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● *Original Contribution*

QUANTITATIVE EVALUATION OF COMBRETASTATIN A4 PHOSPHATE EARLY EFFICACY IN A TUMOR MODEL WITH DYNAMIC CONTRAST-ENHANCED ULTRASOUND

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Abstract—Combretastatin A4 phosphate (CA4P) is a vascular disrupting agent that rapidly shuts down blood supply to tumors. Early monitoring of tumor perfusion plays a crucial role in determining the optimal strategy to managing treatment and guiding future therapy. The aim of this study was to investigate the potential value of dynamic contrast-enhanced ultrasound (CEUS) in quantitative evaluation of tumor perfusion at an early stage in CA4P therapy. Central and peripheral perfusion of tumors was detected by CEUS pre-treatment (0 h) and 2, 12 and 48 h after CA4P injection. Two perfusion parameters, maximum intensity (IMAX) and time to peak (TTP), were calculated from the time–intensity curve. After CEUS, the efficacy of CA4P was immediately confirmed by immunofluorescence assay and hematoxylin and eosin, Hoechst 33342 and fluorescein isothiocyanate–lectin staining. In CEUS of the center region of tumors, IMAX gradually decreased from 0 to 12 h and regrew at 48 h ($p < 0.01$). TTP increased only at 2 h. In the peripheral regions, IMAX did not change obviously from 0 to 12 h ($p > 0.05$) and just increased at 48 h ($p < 0.01$). The TTP of peripheral regions had the same tendency to vary tendency as that of center regions. In addition, microvascular density (MVD), vascular perfusion and necrotic area of the tumor were quantitatively analyzed. A close correlation between IMAX and MVD was observed in the center areas of tumors ($r = 0.72$, $p < 0.01$), whereas the correlation between IMAX and MVD in peripheral areas was weak ($r = 0.37$, $p < 0.01$). However, IMAX was positively correlated with tumor perfusion in both center and peripheral areas of tumors ($r = 0.82$, $p < 0.01$, and $r = 0.63$, $p < 0.01$, respectively). Consequently, IMAX was a reliable indicator of tumor perfusion evaluation by CEUS. The use of CEUS to quantify tumor perfusion could a promising method for the early detection of tumor responses in anti-vascular treatment. (E-mail: xiemx64@126.com)

Key Words: **Dynamic contrast-enhanced ultrasound, Anti-vascular treatment, Tumor perfusion, Combretastatin A4 phosphate, Time–intensity curve, Microvascular density.**

INTRODUCTION

Combretastatin A4 phosphate (CA4P) is a tumor vascular disturbing agent (VDA) that has undergone several preclinical and clinical studies (El-Emir et al. 2005; Rehman and Jayson 2005; Tozer et al. 2005). CA4P depolymerizes the microtubules of endothelial cells, leading to cell membrane blebbing. The alteration of the endothelium induces changes in the blood vessels by contraction of the vascular lumen until a vascular obstruction occurs and, thus, results in extensive blood flow shutdown in the tumor (Jugé et al. 2012). Therefore, the change in tumor vascular perfusion may be a promising biomarker that heralds a positive response to therapy.

Several imaging modalities, including dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) (Jugé et al. 2012), computed tomography (CT) (Bellomi et al. 2007), positron emission tomography (PET) (Groheux et al. 2011) and ultrasound (US) (Lassau et al. 2011; Shao et al. 2013), have been found to effectively monitor early responses to anti-vascular therapy by depicting the reductions in metabolic activity or blood flow. However, as a non-radioactive, convenient and real-time technology, US is thought to be a more promising method among them.

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Conventional Doppler ultrasound methods are not very sensitive to the slow blood flow in tumor vessels and, thus, are not sufficiently specific to detect the subtle effects of CA4P in the early stages of therapy (Delorme et al. 2001). When used with an ultrasound contrast agent, such as SonoVue (Bracco, Milan, Italy), CEUS can facilitate continuous and dynamic observation of tumor vessel perfusion (Klibanov and Hossack 2015). With the use of CEUS, capillary blood flow can be visualized in real time, which makes it possible to detect subtle changes in functional vascular perfusion of tumor microcirculation after anti-angiogenic treatment. Multiple studies have verified that CEUS is more sensitive for detecting tumor responses at early stages of anti-angiogenesis therapy than the assessment of tumor necrosis and tumor volume, which are the two gold standard criteria in most clinical surveillance of tumor treatment using the RECIST protocol (Duffaud and Therasse 2005).

