Quantitative Evaluation of Vascular Microcirculation Using Contrast-Enhanced Ultrasound Imaging In Rabbit Models of Choroidal Melanoma

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As in other tumors, determining blood perfusion level is significant to understand microvascular function and clinical status of the choroidal melanoma.1 However, the clinical methods of microcirculation evaluation in melanoma were limited. New noninvasive methods for quantitatively evaluating microcirculation in choroidal melanoma are therefore urgently needed.

Contrast-enhanced ultrasound (CEUS) is a promising technique for monitoring vascular contrast enhancement patterns in real time. Ultrasound contrast agents are composed of microbubbles, which consist of gas surrounded by a stabilizing shell.2,3 Because their acoustic impedance differs from that of the surrounding tissue, microbubbles strongly reflect sound and can be detected and recorded. Additionally, due to the intravascular location of microbubbles, CEUS might be useful for enhancing imaging signals from vessels.

However, CEUS has rarely been used to evaluate blood microcirculation in choroidal melanoma. Firstly, previous studies have focused on the qualitative analysis of CEUS signals, which might vary dramatically depending on the experience of the doctor evaluating the data.4 The CEUS figures of choroidal melanoma were poor. But CEUS has been successfully used to measure blood volume and flow velocity in many other kinds of tumors.5–7 Secondly, in studies, B16F10 and choroidal melanoma cell line (OCM1) animal models are the most widely used.8–10 But few studies have compared these two models in different contexts. Thirdly, because donated eyes are rare in clinical studies, investigations of correlations between time-intensity curve (TIC) blood perfusion parameters and pathologic characteristics in animal models, which have rarely been conducted, may be necessary.11

Therefore, this article was designed mainly to investigate the application of CEUS to quantify vascular perfusion of choroidal melanoma. Further to investigate the efficiency of CEUS in quantifying vascular perfusion of choroidal melanoma, we compared the primary choroidal melanoma and B16F10 ocular melanoma. It is found that CEUS imaging can be used to detect the clear differences of two ocular melanomas in the aspects of microcirculation patterns, blood circulation volume, and velocity. Moreover, the findings in cell lines and tumor growth pattern, indocyanine green angiography (ICGA) and pathologic findings were in coincidence with the CEUS. In the future, CEUS may have a broader set of applications in the clinically evaluation of microcirculation patters in choroidal melanoma,
which is beneficial for diagnosis, treatment decision making, and prognosis improvement of patients.

**Materials and Methods**

All procedures in this study were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The Shanghai JiaoTong University Institutional Animal Care and Use Committee approved the experiment.

**Cell Culture**

The human OCM1 was kindly provided by Shanghai Ninth People’s Hospital (Shanghai, China) and the mouse skin melanoma cell line (B16F10) was purchased from Shanghai Institutes for Biological Sciences (Shanghai, China). Both cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) with 4.5 g/L glucose and 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA). Cultures were incubated at 37°C with 5% carbon dioxide (CO₂), and medium was replaced every 2 to 3 days. Then, 5 × 10⁷ cells were removed and suspended in 500 μL DMEM media without FBS.

**Subcutaneous Cell Implantation in Mice**

A subcutaneous injection of 100 μL of B16F10 cell suspension containing 1 × 10⁷ cells was subcutaneously injected into the back of a C57BL mouse. Similarly, 100 μL of OCM1 cell suspension containing 1 × 10⁷ cells was subcutaneously injected into the back of a Balb/c nu mouse. Tumors were allowed to grow for 10 to 14 days until they reached 1 to 2 cm in diameter.

**Implantation of Tumor Pieces Into the Suprachoroidal Space in Rabbits**

Twelve 2.0- to 2.2-kg albino rabbits were assigned to each group. Rabbits received daily intramuscular injections of 20 mg/kg cyclosporin A (CsA; Cisen Pharmaceutical, Shandong, China) beginning 1 day before tumor transplantation for immunosuppression. Rabbits were weighed weekly and the CsA dosage was adjusted accordingly. The rabbits were monitored daily for 4 to 5 weeks for signs of CsA toxicity, including loss of appetite, diarrhea, vomiting, and weight loss. Rabbits received 2 mL gentamicin orally once daily to treat diarrhea.

