vein.

Microbubble contrast technology is known to be an extremely useful tool for non-invasive assessment of liver vascular structure [29]. Contrast Enhanced Ultrasound Imaging (CEUS) is used in both preclinical and clinical studies to diagnose liver tumors and/or liver metastases, to monitor disease progression, to deliver drugs and to assess the efficacy of antitumor therapies [16,30,31].

3. Tumor assessment using ultrasound imaging

Through both structural and functional imaging, ultrasound can provide an integrated suite of tools to characterize and monitor tumor changes. For pre-clinical use, the low-cost and real-time capabilities allow for high throughput of analyzed animals. In addition, developed imaging biomarkers are often translatable into humans for use in clinical trials.

3.1 Assessment of tumor volume

The resolution of an ultrasound B-scan image increases with frequency, though depth penetration is diminished due to attenuation. For small-animal imaging, frequencies in the range of 20 to 50 MHz can provide sharp images of anatomical structure for depths down to 1cm. At 40 MHz, the resolution is on the order of 50 μm [32]. In both xenograft and orthotopic rodent models, tumors can be viewed with well defined boundaries, allowing for quantitative measurement of volume size.

Though 3D matrix array probes are slowly becoming more available, accurate volume measurements can be obtained with a mechanical sweep of a 2D array. With the tumor bearing rodent fixed on a platform, a stepping motor translates the transducer across the region of interest. Post-processing of the 2D image sets includes manual outlining of the tumor on individual slices to estimate the volume. For small tumors (less than 2 mm^3), the coefficient of variation for tumor volume measurements has been shown to be on the order of 10% [33]. Delineation of tumor boundaries can be further improved with use of the contrast (Figure 4).

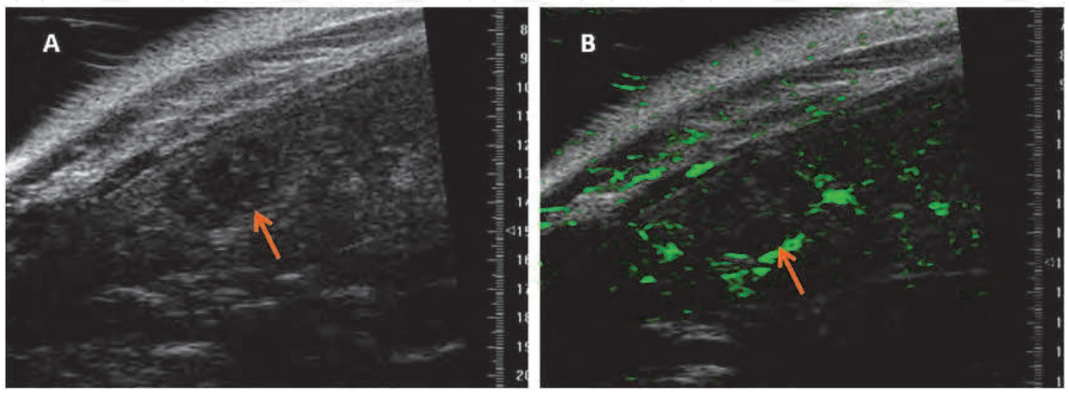

Fig. 4. Contrast enhanced ultrasound improves tumor identification and measurement. The left image (A) was taken without contrast, and although a shadow is visible (arrow), the tumor is difficult to identify. After contrast administration (B) a tumor mass (arrow) is clearly defined and can be readily quantified.

3.2 Assessment of tumor vascularity using Doppler

Since angiogenesis is a major enabling factor of tumor growth, the ability to image and monitor tumor vascularity is of primary interest in oncology applications of US technology. Doppler ultrasound is the standard modality for measuring blood flow. As a transducer emits an acoustic pulse, the echoes from moving scatters are shifted in frequency. In brief, the pulsed-wave Doppler sends short pulse bursts and gates the return signal corresponding to a specific depth. The display of tumor vasculature shows the velocity spectrum as a function of time. This technique is most useful for looking at velocity profiles of flow in macrovessels such as hepatic veins and arteries (Figure 5).

