Measurement of Human Choroidal Melanoma Xenograft Volume in Rats Using High-Frequency Ultrasound

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PURPOSE. The purpose of this study was to test the hypothesis that the volume of primary orthotopic human choroidal melanoma xenografts can be quantified noninvasively in the same nude rat over time using a portable, high-resolution, high-frequency ultrasound (HF-US) system.

METHODS. C918 human choroidal melanoma spheroids were implanted in the superior suprachoroidal space of 26 WAG/RijHsd-rnu nude rats. Fourteen rats were anesthetized 14 days after tumor implantation, and HF-US B-scan images of the tumor-bearing eye were captured at 250-μm intervals. Tumor areas were measured on each image and numerically integrated to calculate volume. Tumor volumes were also estimated from serial histologic sections in six rats. Twelve other rats were anesthetized and weighed every 4 to 5 days after implantation for 2 weeks, and HF-US B-scan image series were acquired for subsequent measurement of tumor volume.

RESULTS. Tumors could be visualized as heterogeneous, relatively hyperechoic regions in the superior portion of the eye. These regions were verified as tumor by comparison with histologic sections, and histologic and HF-US volumes were highly correlated (r = 0.961; P = 0.002). For the determination of HF-US volume, the intraobserver variability was 9.7% ± 5.1% (n = 8), and the coefficient of variation for multiple measurements was 12.1% ± 6.8% (n = 12). Tumor volume could be repeatedly measured in the same rat every 4 to 5 days for 2 weeks without significant weight loss.

CONCLUSIONS. HF-US is a safe, practical method to measure tumor volume in the same nude rat over time in this orthotopic xenograft model of human choroidal melanoma. (Invest Ophthalmol Vis Sci. 2008;49:16–22) DOI:10.1167/iovs.07-0379

The annual incidence of intraocular melanoma in the Western world is approximately 6 cases per million.1 It is the most common intraocular malignancy in adults, accounting for 70% of all adult primary eye cancers.2 Approximately 30% of patients with choroidal melanoma die of metastatic disease within 5 years of diagnosis.3,4

In the past several decades, treatment of uveal melanoma has focused on sparing vision while controlling local tumor growth. Although large tumors are still typically treated by enucleation, medium-sized tumors are now usually treated by radiation therapy or some other eye-sparing treatment.5 Despite the continuing debate about whether small choroidal melanomas should be treated or observed for further growth,6,7 there is a growing trend to treat small uveal melanomas if they present multiple clinical risk factors.8,9 Although eye-sparing therapies such as radiation are equivalent to enucleation in terms of long-term survival,9 these treatments do not always succeed in saving patient vision. Recently, two large studies showed that 3 or 5 years after plaque radiotherapy, only 55% to 66% of all patients maintain visual acuity better than 20/200.10,11

These considerations highlight the need for more effective, vision-sparing treatments of primary uveal melanomas that can be used alone or as adjunctive therapy. Development of effective new therapies will require testing in animal models of primary uveal melanoma. Testing of anticancer therapies usually involves the performance of standard tumor growth delay studies, in which tumor size is monitored before and during a specific treatment.12,13 Such a study would require a noninvasive method to follow the growth of primary orthotopic choroidal melanomas in animal models.

Several orthotopic models of human choroidal melanoma have been developed by implanting human choroidal melanoma tissue or cells into the suprachoroidal space of either the immunosuppressed rabbit14–18 or the immunocompromised mouse.19–23 We recently described an orthotopic model of human choroidal melanoma in the nude, athymic rat.24 Methods are available to estimate tumor size noninvasively in these models, including fundus photography,15,16,18 standard ultrasonography,15,16 and magnetic resonance imaging.15,25,26 Unfortunately, it is difficult to perform multiple, serial measurements in athymic rodents with these techniques. Given that repeated removal of nude athymic animals from their housing facility is prohibited by most animal investigation committees, all equipment used to monitor growth must be kept in the facility or be easily transportable. More important, any system must be able to be used in a hood to maintain sterility. These are serious limitations to most imaging methods.

Recently, portable, high-resolution high-frequency ultrasound (HF-US) systems have become available to image small animals. These include the HF-US system for small animal research (model MHF-1; E-Technologies, Inc., Bettendorf, IA) and the VisualSonics Vevo 770 (VisualSonics, Inc., Toronto, ON, Canada). In this study, we hypothesized that the MHF-1 HF-US system could be used to safely and noninvasively estimate the volume of primary orthotopic choroidal melanoma xenografts repeatedly in the same nude rat.

MATERIALS AND METHODS

Animals

The mutant rat strain WAG/RijHsd-rnu was used in this study because these rats are athymic and permit the xenotransplantation of human choroidal melanoma xenografts repeatedly in the same nude rat.
tumor tissue into the choroid. WAG/RijHsd-rnu rats were bred and housed in the Department of Laboratory Animal Resources (DLAR) facility at Wayne State University. All procedures were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Wayne State University Animal Investigation Committee.