The tumor-bearing OCM1 mice were killed and tumors were dissected into 1 × 2 × 2 mm³ pieces without the capsule or necrotic tissue. The pieces were stored in cold 0.9% normal saline on ice until further use. Tumors were implanted into rabbits as previously described. Anterior chamber paracenteses were made with 1-mL syringes to reduce IOP. Using free forceps, the tumor fragment was placed into the suprachoroidal space as posteriorly as possible to facilitate visualization. The sclerotomy site was carefully closed with 8-0 absorbable sutures to avoid extraskleral extension. Proper tumor position was verified by direct ophthalmoscopy through the dilated pupil. To facilitate tumor growth and enlargement, all tumors were implanted in the superonasal quadrant of the fundus. Rabbit models using B16F10 tumor pieces were generated by the same doctor according to the same method.

**Fundoscopy**

Direct ophthalmoscopy was performed through dilated pupils with 0.5% tropicamide and 0.5% phenylephrine hydrochloride (Santen Pharmaceutical, Osaka, Japan) before surgery to rule out any ocular abnormalities and weekly after surgery to monitor tumor development. After 4 weeks, the fundus was photographed using a VISUCAM 200 (Carl Zeiss Meditec A.G., Germany), and digital images were saved.

**Indocyanine Green Angiography**

ICGA was performed at the end of the second week for B16F10 models and at the end of the third week for OCM-1 models. The pupil was dilated 10 minutes before angiographic examination. Rabbits were anesthetized with injections of 1% Pelltobarbital Natricum diluted in saline at 3 mL/kg, and breathing and changes in heart rate were carefully monitored. The rabbits’ heads were positioned such that the camera’s field was centered and focused directly on the tumor region of the fundus. ICGA was performed using a confocal scanning laser ophthalmoscope (HRA; Heidelberg Engineering, Carlsbad, CA, USA). Twenty-five milligrams of ICG (Dyypc, Liaoning, China) was dissolved in 5 mL aqueous solvent. Each rabbit was given a 1-mL intravenous bolus injection of ICG dye (5 mg) followed by a 5 mL saline flush immediately before angiography. Sequential images were obtained continuously for the first 10 minutes, and additional images were taken approximately every 5 minutes for at least 20 additional minutes. During examination, the light intensity of the fundus was monitored and adjusted to match the luminance to ensure that angiogram features were easily visible. The camera timer automatically recorded inflow times and filling times for all eyes examined. All images were saved digitally and examined a single experienced doctor (KL).

**Microbubbles and Animal Imaging**

B16F10 rabbits were observed for 4 weeks and OCM-1 rabbit models were observed for 5 weeks after tumor fragments were implanted into the eyes. Ultrasound images were taken at the end of the experiment, and the eyes were then removed for immunohistochemistry experiments.

In vivo ultrasound imaging was performed by a single experienced doctor (JT) for all 12 animals in each group during the last week of the experiment. Real-time imaging was performed using a high-resolution imaging system (Iu Elite; PHILIPS, Koninklijke Philips Electronics N.V., Eindhoven, The Netherlands). Ultrasound system parameters were optimized for animal use according to the manufacturer’s instructions. SonoVue contrast agent (Bracco SpA, Milan, Italy) was resuspended in 5mL sterile saline (0.9% sodium chloride) and administered according the manufacturer's instructions; rabbits remained anesthetized throughout the scanning process.

**Two-Dimensional Ultrasound**

Ultrasound gel was applied to the rabbits’ eyes as a coupling agent. The probe was adjusted until the tumor was centered on the ultrasound monitor within the focal zone and was immobilized to reduce any artifacts in the images; the probe position was kept constant throughout the experiment for each animal. The acoustic focus was placed at the center of the tumor and tumor length and width were measured at the level of the largest transverse cross-section.

**Color Doppler Imaging**

Colors for color Doppler imaging (CDI; blue or red) were arbitrarily assigned based on the direction of flow with respect to the transducer; flow toward the transducer was represented.
in red, and flow away from the transducer was represented in blue.

**CEUS Imaging**

A L-3 probe (PHILIPS) was used and was also immobilized to reduce any artifact in the images; the probe position was kept constant throughout the experiment for each animal. Real-time imaging began immediately after intravenous injection of 0.6 ml (0.5 to 1 × 10⁶) of microbubbles into the auricular vein with a 25-G needle. Images were taken in the VisualSonic contrast mode at a frame rate of 6 to 8 frames per second.