Power Doppler, which uses the integrated power of the Doppler signal, does not provide directional flow information, but is angle independent and more sensitive to slow flow than frequency-shift-based color Doppler. By using the clinical scanners, vessels as small as 100 μm diameter can be clearly visualized. Quantification is typically based on calculating the percentage of colorized-pixels in a region of interest. Power Doppler has been shown to have a strong correlation (r=0.98) with tumor blood vessels as determined by histological staining [34]. Regional variations, rim versus tumor core, can also be evaluated as well as heterogeneity of tumor vasculature.

3.3 Assessment of tumor perfusion using contrast imaging

Within the past decade, ultrasound contrast agents have been increasingly used to characterize vascular dynamics. The current generation of contrast agents is composed of 1-10 μm sizedmicrobubbles with a perfluorocarbon or hexafluoride gas core surrounded by a lipid shell. Injected into the blood stream, microbubbles are stable in the circulatory system for over 10

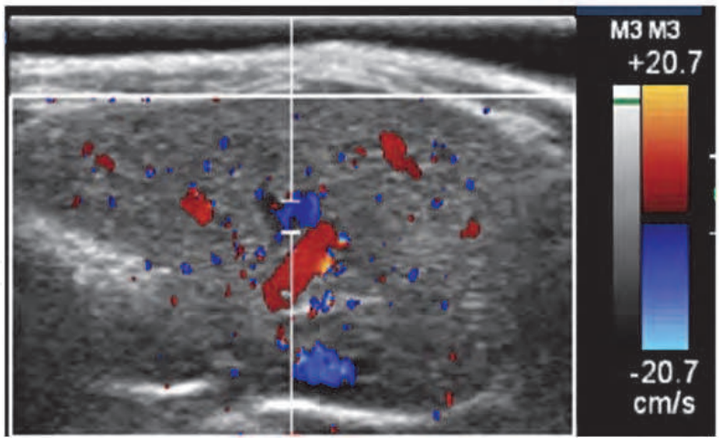

Fig. 5. Example of color Doppler imaging of the normal rat liver. Color Doppler shows directional flow allowing for visualization of the hepatic artery tree and portal vein. A pulse wave Doppler range gate is placed over the colorized vessel to measure flow velocity.

minutes, and unlike MRI contrast agents, the microbubbles remain in intravascular compartment until excreted from the system. Two methods are available when using the contrast agent to assess tumor perfusion; power Doppler and dynamic contrast imaging:

Use of power Doppler. When insonified by a high power ultrasound pulse, microbubbles disintegrate emitting a non-linear signal detectable with Doppler. Power Doppler imaging using contrast agents and a high mechanical index allows detection of vessels down to capillary size and is independent of flow velocities [35]. Total vessel fraction within a tumor can thus be computed from a 3D sweep and the ratio of colorized pixels to the total number of pixels within the tumor can be calculated.

Use of dynamic contrast imaging. The injection of a bolus of contrast can be monitored with low power (less than 0.1) mechanical index. Simultaneous recording of the intensity changes of the contrast agent as a function of time will provide information on blood flow in a region of interest during the entire time of imaging. In brief, the uptake curve can be fitted to an exponential function using the formula $y=A(1-e^{-\beta t})$ where A is proportional to the microvascular cross-sectional area, and β corresponds to velocity [36]. The product of these two parameters can then be used to quantify perfusion. Additional parameters that have been used in tumor assessment include peak enhancement, area under the curve, and wash-in-time [37]. The values obtained can be averaged across the tumor or evaluated on a pixel-by-pixel case to create parametric maps that demonstrate tumor heterogeneity.

The separation of fast flow (from larger functional vessels) and slow flow (from the microvasculature) can provide further insight to vasculature changes from treatment. In a study of an anti-angiogenic drug in a human/mouse chimera model, the quantification of CE-US imaging wash-in parameters revealed that the prevalence of fast blood flow, but not slow flow (associated with small, leaky vessels), was suppressed in treated tumors compared with control tumors [38].

