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Correlation of quantified contrast-enhanced power Doppler ultrasonography with immunofluorescent analysis of microvessel density in spontaneous canine tumours

Stefanie Ohlerth^{a,1}, Melanie Wergin^{a,1}, Carla Rohrer Bley^a, Francesca Del Chicca^a, Dagmar Lalahová^a, Beat Hauser^b, Malgorzata Roos^c and Barbara Kaser-Hotz^{a,2}

^aSection of Diagnostic Imaging and Radio-Oncology, Vetsuisse Faculty, University of Zürich, Winterthurerstrasse 260, 8057 Zürich, Switzerland

^bInstitute of Veterinary Pathology, Vetsuisse Faculty, University of Zürich, 8057 Zürich, Switzerland

^cInstitute of Social and Preventive Medicine, Faculty of Medicine, University of Zürich, 8001 Zürich, Switzerland

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Abstract

Conventionally, tumour vascularity is assessed invasively by immunofluorescent analysis. Quantified contrast-enhanced power Doppler ultrasound has been used to measure tumour angiogenesis non-invasively in humans and experimental animals. The purpose of this study was to correlate quantified contrast-enhanced power Doppler ultrasound with immunofluorescent results in 45 spontaneous canine tumours. With power Doppler, mean vascularity was high in squamous cell carcinomas, moderate in malignant oral melanomas and low in sarcomas. There was high mean vascularity in squamous cell carcinomas and low mean vascularity in sarcomas and malignant oral melanomas. Although Doppler parameters correlated moderately with microvascular density for all tumours ($P = 0.004$, $r = 0.4$), they did not correlate within histology groups. These analyses show that vascularity differs among canine tumour histology groups. However, dependent on the method used, measurement of tumour vascularity can provide different biological information.

Keywords: Doppler ultrasound; Microvessel density; Tumours; Canine

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Introduction

Tumour vasculature plays an important role in cancer research. Formation of new blood vessels, called angiogenesis, allows a tumour to grow beyond the size of the prevascular phase ([Folkman, 1995](#)). It has been shown that highly vascularised tumours have a higher potential to metastasise than tumours with poor vascularity ([Luong et al., 2006](#)). Further, increased vascularity is indicative of poorer survival in a variety of human ([Zetter, 1998](#)) and canine ([\[Preziosi et al., 2004\]](#) and [\[Mukaratirwa et al., 2006\]](#)) neoplasms. Vascularity has also been used as a valuable criterion to differentiate between benign and malignant human tumours, with higher densities of vessels in human breast cancer ([Yang et al., 2002](#)).

Since angiogenesis is important for tumour growth, anti-angiogenic drugs have been developed as treatments that inhibit new blood vessel formation, either directly by inhibiting specific molecules, or indirectly by influencing endothelial cell function ([Rosen, 2000](#)). Conventional radiotherapy has been shown not only to target tumour cells, but also vascular endothelial cells ([Folkman and Camphausen, 2001](#)). Tumour angiogenesis is an important predictor of response to chemotherapy in cancer patients ([Chen et al., 2004](#)). Consequently, the need to evaluate treatment efficacy has resulted in an increased interest in assessment of tumour vasculature.

The standard method for quantifying tumour angiogenesis is by immunofluorescent analysis of intratumoral microvessel density (MVD), which quantifies the number of

vessels per unit volume (Weidner, 1995). However, to determine MVD, tissue samples have to be obtained via invasive biopsy procedures, represent only a certain area within the tumour and results are not immediately available for the clinician. To monitor changes in tumour vascularity in response to ionising radiation, serial histological analysis is impractical in most patients, since tissue must be obtained repeatedly by invasive procedures.

