BIOPHYSICAL IMAGING AND COMPUTATIONAL BIOLOGY

Micro-CT Imaging of Tumor Angiogenesis

Quantitative Measures Describing Micromorphology and Vascularization

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Angiogenesis is a hallmark of cancer, and its noninvasive visualization and quantification are key factors for facilitating translational anticancer research. Using four tumor models characterized by different degrees of aggressiveness and angiogenesis, we show that the combination of functional *in vivo* and anatomical *ex vivo* X-ray micro-computed tomography (μ CT) allows highly accurate quantification of relative blood volume (rBV) and highly detailed three-dimensional analysis of the vascular network in tumors. Depending on the tumor model, rBV values determined using *in vivo* μ CT ranged from 2.6% to 6.0%, and corresponds well with the values assessed using IHC. Using ultra-high-resolution *ex vivo* μ CT, blood vessels as small as 3.4 μ m and vessel branches up to the seventh order could be visualized, enabling a highly detailed and quantitative analysis of the three-dimensional micromorphology of tumor vessels. Microvascular parameters such as vessel size and vessel branching correlated very well with tumor aggressiveness and angiogenesis. In rapidly growing and highly angiogenic A431 tumors, the majority of vessels were small and branched only once or twice, whereas in slowly growing A549 tumors, the vessels were much larger and branched four to seven times. Thus, we consider that combining highly accurate functional with highly detailed anatomical μ CT is a useful tool for facilitating high-throughput, quantitative, and translational (anti-) angiogenesis and antiangiogenesis research. (*Am J Pathol* 2014, 184: 431–441; <http://dx.doi.org/10.1016/j.ajpath.2013.10.014>)

Angiogenesis, the physiological or pathophysiological process of blood vessel formation and growth, plays an important role in both health and disease.^{1–3} As a classical hallmark of cancer, angiogenesis is essential for enabling tumors to grow beyond a size of 1 to 2 mm³.^{4,5} Consequently, inhibiting angiogenesis has been extensively used as a broadly applicable means for attenuating tumor growth.⁶ Numerous antiangiogenic agents have been evaluated over the years, both in animal models and in patients, and six of these formulations are currently approved for clinical use.^{3,7}

To assess the efficacy of antiangiogenic treatments, functional blood vessels in tumors need to be visualized and

quantitatively characterized. At the preclinical level, this is generally done using IHC methods, such as determining the microvessel density in tumors^{8–10} or quantifying the area

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fraction of CD31⁺ vessel structures.^{11,12} Although IHC seems to be the most accurate method for assessing tumor angiogenesis, it has several important limitations, in particular its invasive nature and the inability to visualize three-dimensional structures.

To overcome these shortcomings, IHC is often supplemented by noninvasive imaging techniques, to provide longitudinal *in vivo* information on tumor perfusion and tumor blood vessel functionality.^{13–16} Clinically relevant diagnostic modalities routinely used for anatomical, functional, and molecular angiogenesis imaging include contrast-enhanced (Power Doppler) ultrasound, magnetic resonance imaging, and X-ray computed tomography (CT). Because of its high spatial resolution, reproducibility, user-friendliness, and suitability for high-throughput analyses, CT has become a routinely used method for noninvasively visualizing and quantifying the morphology and the functionality of tumor blood vessels.^{17,18} Preclinically, blood vessels are visualized and quantified mainly by use of contrast-enhanced volumetric CT or micro-CT (μ CT) approaches.^{16,19–23} In this regard, several studies using dynamic contrast-enhanced μ CT imaging for the *in vivo* quantification of tumor angiogenesis and antiangiogenic therapy effects have been published recently, with assessment of vascular parameters such as tumor perfusion, tumor blood flow, vascular permeability, or relative blood volume (rBV).^{24–26} Importantly, however, there has been a lack of systematic study comparing the accuracy, the congruence, and the correlation of functional (steady-state) μ CT imaging to IHC for determining rBV in

tumors. In addition, in no previous studies has the anatomical information that can now be obtained at extremely high resolution by *ex vivo* μ CT been used to quantitatively analyze the three-dimensional micromorphology of tumor blood vessels and to correlate it with the degree of tumor aggressiveness and angiogenesis.