Tumor efficacy in response to CA4P activity has been investigated, mainly by MRI (Jugé et al. 2012). However, little is known about the assessment of different regions of tumor tissue at the early stage after administration of CA4P. In this study, we used CEUS to quantitatively evaluate the early efficacy of CA4P in a mouse colon model by quantifying intra-tumoral perfusion in both central and peripheral regions of tumors. The accuracy of CEUS was proved by correlation analysis of CEUS results and histopathology.

METHODS

Cell culture and animal models

All experimental protocols were carried out in accordance with the Rules of Animal Care and Use Committees of Huazhong University of Science and Technology (HUST). Maximum effort was exerted to reduce the number of animals used and to minimize discomfort. The mouse colon carcinoma cell line CT26 (syngeneic with BALB-C mice) was purchased from American Type Culture Collection (ATCC) and maintained in RPMI medium 1640 containing 10% fetal calf serum and antibiotics at 37 °C in a humidified 5% CO_2 atmosphere. For inoculation, approximately 7×10^7 CT26 cells suspended in phosphate-buffered saline were injected subcutaneously into the right flanks (at the level of the liver) of BALB-C female mice (n = 80) weighing 18 to 20 g. After implantation, tumor growth was imaged at day 11 for the ectopic model (Jugé et al. 2012), when the tumors had reached a volume of 100–200 mm^3, in the intermediate angiogenic stage (Jugé et al. 2012).

Combretastatin A4 phosphate treatment

Combretastatin A4 phosphate therapy was started 11 d after implantation. Forty-two mice (14 animals per treated group) were injected intraperitoneally with 100 mg/kg CA4P disodium salt (Meilun, Dalian, China) dissolved in sterile saline. CEUS imaging was performed 2, 12 and 48 h after CA4P injection was begun. Additionally, the pre-treatment mice (n = 14) and a control group not treated with CA4P at 0, 2, 12 and 48 h (six animals per group) were examined with CEUS in the same way. The mice in each group were sacrificed immediately after CEUS imaging (Fig. 1a).

CEUS imaging

Ultrasound imaging was performed on different groups. Ultrasound imaging of tumors was performed using a Philips IU22 ultrasound system (Philips Healthcare, Bothell, WA, USA) with a 9- to 12-MHz linear probe. The contrast imaging mode was used for evaluation of tumor perfusion (mechanical index, 0.07; focal length, 20 mm; frame rate, 20 Hz). These settings were maintained through the experiment. The maximum tumor cross-section plane was imaged by CEUS, and the transducer was held manually in this position throughout the examination. In the image of maximum tumor cross section, the periphery of the tumor was established to be the outer one-third radius of the tumor, and the center area was the inside two-thirds of the tumor. The greatest longitudinal, transverse and anteroposterior dimensions of tumors were measured in gray-scale images using calipers before contrast agent injection. Tumor volume was calculated using the formula for a prolate ellipsoid: volume = $\pi/6 \times$ length $\times$ width $\times$ depth (Lunt et al. 2011; Wang et al. 2013; Zhou et al. 2011).

The microbubble-based contrast agent SonoVue (Bracco, Milan Italy) was used for CEUS. SonoVue was dissolved in physiologic saline to 5 mL and injected as a bolus (0.1 mL/20 g) through the tail vein with a 24-gauge needle, followed by 50 μL of isotonic NaCl solution within 1 s. A dual-mode examination (real-time B-mode and CEUS) was continuously recorded for 60 se on clips starting just after contrast agent injection.

Imaging analysis

Contrast images of each tumor were recorded in DICOM format for offline processing to create a time–intensity curve (TIC) with the use of SonoLiver software (the latest version 1.0; TomTec, Munich, Germany). Five regions of interest (ROIs) were manually drawn on the CEUS image (Fig. 1b). First, the delimitation ROI (*cyan*) outlined the lesion, which encompassed the entire tumor and the healthy liver. Second, an analysis ROI (*green*) of the whole tumor (ROI_{whole}) encompassed the entire tumor, consisting of the viable and necrotic portions within the tumor. Third, a reference ROI ($ROI_{reference}$, *yellow*) was drawn adjacent to the normal liver tissue. Then, the software derived a parametric image of ROI_{whole} through dynamic vascular