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Contrast-enhanced ultrasonic parametric perfusion imaging in the evaluation of antiangiogenic tumor treatment

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ABSTRACT

Purpose: To assess the validity of contrast-enhanced ultrasonic parametric perfusion imaging in the evaluation of antiangiogenic tumor treatment by using histology as the reference standard.
Materials and methods: H22 hepatoma-bearing mice were treated with thalidomide or placebo by intraperitoneal injection. Contrast-enhanced ultrasound was performed on day 8 after bolus injection of SonoVue. Three different parametric perfusion images were calculated based on the following parameters: area under the curve (AUC), maximum intensity (IMAX) and perfusion index (PI). A score from 1 to 5 (1 = low, 5 = excellent) was used for analysis of parametric perfusion images by two independent readers. Immunohistochemical analysis was performed for evaluation of microvascular density (MVD).
Results: Treatment with thalidomide resulted in a significant decrease in perfusion scores assigned to AUC, IMAX and PI parametric images as compared with control tumors ($P < 0.001$). Immunohistochemistry showed significant decreases of MVD in treated tumors as compared with control tumors ($P = 0.002$). MVD was positively correlated with the perfusion scores assigned to AUC parametric images ($r = 0.568$, $P = 0.009$), IMAX parametric images ($r = 0.614$, $P = 0.004$) and PI parametric images ($r = 0.636$, $P = 0.003$).
Conclusion: Contrast-enhanced ultrasonic parametric perfusion imaging provides a noninvasive tool to directly visualize tumor perfusion changes after antiangiogenic tumor treatment.

1. Introduction

Angiogenesis, the growth of new capillary blood vessels from pre-existing vasculature, is a precondition for growth and the spread of malignant tumors [1]. Selective inhibitors of tumor angiogenesis, therefore, have emerged as a new class of drugs besides established anticancer therapies and a large number of antiangiogenic agents have entered clinical development. However, clinical cancer trials have shown that antiangiogenic agents may not significantly reduce tumor size [2,3], especially soon after initiation of therapy, because antiangiogenic agents do not act directly on tumor cells but on the endothelial cells. Therefore, classic end point analyses based on morphologic criteria (e.g. tumor size regression) may be insensitive to therapy or provide markedly delayed indications of response, even when the therapeutic effect is substantial. With increasingly clinical uses of antiangiogenic agents, there is an urgent need for an imaging technique that can quantify functional as well as structural properties of tumor vascularity to early evaluate their clinical efficacy [4].

Several non-invasive image modalities are being developed in an attempt to monitor tumor response to antiangiogenic treatment, including dynamic contrast material-enhanced magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), and ultrasound [5–7]. Of these, contrast-enhanced ultrasound imaging, utilizing commercially available encapsulated gas-filled microbubbles, plays an important role in this field because it is a real-time, high-spatial resolution imaging technique that can easily be applied to small animal models and human beings without the risk of ionizing radiation.

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Another advantage of contrast-enhanced ultrasound is that it is highly sensitive to extremely low concentrations of microbubbles regarded as a pure vascular agent, which make it have the potential to help visualize the tumor vasculature and quantify tumor perfusion parameters [8,9]. By using the technique that involves the color-coding of tissue perfusion information obtained after the administration of contrast agents, the perfusion parameters of a dynamic study can be directly presented on a single image, which is named as parametric perfusion imaging. Recently, contrast-enhanced ultrasonic parametric perfusion imaging has been used to help characterize liver lesions [10] and identify cerebral perfusion deficits in acute ischemic stroke [11]. To the best of our knowledge, the value of contrast-enhanced ultrasonic parametric perfusion imaging has not been tested in assessing tumor response to antiangiogenic therapy. The present study was designed to assess the validity of contrast-enhanced ultrasonic parametric perfusion imaging in the evaluation of antiangiogenic tumor treatment by using histology as the reference standard.

2. Materials and methods

2.1. Animal model

Experimental protocols were approved by the animal care and use committee of our university under the guidelines of the National Institutes of Health for the care of laboratory animals. A highly malignant mice hepatoma cell line H22 (obtained from State Key Laboratory of Oncology in Southern China) was used to establish an implanted tumor by subcutaneous injection of 2×10^6 H22 cells at the right axillary fossa in 20 Kun-Ming mice weighed from 16 to 18 g [12].

Ten mice were treated with thalidomide (Changzhou Pharmaceutical Factory, Jiangsu Province, China) suspended in 0.5% carboxylmethylcellulose (CMC), administered by intraperitoneal injection (200 mg/kg) once daily beginning 24 h after tumor implantation. Ten mice were administered 0.5% CMC (vehicle media of thalidomide) using the same timing and dosing schedule. Mice were weighed before imaging and imaging was performed on day 7 after initiation of therapy.

