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FULL PAPER

Evaluation of neovascularization with spectral computed tomography in a rabbit VX2 liver model: a comparison with real-time contrast-enhanced ultrasound and molecular biological findings

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Objective: To validate the feasibility of using the parameters of spectral CT and CT perfusion and the dynamic features of real-time contrast-enhanced ultrasound (CEUS) to evaluate the vascularization of VX2 hepatic tumours.

Methods: Spectral CT imaging, CT perfusion and CEUS analysis were performed on rabbits implanted with VX2 hepatic tumours, 7 and 14 days after implantation. The perfusion parameters of CT, normalized iodine concentration (NIC) and dynamic features of CEUS were measured in the rim of the tumour (TR) and the normal liver region. The expression of vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2) was also determined.

Results: Increased perfusion parameters of CT were found in the TR. In addition, the NIC was elevated in TR during the arterial phase, and the peak intensity of the CEUS of the TR was reached significantly earlier than that

on normal liver region. At 14 days, the perfusion parameters of CT (blood volume, permeability surface and hepatic arterial fraction) offered higher accuracy and stability in differentiating the TR from the normal liver region. Furthermore, CEUS was more accurate in diagnosing tumours <1.0 cm in diameter. In addition, VEGF and FGF2 expression was higher in the TR and were positively correlated with CT and CEUS parameters, except mean transit time, rise time, washout time and peak time.

Conclusion: Use of spectral CT with perfusion techniques, iodine-based material-decomposition analysis and dynamic CEUS changes may reflect the angiogenesis and haemodynamic information of hepatic tumours.

Advances in knowledge: It is feasible to assess vascularization in hepatic cancer using CT or CEUS.

INTRODUCTION

Primary hepatic carcinoma is one of the most common malignant tumours, with a steadily increasing mortality rate over the past few years.¹ The newly emerging techniques of spectral CT imaging and CT perfusion reveal the haemodynamic features in the tumour tissues and play an important role in the detection and differential diagnosis of a solid mass in the liver.^{2,3} Dual-energy CT performed during two consecutive scans or with a dual X-ray source or dual-detector assembly has existed for a number of years. It was demonstrated that the additional information was provided by using dual-energy material separation technique, which had potential clinical applications.^{4,5} By revealing the blood supply and the haemodynamic features of the tumours, the real-time contrast-enhanced ultrasound (CEUS) provides high accuracy and specificity for diagnosing small hepatic tumours and enables the detection of early malignant diseases.

A rabbit model bearing VX2 hepatic tumours has been widely used in evaluating imaging and interventional therapy.⁶ The objectives of this study were to characterize the blood supply features of hepatic tumours using dynamic CEUS, CT perfusion parameters and normalized iodine concentration (NIC) changes by spectral CT imaging, and to correlate the angiogenesis and haemodynamic information of hepatic tumours with the expression of vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2).

METHODS AND MATERIALS

Animals

This study was conducted in accordance with the guidelines of the National Institutes of Health for the care and use of laboratory animals and was approved by the local animal ethics committee. A flowchart for the experimental study is shown in [Figure 1](#). 35 healthy New Zealand white

Figure 1. Flowchart of the experimental study. CEUS, contrast-enhanced ultrasound; FGF2, fibroblast growth factor 2; H&E, haematoxylin and eosin; MD analysis, material-decomposition analysis; VEGF, vascular endothelial growth factor.

rabbits (male or female, 2.0–3.0 kg) were supplied by the Henan Medical Animal Research Center (Zhengzhou, China).

Preparation of the VX2 tumour cells

Rabbits bearing VX2 tumours were provided by the Department of Intervention in the First Affiliated Hospital of China Medical University (Shenyang, China). A solid palpable mass had developed in the inguinal region by about 23 days after implantation. The rabbits were anaesthetized with an injection of 10% chloral hydrate (7–8 ml kg⁻¹); subsequently, the grey tumours were carefully dissected and cut into small pieces under aseptic conditions.

A rabbit VX2 hepatic tumour model

The healthy rabbits were anaesthetized, and then the left lobe of the liver was exposed through a midline abdominal incision. The liver parenchyma was punctured using smooth forceps to create a small nick. Fresh VX2 tumour masses (approximately 2 mm³) were implanted into the little nick, and then the wound was closed. The rabbits were treated with gentamicin at a dose of 80,000 units by intraperitoneal injection on the day of the operation and also intramuscularly for 3 days afterwards.

Spectral CT examination

After 7 and 14 days of tumour inoculation, multislice spiral CT perfusion was performed with a spectral CT scanner (Discovery™ CT750 HD; GE Healthcare, Waukesha, WI). The CT perfusion was performed with “toggling-table” scan mode, which covered from the diaphragm to the inferior border of the liver. A contrast medium containing 350 mg ml⁻¹ of iodine (Omnipaque™; GE Healthcare, Cork, Ireland) was injected *via* the auricular vein at a flow rate of 0.7 ml s⁻¹. The dose of contrast medium was calculated by body weight (1 ml per kg body weight). The scan parameters included 100 kV, 80 mA, 5-mm slice thickness, 0.6 s rotation speed, a total scan time of 50 s and collimation 64.00 × 0.63 mm. Artefacts caused by rabbit respiration were controlled with a tight, homemade belly band. All the images were analysed using a dedicated workstation (Advantage Workstation 4.3; GE Healthcare).