**Image Analysis**

Digital image files were saved and analyzed offline using commercially available SonoLaver v1.1.15.0 software (Bracco, Milan, Italy). Images were analyzed in random order by a single radiologist with 5 years of experience who was blind to group assignments. Image parameters, including peak intensity (IMAX), rise time (RT), time to peak (TTP), mean transit time (mTT), and quality of fit (QOF) values, were exported as Excel files (Microsoft, Redmond, WA, USA). TIC curves were drawn automatically by the software using a Gamma-variate function model. Hot spots were drawn and the reference, region of interest (ROI) of analysis (the hole mass area), and two other ROIs in solid tissue within the lesions which served as parametric 1 and 2 were analyzed. Area under the curve (AUC), wash-in slope, and wash-out slope were calculated automatically by the software using a Gamma-variate function model. Peak heights less than 5% were disregarded when calculating effective perfusion as represented by AUC.

**Tumor Immunohistochemistry**

The rabbits were euthanized with an overdose of 1% pentobarbital at the end of the experiment. The enucleated eyes were fixed in 4% paraformaldehyde for 24 to 48 hours and then paraffin embedded and dehydrated in increasing concentrations of alcohol. Serial 4-μm sections were prepared and stained with hematoxylin-eosin (H-E) and periodic acid-schiff staining (PAS). For microvessel density (MVD) immunostaining, a mouse monoclonal antibody CD31 (ab9498; Abcam, Cambridge, MA, USA) was used. All tumor blood microvessels were visualized with standard immunoperoxidase staining techniques. Slides were examined at ×100, ×200, and ×400 magnification (BX 55; Olympus, Tokyo, Japan).

**Statistical Analysis**

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS; IBM Corp., Armonk, NY, USA) 21.0 software. Means, SD, and standard error of the mean (SEM) were calculated for all parameters. After homogeneity of variance was evaluated, Kruskal-Wallis tests followed by Nemenyi tests and Bonferroni corrections were used to assess differences between the groups due to the skewed data distributions. The correlation was calculated using the Pearson model. A P value of less than 0.05 indicated a statistically significant difference.

**RESULTS**

**Tumor Formation in the Two Animal Models**

Tumors formed in all mice in each group. The OCM1 cells and B16F10 cells were both adherent and consisted of a mixture of spindle-shaped and epithelioid cells. Different from OCM1 cells, B16F10 cells were filled with brown-pigmented granules consistent with melanin. Five B16F10 tumor-bearing C57BL mice developed subcutaneous masses with diameters of 2 cm after 2 weeks, while five OCM1 tumor-bearing nude mice developed masses with diameters of 1 cm after 3 weeks. This indicates that subcutaneous tumor formation was more aggressive in B16F10 tumors.

And the ocular tumor formation rate was higher in B16F10 melanoma than OCM1 melanoma, which indicates the easier formation of ocular mass in B16F10 tumors. The survival rate for rabbits was 83.33% (10/12, SEM = 10.76%) in each group. All of the rabbits that died suffered from severe anorexia, diarrhea, and weight loss by the end of the second week, and none of these rabbits survived longer than 15 days. The tumor formation rate was 60% (6/10, SEM = 12.64%) in OCM1 group rabbits and 90% (9/10, SEM = 9.49%) in B16F10 group rabbits as indicated by direct ophthalmoscopy and ultrasound. Choroidal melanoma tumors grew slowly during the first 2 weeks, and growth accelerated during the fourth week. CsA injections were initially accompanied by loss of appetite, after which the rabbits' weights remained stable for the duration of the experiment.

**Monitoring Tumor Growth With Direct Ophthalmoscopy and Fundus Photography**

Under direct observation, the ocular OCM1 melanoma show as nonpigmented mass and grew slower than ocular B16F10 melanoma. Direct fundoscopy was performed weekly during the 4 to 5 weeks of the experiment to detect intraocular tumors. In OCM1 rabbits, choroidal melanoma was easily detectable 2 weeks after tumor implantation. In contrast, in B16F10 rabbits, tumors could be detected easily through the pupil without fundoscopy due to the dark black appearance of the pigmented focal tumor tissue against the red fundus (Fig. 1). The tumors then grew in height into a mushroom- or dome-shaped mass. Focal retinal detachment also occurred in eyes with relatively big tumors. No severe complications, such as vitreous hemorrhage, choroidal detachment, intraocular inflammation, or cataracts were observed.