Human Choroidal Melanoma Cell Line
We used the human choroidal melanoma cell line C918. This cell line was derived from a patient tumor at the University of Iowa in the 1990s. The cells were maintained in RPMI medium + 10% fetal bovine serum + antibiotic. We previously demonstrated the ability of these cells to reliably generate tumor xenografts in the choroid of WAG/RijHsd-rnu nude, athymic rats.

Growth of Orthotopic Human Choroidal Melanoma Xenografts
C918 spheroids were grown and implanted into the suprachoroidal space of 26 rats as detailed previously. C918 tumor spheroids (three-dimensional aggregations of tumor cells) were grown in Petri dishes on 1% agar using a modified version of the method described by Yuhas et al. Spheroids were implanted into the choroid when they measured 200 to 500 µm in diameter. Tumor implantation was performed under sterile conditions in a BSL2 safety hood in the Wayne State DLAR facility. Rats were anesthetized with a ketamine/xylazine mixture (40/4 mg/kg administered intraperitoneally), and preparacaine HCl (0.5%) was applied topically to the right eye as local anesthesia. The rat was placed on a heating pad over a heating blanket. After topical application of 0.5% proparacaine HCl, the superior surface of the right eye was partially visible. The probe was then moved to the temporal edge of the eye, and an image was captured using the accompanying software. The probe was oriented so that the cornea was to the left of the B-scan image, and the probe was moved vertically until the sclera and lens were visible. The probe was then moved to the temporal edge of the eye, and an image was captured using the accompanying software. The probe was moved nasally in 250-µm steps using the micromanipulator, and images were captured at each step. We attempted to record at least three such image series across the eye during each imaging session. All images were stored for future analysis.

To image the ocular tumor, sterile ultrasound gel (Aquasonic 100; Parker Laboratories, Fairfield, NJ) was placed on the eye, and the membrane-covered probe was positioned over the gel (Fig. 1). The probe was oriented so that the cornea was to the left of the B-scan image, and the probe was moved vertically until the sclera and lens were visible. The probe was then moved to the temporal edge of the eye, and an image was captured using the accompanying software. The probe was moved nasally in 250-µm steps using the micromanipulator, and images were captured at each step. We attempted to record at least three such image series across the eye during each imaging session. All images were stored for future analysis.

Determination of Tumor Volume from Ultrasound Images
The series of scans saved by the E-Technologies software were converted into tiff images using a program written and run in analytical software (MatLab; MathWorks, Natick, MA). The images were loaded as a stack into imaging software (Scion Image; Scion Corporation, Frederick, MD), and the relatively hyperechoic region on each image was
measured using the accompanying outlining tools. The areas were saved in a data file that was used as input for a computer program (MatLab; MathWorks) that calculated tumor volume by numerically integrating the areas using Simpson’s 1⁄3 rule, Simpson’s 3⁄8 rule, or the trapezoidal rule, depending on the number of images available for analysis. For each imaging session, one to five series of images were analyzed, and the average volume was used as the tumor volume for that day.

Before the start of the study, the observer practiced identifying the tumor on HF-US images. Comparisons of HF-US B-scans and corresponding histologic sections were used to help distinguish tumor from neighboring normal ocular tissue. The appearance of the sclera on an HF-US scan was observed from HF-US scans of normal rat eyes. After this training, the areas from individual HF-US scans included in the study were calculated.

**Estimation of Tumor Volume from Histologic Sections**

To estimate the volume of human choroidal melanoma xenografts from histologic sections, serial 10-μm sections were cut from a paraffin block of six tumor-bearing eyes that were embedded after 14 days of tumor growth. Every third slide was stained with hematoxylin and eosin using a standard protocol. Slides were viewed under a microscope (Axiophot; Zeiss, Thornwood, NY), and tumor-containing sections were identified. Sections separated by 200 to 400 μm were photographed with a digital camera (Axiocam; Zeiss) and software. Tumor areas were measured from the digitized images using imaging software (Scion Image; Scion Corporation), and the volume was calculated using the same software described for the analysis of the HF-US images.

**Statistical Analysis**

Because all compared data passed normality tests, statistical comparisons were made using standard parametric tests. Rat weights and tumor volumes at different time points were compared using a Student’s paired t-test. Volumes determined from histologic sections and HF-US images were compared using standard linear regression. In all tests, \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**High-Frequency Ultrasound Imaging of C918 Human Choroidal Melanoma Xenografts**

C918 xenografts appeared as relatively hyperechoic regions beneath the superior sclera (Fig. 2A). The superior portion of the cornea could also be imaged, and the border of the lens appeared as a hyperintense arc in the center of the eyes. Tumor images were heterogeneous and often showed evi-
The hyperechoic region could be identified histologically as tumor (Fig. 2B).

