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● *Technical Note*

SEMI-AUTOMATED SEGMENTATION OF THE TUMOR VASCULATURE IN CONTRAST-ENHANCED ULTRASOUND DATA

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Abstract—The vascular architecture in tumors contains relevant information for tumor classification and evaluation of therapy responses. To develop a reliable and user-independent analysis tool, a foreground detection algorithm was combined with a maximum-intensity projection to obtain a high signal-to-noise image from contrast-enhanced B-mode data sets, enabling vessel segmentation by thresholding. Parameters describing the density of the vascular network, the number of vessels and the number of branches were extracted. The highly angiogenic A431 tumors had a relative blood volume of 49%, a mean pixel distance to the next vessel of 1.8 ± 0.3 px, 51 ± 29 individual vessels and 478 ± 184 branching points, whereas the more mature and heterogeneous vascularized myxoid liposarcoma (MLS) and A549 tumors had values of 30%, 3.7 ± 2.7 px, 65 ± 12 and 220 ± 159, and 13%, 7.4 ± 2 px, 31 ± 9 and 59 ± 40, respectively. Thus, our semi-automated analysis method enables the extraction of quantitative vascular features that may help to simplify and standardize tumor characterization. (E-mail: fkiessling@ukaachen.de)

Key Words: **Ultrasonography, Microbubble, Tumor, Microvasculature.**

INTRODUCTION

Contrast-enhanced ultrasound (CEUS) is a safe, inexpensive and widely available imaging technique that provides anatomical, functional and molecular information on the vasculature (Baetke et al. 2016). The application of contrast agents enables the visualization of the microvasculature in tumors, which is often very sensitively responding to anti-angiogenic therapies, and has been reported to have prognostic relevance for many types of cancers (Borre et al. 1998; Cheng et al. 2014; Cosgrove 2003; de Jong et al. 2000; Lassau et al. 2010; Uzzan et al. 2004; Wang et al. 2014). Furthermore, morphological characteristics of the vasculature have been identified as helpful imaging biomarkers for the classification of tumors (Xu et al. 2006; Yang et al. 2007, 2013).

To assess characteristics such as microvessel density and vascular morphology, several different image analysis strategies have been investigated (Koda et al. 2004; Xu et al. 2006; Yang et al. 2007, 2013). A very promising technique is superharmonic imaging, which is capable of generating images having a significantly higher contrast-to-tissue ratio in comparison to conventional ultrasound (US) imaging modes (Bouakaz et al. 2003; Kruse and Ferrara 2005). However, this requires a dedicated transducer setup, which is not yet available for clinical use. A more commonly performed technique is the temporal maximum-intensity projection (MIP), also called maximum intensity over time (MIOT) (Palmowski et al. 2010; Wilson et al. 2008). This technique does not require any modification of the US system, as it is a pure image post-processing approach. Modified versions of this technique are already available in clinical US systems and are referred to as micro-flow imaging or microvascular imaging (Du et al. 2008; Yang et al. 2007). The resulting images provide a more complete view of the vascular architecture than single conventional cross-sectional images and are used to diagnose and differentiate various benign and malignant neoplasms, for example, in liver and breast (Du et al. 2008; Wilson et al. 2008; Yang et al. 2007, 2013).

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With respect to cancer systems biology and the upcoming field of radiomics, more and more quantifiable imaging parameters are being extracted to obtain a more holistic description of tumor phenotypes and their inter- and intra-tumoral heterogeneity. For different imaging modalities, algorithms have been developed that aid in the extraction of imageable phenotypic characteristics and relate them to diagnosis, prognosis and prediction of therapy response (Aerts et al. 2016; Coroller et al. 2016; Lambin et al. 2012). To date, US data have not been used for such analysis, possibly because US data are mostly non-tomographic and more difficult to standardize. However, not only have US data not been used, but quantitative vascular features from other modalities have hardly been considered, even though it is known that vascular features are relevant for monitoring anti-angiogenic therapies, tumor classification and the accumulation of intravenously injected (nano)medicines (Koukourakis et al. 1999; Lassau et al. 2007; Sorace et al. 2012; Theek et al. 2014; Yang et al. 2013).