4. Ultrasound imaging of tumor growth kinetics

4.1 Changes in tumor volume and tumor composition

In both the xenograft and orthotopic models B-mode imaging using high-frequency ultrasound can provide valuable data on the volume of the tumor. Traditional tumor volume measurement in xenograft models uses caliper measurement of the tumor width

(W) and tumor length (L). An ellipsoid shape is assumed, and the volume computed as $V=1/2\ (L \times W^2)$. Using this technique, inter-observer variation is 15% [39]. Measurement of tumor volume using calipers is not possible in orthotopic animal models known to much closer resemble local and tumor vascular events seen in HCC patients relative to xenograft models [17]. Volume size estimates from a 3D ultrasound image set are more accurate due to the ability to account for non-ellipsoidal shapes. Currently, there are commercially available software that can view individual slices and manually outline the tumor on individual slices in both xenograft and orthotopic tumors in rats and mice. By interpolating between slices, an accurate representation of the 3D tumor shape is provided along with the volume estimation. For fine sweeps, not every slice needs to be evaluated. We have found that with a slice separation in the range of 100 to 300 μm, manual tumor delineation on every 10^{th} slice provides accurate volume estimation (unpublished data).

Along with tumor delineation, ultrasound grayscale images can also allow for detection of change in tumor composition including tumor necrosis. Necrotic regions in tumor xenograft appear as fluid filled pockets within the tumor (Figure 6). By using the same techniques utilized to assess the tumor volume, the necrotic volume can also be estimated.

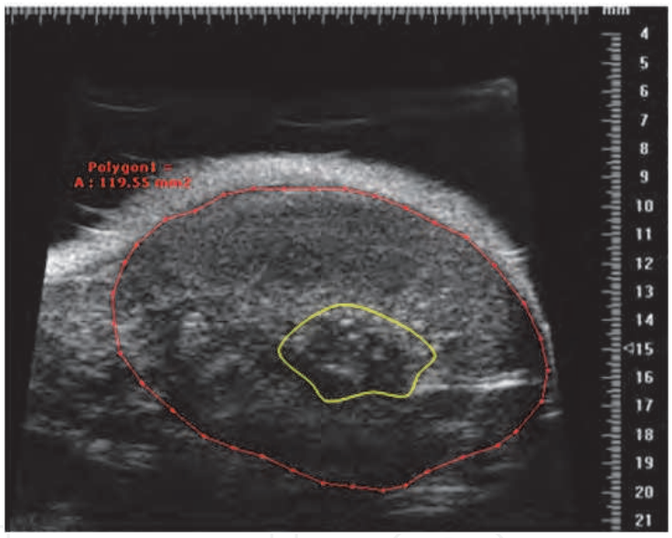

Fig. 6. Example-scan of a xenograft tumor composed of human Huh7.5 cell line outlined in red with necrotic core outlined in yellow.

4.2 Change of tumor vasculature following drug treatment

Dynamic contrast methods may present some difficulties in the rodent liver due to breathing motion. The high frequency ultrasound systems allow for respiratory gating, but the tumor bearing liver in rodents can still be difficult to image with sufficient accuracy. Early testing of anti-angiogenic compounds in both xenograft and orthotopic models have utilized US imaging in order to provide the proof of mechanism and facilitate design of follow-up clinical trials [40,41].

Ultrasound has been shown to help in quantifying changes in tumor perfusion in response to combination therapy [14]. In this study both, an anti-angiogenic mono-therapy and an anti-angiogenic plus a tyrosine kinase inhibitor combination were tested. Under approval of the IACUC, male nude rats (Taconic) were implanted with Huh7.5 human hepatocarcinoma cells

suspended in Matrigel. During the first phase of the study (days 5 - 21 after cell inoculation) rats were treated with either vehicle, sunitinib (Sutent®, Pfizer Inc.) or sunitinib plus a FAK/Pyk2 tyrosine kinase inhibitor (PF-562,271). In the second phase of the study the rats switched treatments so that half of vehicle treated rats remained on vehicle, the other half of vehicle treated rats switched to sunitinib + FAK/Pyk2 compound, rats receiving sunitinib alone switched to vehicle while rats receiving sunitinib + FAK/Pyk2 switched to vehicle.

