Three-dimensional High-Frequency Ultrasound Imaging for Longitudinal Evaluation of Liver Metastases in Preclinical Models

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Abstract
Liver metastasis is a clinically significant contributor to the mortality associated with melanoma, colon, and breast cancer. Preclinical mouse models are essential to the study of liver metastasis, yet their utility has been limited by the inability to study this dynamic process in a noninvasive and longitudinal manner. This study shows that three-dimensional high-frequency ultrasound can be used to noninvasively track the growth of liver metastases and evaluate potential chemotherapeutics in experimental liver metastasis models. Liver metastases produced by mesenteric vein injection of B16F1 (murine melanoma), PAP2 (murine H-ras–transformed fibroblast), HT-29 (human colon carcinoma), and MDA-MB-435/HAL (human breast carcinoma) cells were identified and tracked longitudinally. Tumor size and location were verified by histologic evaluation. Tumor volumes were calculated from the three-dimensional volumetric data, with individual liver metastases showing exponential growth. The importance of volumetric imaging to reduce uncertainty in tumor volume measurement was shown by comparing three-dimensional segmented volumes with volumes estimated from diameter measurements and the assumption of an ellipsoid shape. The utility of high-frequency ultrasound imaging in the evaluation of therapeutic interventions was established with a doxorubicin treatment trial. These results show that three-dimensional high-frequency ultrasound imaging may be particularly well suited for the quantitative assessment of metastatic progression and the evaluation of chemotherapeutics in preclinical liver metastasis models. (Cancer Res 2005; 65(12): 5231-7)

Introduction
Metastasis, the dissemination and growth of cancer cells in a secondary organ, is the leading cause of cancer mortality. The liver is a frequent metastatic site for melanoma, colon, and breast cancer and therefore an important area of metastasis research. Preclinical animal models, such as the mouse, are essential to the study of liver metastasis, yet their utility has been limited by difficulty in tracking the progression of metastases through time. Noninvasive longitudinal imaging would decrease experimental variability, provide a more accurate assessment of metastatic progression and the efficacy of therapeutic interventions, and allow the study of dynamic processes such as tumor vascularization and dormancy.

A multitude of preclinical imaging modalities are under development, including magnetic resonance imaging (MRI), X-ray computed tomography (CT), positron emission tomography (PET), and fluorescent and bioluminescent imaging, yet no single modality should be considered a comprehensive solution for cancer microimaging applications. Each modality possesses a unique combination of strengths and weaknesses that affect their selection for use in a particular study. In general, desirable characteristics in a noninvasive imaging modality would be high resolution to allow detection of minimal disease, cost-effectiveness and rapid image acquisition to facilitate throughput, inherent contrast between the liver parenchyma and tumor to avoid genetically encoded or endogenously given contrast agents, and applicability to a range of liver metastasis models. MRI offers high-resolution imaging yet may be time consuming and relatively expensive to purchase and operate (1). X-ray CT also offers high resolution, but poor soft tissue contrast necessitates the use of radiopaque contrast agents and radiation dosage may limit longitudinal imaging (1). The resolution of PET does not match that of MRI or CT, and the requirement for production and containment of radionuclides can make costs prohibitive (1). Fluorescent and bioluminescent imaging offer a relatively cost-effective way to study liver metastases but suffer from poor resolution and the requirement to transfet endogenous reporter genes into the cell line of interest (1, 2). The expression of foreign reporter proteins may lead to increased immunogenicity and thus must be carefully examined for their effect on the metastatic model being studied (3–5).

Ultrasound is an attractive option for preclinical imaging due to the cost and time efficiencies of this modality. Previous studies using high-frequency ultrasound imaging of murine cancer models showed the feasibility of this technique to track s.c. tumor progression (6). That study concluded that further application of ultrasound imaging would require a fast method for generating three-dimensional images. A new high-frequency scanner that employs three-dimensional image acquisition methods and reconstruction software developed in our laboratory has addressed this limitation (7). These developments raised the possibility of using high-frequency ultrasound in the evaluation of clinically relevant metastatic models, which are difficult to study in a noninvasive fashion.