Here, using four tumor models with different vascular characteristics and a combination of *in vivo* and *ex vivo* μ CT, we show that functional μ CT imaging allows highly accurate quantification of the rBV in tumors, with values correlating very well with those obtained using IHC, and that anatomical imaging allows ultra-high-resolution structural analyses of the three-dimensional micromorphology of the tumor vasculature (Figure 1). Anatomical μ CT can depict vessel diameters, vessel distribution, and vessel branching at sizes as small as 3.4 μ m; thus, the visualization and quantification of such microvascular characteristics can be used to discriminate between tumors with low versus high degrees of aggressiveness and angiogenesis.

Materials and Methods

Tumor Models

Four different mouse xenograft models were used, based on A431 epidermoid carcinoma, Calu-6 anaplastic lung carcinoma, MLS ovarian carcinoma, and A549 non-small cell lung carcinoma human cell lines. All cell lines were obtained from the American Type Culture Collection (Manassas, VA);

Figure 1 Study design. Four tumor models (A431, Calu-6, MLS, and A549) with differing degrees of angiogenesis were used to demonstrate the potential of combining *in vivo* and *ex vivo* μ CT for highly accurate functional and ultra-high-resolution anatomical imaging of tumor angiogenesis. Using contrast-enhanced *in vivo* μ CT, the rBV in tumors approximately 6 × 6 mm in size was determined noninvasively. The rBV values were then compared with those obtained with IHC by quantifying the area fraction of CD31⁺ blood vessels on three different sections for each tumor; both congruence and correlation were highly significant. After *ex vivo* imaging with Microfil perfusion, the three-dimensional micromorphology of tumor blood vessels was visualized at a resolution of approximately 3.4 μ m; this allowed a highly detailed and quantitative analysis of the anatomical properties of the vascular network in tumors, as well as correlation of vessel size, vessel distribution, and vessel branching with the degree of angiogenesis by quantifying the amount of α SMA⁺ (mature) blood vessels using IHC.

cells were resuscitated before use in *in vivo* experiments and were regularly tested for mycoplasma infection using Hoechst 33258 staining (1:400; Sigma-Aldrich, Steinheim, Germany) and fluorescence microscopy. Tumors were induced by inoculating 4×10^6 A431 cells, 2×10^6 Calu-6 cells (in Matrigel; BD Biosciences, Heidelberg, Germany; San Jose, CA), 2.5×10^6 MLS cells or 3×10^6 A549 cells subcutaneously into the right flank of 8-week-old female CD-1 nude mice (Charles River Laboratories International, Wilmington, MA) ($n = 5$ mice per model). Tumor growth was monitored by means of caliper measurements throughout the experimental period; tumor volumes were calculated as $(l \times w^2)/2$, where l is the largest diameter (length) and w is the smallest diameter (width). Experiments were performed when tumors reached a volume of approximately 100 mm^3 . Animals were inhalation-anesthetized with 1.5% isoflurane in oxygen-enriched air with a face mask during all experimental procedures. All animal experiments were approved by the local and institutional ethics committees.

In Vitro VEGF Production

To determine VEGF production by the A431, Calu-6, MLS, and A549 human carcinoma cells, a human VEGF enzyme-linked immunosorbent assay kit (ELISA; RayBiotech, Norcross, GA) was used. To this end, 2×10^5 cells were seeded in Petri dishes; 3 days later, when cells reached approximately 90% confluency, supernatants were harvested and transferred to an anti-human VEGF-coated microtiter plate, followed by application of horseradish peroxidase-conjugated streptavidin for 45 minutes and TMB One-Step substrate reagent (RayBiotech) for 30 minutes. Color intensity was determined at 450 nm.