Colour and power Doppler ultrasonography are non-invasive techniques for assessment of vascularity and blood volume in tumours ([Bodner et al., 2002] and [Eriksson et al., 1991]). The entire tumour can be surveyed without affecting the tumour itself. Ultrasonography also allows serial measurements of the same tumour over time during a treatment regimen. Power Doppler ultrasonography has been shown to be more sensitive than colour Doppler for detecting low velocities and for depicting small vessels ([Lencioni et al., 1996] and [Tschammler et al., 2002]). Contrast-enhanced Doppler ultrasonography further improves the visualisation of vascularisation in tumours. The intravenous administration of a microbubble-based contrast agent can increase the Doppler signal intensity up to 25 dB. This increases the sensitivity for low-flow situations and provides a better depiction of vascularity than non-contrast-enhanced Doppler ultrasonography (Schroeder et al., 2003).

The purpose of the present study was to correlate the non-invasive quantification of vascularity and blood volume via contrast-enhanced power Doppler ultrasonography with the immunofluorescent analysis of MVD in spontaneous canine tumours.

Materials and methods

Patient selection

The study was performed using 45 dogs with 45 spontaneous tumours presented to the Section of Diagnostic Imaging and Radio-Oncology at the Vetsuisse Faculty of the University of Zürich. Dogs were included in the study if: (a) the tumour was superficial and accessible for ultrasound; (b) tissue core biopsy samples were available for histological examination; (c) tumour size allowed tissue core biopsy samples with a length of 22 mm to be taken, and (d) dogs were in good clinical condition and able to undergo general anaesthesia.

After owner consent was obtained, each patient underwent a physical examination and thoracic radiography. Breed, sex, weight and age of dogs were recorded. If indicated, fine needle aspiration of regional lymph nodes, abdominal ultrasound and computed tomography of the primary tumour were done. For tumour diagnosis,

routine histological evaluation of tumour specimens was performed.

Anaesthesia was initiated by use of midazolam (2 mg/kg, IV; Dormicum; Roche Pharma SA) or diazepam (0.2 mg/kg, IV; Valium; Roche Pharma SA), followed by propofol (Propofol; Fresenius Kabi AG) given to effect; in addition butorphanol (0.1–0.3 mg/kg, IV; Morphasol, Gräub AG) was administered to some dogs. Anaesthesia was maintained with isofluran (Isoflo; Abbott AG) delivered in oxygen through an endotracheal tube. Heart rate and oxygen saturation, as measured by pulse oximetry (SpO₂), were monitored continuously during anaesthesia to assess the status of each dog. At the time of Doppler ultrasound examination, the heart rate of each dog was recorded. All patients received IV lactated Ringer's solution at the rate of 10 mL/kg/h.

The length (*a*), width (*b*) and depth (*c*) of the tumours were determined from caliper measurements during anaesthesia. Tumour volume was calculated using the rotation ellipsoid formula ($\pi abc/6$).

Ultrasonographic imaging of tumours

Whenever necessary, the hair overlying the tumour was clipped. For each ultrasonographic examination, acoustic gel (Aquasonic 100, Parker) was applied to the skin and imaging was performed by use of a 5–12 MHz linear transducer (ATL 5000; Philips AG). For power Doppler ultrasonography, settings were constant for all examinations (81% colour gain, medium wall filter, 500 Hz pulse repetition frequency, frame rate 10–12 Hz). The maximal image depth was 25 mm. The ultrasound contrast agent used was a first-generation microbubble suspension (400 mg/mL Levovist; Schering) that was administered at a dose rate of 80 mg/kg.

Initial ultrasound scanning of the entire tumour was performed in B-mode to define its boundaries and morphological features. A rectangular sample volume was then placed over a selected region of the tumour and the surrounding tissue, denoting the region in which power Doppler ultrasonographic data would be acquired. A sliding scan of the entire tumour was performed to subjectively assess overall tumour vascularity. Finally, an area that subjectively represented the tumour's typical vessel density was chosen. The probe remained in the same location for ultrasonographic examination after administration of contrast medium. A bolus injection of the microbubble contrast agent was administered by hand via a catheter placed in a peripheral vein.

After blooming artefacts had degraded, a minimum of five power Doppler images were captured digitally and analysed ([Schärz et al., 2005](#)). Two parameters were computed for post-contrast power Doppler by calculating the median of the five images. Fractional area (FA) represents a vascularity index and indicates the percentage area of the tumour occupied by large blood vessels. Colour-weighted fractional area (CWFA) determines the mean blood volume within the tissue.