We report here the application of a high-frequency (40 MHz) ultrasound system with three-dimensional imaging capabilities to
the study of murine liver metastasis. We showed that the resolution of high-frequency ultrasound allowed detection of liver metastases at a minimum size that compared favorably with that of MRI, CT, or optical methods (8–11). The applicability of this technique was shown by identifying and tracking liver metastases from four tumor cell lines of different tumor origins. The importance of three-dimensional volumetric imaging to reduce uncertainty in volume determination was established by comparison of three-dimensional segmented volumes with the commonly assumed ellipsoidal volume calculated from diameter measurements. The utility of three-dimensional high-frequency ultrasound in the evaluation of chemotherapeutics was shown in a preclinical trial with doxorubicin. These results show that the cost and time efficiencies of traditional ultrasound coupled with the three-dimensional capabilities and high resolution of this high-frequency ultrasound system make this modality particularly well suited to the study of liver metastasis in a wide range of preclinical models.

Materials and Methods

Cell culture and experimental metastasis models. B16F1 (12) and HT-29 (Cat# HTB-38, American Type Culture Collection, Manassas, VA) cells were maintained in oMEM with t-glutamine, ribonucleosides, and deoxyribonucleosides (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma, Mississauga, Ontario, Canada). PAP2 cells (13, 14) were maintained in DMEM (Invitrogen) supplemented with 10% FCS (Invitrogen). MDA-MB-435/HAL cells were maintained in EMEM (Invitrogen) supplemented with 2 mmol/L t-glutamine, 100 mmol/L nonessential amino acids, 25 mmol/L HEPES buffer, 1 mmol/L sodium pyruvate (Invitrogen), and 1× MEM vitamin solution (Sigma). The MDA-MB-435/HAL line was derived from the MDA-MB-435 cell line by an selection with doxorubicin. These results show that the cost and time efficiencies of traditional ultrasound coupled with the three-dimensional capabilities and high resolution of this high-frequency ultrasound system make this modality particularly well suited to the study of liver metastasis in a wide range of preclinical models.

Results

Identification of murine liver metastases using high-frequency ultrasound. To validate ultrasound imaging for the detection of murine liver metastases, mice were noninvasively imaged; once suspected metastases were detected by ultrasound, the animals were sacrificed due to escalating tumor burden, as assessed by ultrasound imaging, or when at least four imaging time points had been acquired to construct a growth curve. Approximately 5 minutes was required to locate, identify and image the liver metastases of each mouse. If the time spent on setup, animal handling, anesthesia, and recovery is included, the average duration of an imaging session was 15 minutes per mouse.

Histology. At sacrifice, the mouse liver was excised and fixed in 10% neutral buffered formalin. Visual inspection validated the tumor size and location depicted by ultrasound imaging. For histologic confirmation, formalin-fixed, paraffin-embedded livers were sectioned (4 μm) and stained with H&E.

Ultrasound imaging. For ultrasound imaging, the Vevo 660 high-frequency ultrasound system (VisualSonics, Inc., Toronto, Ontario, Canada) was used. The Vevo 660 is the second generation of a system described previously (17). The Vevo 660 ultrasound probe has a 40-MHz center frequency with a 6-mm focal depth. The spatial resolution at the focus is 40 × 80 × 80 μm³. Before the first imaging session, the mouse's abdomen was depilated with commercial hair removal cream. During imaging, the mouse was kept under anesthesia with 1.5% isoflurane in oxygen and restrained on a heated stage. During imaging with the immunodeficient NIH III and SCID mice, the animals were handled and imaged in a HEPA-filtered workstation (Microzone Corp., Ottawa, Ontario, Canada). Ultrasound is strongly reflected by the ribcage, which hinders imaging of any tissue located beneath the ribs, such as the lungs and a portion of the liver. Thus, the volume of liver tissue accessible for ultrasound imaging may vary between animals and between imaging sessions for the same animals. In general, we found that a significant volume of the left lateral, left medial, and right medial liver lobes were routinely accessible for imaging. During imaging, two-dimensional images were acquired in the sagittal plane after ultrasound contact gel was applied to the abdomen. For three-dimensional imaging, parallel two-dimensional images were acquired by stepping the transducer in 30-μm intervals in the out-of-plane dimension. Using software developed in our laboratory, two-dimensional images were interpolated and reconstructed online to create a three-dimensional volumetric image (7). The system can acquire and produce a typical three-dimensional image in <20 seconds. The three-dimensional reconstruction software is available through VisualSonics, or through the authors for research purposes.