In Vivo μ CT

For *in vivo* μ CT, a dual-energy gantry-based flat-panel X-ray microtomography scanner was used (TomoScope 30s Duo; CT Imaging, Erlangen, Germany); this is a desktop cone-beam scanner with integrated radiation shielding, object diameter 40 mm, two source-detector systems, and beam-hardening filter. Details of the scanning system and scanning protocol have been described previously.²⁰ Tumor-bearing mice were scanned before and after intravenous administration of $100 \mu\text{L}$ of an iodine-based blood-pool contrast agent (prepared in-house) characterized by prolonged circulation kinetics and optimized for *in vivo* μ CT imaging.²⁷ The contrast agent was injected as a bolus into the lateral tail vein. Each *in vivo* μ CT scan resulted in 2880 projections over a 6-minute time frame.

A Feldkamp-type reconstruction algorithm (CT Imaging), including ring artifact correction, was performed, with a voxel size of $35 \mu\text{m}^3$ ($35 \times 35 \times 35 \mu\text{m}$). The reconstructed data sets were analyzed using Imalytics preclinical software version 1.2.1.4 (Philips Research, Aachen, Germany), which allows interactive segmentation and visualization of large data sets, including the adjustment of volumes of interest in any slicing

orientation. After an elliptical volume of interest was defined, the rBV was determined based on the mean radiodensity, in Hounsfield units, of the segmented tumor and of a large reference blood vessel (the abdominal part of the aorta) before (0% rBV) and after (100% rBV) injection of the blood-pool contrast agent (Figure 1). The three-dimensional morphology of tumor blood vessels was visualized using MeVisLab software version 2.3.1 (MeVis Medical Solutions, Bremen, Germany).

Ex Vivo μ CT

After *in vivo* μ CT imaging, tumor-bearing mice were intracardially perfused with Microfil injection compounds MV-112 and MV-Diluent (Flow Tech, Carver, MA), a lead-containing silicone rubber CT contrast agent that polymerizes within the vascular compartment.²⁸ Before Microfil perfusion, mice were perfused with 20 mL of PBS for complete blood removal and 20 mL of 4% paraformaldehyde for vessel fixation. Perfusion was performed by direct infusion into the left ventricle (after incising of the inferior vena cava) at physiological pressures, using a perfusion pump. After Microfil perfusion, tumors were excised and formalin-fixed.

The tumors were scanned using a non-gantry-based SkyScan 1172 μ CT system (SkyScan, Kontich, Belgium). Tumors were positioned on a computer-controlled rotation platform and were scanned 180 degrees around the vertical axis, in rotation steps of 0.3 degrees at 60 kV. Acquisition times ranged from 2 to 4 hours. The generated isotropic pixel sizes ranged from 3.4 to $5.3 \mu\text{m}$. After three-dimensional volume rendering of reconstructed high-resolution μ CT data sets, blood vessels were visualized using Imalytics preclinical software and vessel size as well as vessel distribution were analyzed in 10 randomly chosen fields of view (FOV = $500 \times 350 \mu\text{m}$) in the periphery versus the core of A431, Calu-6, MLS, and A549 xenografts. For each blood vessel, the diameter was determined in one of three possible planes (transverse, sagittal, or coronal) in which the cross-sectional area was relatively round. Values were compared with those obtained using IHC.

After threshold-based blood vessel segmentation, branching points and the three-dimensional micromorphology of tumor blood vessels were systematically analyzed using high-resolution *ex vivo* μ CT and three-dimensional volume rendering. Similar thresholds were used for all tumors. To this end, all branching points within five representative vessels per FOV were manually counted, and the number of blood vessel branches per primary blood vessel was quantified (Figure 1).

Immunohistochemistry

Paraffin-embedded tumor sections were stained using CD31 (Dianova, Hamburg, Germany) and α -SMA-biotin (PROGEN Biotechnik, Heidelberg, Germany) antibodies. Secondary antibodies were obtained from Dianova. Nuclei were

