Original Investigation

# Angiogenesis Research in Mouse Mammary Cancer Based on Contrast-enhanced Ultrasonography: Exploratory Study

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**Rationale and Objectives:** The objective of this study was to investigate the contrast-enhanced ultrasound (CEUS) characteristics of tumor angiogenesis in mouse mammary cancer.

**Materials and Methods:** Twenty-four mice were examined with ultrasound and CEUS at 2–12 days after implantation. Four to five mice were assessed daily, and one to three mice were then sacrificed for histology. All of the histologic slides were reviewed and correlated with CEUS findings.

**Results:** A total of 46 cases of ultrasound examination had been performed in 24 mice. The mice were classified into three groups according to the tumor growth: group 1 (2~6 days after implantation, $n = 20$ cases), group 2 (7~9 days after implantation, $n = 15$ cases), and group 3 (10~12 days after implantation, $n = 11$ cases). In group 1, all tumors presented as a homogeneous hypoechoic mass with no color Doppler signals. However, three CEUS patterns were observed: 14 tumors presented as type I (peripheral ring enhancement with no enhancement within the tumor), 4 tumors presented as type II (peripheral ring enhancement with deep penetration), and 2 tumors presented as type III (homogeneous or heterogeneous enhancement in the entire tumor). In group 2, there was only difference in the echo (heterogeneous or not) and color Doppler signals (with or without) among the tumors in conventional ultrasound, but four CEUS patterns were observed and most presented as type III (53.3%, 8/15). In group 3, most tumors presented as a heterogeneous solid mass (81.8%, 9/11) with color signals (100%, 11/11), and almost all tumors presented as enhancement of type IV (peripheral ring enhancement with focal nodular enhancement) (90.9%, 10/11).The histologic results showed that the enhanced areas mainly corresponded to tumor cells, large tortuous vessels, and an inflammatory cell infiltrate. Nonenhanced areas corresponded to large areas of necrotic tissue or tumor cells, which arranged loosely with the small zone of necrosis.

**Conclusions:** CEUS could image the progression of vessel formation. Moreover, most importantly, CEUS is able to identify angiogenesis before the change of tumor color Doppler, and presents different enhanced patterns at different tumor growth times, which corresponded to tumor histologic features.

**Key Words:** Breast cancer; animal model; contrast-enhanced ultrasound; tumor angiogenesis.



Breast cancer angiogenesis plays an essential role in tumor growth, invasion, and metastasis. The assessment and follow-up of the angiogenic process are important for breast cancer diagnosis and for evaluating therapeutic efficacy (1,2). Compared to a genetically engineered mouse model and a dimethylbenz(a)anthracene-induced animal model, a transplanted mouse model has the characteristics of stable tumor incidence rate and reproducibility, which made it suitable for tumor study. Therefore, transplanted mouse models are widely used in the field of pharmaceutical development and research, such as the field of targeted tumor therapies (3,4). Therefore, a kind of effective imaging method to assess dynamic changes in tumor angiogenesis in vivo is urgently needed.

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The recent developments of microbubble contrast agents and the ability to use microvascular imaging techniques (MVIs) have improved the detection of characteristic neovascular morphologic features by depicting microvessel perfusion (5). Furthermore, recent studies of breast tumors have indicated a significant correlation between direct pathologic vascularity assessments, such as the microvessel density (MVD), and postcontrast ultrasonic vascularity measurements, particularly for vessels 20–39 μm in diameter ($r^2 = 0.16$; $P = .01$) (6). This correlation suggests that contrast-enhanced ultrasound (CEUS) may provide a noninvasive measure of breast tumor neovascularity. In the present study, we establish a mouse mammary cancer model and monitor the tumor size and blood dynamics characteristics in different growth time points with ultrasound and CEUS to assess tumor angiogenesis dynamics in vivo.

## MATERIALS AND METHODS

### Mice Mammary Cancer Samples

The Institutional Animal Care and Use Committee approved the present study of cancer in mice. All procedures involving mice were conducted in accordance with the National Institutes of Health guidelines concerning the use and care of experimental animals.