Volume measurements. To determine tumor volume, the boundaries of a metastasis were outlined within parallel planes separated by 50 μm in the volumetric image. The total metastasis volume was calculated by summing the outlined areas and multiplying by the interslice distance (18). Segmented volumes were compared with ellipsoid volumes estimated using the formula \( V = \pi abc / 6 \). The measurements for diameters \( a, b, \) and \( c \) were obtained from the three-dimensional volumetric images. The sagittal plane showing the greatest tumor diameter was selected, and the greatest diameter \( a \) measured. The diameter \( b \), perpendicular to \( a \), was then measured. The volume was then rotated and the transverse plane showing the largest tumor diameter was selected. The diameter \( c \), perpendicular to both \( a \) and \( b \), was then measured. To determine the % difference between the ellipsoid and three-dimensional segmented volumes the following formula was used: \( 100\% \times (\text{ellipsoid volume} - \text{segmented volume}) / (\text{ellipsoid volume} + \text{segmented volume}) / 2 \).

Longitudinal growth measurements. For longitudinal imaging, the initial imaging time point was based on previous indications of when micrometastases could first be detected by ultrasound. Individual liver metastases were identified and a three-dimensional image recorded. Individual liver metastases were identified on successive imaging dates by their particular liver lobe location, tumor shape, and proximity to landmark structures such as major blood vessels or the liver edges. Landmarks were all internal to the liver, because the liver lobes move in relation to any external landmarks such as the ribs. Animals were sacrificed due to escalating tumor burden, as assessed by ultrasound imaging, or when at least four imaging time points had been acquired to construct a growth curve. Approximately 5 minutes was required to locate, identify and image the liver metastases of each mouse. If the time spent on setup, animal handling, anesthesia, and recovery is included, the average duration of an imaging session was 15 minutes per mouse.

Treatment protocols. The B16F1 liver metastasis model was used to assess the ability of high-frequency ultrasound to evaluate cytotoxic chemotherapeutic agents. At day 7 after cell injection, the first treatment with doxorubicin was given. Doxorubicin (Pharmacia, Mississauga, Ontario, Canada) was given at a previously described treatment schedule (1 mg/kg, 0.1 mL, i.p.) every second day until day 17 after cell injection, for a total of six treatments (19). Control animals received saline control injections (0.1 mL, i.p.). Ultrasound imaging was done from day 8 after cell injection, the earliest time B16F1 liver metastases could be detected in the images, until the end of the experiment.

Discussion

Identification of murine liver metastases using high-frequency ultrasound. To validate ultrasound imaging for the detection of murine liver metastases, mice were noninvasively imaged; once suspected metastases were detected by ultrasound, the animal was sacrificed. Gross pathology and histologic sections verified the presence of a tumor, its size, and location. Ultrasound reliably detected murine liver metastases from the four tumor cell lines tested, B16F1, HT-29, MDA-MB-435/HAL, and PAP2, with excellent agreement among ultrasound images, gross pathology, and histologic sections (Fig. 1A-D). Ultrasound imaging proved highly sensitive to small metastases with a minimum detection size (maximum diameter \( \rightarrow \) segmented volume) of
0.22 mm → 0.01 mm³, 0.47 mm → 0.03 mm³, 0.66 mm → 0.08 mm³, and 0.78 mm → 0.17 mm³ for B16F1, HT-29, MDA-MB-435/HAL, and PAP2 tumors, respectively. As a point of reference, a volume of 0.01 mm³ would be produced by ~700 cells, based on the assumption of a spherical cell volume and a cell diameter of 15 μm.