**ICGA**

OCM1 melanoma vessels arranged in parallel with small branch networks allowing communication between the large vessels. Differently, vessels of ocular B16F10 melanoma were much more tortuous and dilated. Tumor-bearing eyes in two groups were characterized by obviously dilated intratumoral blood vessels, which were deflected or irregularly tortuous; these features are also present in ICGA images of human choroidal melanoma (Fig. 2). However, while OCM1 tumor vessels were also dilated, they were less irregular and crossed the mass in parallel at different depths. In comparison, B16F10 vessels were much more tortuous and formed focal entanglements on the surface of the mass; vessels in the deep tissues/layers of these tumors were not clearly visible because the thick and opaque tumor cells partially blocked their fluorescence. Leakage from the abnormal choroidal vasculature occurred in late stages in both animal models.

**Ultrasound Imaging Features**

Using gray scale B-scan, tumor size was larger in ocular B16F10 melanoma than OCM1 melanoma, and both kinds of melanoma were characterized by solid choroidal lesions with nonuniform echo. The relatively low intensity might represent apoptosis or necrosis within the mass, while relatively high intensity might represent calcification within the mass (Figs. 5a, 5b). The
Lesions were located nasally compared with the implantation site. Ultrasounds revealed mushroom-shaped mass lesions with choroidal excavation at the lesion basement. Focal retinal detachment and the presence of subretinal fluid were observed around the mass. Tumor lengths and widths were measured at the level of the largest transverse cross section. The mean dimensions of ocular OCM1 tumors were 0.749 ± 0.135 cm (SEM = 0.471) in length and 0.465 ± 0.151 cm (SEM = 0.463) in width, while B16F10 tumors were 0.998 ± 0.320 cm (SEM = 0.143) in length and 0.830 ± 0.225 cm (SEM = 0.255) in width.

Mixed signals of neovascularization in OCM1 melanoma were showed under color Doppler ultrasound, similar with that in B16F10 ocular melanoma. The majority of the colored areas were in the peripheries of the tumors, while the central portions of the tumors appeared relatively anechoic (Figs. 3c, 3d). However, the low-intensity areas in gray-scale images of the lesions did not correspond to the larger anechoic portions of the Doppler images. A pulsatile arterial wave at the bottom of the picture was used for spectral analysis of color Doppler signals (Figs. 3e, 3f).

An example of ultrasound imaging of uveal melanoma in the rabbit eye before and after ultrasound contrast agent (UCA) administration is shown in Figures 4 and 5. After injection, the UCA first filled the posterior pole of the eyeball, corresponding to the large vessels. The tumor and the posterior eyeball filled at the same time. Analysis of uniform mass pixels indicated that UCAs first filled portions of the choroidal melanoma with abundant blood supplies and then extended to portions with lower blood supplies. The tumor appeared as a relatively hyperchoic and heterogeneous region with signs of calcification and necrosis. In addition, anechoic areas identified by CEUS corresponded well to the gray-scale findings, indicating that these areas contained fewer vessels that were not filled with contrast agent.

Descriptive statistics of wash-in time, time to peak, peak intensity, area under the time-intensity curve, and ascending and descending slope are summarized in Tables 1 to 3 and Supplementary Table S1. IMAX and AUC were higher and mTT was lower for melanoma compared with the normal eye wall (P < 0.05). Wash-in and wash-out slopes were bigger in the green and purple TIC curves, which represent the tumor ROI, for tumors compared with the reference (Fig. 6a). So both choroidal melanomas had higher blood supply and blood flow velocities than normal eye wall. But, more importantly, the difference in accurate perfusion patterns was detected in two kinds of choroidal melanoma. It is notable that AUC and mTT were lower for OCM1 masses than for B16F10 masses (P < 0.05) (Supplementary Table S2).

The curve parameters for individual tumors are also displayed as a color-coded map to the right of the original image, which allowed fast and easy identification of hot and cold spots (i.e., areas with abnormally high or low perfusion characteristics; Fig. 6b).