2.2. Ultrasound imaging protocol

Ultrasound imaging was performed on day 7 after initiation of therapy. For the ultrasound imaging studies, each mouse was anesthetized by i.p. injection of pentobarbital sodium (75 mg/kg, Sigma, USA). The hair over the imaging site was shaved and coupling gel with a stand-off gel pad was placed on the skin for scanning. Ultrasound images of tumors were obtained on anesthetized animals using an Acuson Sequoia 512 (Siemens, Mountain View, USA) ultrasound equipment associated with a 15L8 linear array transducer (7.0–14.0 MHz). All ultrasound examinations were performed by one investigator (J.H.Z.), who was blinded to the treatment information. Contrast pulse sequence imaging (CPS) was used for evaluation of tumor perfusion (mechanical index: 0.25, frame rate: 5 Hz, dynamic range: 80 dB, depth: 3 cm). Settings were adjusted at the beginning and maintained constant during the experiments.

Before contrast agent injection, the greatest longitudinal, transverse, and anteroposterior dimensions of tumors were measured in fundamental gray-scale imaging using calipers. Tumor volume was calculated using the formula for a prolate ellipsoid: volume = $\pi/6 \times$ length $\times$ width $\times$ depth. The largest cross-section plane of the tumor was imaged with the transducer held in this position throughout the examination.

Lipid-based ultrasound contrast agent SonoVue (Bracco, Italy) dissolved with physiologic saline to 5 ml was used in this study. SonoVue was administered as a bolus (0.1 ml/20 g) into the retroorbital vein using a 27-gauge needle within 1–2 s. To minimize variations in the injection rate, the bolus injection was systematically performed as a brief injection by the same operator. Imaging was recorded on cine clips starting immediately after the contrast agent injection and continuing for 60 s.

2.3. Image analysis

The clips were downloaded as DICOM format for offline processing with the use of SonoLiver software (TomTec Imaging Systems, Germany) on a normal personal computer using a bolus kinetic model. A region of interest (ROI) was drawn along the perimeter of each tumor to generate parametric perfusion images based on video intensity and the ROI was automatically positioned by the software over all implants on subsequent images, with minor adjustments to correct for respiratory motion when necessary.

From a bolus kinetics image series, three different parametric perfusion images were calculated for analysis in this study: (1) area under the curve (AUC) image, (2) maximum intensity (IMAX) image and (3) perfusion index (PI) image. The local parameter of AUC was defined as area under curve from the point of contrast agent arrival to infinite time for each image pixel. The local parameter of IMAX was defined as the maximum increase in the signal intensity produced by the injection of the contrast agent for each image pixel. The local parameter of PI was calculated for each image pixel by dividing the AUC by the mean transit time (MTT, defined as the average time required for the contrast agent to pass through the image pixel). To calculate the parametric perfusion images of a given parameter, the maximum value at each image pixel was identified. By plotting the value of a given parameter for a defined image pixel, a color-encoded image known as a parametric perfusion image can be built with increasing values presented in ascending order of blue, green, yellow and red. A perfusion score from 1 to 5 (1 severely low, 2 moderately low, 3 mildly low, 4 good, 5 excellent) was used for evaluation of the parametric perfusion images. Score 1 mainly consists of a mosaic of blue and green. Score 2 mainly consists of a mosaic of blue, green and yellow. Score 3 mainly consists of a mosaic of blue, green, yellow and red. Score 4 mainly consists of a mosaic of yellow and red. Score 5 is defined as almost red color tone. After observing the color distribution of each parametric perfusion image in the entire tumor area, two independent readers who were blinded to the treatment information subjectively assigned the perfusion score for each tumor.

2.4. Histology analysis

After ultrasound imaging, mice were sacrificed and tumors were removed and fixed in 10% formalin. Tumor tissue sections equivalent to the ultrasound imaging plane were prepared for immunohistochemical evaluation of endothelial cell (CD34) density. Antigen-retrieval procedure using citrate acid (pH of 6.0) was performed. Primary antibody incubation was performed using a rat antimouse CD34 antibody (clone MEC14.7, Abcam, UK) at 1:200 dilution overnight at 4 °C. After rinsing with phosphate buffered saline (PBS), a secondary rabbit antirat antibody (Zhongshan Goldenbridge Biology, Beijing, China) was added and diaminobenzidine (DAB) for color development.

The measurements of microvascular density (MVD) by counting the CD34-stained vessels under light microscopy were performed independently by two experienced observers, who were blinded to the tumor treatment and ultrasound findings according to an established method by Weidner et al. [13]. After the hot spots were identified under ×40-power microscope, three fields were ran-