High-frequency ultrasound can identify areas of liquefactive necrosis within metastases. During imaging of B16F1 liver metastases, it was noted that although the metastases were always clearly delineated from the surrounding parenchyma, the metastases showed a large amount of heterogeneity in their ultrasound backscatter. In a number of metastases, distinct anechoic regions (no texture and dark in appearance) were evident. Histologic examination of these anechoic regions revealed that they are regions of liquefactive necrosis (Fig. 1E). Liquefactive necrosis is expected to be anechoic because the breakdown of necrotic cells eliminates the majority of ultrasound scattering structures from that region of the tumor.

Tracking the growth of individual liver metastases by noninvasive ultrasound imaging. To show the use of ultrasound imaging in the longitudinal study of liver metastases, mice were noninvasively imaged at 2- to 3-day intervals. At sacrifice, gross pathology and histologic sections verified tumor sizes and locations depicted during ultrasound imaging. The B16F1 metastases developed rapidly, forming detectable metastases as early as 10 days after cell injection in this experiment (Fig. 2A). The B16F1 metastases showed exponential growth with an average volume doubling time of 1.2 ± 0.2 (mean ± SD) days. The mean correlation coefficient for fitting an exponential curve was 0.966 ± 0.047. The HT-29 and MDA-MB-435/HAL liver metastases were much slower to develop, forming detectable metastases at a minimum of 33 days after cell injection (Fig. 2B). The HT-29 and MDA-MB-435/HAL metastases also showed exponential growth with doubling times ranging from 3.7 to 4.8 days for the HT-29 metastases and 5.7 to 10.4 days for MDA-MB-435/HAL metastases. The correlation coefficients ranged between 0.972 and 0.993 for HT-29 and between 0.635 and 0.889 for MDA-MB-435/HAL. The metastasis HT-29-4 was not included in the range of doubling times because its volume did not increase over the 10-day interval that it was imaged. Representative two-dimensional images from the longitudinal imaging of an individual B16F1 metastasis, B16F1-E, are shown (Fig. 2C). Individual PAP2 liver metastases could not be evaluated for longitudinal growth because in this highly aggressive model numerous metastases form and quickly fuse. In such cases, ultrasound could be used to monitor increasing tumor burden, instead of the growth of individual metastases, as an indicator of tumor progression.

Two-dimensional measurement provides frequent overestimation or underestimation of tumor volume as compared with three-dimensional measurement. Tumors are often assumed to have an ellipsoid shape, which allows a volume to be calculated from the maximum widths and length in two-dimensional images. Tumor volumes calculated from this two-dimensional method...
and from three-dimensional segmentation were compared to determine if the assumption of an ellipsoid shape was valid for liver metastases. In the liver metastasis models examined here, there were large differences in the measured tumor volumes between the three-dimensional and two-dimensional methods. The mean percent difference for B16F1 liver metastases was \( \frac{8.8}{23.5\%} \) (range, \( \frac{90.1\%}{53.2\%} \); Fig. 3A). The negative mean indicates that the ellipsoid volume was on average smaller than the three-dimensional segmented volume. For the MDA-MB-435/HAL metastases, the mean percent difference was \( \frac{15.0}{25.3\%} \) (range, \( \frac{45.2\%}{23.1\%} \)) and for the HT-29 metastases \( \frac{7.9}{43.8\%} \) (range, \( \frac{106.5\%}{80.5\%} \)). Three-dimensional surface rendering of the liver metastases allowed visualization of the irregular shapes of some tumors (Fig. 3B-C).

Three-dimensional ultrasound can be used to monitor therapeutic response of individual metastases. To assess the ability of high-frequency ultrasound to evaluate the efficacy of cytotoxic chemotherapeutic agents, the B16F1 liver metastasis model was used. By noninvasively tracking the development of individual liver metastases, it was shown that doxorubicin significantly increased the doubling time of B16F1 metastases from \( 1.4 \pm 0.4 \) to \( 1.7 \pm 0.4 \) days (\( t \)-test, \( P = 0.038 \); Fig. 4A-B). The increased doubling time is apparent in the significant difference between the average tumor volumes of doxorubicin-treated metastases and the control metastases (Fig. 4C).