counterstained using Hoechst nuclear dye, and sections were mounted using Mowiol polyvinyl alcohol medium. Fluorescence microscopy was performed using an Axio Imager M2 light microscope with an AxioCam MRm revision 3 high-resolution camera (both from Carl Zeiss Microimaging, Göttingen, Germany). For quantifying rBV, six images (three from the periphery and three from the core) were investigated (FOV = $500 \times 350 \mu\text{m}$), from three representative sections per tumor and five tumors per model. Both nonfilled CD31⁺ area fractions and semiautomatically filled CD31⁺ vascular structures were determined using a custom macro implemented for open-access image analysis software (ImageJ version 1.43u; NIH, Bethesda, MD) (Supplemental Figure S1). This approach, which has been described previously,²⁹ is based on the detection and filling of CD31⁺ vascular structures. Vessel size was analyzed by determining diameters in 10 representative vessels in the tumor core versus periphery. To standardize the diameter quantifications in two-dimensional IHC slices, the shorter of two orthogonally arranged possible diameters was chosen. Images were analyzed and quantified using ImageJ software.

Statistical Analysis

Statistical and correlation analyses were performed using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA). Two-tailed *t*-test and Fisher's *z*-test were used to assess statistical significance. *P* < 0.05 was considered to represent statistical significance. Data are expressed as means $\pm$ SD.

Results

Biological and Immunohistochemical Characterization of the Tumor Models

Anatomical and functional CT imaging of tumor angiogenesis was performed in four mouse xenograft models with significantly different *in vivo* growth characteristics and vascularization: A431, Calu-6, MLS, and A549 tumors. Representative histopathological images for the four models are shown in Figure 2, A and B. The endothelial cell marker platelet endothelial cell adhesion molecule-1 (PECAM-1; alias CD31) provides information on blood vessel density and distribution, and the myofibroblast and pericyte marker α -smooth muscle actin (α SMA) provides information on blood vessel maturation. H&E staining revealed that tumors did not contain large necrotic areas (such as could give rise to false-negative results on the rBV). Tumor growth, which generally correlates with tumor aggressiveness, was fastest for A431 tumors, followed by Calu-6 and by MLS (Figure 2C). A549 tumors grew significantly more slowly than the other three xenograft models.

The total number of blood vessels per FOV was consistently highest in A431 tumors and lowest in A549 tumors (26 ± 9 versus 9 ± 2 vessels per FOV, respectively) (Figure 2D),

indicating that A431 tumors are not only more aggressive, but also more angiogenic than A549 tumors. In corroboration, the highest expression levels of VEGF were detected in A431 tumors, and the lowest levels in A549 (Supplemental Figure S2). Conversely, the overall degree of vessel maturation was highest in A549 and lowest in A431 tumors ($73 \pm 11\%$ versus $14 \pm 4\%$ α SMA⁺ vessels, respectively) (Figure 2D). IHC analysis of the distribution of blood vessels within the A431, Calu-6, MLS, and A549 xenografts revealed that the tumors which intrinsically produce very high levels of VEGF (ie, A431 and MLS) were much more homogeneously vascularized than the tumors which express low levels of VEGF (ie, Calu-6 and A549), in which vessels were located mostly in the tumor periphery (Figure 2B).

Anatomical and Functional *in Vivo* μ CT Imaging of Tumor Angiogenesis

Next, we visualized the three-dimensional micromorphology of tumor blood vessels in these four tumor models, and we quantified the rBV using *in vivo* dual-energy μ CT and an iodine-containing contrast agent optimized for blood-pool imaging.^{15,20,30} Dual-source flat-panel *in vivo* μ CT allowed noninvasive visualization of blood vessel branches up to the third order in MLS and A549 xenografts, whereas in the more aggressive and more angiogenic A431 and Calu-6 tumors only first-order blood vessel branches (in A431) or up to the second order (Calu-6) could be visualized (Figure 3, A–D). The resolution (ie, the smallest vessel size that could be noninvasively visualized) using this technique was approximately $40 \mu\text{m}$.