To decrease user dependence and to standardize and improve the analysis of CEUS scans, we developed a new semi-automated vessel segmentation algorithm for standard CEUS B-mode data that extracts various quantifiable parameters of the tumor vasculature. To this end, we tested the capability of the algorithm to identify differences in the vascular structure of A431, myxoid liposarcoma (MLS) and A549 xenograft tumors in mice. The extracted vascular biomarkers presented in this study are considered to be useful for tumor staging, therapy decision support and evaluation of therapy responses.

METHODS

Animals

All experiments were performed in accordance with national and local animal welfare regulations and approved by the national animal ethics committee. Four million A431, MLS or A549 cells were subcutaneously injected into the flank of 6- to 8-week-old nude CD-1 mice (Crl:CD1-Foxn1nu, Charles River, Sulzfeld, Germany) (n = 3 for each tumor cell line). The mice were kept on controlled light/dark cycles, with standard pellets for laboratory mice (Sniff GmbH, Soest, Germany) and water *ad libitum* (according to FELASA [Federation of European Laboratory Animal Science Associations] guidelines). When the tumors reached 6–8 mm in diameter, the mice were subjected to CEUS imaging.

Immunohistochemistry

Five-micrometer-thick sections of formalin- fixed and paraffin-embedded tumors were used for immunofluorescence staining. A xylol/ethanol series was performed to deparaffinize the sections. Afterward, the sections were cooked in citrate buffer (pH 6) for 30 min at 98 °C and allowed to cool down for 20 min. A rat anti-mouse CD31 primary antibody (1:10, Dianova, Hamburg, Germany) was used as a marker to target endothelial cells, followed by a donkey anti-rat Cy-3-labeled secondary antibody (1:500, Dianova). Nuclei were counterstained using 4,6-diamidino-2-phenylindole (DAPI, 1:500, ThermoFisher Scientific, USA). Five images per tumor were acquired using the AxioImager M2 (Carl Zeiss AG, Oberkochen, Germany). For quantification of the CD31 area fraction, AxioVision 4.8.3.0 (Carl Zeiss AG, Oberkochen, Germany) was used. Statistical significance of differences between the three tumor models was assessed by a one-way analysis of variance with Tukey's multiple comparison test. p Values < 0.05 were considered to indicate statistical significance.

Microbubble synthesis

Poly(butyl cyanoacrylate microbubbles (MBs) were synthesized as described by Fokong et al. (2011). The monomer *n*-butyl cyanoacrylate was added dropwise into a watery solution containing 1% (w/v) Triton X-100, having an acidic pH of 2.5. After addition of the monomer, the solution was vigorously stirred for 1 h. To isolate intact MBs with a mean diameter of ~2 μm, several washing and centrifugation steps were performed.

Contrast-enhanced ultrasound imaging

During the entire imaging procedure, the mice were anesthetized using 2% (v/v) isoflurane. Anesthetized mice were placed on a heating pad to maintain a stable body temperature, and a catheter was placed into one of the lateral tail veins. A bolus of 1×10^7 MBs in a volume of 50 μL was intravenously injected. After the injection, an imaging sequence with 50 fps, containing a high-mechanical-index pulse, was recorded using the Vevo2100 imaging system (FujiFilm VisualSonics Inc., Toronto, ON, Canada) and the MS550 transducer, operating at 40 MHz.

Image processing

All computations were performed on a computer with an Intel Xeon CPU X5677 and 96 GB RAM; Windows 7 was the operating system. Raw radiofrequency data of CEUS scans were loaded into MATLAB 2015 a (The MathWorks, Natick, MA, USA) using an algorithm supplied by FujiFilm Visualsonics Inc., which we adapted to our needs. After manual delineation of the tumor boundaries by the user, all subsequent post-processing steps to segment the vasculature were performed using a custom algorithm developed at our institute using MATLAB 2015 a (see Fig. 1).

Tumor segmentation. The first B-mode image of every cine loop was used to manually delineate the tumor margin and to create a binary mask (I_{bin}) of the region of interest.