**Tumor Immunohistochemistry**

Macroscopically, intraocular tumors were visible as mushroom-shaped masses that expanded toward the vitreous; B16F10 melanoma was pigmented and OCM1 melanoma was nonpigmented (Fig. 7). OCM1 ocular melanoma was enlarged within uvea without prominent zones of necrosis, while B16F10 ocular melanoma contained large areas of necrosis and pigmented tumor cells within the tumor. OCM1 melanoma tumors, which contained different cell types, were primarily composed of epithelioid cells (70%–80% of the tumor field). In the OCM1 group, seven tumors (87.50%) contained networks of interconnected PAS-positive hollow loops in the extracellular matrix, which were less common in the B16F10 group (Figs. 7c, 7d). No endothelial cells were identified within the matrix-embedded channels using a CD31 immunohistochemical panel. Unlike OCM1 melanoma, B16F10 tumor masses contained “insular cancer” with central vessel for blood supply. Again, no endothelial cells were identified using CD31. In addition, B16F10 melanoma primarily consisted of epithelioid cells (~40%), and the cells were pigmented. Large areas of necrosis were present in both pigmented and nonpigmented cells throughout the B16F10 solid tumor tissue (Fig. 7d).

**Discussion**

In this study, we generated animal models with localized solid soft-tissue human melanoma tumors. The two models differed in growth patterns and vascular microcirculation. CEUS and quantitative analysis of images using IMAX, AUC, RT, TTP, mTT, wash-in slope, and wash-out slope were used to thoroughly examine vascular perfusion in these rabbit choroidal melanoma models. Surgical implantation of solid fragments was suitable...
FIGURE 2. Microcirculation patterns in OCM1 and B16F10 melanoma. ICGA for OCM1 choroidal melanoma located in the upper nasal quadrant of the right eye. (a) Fundus photograph. Note the nerve fiber with white reflection and the dilated choroidal vessels on the top of the tumor. (b, c) ICGA from the early stages after injection. The large vessels on the top of the tumor were filled, and the vessels were arranged in parallel. Note the small filled vessel networks that allow communication between the large vessels. (d, e) ICGA from a later experimental stage. Note the leakage of the melanoma vessels. (f) ICGA for B16F10 choroidal melanoma located in the upper nasal quadrant of the right eye. Fundus photograph. Note the presence of nodular tissue under the mass, which might result from the dispersion of small groups of melanoma cells during rapid tumor enlargement. (g, h) ICGA from the early stages after injection. The large vessels on the top of the tumor were remarkably irregular and tortuous. The black spot in the center might result from the blockage of reflection from the vessels due to the heavy pigmentation of the tumor. (i, j) ICGA from a later experimental stage. Note the leakage of the melanoma vessels. (k) ICGA for another animal. Note the large, tortuous vessels.

TABLE 1. Comparison of Microcirculation Patterns Between Controls and OCM-1 Melanoma Tumors

| Group      | IMAX  | RT    | TTP    | mTT    | AUC    | Wash In Slope | Wash Out Slope |
|------------|-------|-------|--------|--------|--------|---------------|----------------|----------------|
| OCM-1      |       |       |        |        |        |               |                |
| Mean       | 351.3300 | 3.1575 | 3.8317 | 11.7479 | 3987.8125 | 108.0883       | −8.9338        |
| N          | 24    | 24    | 24     | 24     | 24     | 24            | 24             |
| SD         | 178.1720 | 1.01541 | 0.93579 | 8.52534 | 1897.94482 | 77.96012       | 10.53878       |
| Reference  |       |       |        |        |        |               |                |
| Mean       | 100.0000 | 4.8363 | 5.4663 | 19.5238 | 1959.1125 | 17.1513        | −1.4515        |
| N          | 8     | 8     | 8      | 8      | 8      | 8             | 8              |
| SD         | 0.00000 | 1.45062 | 1.54955 | 8.62964 | 754.10751 | 6.63845        | 0.39955        |
| P Value    | <0.01 | <0.01 | <0.01  | <0.01  | <0.01  | <0.01         | <0.01          |

The pooled mean values for each group in each tumor region were calculated using the values of Analysis, Parameter 1, and Parameter 2; these data points were collected for three different tumor areas in each animal and collectively represented the blood flow characteristics for that tumor. Differences between the mean value for the reference and the pooled mean value for the tumor are shown.
for obtaining local elevated choroidal melanoma, which is consistent with Mueller and colleagues’ findings. Several factors might affect the success of such implants, including the cell line used, the implantation position in the fundus, necrosis and calcification in the tumor pieces, rabbit life span, and host immunity.