To assess the accuracy of *in vivo* μ CT imaging for quantitative angiogenesis characterization (relative to standard angiogenic profiling via IHC), noninvasively obtained rBV values were correlated with filled versus nonfilled area fractions of CD31⁺ vascular structures (Figure 4 and Supplemental Figure S1). To properly reflect differences in blood vessel distribution in the core versus the periphery of tumors, IHC images were obtained in an equal proportion: six images per section (three for the core and three for the periphery), three different sections per tumor, and five different tumors per model (Figure 1). rBV values obtained using *in vivo* μ CT ranged from $2.6 \pm 0.5\%$ (A549) to $6.0 \pm 1.5\%$ (MLS); the intermediate values were $3.6 \pm 0.4\%$ (Calu-6) and $4.7 \pm 0.9\%$ (A431) (Figure 4A). IHC using filled CD31⁺ vessel structures consistently rendered rBV values of $2.5 \pm 0.4\%$ for A549, $3.2 \pm 0.5\%$ for Calu-6, $4.1 \pm 1.2\%$ for A431, and $5.0 \pm 1.4\%$ for MLS (Figure 4A). A highly significant correlation between rBV values determined using *in vivo* μ CT and IHC was observed, not only for all 20 tumors in total (*P* < 0.0001) (Figure 4B), but also for all four tumor models (*P* = 0.0239 to 0.0019) (Figure 4C). These differences in functional blood vessels within the different tumor models are consistent with the *in vitro* VEGF levels (Supplemental Figure S2), indicating that contrast-enhanced *in vivo* μ CT imaging not only can accurately determine small differences

Figure 2 Characterization of tumor growth and angiogenesis. **A** and **B**: Representative histological and immunofluorescence images of A431, Calu-6, MLS, and A549 tumors with H&E staining for histology (**A**) or with immunofluorescence staining (**B**) for CD31 (green) as a marker of blood vessels, for α SMA (red) as a marker of pericytes, and for Hoechst nuclear dye (blue). **C** and **D**: Differences in tumor growth (**C**) and blood vessel density and maturity (**D**) characterize the four models. Data are expressed as means $\pm$ SD. $n = 5$ tumors per model. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, two-tailed t -test. Original magnification: $\times 100$ (**B**, insets).

in tumor vascularization, but also can quantitatively characterize the angiogenic activity of tumors with different growth characteristics. Finally, not filling the lumen of CD31⁺ vascular structures in formalin-fixed tumor sections resulted in a significant underestimation of the rBV in IHC, with no correlation between *in vivo* μ CT and IHC (Figure 4, D and E).

Anatomical *ex Vivo* μ CT Imaging of Tumor Angiogenesis

These results convincingly demonstrate that *in vivo* μ CT allows a highly accurate noninvasive determination of the

rBV in tumors. However, the spatial resolution of gantry-based *in vivo* μ CT scanners ends at approximately 35 μ m per voxel (depending on scan protocols, scan times, biologically relevant X-ray doses, and contrast agents).¹⁶ The vast majority of newly formed blood vessels in tumors are smaller than 35 μ m, but we overcame this limitation by visualizing and quantitatively characterizing the microarchitecture of vascular structures in Microfil-perfused A431, Calu-6, MLS, and A549 tumors using ultra-high-resolution *ex vivo* μ CT tomography. Representative three-dimensional images of *ex vivo* imaged blood vessel networks, as well as two-dimensional μ CT images of the

Figure 3 *In vivo* and *ex vivo* μ CT imaging of tumor angiogenesis. **A–D:** Contrast-enhanced *in vivo* μ CT was used for anatomical and functional visualization and quantitative characterization of tumor angiogenesis. Numbers represent the vessel branching order. In the xenograft models with large numbers of mature blood vessels (MLS and A549), blood vessel branches up to the third order could be visualized; in models characterized by more angiogenic and less mature blood vessels (A431 and Calu-6), only first- and second-order blood vessel branches could be identified. The numbers within the images exemplify the rising order of vascular branches. **E–H:** After Microfil perfusion and vascular casting, ultra-high-resolution *ex vivo* μ CT was performed with three-dimensional volume rendering, enabling the visualization and quantitative characterization of blood vessels with diameters as small as 3.4 μ m, and blood vessel branches up to the seventh order. **I–L:** Two-dimensional cross-sectional images (*x*, *y*, and *z* planes) provide highly detailed information on blood vessel diameter, blood vessel density, and blood vessel distribution; tumors are the same as in panels **E–H**.

same tumors in transversal, sagittal, and coronal planes, are presented in **Figure 3**, **E–L**. Using this technique, blood vessels as small as 3.4 μ m could be visualized, and the three-dimensional vascular network in the xenograft models used could be visualized at very high resolution.