Discussion
The importance of preclinical animal models in oncological research has supported the development of small animal imaging modalities, including MRI, X-ray CT, PET, and fluorescent

![Figure 2](image-url)

**Figure 2.** Tracking the growth of individual liver metastases by noninvasive ultrasound imaging. A, growth curves of B16F1 liver metastases plotted on a semilogarithmic scale. B, growth curves of HT-29 and MDA-MB-435/HAL liver metastases plotted on a semilogarithmic scale. C, representative two-dimensional ultrasound images of B16F1-E. Sizes of B16F1-E (maximum diameter \( \rightarrow \) segmented volume) are 0.50 mm \( \rightarrow \) 0.06 mm\(^3\) (day 10), 1.07 mm \( \rightarrow \) 0.61 mm\(^3\) (day 14), and 2.09 mm \( \rightarrow \) 3.79 mm\(^3\) (day 18). Bar on the ultrasound images, 1.00 mm.

![Figure 3](image-url)

**Figure 3.** Discrepancy between tumor volumes obtained from three-dimensional segmentation or diameter measurement with the assumption of an ellipsoid shape. A, \( \% \) difference between the ellipsoid volume and the three-dimensional segmented volume for individual B16F1 metastases is plotted versus the mean volume of those two measurements. Solid bars, mean \( \pm 2 \) SD. B, three-dimensional surface rendering of a B16F1 metastasis in which the ellipsoid and three-dimensional segmented volumes are in close agreement (ellipsoid = 5.58 mm\(^3\), three-dimensional = 5.79 mm\(^3\), \% difference = \(-3.70\%\)). C, three-dimensional surface rendering of a B16F1 metastasis in which the ellipsoid and three-dimensional segmented volumes are not in close agreement (ellipsoid = 3.51 mm\(^3\), three-dimensional = 5.03 mm\(^3\), \% difference = \(-35.60\%\)).
bioluminescent based systems. Each of these modalities occupies a niche in noninvasive imaging, based on the unique requirements and constraints of a particular research study. Important factors in choosing the appropriate imaging modality for a particular study may include the anatomic site being imaged, the desired resolution and animal throughput, availability of targeted contrast agents, and cost. In this report, we describe the use of high-frequency ultrasound imaging for the detection and longitudinal tracking of murine liver metastases. High-frequency ultrasound offers distinct advantages as a cost-effective, rapid, high-resolution, and noninvasive imaging system. The ultrasound imaging described here was done without exogenous contrast agents or genetic manipulation of the cell lines being studied. This offers significant advantages both in terms of animal throughput and in the number of animal models able to be studied.

As shown in this report, all four cell lines tested in experimental liver metastasis models, B16F1, HT-29, MDA-MB-435/HAL, and PAP2, showed inherent ultrasound contrast relative to the surrounding liver parenchyma. These cell lines are derived from different primary tumor types, murine melanoma, human colon carcinoma, human breast carcinoma, and oncogene-transformed murine fibroblasts, which shows the wide applicability of this technique. In all cases, the liver metastases were hypoechoic, appearing darker than the surrounding tissue on ultrasound images. Ultrasound imaging was shown highly sensitive with metastases from all four tumor models detected with maximum diameters of <0.78 mm. The consistent background image texture of normal liver parenchyma likely contributed to the detection of small tumors; thus, ultrasound seems particularly well suited for imaging liver metastasis models. To determine the absolute detection limit for any particular cell line, more frequent imaging on a greater number of mice would need to be done.

The ability to track the growth of individual liver metastases over time was shown for B16F1, HT-29, and MDA-MB-435/HAL tumor cell lines. The use of the human cell lines HT-29 and MDA-MB-435/HAL is particularly noteworthy because immunodeficient animals, which must be protected from infection, are used for these metastasis models. The ultrasound system was easily adapted to this requirement by restricting the mice and ultrasound probe to a HEPA-filtered environment. This process would not be possible with larger, less portable imaging modalities.