Because the penetration depth was limited by use of a 35-MHz probe, a key imaging parameter was the distance of the membrane from the surface of the superior sclera (Fig. 1). In several rats early in the study, we tested the effect of probe distance from the eye on the calculated volume. As shown in Figure 3, if the probe head was more than 5 mm from the eye, the penetration depth was limited, and the volume was underestimated. Any images collected with a membrane-sclera distance greater than 5 mm were not included in subsequent analysis. Thereafter, we attempted to position the probe 3 mm or less from the eye, but this was not always possible. The ability to position the probe was dependent on how well the eye was proptosed and on the position of the speculum. Fortunately, eyes with larger tumors were often more easily proptosed, which permitted the probe to be moved closer to the surface of the eye and resulted in deeper scan penetration.

For all 156 image series collected in the study, the probe was positioned 3.19±0.85 mm from the nearest point of the sclera (range, 1.3–4.6 mm; n = 156).

As the probe was moved nasally across the right eye in 250-μm steps (Fig. 1), the hyperechoic region changed in size (Fig. 4). In early studies, we sometimes acquired five or more series of images during each measurement session because we were attempting to determine the optimal scanning parameters. Eventually, we decided to obtain three series of images, which would permit us to obtain an average value of the tumor volume. During some measurement sessions, we were unable to obtain three series because the rat awoke from anesthesia after the first or second scan series. During this study, one to five series of scans were recorded per measurement session, with an average of 3.1±1.0 series (n = 50 sessions). A series consisted of 28.2±2.8 individual B-scan images (n = 156 series; range, 21–37 B-scans), for a total scanning distance of 7.0±0.7 mm. This ensured that the series captured images across the entire eye, which was approximately 6 mm in diameter. A single such series took only 2 to 3 minutes to capture.

Tumor Volume Measurement by HF-US

Tumor volume was calculated from series of images such as those shown in Figure 4. In our study on tumor volume after 2 weeks of growth, tumor was evident in, on average, 21 (20.6±3.9 scans; n = 41 series) of the 28 consecutive B-scans constituting a scan series. This translated to a distance of 5.15±0.98 mm in the temporonasal direction. The average C918 volume after 2 weeks was 4.04±1.97 mm³ (n = 14 rats).

When more than one image series could be collected (12 of 14), SD of the volume measurements could be calculated. The
FIGURE 5. Volume determined from HF-US images as a function of the corresponding volume estimated from histologic sections. Error bars show the standard deviations for measurements from 2 to 5 image series. The dashed line is the identity line. The solid line is the regression line: HF-US volume (mm$^3$) = 1.126 (histologic volume) + 0.255, $r^2 = 0.923$, $n = 6$, $P = 0.002$.

The interseries coefficient of variation (relative SD) was independent of the tumor size, and its mean value was 12.1% ± 6.8% ($n = 12$). To evaluate the intraobserver variability, eight image series were analyzed by the same observer on three different days in a blinded fashion. The mean intraobserver coefficient of variation was 9.7% ± 5.1% ($n = 8$).

In six rats, tumor volume after 14 days of growth was also estimated from histologic sections. The volumes calculated by the two techniques were significantly correlated ($r = 0.961; P = 0.002$), though the HF-US volumes tended to be larger (Fig. 5).

Repeated Measurement of Tumor Volume in the Same Rat

It was possible to safely and repeatedly image the tumor-bearing eye of all 12 rats every 4 to 5 days for 2 weeks. Tumor was evident in 14.8 bearing eye of all 12 rats every 4 to 5 days for 2 weeks. Tumor

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The appearance of the tumor changed with each imaging session (Fig. 6). Immediately after implantation, the spheroids were usually visible as a small relatively hyperechoic mass (day 0). As early as 5 days after spheroid implantation, HF-US revealed the tumors as an organized mass. When the eye was imaged on later days, the tumor showed evidence of further growth.

All 12 tumors showed evidence of growth over the 2-week period, though variability among the rats was noted (Fig. 7A).

Six of the tumors began to grow exponentially by day 14, and the other six grew approximately linearly or showed a plateau in growth after 2 weeks. The average tumor volumes on days 5, 9, and 14 after implantation were 1.19 ± 0.78 mm$^3$, 2.28 ± 0.90 mm$^3$, and 5.45 ± 2.79 mm$^3$ ($n = 12$), respectively. The volume on a given day was significantly larger than the volume from the previous measurement session ($P \leq 0.002; n = 12$). For all 26 rats in the study, the average C918 volume after 2 weeks was 4.69 ± 2.44 mm$^3$ ($n = 26$).