The melanoma (B16F10 cells) were used to establish one choroidal melanoma model in this study. Previous studies reported some important advantages of the B16F10 melanoma model; for example, the commercially available and easy tumor formation, its origin and genetic characteristics are well documented, the time course of its metastasis is highly predictable, and the mechanisms underlying this metastasis and related immune response have been extensively researched. However, because the orthotopic transplants used typically display pathogenic, morphologic, and molecular characteristics similar to those of the original tumor, it is still questionable to commonly use B16F10 choroidal melanoma models for the further of human choroidal melanoma except for some very specific purposes.

ICGA imaging of ocular melanoma allowed for thorough characterization of the microvasculature patterns on the tumor

<p>| Table 2. Comparison of Microcirculation Patterns Between Controls and B16F10 Melanoma Tumors. |</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>IMAX</th>
<th>RT</th>
<th>TTP</th>
<th>mTT</th>
<th>AUC</th>
<th>Wash-In Slope</th>
<th>Wash-Out Slope</th>
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<td>27</td>
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<tr>
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<td>2.51009</td>
<td>5.43025</td>
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Values are defined and arranged as in Table 3.
surface and in superficial tissue. First, consistent with the findings of Mueller et al., tumor vessels could be easily identified in early stages, and the amount of dye in the vessels slowly decreased in later stages. Here, we observed this late leakage in the tumor region in both ocular masses, but it was not observed in melanoma patients by Mueller et al. Second, networks of vasculogenic-mimicking back-to-back loops were presented between the large parallel vessels within OCM1 tumors, but not within B16F10 melanomas. According to Maniotis et al., the loops forming networks between the vessel branches represented the microcirculation patterns of the melanoma. And the microcirculation patterns of choroidal

FIGURE 3. (a, c, e) Gray-scale and Doppler ultrasound of OCM1 melanoma. (b, d, f) Grey-scale and Doppler ultrasound of B16F10 melanoma. Both types of intraocular tumors appeared as solid choroidal lesions with nonuniform echo; tumor length and width were measured in the largest plane of the tumor under ultrasound. Mixed signals of neovascularization and the pulsatile arterial wave were observed under Doppler ultrasound.
Figure 4. CEUS imaging of OCM1 melanoma. (a) The UCAs reached the posterior pole of the eye first. (b–d) Large vessels in the melanomas appear as bright cords, which filled as rapidly as the posterior vessels were and regularly arranged throughout the mass. Low intensity areas (white star) might represent calcification in the tumor. (e, f) Contrast faded faster from the center outward in the melanoma compared with the normal eyewall.

Table 3. Comparison of Microcirculation Patterns Between OCM-1 and B16F10 Melanoma Tumors

<table>
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<tr>
<th>Group</th>
<th>IMAX</th>
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<th>mTT</th>
<th>AUC</th>
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melanoma were relevant to the vascular structure deep in the tumor. So the networks showed in ICGA images of OCM1 melanoma may represent the PAS-positive vasculogenic mimicry in histopathologic within the tumor, which was not found in B16F10 melanoma in this study. The ICGA images as Frenkel et al.\textsuperscript{15} demonstrated, rapid ICG infusion allows for observation of rapid blood circulation, but not stagnant blood pools.

The high predictive power of fine needle aspiration biopsy (FNAB) for choroidal melanoma was proven. Original descriptions of diagnostic FNAB for undetermined intraocular mass was made by Jakobiec in 1979,\textsuperscript{16} feasibility of FNAB of uveal melanoma was firstly reported by Augsburger et al. in 2002,\textsuperscript{17} and safety of prognostic FNAB for uveal melanoma has been reported by some studies from 2008.\textsuperscript{18,19} As their occurrence of complications has not been reported during the follow-up in the scientific literature, studies widely assured the safety and reproducibility of FNAB for patients with rare complication.\textsuperscript{18–20} Now FNAB was accepted as a routinely recommended for individualized prognostication examinations. These prognostication examinations on the obtained specimens mainly included cytopathologic assessments, including fluorescent in situ hybridization (FISH), cytogenetic analyses, and gene expression profile (GEP).