The longitudinal imaging trials showed the importance of noninvasive imaging in allowing analysis on a per metastasis instead of a per mouse basis. For example, in contrast with the exponential growth seen with other liver metastases in the same animal, the metastasis HT-29-4 did not show a significant increase in tumor volume during the 10 days it was imaged. The volume of this metastasis was constant at 0.03 mm³. The identification of metastases with variable growth patterns would allow further investigation, such as microdissection and microarray analysis, to elucidate the molecular basis of such variations. The monitoring of dormant metastases would also permit the study of host-tumor interactions, such as the role of angiogenesis in the switch from a dormant to progressive phenotype (20). The detection sensitivity of high-frequency ultrasound allows the investigation of processes, such as tumor dormancy and angiogenesis, which may occur very early in the metastatic process.

The longitudinal imaging of B16F1 liver metastases revealed striking changes in ultrasound image texture during tumor development. The most obvious of these changes was the

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development of distinct anechoic regions that were shown to be areas of liquefactive necrosis. The ability to detect the formation of necrosis may be useful in the assessment of vascular targeting agents and antiangiogenic compounds (21, 22). Incorporating Doppler blood flow imaging into studies using the high-frequency ultrasound system may further enhance the assessment of tumor vasculature and hemodynamics (23). The cellular or structural characteristics that cause the more subtle changes in ultrasound backscatter are currently under investigation in several laboratories (24–26).

These studies have focused on imaging the development of individual metastases because of the unique research opportunities that this approach presents. In time, the models used here will form large coalescing metastases, which can no longer be monitored for individual growth characteristics. At this stage, ultrasound imaging could be used to measure relative tumor burden between animals. This application will require further study to determine how tumor burden in acoustically accessible liver areas represent the state of the entire liver.

Because s.c. tumor growth is frequently monitored by caliper measurement and calculation of an ellipsoid volume, we sought to determine if the approximation of an ellipsoid volume was sufficient for monitoring the growth of liver metastases. It was shown that tumor volumes calculated from three-dimensional and two-dimensional methods yielded vastly different results for many metastases. For B16F1 metastases, the mean percent volume difference between the two methods was \(-8.8 \pm 23.5\%\). The large SD indicates that the two-dimensional method often gives large overestimations or underestimations of tumor volume when compared with the three-dimensional method. Because the true volume of the metastases could not be determined, it cannot be definitely stated that one method is more accurate than the other. However, it is reasonable to suggest that the three-dimensional method is more accurate because there is no assumption of a defined shape, and a three-dimensional image allows the operator greater time and control when defining tumor borders. Definition of tumor borders can be done offline with a three-dimensional image, whereas the operator of a two-dimensional system must identify maximum tumor diameters during imaging. Furthermore, previous work with a clinical ultrasound system has shown that the three-dimensional method is more accurate than the two-dimensional method when measuring the volume of regular and irregular shaped phantoms (18, 27). The inaccuracy and variability brought about by assuming a defined shape could hinder the ability to track volume changes in slowly growing metastatic models, the ability to track subtle responses to therapeutic treatment, and the ability to determine if a metastasis is going through a period of dormancy. The elimination of this uncertainty presents a compelling case for using an imaging modality with three-dimensional imaging capabilities.

The use of longitudinal ultrasound imaging in preclinical trials was shown with the anthracycline chemotherapeutic doxorubicin. Longitudinal assessment of individual liver metastases showed that doxorubicin significantly decreased tumor growth rate and tumor volume in the B16F1 liver metastasis model. Significant differences in tumor volume were evident at day 12 after cell injection, after only three doxorubicin treatments, when the average tumor volume in the control group was \(<1.00\ mm^3\) and in the treated group was \(<0.25\ mm^3\). The ability of high-frequency ultrasound to track the progression of micrometastases noninvasively allows the evaluation of therapeutic efficacy on sequential stages of tumor development, from early formation to the development of large vascularized metastases, in a single experiment.

In summary, this report is the first to describe the use of three-dimensional high-frequency (40 MHz) ultrasound imaging in the noninvasive detection and longitudinal evaluation of murine liver metastases. This development is significant in that ultrasound offers rapid, cost-effective, high-resolution imaging that can be applied to a wide range of liver metastasis models without the requirement for contrast agents. Compared with traditional histologic methods, ultrasound imaging may provide a more accurate assessment of tumor progression and chemotherapeutic response, which will open new avenues of investigation into dynamic processes such as tumor vascularization and tumor dormancy.

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References


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