Microfil perfusion-based vascular casting combined with *ex vivo* μ CT imaging allowed highly detailed three-dimensional visualization of blood vessel distribution within the core and the periphery of tumors, as well as systematic and quantitative analysis of angiogenesis-related vascular parameters, such as vessel size, vessel distribution, and vessel branching (**Figures 5** and **6**). A technique assessing all of these parameters within intact tumors has not yet been described, but a quantitative characterization of the three-dimensional micromorphology of tumor blood vessels, especially with regard to their branching characteristics, would hold great potential for better understanding tumor angiogenesis and monitoring the efficacy of antiangiogenic therapies. In an empirical approach, we systematically investigated vessel size, vessel distribution, and vessel branching using Microfil perfusion-based vascular casting, high-resolution *ex vivo* μ CT, and volume-rendering techniques for quantitative characterization of tumor angiogenesis.

Consistent with their increased growth rate and higher degree of angiogenesis (**Figures 2** and **3**), a clear tendency toward reduced vessel size and reduced vessel branching was observed for A431 xenografts, relative to A549. Mean vessel size for A431 was 11 ± 3 μ m (core) and 23 ± 6 μ m

(periphery), compared with 15 ± 4 μ m and 35 ± 11 μ m, respectively, for A549. These values matched very well those obtained using IHC: 8 ± 3 μ m (core) and 20 ± 6 μ m (periphery) for A431, compared with 13 ± 4 μ m and 32 ± 13 μ m, respectively, for A549 (**Figure 5**, **A** and **B**). Consistently, comparing vessel diameters obtained by *ex vivo* μ CT and IHC confirmed a highly significant correlation between the two modalities ($P < 0.0001$) (**Figure 5C**), demonstrating that *ex vivo* μ CT is highly suitable for quantitative and ultra-high-resolution anatomical analyses of tumor angiogenesis. Findings from qualitative and quantitative comparisons of blood vessels between exactly corresponding *ex vivo* μ CT and IHC data sets of moderately vascularized A549 tumors and highly vascularized MLS tumors confirmed these findings (**Figure 6**). In addition, the percentages of blood vessels within a particular size range were analyzed using *ex vivo* μ CT; in the more aggressively growing and more angiogenic tumors (A431 and Calu-6), vessels were much smaller and much more homogeneous in size than in the less aggressively growing and less angiogenic tumors (MLS and A549) (**Figure 5D**).

Consistently, upon systematic analysis and quantification of the three-dimensional micromorphology of tumor blood vessels, more aggressive and more angiogenic tumors were found to have not only a much lower overall number of blood vessel branches, but also much lower orders of blood vessel branching (**Figure 5**, **E** and **F**). Quantification of the total number of branches per primary blood vessel in

Figure 4 Congruence and correlation of functional *in vivo* μCT with IHC. **A:** Comparison of the model-dependent tumor rBV values determined using *in vivo* μCT and IHC. To properly assess the rBV in IHC, the vessel lumen was semiautomatically filled and quantified in six different FOVs (three for the core and three for the periphery) in three representative sections from each of five tumors per tumor model. **B:** Correlation of the rBV values obtained on *in vivo* μCT and IHC after semiautomated vessel filling. For the 20 tumors, the correlation was highly significant ($P < 0.0001$), and the linear regression ($\alpha = 47$ degrees) indicates a highly accurate and congruent determination of the rBV by *in vivo* μCT versus IHC. **C:** Correlation of the rBV values determined using *in vivo* μCT versus IHC was significant for all four tumor models ($P < 0.05$). **D:** Quantification of nonfilled CD31⁺ area fractions in IHC, exemplifying that, compared with filled vessel structures and to *in vivo* μCT, no significant differences were observed between the four tumor models used and that absolute rBV values are strongly underestimated. **E** and **F:** Correlation of nonfilled CD31⁺ area fractions in IHC with rBV values determined using *in vivo* μCT (**E**) and with semiautomatically filled CD31 area fractions (**F**), exemplifying poor congruence and correlation. Data are expressed as box plots $\pm$ SD (**A**, **C**, and **D**).