Immunofluorescence staining of microvessels

After the ultrasound procedure, a tissue core biopsy was taken with a 14 G a needle using an automated device (Magnum Biopsy gun; Bard). Samples always were obtained in the centre of the area of the power Doppler analysis under ultrasound guidance at an insertion angle of 45–60° to the image plane of the linear transducer. The sample depth was always 22 mm and therefore consistent with the ultrasound image depth of 25 mm. Tumours with a bone interface were only sampled in the soft tissue part. The specimens were immediately frozen with isopentane and liquid nitrogen and then stored at –80 °C. Tissue samples were cut into 7 µm thick sections with a cryostat microtome (Leica; CM1850), applied to slides and air dried for 20 min at room temperature before staining.

Slides were fixed in 4% paraformaldehyde for 30 min and blocked with normal goat serum for 1 h before incubating overnight at 4 °C with a commercially available rabbit–anti-human F8RA antibody (1:200; Dako Polyclonal; Dako) tested in canine tissues ([von Beust et al., 1988](#)). This step was followed by incubation with a secondary goat–anti-rabbit antibody labelled with fluorescein isothiocyanate (FITC; 1:2000; Sc3839; Santa Cruz Biotechnology) for 1 h at room temperature. Slides were mounted with Dako Fluorescent Media and examined with an inverse confocal laser scan microscope (400× total magnification, LSM 410; Carl Zeiss).

Computerised image analysis (Qwin; Leica Microsystems AG) was performed on five digital images from randomly chosen visual fields (high power fields). The count of microvessels per high power field was recorded and the mean of five fields was calculated. The programme was optimised for quantitative microscopy, producing precise results with megapixel accuracy ([\[Sims et al., 2002\]](#) and [\[Law et al., 2003\]](#)). The software was adapted to the project and the accuracy of the vessel count was tested before use. Statistical analyses were performed with computer software programmes (StatView 5.0.1, SAS Institute Inc. and SPSS 10.0, SPSS Schweiz AG).

Values of $P < 0.05$ were considered to be significant.

Results

Twenty-five sarcomas, 10 oral squamous cell carcinomas and 10 malignant oral melanomas were included in the study (Table 1). The age of the dogs ranged from 2 to 15 years (mean 9.4 years). Twenty different breeds, along with mixed breed dogs, were included. The bodyweight ranged from 2.8 to 75 kg (mean 29.6 kg). Twenty-eight dogs were male and 17 female.

Table 1.

Location and histological classification of 45 spontaneous canine tumours examined by contrast-enhanced power Doppler ultrasonography

Histological classification	Location	Number of tumours
Fibrosarcoma	Oral cavity	5
	Head	2
	Limb	1
Osteosarcoma	Oral cavity	4
Histiocytic sarcoma	Oral cavity	2
Spindle cell sarcoma	Oral cavity	2
	Trunk	1
Myxosarcoma	Oral cavity	2
Haemangiopericytoma	Limb	4
	Trunk	1
Undifferentiated sarcoma	Oral cavity	1
Squamous cell carcinoma	Rostral oral cavity	10
Malignant melanoma	Oral cavity	10

Thirty-six dogs were anaesthetised with midazolam and four dogs with diazepam; five dogs were given butorphanol additionally; subsequently, every dog received propofol. At the time of Doppler ultrasonographic examination, the mean heart rate was 115 beats per minute (bpm) (SD 23 bpm; reference range 84–110 bpm) (Haskins, 1996). The mean tumour volume was 83.5 cm³ (SD 170.3 cm³).

Descriptive statistics for post-contrast power Doppler parameters and MVD are shown

in Table 2. Post-contrast power Doppler FA and CWFA were significantly correlated with MVD for all tumours ($P = 0.006$, $r = 0.4$, and $P = 0.005$, $r = 0.41$, respectively). If correlations were calculated for tumour histology groups, no significant associations were found ($P = 0.47$ – 0.96) (see Fig. 1 and Fig. 2). Highly significant differences were found between sarcomas, oral squamous cell carcinomas and malignant oral melanomas for the mean values of post-contrast power Doppler FA and CWFA ($P = 0.0001$, 1-way ANOVA and post-hoc Bonferroni–Dunn test).