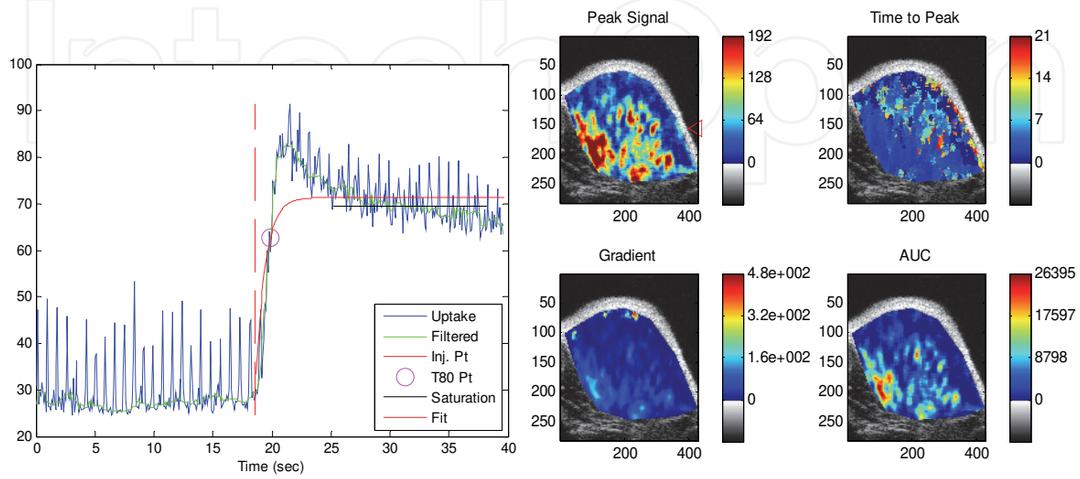

Fig. 7. Examples of parametric maps and uptake curves generated on HCC xenografts using contrast enhanced ultrasound imaging (CEUS).

Using the high-frequency Vevo770 (VisualSonics, Toronto, ON), 3D grayscale volumes were collected along with B-scan cineloops of the center slice of the tumor. These cineloops were collected at 50% power and 100 µl of contrast (MicroMarker, VisualSonics) was given as a slow bolus. Ultrasound imaging, blood and tumor measurements by caliper were collected on day 10, 14, 21, and 28 post cell injection, under isoflurane anesthesia. At the end of the study, day 36, the rats were euthanized and the livers were collected and processed for histology. Ultrasound images were analyzed using in house developed methods based in Matlab (Mathworks). Post processing involved both motion correction and filtering.

Temporal uptake curves were obtained for each pixel, and pseudo-colored parametric maps were created to reflect spatial variation. Regions where the peak signal was less than a predefined threshold were labeled non-perfused. Region averages were then computed to provide data on total peak signal and peak signal without necrosis/non-perfused (Figure 8). Therefore, early testing of anti-angiogenic compounds for proof of mechanism using xenograft model provide valuable data that can be used to improve the design of the studies using orthotopic models as well as to devise a better plan human trials. Non-imaging assessment in the study published by Bagi et al. [13] showed there was a good correlation between results obtained by CEUS and tumor size measured by caliper as well as with serum alpha-fetoprotein levels (tumor cell biomarker).