A431, Calu-6, MLS, and A549 xenografts yielded values of 11 ± 5 , 19 ± 6 , 52 ± 37 , and 130 ± 67 , respectively (Figure 5E). Analogously, analysis of the percentages of blood vessel branches of the first to the seventh order (Figure 1) revealed that, in the more rapidly growing and more angiogenic A431 and Calu-6 tumors, the majority of vessels branched only once or twice, whereas in the more slowly growing and mature MLS and A549 tumors (Figure 2), a significant number of vessels branched four to seven times (Figure 5F). These findings exemplify the potential of using ultra-high-resolution *ex vivo* μCT for detailed visualization and quantitative characterization of the three-dimensional micromorphology of

tumor blood vessels and for differentiating tumors with high versus low degrees of angiogenesis (Figure 3, E–H, and Supplemental Videos S1, S2, S3, and S4).

Discussion

Our aim was to systematically demonstrate that contrast-enhanced *in vivo* μCT can be used to accurately quantify rBV in tumors, and to exemplify that *ex vivo* μCT allows quantitative and ultra-high-resolution analyses on the three-dimensional micromorphology and the angiogenic

Figure 5 Quantitative *ex vivo* μ CT imaging of tumor angiogenesis. **A** and **B**: Quantification of vessel diameters in the core and periphery of the four tumor models using IHC (**A**) and *ex vivo* μ CT (**B**), exemplifying that vessels in more aggressive tumors (A431 and Calu-6), which are characterized by a more angiogenic and less mature vasculature, are smaller than vessels in less angiogenic and more mature tumors (MLS and A549). **C**: Correlation of vessel diameters determined using *ex vivo* μ CT and IHC. **D**: Quantification of blood vessel diameter distribution within a particular size range, confirming that vessels in more aggressive and more angiogenic tumors were smaller and more homogeneous in size than in less aggressive and less angiogenic tumors. **E** and **F**: *Ex vivo* μ CT-based quantification of the total number of blood vessel branches (**E**) and of the percentage of branches by increasing order (**F**), exemplifying that less aggressive and more mature tumors (MLS and A549) contained more branches and higher-order branches per vessel than less mature tumors (A431 and Calu-6). Data are expressed as means $\pm$ SD (**A**, **B**, and **D–F**). ** $P < 0.01$, *** $P < 0.001$.

characteristics of tumor blood vessels. To this end, using four different tumor models, we systematically compared the functional and anatomical information obtained using *in vivo* and *ex vivo* μ CT with that obtained from IHC. The four xenograft models differed significantly in aggressiveness and angiogenesis. Rapidly growing A431 tumors contain many small and mostly immature blood vessels, which are homogeneously distributed throughout the tumor. Calu-6 tumors are characterized by an intermediate growth rate and by an intermediate number of blood vessels, which are small, mostly immature and relatively heterogeneously distributed throughout the tumor; MLS tumors are also characterized by an intermediate growth rate, but (because of an extensive stromal compartment) the majority of blood vessels appear to be mature, and are distributed homogeneously throughout the tumor. Slowly growing A549 tumors contain a relatively small number of blood vessels, the vast majority of which are large and mature, and are distributed relatively heterogeneously throughout the tumor (Figure 2). With these four models, we show that contrast-enhanced

in vivo μ CT is an excellent tool for noninvasively visualizing and quantifying the rBV in tumors. Together with the highly detailed anatomical information that can be obtained about the three-dimensional microvascular network within tumors using *in vivo* and *ex vivo* μ CT, these findings indicate that μ CT is a highly useful and highly versatile tool for characterizing tumor angiogenesis.