Table 2.

Descriptive statistics for post-contrast power Doppler parameters, including vascularity (fractional area) and blood volume (colour-weighted fractional area), and microvessel density, in 45 spontaneous canine tumours

	FA (%)			CWFA			MVD		
	Me	SD	Range	Me	SD	Range	Me	SD	Range
Sarcoma	10.2	1.3	0.5–38.8	6.8	7.6	0.3–27.9	1.6	1.2	0.2–4.8
Oral squamous cell carcinoma	40.2	8.0	12.9–69.2	36.0	5.3	6.5–86.3	4.5	3.3	2.7–6.1
Oral malignant melanoma	24.8	2.1	0.2–68.7	23.1	4.0	0.05–81.4	1.7	1.0	0.9–4.3

SD, standard deviation; FA, fractional area; CWFA, colour-weighted fractional area; MVD, microvessel density.

[Full-size image \(65K\)](#)

Fig. 1. Post-contrast power Doppler ultrasound image (A) and corresponding photomicrographs of immunofluorescent stained sections (B) of an oral squamous cell carcinoma. Whereas microvessel density is high (5.9), Doppler measurements of vascularity and blood volume are only moderate (FA 35.8%, CWFA 20.2). White arrows indicate the hyperechoic contour of the mandible, which appears irregular and partially destroyed.

[Full-size image \(63K\)](#)

Fig. 2. Post-contrast power Doppler ultrasound image (A) and corresponding photomicrographs of immunofluorescent stained sections (B) of another oral squamous cell carcinoma. In contrast with [Fig. 1](#), this tumour has a moderate microvessel density of 2.8 (A) and high Doppler values with FA of 69.2% and CWFA of 63.6 (B). White arrows indicate the hyperechoic contour of the partially destroyed mandible.

With power Doppler, squamous cell carcinomas were highly vascularised and perfused, whereas in malignant oral melanomas, vascularity and blood volume were moderate and in sarcomas they were low. However, there was overlap in the range denoted by the standard deviations between the different types of tumours. There was high MVD in squamous cell carcinomas and low MVD in sarcomas and malignant oral melanomas. Overlap in the range was less distinct. Differences between mean values of MVD were highly significant for sarcomas and malignant oral melanomas in comparison with squamous cell carcinomas ($P < 0.0001$, 1-way ANOVA and post-hoc Bonferroni–Dunn test).

Discussion

The purpose of the present study was to correlate vascularity measurements via non-invasive quantified contrast-enhanced power Doppler ultrasonography with the immunofluorescent analysis of MVD in spontaneous canine tumours. The analysed Doppler parameters correlated significantly with MVD for all tumours, but not within the tumour histological groups. This moderate correlation for all tumours may be explained statistically by the fact that mean vascularity was low in sarcomas, moderate in melanomas and high in squamous cell carcinomas. However, within the various tumour histology groups, measured values scattered widely and, therefore, no correlation was found.

Immunofluorescent analysis of tumour vascularity represents the conventional method of assessment of intratumoral MVD. Disadvantages of this method are that tissue samples have to be obtained via invasive biopsy procedures and results are not immediately available for the clinician. To monitor the response of tumours to treatment, serial histological analysis is also impractical with most patients, because

tissue must be obtained repeatedly by invasive procedures. Therefore, non-invasive imaging methods, e.g. contrast-enhanced power Doppler ultrasound, have been evaluated for the assessment of tumour vascularity. Diverging results were reported in the literature.

Correlation of Doppler ultrasonographic measurements with the quantified results of immunofluorescent staining has been reported to be high in human breast tumours (Sehgal et al., 2000) and murine glioblastomas (Donnelly et al., 2001). However, results of other studies indicated moderate to poor correlations ([Cheng et al., 1999], [Fleischer et al., 1999] and [Yang et al., 2002]). The discrepancy, in part, might have been due to differences in techniques used. In most studies involving immunofluorescent analysis of intratumoral MVD, an area of the tumour that appears subjectively to contain the most microvessels is chosen; these areas are so-called neovascular hot spots. Within a 0.74 mm² region of the selected area, all microvessels are counted by use of a microscope to assess MVD ([Weidner, 1995] and [Fleischer et al., 1999]). This might not reflect Doppler measurements, which evaluate global vascularity.