5. Summary

Preclinical experimentation allows for simultaneous longitudinal implementation of imaging techniques along with use of serum biomarkers to monitor the growth and

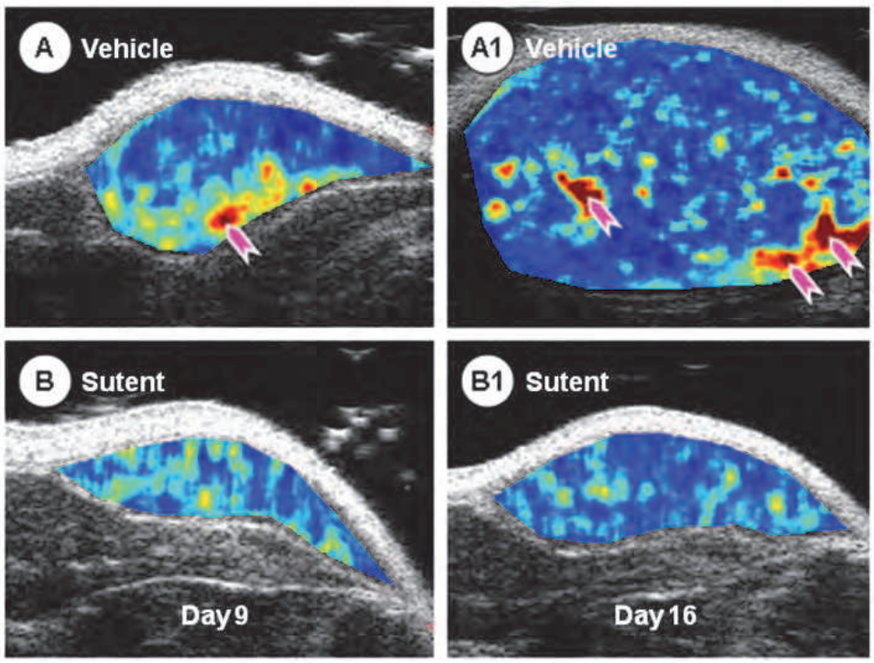

Fig. 8. Representative images obtained by CEUS depicting HCC xenograft treated with vehicle (A) or sunitinib (B) at 2 different time-points. There is a clear difference in tumor size and vascularity between control and treated tumor.

response to treatment of HCC tumors. Although, xenograft and orthotopic models are complementary, and both models have a place in the screening strategy of novel therapies based on the complex vascular events and microenvironment of the liver that plays a role in tumor growth and spreading, only orthotopic liver tumor models can provide the level of complexity that is needed to reliably evaluate the antitumor effects of compounds under investigation in preclinical studies.

The first step to establish the clinical diagnosis of HCC in patients consists of a blood test for elevated AFP concentrations followed by structural imaging utilizing one of several imaging modalities that are currently available including ultrasonography. Ultrasonography has been validated for preclinical and clinical use and it has been one of the most valuable translational tools to assess the efficacy of novel therapies in animal models of HCC as well as in the patients. Additional technological advances toward developing safe contrast agents continue to add value to current ultrasonography methods. Therefore, the thorough preclinical research of HCC should include both, predictive animal models and reliable technologies.

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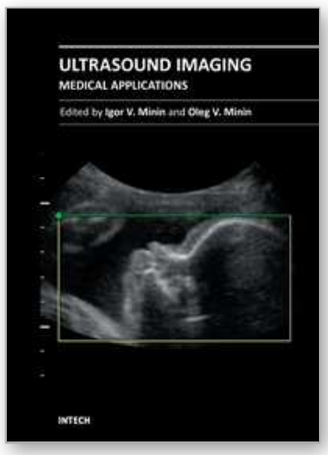

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This book provides an overview of ultrafast ultrasound imaging, 3D high-quality ultrasonic imaging, correction of phase aberrations in medical ultrasound images, etc. Several interesting medical and clinical applications areas are also discussed in the book, like the use of three dimensional ultrasound imaging in evaluation of Ashermanâ€™s syndrome, the role of 3D ultrasound in assessment of endometrial receptivity and follicular vascularity to predict the quality oocyte, ultrasound imaging in vascular diseases and the fetal palate, clinical application of ultrasound molecular imaging, Doppler abdominal ultrasound in small animals and so on.

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