Anesthesia and handling of the rats every 4 to 5 days did not result in significant loss in body weight (Fig. 7B). The initial weight of the rats ($n = 12$) was 172 ± 25 g. The rats’ weights did not change on days 5 and 9 ($P > 0.05$) but had significantly increased to 179 ± 28 g by day 14 ($P = 0.006$).

DISCUSSION

HF-US Imaging of C918 Human Choroidal Melanoma Xenografts

HF-US imaging of the rat eye permitted the visualization of human choroidal melanoma xenografts growing in the supraciliary space in this nude rat model at high resolution. The orthotopic xenografts were relatively hyperechoic compared with most other structures in the eye (Figs. 2, 4). The high-resolution images obtained in this study were homogeneous in intensity and permitted discrimination of structures within the tumor, but the exact nature of these structures could not be determined.

Ultrasound has previously been used to image choroidal melanoma xenografts growing in the immunosuppressed rabbit, but typically tumors were only imaged at one time point. Recently, a three-dimensional VHF ultrasound system was used to measure tumor volume and ultrasound backscatter in athymic nude mice bearing orthotopic human choroidal melanoma M619 xenografts (Deobhakta AA, et al. IOVS 2006; 47:ARVO E-Abstract 4702). The present study is the first to present full series of high-resolution ultrasound images of an orthotopic human choroidal melanoma xenograft in the same animal at different time points.

The ability to measure sequences of high-resolution HF-US images of the xenograft in a short period is a major advantage of this system. It permitted multiple series of images to be collected while the rats were under short-lasting anesthesia, which could be repeated every 4 to 5 days for up to 2 weeks, with no significant weight loss. This

FIGURE 6. HF-US images from the same tumor-bearing eye at different times after spheroid implantation. All images are from near the middle of the eye.

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speed of image acquisition is an advantage over some other imaging methods, such as magnetic resonance imaging. In addition, all these measurements could be performed in a sterile environment within the animal facility, minimizing the risk for infection in these immunocompromised rats and enabling us to follow tumor growth for 2 weeks. This would not have been possible with imaging modalities that do not use portable equipment or are not easily performed in a laminar flow hood.

**Volume Measurement of C918 Human Choroidal Melanoma Xenografts**

Series of HF-US images were used to calculate tumor volumes, which were similar to volumes determined from serial histologic sections. The volumes determined from the two methods were highly correlated, but the HF-US volumes tended to be larger (Fig. 5). This result is not too surprising because histologic sections are prone to shrinkage during processing and would be expected to result in smaller volumes than are found in vivo. Of course, it is also possible that the tumor area was slightly overestimated in individual HF-US scans. Although the boundary between the lens and the tumor was clearly delineated, the boundary between the tumor and the sclera was not always as definite. We attempted to minimize this error by training the observer early in the study to distinguish sclera from tumor based on B-scan images of normal eyes and comparison of HF-US tumor images and histology. It should also be noted that we could not distinguish the retina from the tumor in the HF-US scan, so the retina was typically included in the estimate of the tumor. This is evident in Figure 2.

After training, the reproducibility of the volume estimated by the same observer on the same series of B-scan images was very good. Mean intraobserver variability in this study was 9.7%, which is similar to the value of 7.7% found in a study measuring uveal melanoma volume in human patients using three-dimensional ultrasound.30

Typically we collected three series of HF-US images across the eye, permitting us to obtain an average tumor volume for each measurement session. The mean coefficient of variation was 12.1%. In a study using three-dimensional ultrasonography to estimate choroidal melanoma volumes in human patients, the interscan variability was 8.2%.50 Thus, the repeatability of our tumor volume measurements compares favorably with the human ultrasound measurements, especially considering the small size of the rat eye compared with the human eye.

**Serial Volume Measurement of C918 Human Choroidal Melanoma Xenografts**

The ability to measure tumor volume in the same rat every 4 to 5 days for 2 weeks is extremely important for future applications of this technique. It means that tumor growth can be followed serially in an individual animal without removing it from the sterile environment of the housing facility. In future studies, we will attempt to follow tumor growth in the same rat for longer than 2 weeks. Given the variability in C918 growth across tumors (Fig. 7A), tracking volume in the same rat over an extended period should permit us to perform standard tumor growth delay studies to evaluate new treatments of choroidal melanoma using fewer rats.

**SUMMARY**

In summary, for the first time, we have serially estimated the volume of orthotopic human choroidal melanoma xenografts in an animal model using high-frequency ultrasonography. We showed that the tumor could be safely imaged in the rat every 4 to 5 days for 2 weeks and that tumor growth could be tracked in the same animal. This technique should be useful to perform standard tumor growth delay studies in this model and will permit the impact of various treatments of primary choroidal melanoma to be better evaluated.

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**References**