A more important reason for the discrepancy is that, with histological assessment, tumour vessels (approximately 15 µm in diameter) are detected, whereas larger vessels (approximately 100 µm in diameter) are detected via contrast-enhanced power Doppler ultrasonography (Fleischer et al., 1999). Furthermore, with immunofluorescent staining, patency of vessels does not play a role, whereas, with Power Doppler ultrasound, a Doppler signal can only be detected in functional, i.e., patent vessels. For these reasons, significant correlations may not have been found in the present study. It appears that measurement of tumour vascularity with contrast-enhanced power Doppler ultrasound and MVD provided different biological information.

On the other hand, results of the present study may be limited, since only a single biopsy was obtained in each tumour. In previous studies that showed a better correlation between imaging and histology measurements ([Sehgal et al., 2000] and [Donnelly et al., 2001]), it was not feasible to match the two sampling sites. To minimise sampling variations, Doppler ultrasound and histological measurements, based on excisional biopsies, were made in different sections throughout the tumour and means were calculated. In the present study, the aim was to match the ultrasound image plane and histological section by taking ultrasound-guided tissue core

samples and by adapting biopsy sample length to the ultrasound image depth. However, one biopsy sample may still not reflect the complete ultrasound image or the heterogeneous character of tumours. It was difficult to obtain several biopsy samples from one tumour, since many tumours were small. Moreover, it was difficult to obtain the owner's consent for multiple biopsies because of a higher complication rate (e.g. bleeding, pain, bacterial infection). Most of the dogs with tumours in this study underwent radiation therapy and, therefore, excisional biopsies were usually not available.

In the present study, significant differences in mean vascularity (FA) and blood volume (CWFA) were found among tumour histology groups. Sarcomas had low and squamous cell carcinomas had high vascularity and blood volume, whereas oral malignant melanomas were moderately vascularised and perfused. In human tumours, colour and power Doppler studies have been performed to differentiate between benign and malignant tumours and to determine the prognostic value of vascularity and perfusion. In humans, a significant association between high vascularity (measured via colour Doppler ultrasonography) and tumour aggressiveness, metastasis rate and decreased patient survival has been shown for a variety of tumours ([[Delorme et al., 1997](#)], [[Newman et al., 1995](#)] and [[Schroeder et al., 2003](#)]).

In the sarcomas, malignant oral melanomas and squamous cell carcinomas examined in this study, it could be hypothesised that, in part, a higher vascularity and blood volume are related to different biological behaviour, such as local aggressiveness, metastasis rate and survival time. Because the range of Doppler values was large in the different histology groups, this may also indicate, as in humans, that tumours with the same histological appearance may exhibit different biological behaviours, depending on their vascularity and blood volume. This has already been shown in histological studies in canine cutaneous squamous cell carcinomas, mast cell tumours, osteosarcomas and mammary tumours, where significant positive correlations between intratumoral MVD and malignancy and patient survival were observed ([[Coomber et al., 1998](#)], [[Graham and Myers, 1999](#)], [[Maiolino et al., 2001](#)] and [[Preziosi et al., 2004](#)]).

Although no correlation between MVD and power Doppler parameters was found in this study, the ability of power Doppler to detect significant differences in vascularity and blood volume in the tumours investigated may indicate that this technique is well

suiting to monitor the response of canine tumours to various treatment modalities.

Conclusions

Vascularity and blood volume assessed with contrast-enhanced power Doppler ultrasound and immunofluorescent analysis of MVD varied between, but also within, different types of canine neoplasms. This may be related to the different biological behaviours of these tumours. Parameters were not significantly correlated, which may indicate that measurements of tumour vascularity can provide different biological information, dependent on the method used.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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