However, FNAB alone is difficult to provide analysis of the microcirculation patterns within the choroidal melanoma. It is widely accepted microcirculation features by histopathologic analysis could provide important prognostic estimate for uveal melanoma. As Correa and Augsburger\textsuperscript{21} have reported, FNAB may more commonly yield sufficient material for genetic analysis or cytopathologic assessments. However, it was argued that maybe FNAB alone is inadequate to obtain representative volume of tumor tissue for histopathologic analysis.\textsuperscript{22} So FNAB
hot circulation within the melanoma is shown with 
complementary to clinical judgement of the choroidal melano-
the evaluation of the microcirculation could be profound and 
able and comparable perfusion kinetics, which could be an 
evaluate blood perfusion level, in particular providing measur-
vessels inside the tumor in vivo. In this study, the CEUS was 
through assessment the blood volume and velocity of the 
so the combination of molecular testing by FNAB and 
material may not be representative of the tumor microcircu-
and nonuniform blood 
circulation within the melanoma is shown with hot and cold spots.

quantifying vascular perfusion of choroidal melanoma, we compared the primary OCM1 choroidal melanoma and B16F10 ocular melanoma. It was found that AUC, RT, TTP, and mTT values were higher for ocular B16F10 melanoma than that for OCM1 melanoma.

Figures 6. Analysis of time-intensity curve. (a) Note the shape of the TIC curve and the accumulation of parameters. (b) Nonuniform blood performance level of choroidal melanoma is helpful to understand microvascular function and its clinical status.

In this study, CEUS was used to accurately find choroidal melanoma lesions, clearly display lesion shape, and effectively observe tumor-bearing vessels with its higher temporal resolution. CEUS shows the big volume of blood perfusion and fast velocity of blood flow within the malignant choroidal melanoma. IMAX and AUC values were higher, while mTT was lower, for both ocular masses compared with the reference. These results suggest that the tumor tissue received a larger volume of blood more rapidly than normal eye wall tissue. Quantitative analysis of CEUS thus provided more precise information regarding blood perfusion in choroidal melanoma. Results obtained by Zhang et al. in a mouse model of uveal melanoma are similar to our CEUS findings in MVD. However, the eyes of B16LS9 mice are much smaller than and quite different from the human eye. Kang et al. also used CEUS to examine the 9.1 choroidal melanoma model, but coarse analysis of CEUS image features did not provide sufficient information to characterize tumor blood flow in that study; the observed correlations between sonographic tumor size, histologic tumor size, and blood volume were not conclusive.

Moreover, it is firstly studied that CEUS imaging can be used to detect the specific differences between two ocular melanomas in the aspects of microcirculation patterns, blood circulation volume, and velocity. To investigate the efficiency of CEUS in quantifying vascular perfusion of choroidal melanoma, we compared the primary OCM1 choroidal melanoma and B16F10 ocular melanoma. It was found that AUC, RT, TTP, and mTT values were higher for ocular B16F10 melanoma than that for OCM1 melanoma.

In B16F10 choroidal melanoma, higher AUC values may reflect larger tumor volumes and higher blood flow volumes, while prolonged filling times may reflect the low blood velocity. It might be a result of the low density and soft quality of pigmented masses as well as larger tumor sizes and areas of necrosis. Therefore, compared with OCM1 melanoma, microcirculation of choroidal B16F10 melanoma contains higher blood volume and flow velocity. In addition, homogeneous enhancement was observed more often in OCM1 tumors than in B16F10 tumors. So the blood perfusion pattern in melanoma originating in mouse skin differed from that in a human ocular origin. So we speculated that CEUS could be used to differentiate choroidal melanoma from different origins. But the CEUS imaging characteristics of other various primary choroidal melanomas needed to be studied further.

Many factors can affect CEUS images. Portions of the solid tumors showed no evidence of filling, which was consistent with gray scale and histopathology findings indicating calcification and necrosis. IMAX, mTT, and AUC values for tumors are greatly affected by the dose of contrast agent used; higher volumes and concentrations of contrast agent prolonged the contrast imaging period and increased the contrast intensity in tumor images. Here, SonoVue was used within 2 hours after it had been opened to prevent degradation. The physical condition of the animals, especially with regard to blood circulation, could also affect lesion filling and clearance of the contrast agent through the lungs. Weaker circulation could significantly prolong rise time and time to peak intensity and reduce IMAX for the filling compared with animals with normal circulation. Finally, probe focus during imaging was offset from the tumor lesion to reduce potential destruction of microbubbles due to the echo wave.