Our present findings extend previous efforts on anatomical and functional μ CT imaging of tumor angiogenesis in several ways. First, in terms of functional *in vivo* μ CT, in no previous studies have rBV values obtained noninvasively using steady-state μ CT been quantitatively and systematically compared with those obtained using IHC in multiple xenograft models differing in their vascular characteristics. Several previous analyses have shown a correlation between the rBV determined using dynamic (μ)CT or steady-state magnetic resonance imaging and the microvessel density determined using IHC, both in animal models and in patients,^{24–26,31–37} but the present study is the first to systematically demonstrate a highly significant congruence

Figure 6 Qualitative and quantitative comparison of tumor blood vessels in corresponding *ex vivo* μCT and IHC data sets. **A** and **B**: Immunofluorescence images and corresponding two-dimensional cross-sectional images from high-resolution *ex vivo* μCT imaging after Microfil perfusion and vascular casting of moderately vascularized A549 (**A**) and highly vascularized MLS (**B**) tumors. **Boxed regions** in the whole-tumor images (**top row**) are shown at higher magnification in the **middle** and **bottom rows**. Immunohistochemical staining was performed using antibodies against CD31 (blood vessels; red), αSMA (pericytes; green), and Hoechst nuclear dye (blue). **C** and **D**: Correlation of vessel diameters determined using corresponding IHC and *ex vivo* μCT data sets, exemplifying a highly significant correlation ($P < 0.0001$) and linear regression α values close to 45 degrees for both A549 (**C**) and MLS (**D**) tumors.

between rBV values determined using three-dimensional steady-state μCT and two-dimensional IHC (Figure 4). Second, in terms of anatomical μCT imaging, we for the first time provide *ex vivo* images of the vascular network in subcutaneous tumor xenografts at a resolution of less than 4 μm. These images exemplify the huge potential of vascular casting and ultra-high-resolution *ex vivo* μCT for quantitatively analyzing the three-dimensional micromorphology of tumor blood vessels (Figure 3). Third, we demonstrate for the first time that blood vessel diameters, blood vessel distribution, and blood vessel branching in tumors can be accurately and quantitatively analyzed using *ex vivo* μCT, with two-dimensional imaging information on vessel size and vessel distribution corresponding very well with that observed in IHC and with three-dimensional information on blood vessel branching corresponding very well with the

degree of aggressiveness and angiogenesis of the different tumor models used (Figure 5). Importantly in this regard, the application of architectural and functional vessel imaging to identify, monitor, and predict outcomes for cancer patients who respond to antiangiogenic therapies has been described.^{38,39} In the present study, we could not perform validation of the high-resolution μCT-based branching analyses using the acquired IHC data set, because of the two-dimensionality of conventional histological procedures. This limitation could be overcome by using advanced multiphoton or super-resolution microscopy techniques, which we plan to address in future studies.

Compared with other clinically relevant imaging modalities that can be used for visualizing and analyzing tumor angiogenesis, such as ultrasound and magnetic resonance imaging,^{13,40–42} combining *in vivo* and *ex vivo* μCT

imaging has both advantages and disadvantages. The advantages include observer-independence, user-friendliness, the quantitative nature for both functional and micromorphological tumor blood vessel characterization, low costs, and high resolution, as well as suitability for high-throughput analyses. The disadvantages include X-ray exposure, the need for contrast agents, and the relatively low contrast-agent sensitivity of CT.^{13,16–19} In particular, in longitudinal studies assessing effects of antiangiogenic and/or antitumorigenic therapy, appropriate X-ray doses that do not affect tumor biology and tumor development should be taken into account, along with the individual accumulated load for iodine-based contrast agents.

Overall, when balancing these various pros and cons, and taking into account the findings reported here, it can be concluded that μ CT is a simple, rational, reliable, and broadly applicable tool for visualizing and quantifying tumor angiogenesis, in particular in small-animal models such as mouse or rat. This is not only because μ CT provides rapid and highly detailed anatomical information on the size and shape of tumors, but also because it allows high-throughput, highly accurate, and highly quantitative functional analyses on the rBV in tumors. In addition, with respect to pathophysiological conditions of circulation in tumors, μ CT can be used to visualize and quantitatively characterize the three-dimensional micromorphology of tumor blood vessels, thereby differentiating between tumors with high versus low degrees of aggressiveness and angiogenesis. Consequently, the combination of anatomical and functional μ CT seems to hold significant potential for facilitating translational anticancer research.

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Supplemental Data

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