A total of 24 female laboratory 615 mice (mean weight 16–18 g) were used in the experiment. Mammary cancer cells (Ca761, obtained from Dr. Y.-Q. L., Cell Resource Center, Basic Medical Sciences Institute, CAMS, Beijing, China) were grown in tissue culture. Approximately $2 \times 10^6$ tumor cells were suspended in 0.1 mL of culture medium and subcutaneously injected into the left thigh of each mouse to establish the animal model. Subsequently, all 24 mice were imaged from 2 to 12 days after implantation. Four to five mice were imaged every day, and then one to three mice were sacrificed for histology, and the rest continued to undergo ultrasound examination every other day.

### Acquisition of Ultrasound and Pathology Data

For the ultrasound study, the mice were anesthetized with 10% chloral hydrate (10 mg/kg, Cell Resource Center, Beijing, China) via an intraperitoneal injection, and the left thighs were shaved. The contrast agent was SonoVue (Bracco, Italy), which consisted of a lyophilized powder of phospholipid-stabilized microbubbles with a mean diameter of 2.5 μm containing sulfur hexafluoride ($SF_6$) gas. The solution was reconstituted by the addition of 5 mL of sterile saline. The ultrasound gel was then centrifuged to remove air bubbles. The space surrounding the animal was completely filled with the warmed gel to provide contact and to keep the animal warm during the imaging session. All of ultrasound examinations were performed by one experienced radiologist using a 12- to 5-MHz linear array on an iU22 scanner (Philips Medical System Ultrasound, Bothell, WA).

With conventional ultrasound, the tumor was localized and imaged along the maximal diameter of the lesion to measure its width and depth (volume = 1/2 width × depth$^2$). The same scanning plane of the maximal tumor diameter was then maintained for CEUS. CEUS examination started when 0.05 mL of SonoVue contrast agent was manually retro-orbitally injected as a bolus. The selected imaging plane remained unchanged during the examination, and the dynamic images were recorded for at least 2 minutes. The following settings were used for the CEUS examination: the selected plane included the lesion and its surrounding normal tissue, if possible; the mechanical index was 0.07; the depth of the imaging was 2.5 cm; and the single focus was placed at the bottom of the image. All imaging parameters were kept constant before and after contrast administration, no more than two injections were administered during a single imaging session, and the time between injections was at least 10 minutes to allow the agent to clear the blood pool. In addition, the probe was stabilized manually, and no pressure was exerted.

After the completion of the experiments, the mice were euthanized with an intracardiac injection of pentobarbital (20–30 mg/mL). Tumors were surgically removed and specimens were sectioned in the same planes as the ultrasound images. Each specimen was labeled by specimen ink, marking the true front, back, right, and left. Then specimens were fixed in formaldehyde, embedded in paraffin, cut into slices of 5-mm thickness parallel to the longest axis, and stained with hematoxylin and eosin. The histologic interpretations were performed by one pathologist (Dr. , with 6 years of experience in breast disease).

### Image Analysis

All ultrasound images were analyzed and assessed in consensus by two physicians (Dr. M.W., with 5 years of ultrasound experience, and Dr. K.-N.L., with 4 years of ultrasound experience). The shape, orientation, echogenicity, distribution of the echo, margin, and color Doppler signals of the lesions were assessed for conventional ultrasound. For CEUS, the images obtained at the time of maximal enhancement were divided into four categories based on the distribution of enhanced areas of the lesion: type I—peripheral ring enhancement with no enhancement within the tumor (Fig 1a), type II—peripheral ring enhancement with deep penetration into the tumor (Fig 1b), type III—entire tumor diffusely enhanced irrespective of homogeneity or heterogeneity (Fig 1c), and type IV—peripheral ring enhancement with focal nodular enhancement in the tumor (Fig 1d). If the enhancement patterns within the same lesion were mixed, only one category was assigned to that lesion based on the most prominent enhancement pattern in the lesion. For example, the mass was classified as having regional enhancement of type IV if less than 50% of the mass was enhanced. Moreover, if more than 50% of the area was enhanced inside the mass, it was classified as an enhancement pattern type III. The distinction was mainly based on subjective evaluation in consensus by the two physicians.