A large number of patterned solid and hollow matrix channels were observed in PAS-stained paraffin slides of some OCM1 melanomas. In 1999, Manitotis et al. originally reported vasculogenic mimicry (VM) patterns in highly aggressive uveal melanomas, which formed by tumor cells
instead of endothelial cells. Since then, VM has been observed in several other aggressive tumor types. Although these masses were larger in volume than those without PAS-positive hollow matrix channels, the correlation between sonographic blood volume and histologic tumor vascularity was not significant. In a previous study, back-to-back hollow channels contained Matrigel or dilute Type I collagen without endothelial cells or fibroblasts, and were absent in normal melanocytes or poorly invasive melanoma. Some of the hollow channels permitted red blood cells to pass through. As a result, mean vessel density counts, which were dependent on endothelial cells stained for CD31/CD34/factor VIII, were not fully representative of effective microcirculation. A new pathologic staining method was therefore required to accurately quantify effective microcirculation in human ocular VM.

In our study, VM structures differed between the two kinds of melanoma. Different from OCM1 melanoma, the B16F10 tumor was supplied by the vessels in the center of the cancer nests. Because these vessels were not positive for CD31 or CD34 staining, they were probably not normal vessels covered with endothelial cells. Chen et al. found that VM was present in B16 ocular melanoma in C57BL mice, and red blood cells were able to pass through the VM. In OCM1, VM was abundant in the matrix and collagen as indicated by PAS staining, while in B16F10 VM was covered endothelial-like cells and was less prominent in the extracellular matrix. Moreover, the number of hollow VM tubes was also higher in OCM1 melanoma than in B16F10. The reasons for these differences VM between the animal models remain unclear, and the mechanisms underlying VM development in these tumors require further study.

Some limitations should be considered when interpreting our results. First, the sample sizes are small. Second, the growth pattern and development of choroidal melanoma may differ between the rabbit model used here and human patients. CEUS data obtained from patients will help determine the clinical applicability of our findings. Third, only malignant tumors were examined in this study, and additional studies are needed to establish a stable animal model of benign choroidal tumors to allow for comparison of circulation patterns. Finally, additional challenges need to be addressed before fully quantitative eye imaging protocols can be developed.

Due to the limitations of our study, it needs to be investigated and verified on patients of choroidal melanoma in different hospitals before this quantitative imaging could be accepted as a routine tool. And it is still necessary to continuously explore the effects of CEUS based on new problems, such as in the therapeutic process of choroidal melanoma. The targeted microbubbles can specifically bind with ligands to tissue receptors reflective of neoplasia tissue, to provide precise information of microcirculation function and activity in molecular way. Now targeted CEUS has been studied and applied in some tumors, such as breast cancer and ovarian cancer, but it has not been studied in choroidal melanoma. Also, future developments for CEUS include targeted drug and gene delivery, although it is probably still many years away.

**CONCLUSIONS**

In this study, we successfully developed two different rabbit models of human choroidal melanoma by surgically implanting tumor fragments into the suprachoroidal space. CEUS could be used to effectively detect the microcirculation patterns and quantify blood circulation volume and velocity in ocular melanoma in these two animal models. We found that B16F10 melanoma had higher blood flow volumes and slower blood flow velocity than OCM1. Our findings might help advance the clinical use of CEUS and TIC curve analysis to examine blood supply in choroidal melanoma in order to assist in accurate diagnosis and evaluation of prognosis and treatment efficacy in the future. The development of the CEUS targeted with specific molecule could provide effective

**FIGURE 7.** Differences in tissue structure and vascular channel formations between OCM1 and B16F10 melanoma. (a–c) OCM1 melanoma was nonpigmented. Necrosis and/or calcification appears as red areas within the tumor. Microcirculation was abundant in the tumor. Vascular mimicry was formed by tumor cells and extracellular matrix. Red blood cells were able to pass through the PAS-positive hollows (black arrow). (d–f) B16F10 melanoma formed dark brown pigmented areas within the tumor. Tumor cells formed "nests" with large vessels supplying blood to the center of the "nests."
evaluation of the function and status to help the doctors judge the progression and prognosis of the choroidal melanoma. But continued improvement of functional imaging techniques in ophthalmology is needed.

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References