The two-phase contrast enhancement spectral CT imaging was started 3–5 h after the CT perfusion study to eliminate contrast medium interference. The scan mode was: tube voltage 80 and 140 kV with fast kilo voltage-switching technique; tube current Auto milliampere (mA) with noise index 8.0; slice thickness 5 mm, gantry speed 0.6 s per rotation, pitch 0.984:1. The contrast medium containing 350 mg ml⁻¹ of iodine (Omnipaque) was injected with a flow rate of 0.5 ml s⁻¹ and volume of 4 ml. The saline tracer injection rate was similar to contrast medium.

Spectral CT image post-processing

In post-processing, the 40–140 keV monochromatic CT images were reconstructed using Gemstone Spectral Imaging analysis software (GE Healthcare, Waukesha, WI). The regions of interest (ROIs) included the rim of the tumour and the normal liver region from the optimal contrast-to-noise ratio images. The CT value under different kilo electron voltage (keV), the corresponding energy spectral curve and the base material-decomposition images were calculated. A circular ROI was placed on the tumour according to the size and necrosis core of the tumour. Because the necrosis was not observed for the tumour with diameter <1 cm in our study, the ROI was simply put on the tumour with a diameter as large as possible. For the tumour with diameter >1 cm where a ring enhancement was observed, a round ROI was placed on the ring enhancement region. The centre necrosis and blood vessels were carefully avoided during the placement of ROI. The NIC was obtained by dividing the iodine concentration in the liver (IC_{liver}) by that in the aorta (IC_{aorta}) for both the arterial phase (AP) and the portal venous phase (VP): normalized iodine concentration = IC_{liver}/IC_{aorta}. The NIC was introduced to minimize variations among subjects as well as to reduce the scanning time. The measurement of ROI was repeated three times, and the average of three measurements was used for final analysis.

The CT perfusion imaging data were reconstructed and analysed using GE perfusion 4.0 software (GE Healthcare). The ROIs were placed on the rim of the tumour (TR) and the normal liver region. The selection criteria for the ROIs were the same as that in spectral imaging. The time–density curve (TDC) and the CT perfusion parameters of blood flow (BF), blood volume (BV), permeability surface (PS), mean transit time (MTT) and hepatic arterial fraction (HAF) were measured automatically: hepatic arterial perfusion (HAP) = BF × HAF, and portal venous perfusion (PVP) = BF × (1 – HAF). The peak time of the TDC in the renal cortex was used to distinguish the hepatic AP and the portal VP. All the data were measured on three separate occasions and used to obtain a mean value.

Ultrasound examination and post-processing

Colour Doppler ultrasonography and CEUS were performed on Day 7 and Day 14 after tumour inoculation using a Philips IU22 Colour Doppler Diagnostic Apparatus (Philips, Eindhoven, Netherlands). The liver was detected with two-dimensional (2D) ultrasound, which was used to record the location, diameter, shape and colour Doppler flow images inside and outside of the tumour. When a lesion was localized, a verification process that was as precise as possible enabled the lesion to be characterized using CEUS and CT. Special vascular structures, such as branching of the portal vein, the liver veins, the coeliac artery or

the mesenteric vessels, were therefore chosen as anatomical landmarks. This method of identification is feasible by positioning the lesions close to the scanning position landmarks in different imaging modalities. After targeting the lesions as precisely as possible, the lesions (also in sectional planes running at an angle) in different contrast medium phases of the CEUS could be assigned to CT. If improved tumour detection occurred during the course of the dynamic enhancement, a second bolus injection with SonoVue (Bracco, Milan, Italy) was administered to perform scanning of the tumour 5 min after the first injection.

After a bolus injection of SonoVue (0.1 ml kg^{-1} body weight) and normal saline (2 ml), the dynamic features of CEUS in both tumours and normal livers were obtained by contrast-tuned imaging with a total detection time of 50 s, detector frequency of 11 MHz and mechanical index of 0.05.

The peak time of the time–intensity curve in the renal cortex was used to make a distinction between the hepatic AP and the portal VP. The whole dynamic images were analysed by Qlab v. 8.1 (Philips, Eindhoven, Netherlands). ROIs with a diameter of 3 mm and a depth of 3 cm were placed on the TR and also at the normal liver region with the same selected criterion as CT perfusion. The time–intensity curve of the ROIs was then rendered, and the peak intensity (PI), rise time (RT), peak time (PT) and washout time (WT) were measured.

Molecular biological analysis

Liver specimens of both tumours and normal regions were obtained by CT-guided biopsy on the seventh day after CT and ultrasound examination. On Day 14, the rabbits were sacrificed with an overdose of pentobarbital *via* auricular vein injection. The liver specimens were sliced transversely at 1-mm intervals to match the orientation of previously acquired spectral CT and perfusion CT images. Haematoxylin and eosin staining was used to detect VEGF messenger RNA (mRNA) and FGF2 proteins. Histological characterization of the tumour was accomplished *via* visual inspection, and the findings were correlated with the findings on the quantitative parameters measurement from CT and ultrasound.

Livers from the euthanized rabbits were harvested for RNA extraction and real-time polymerase chain reaction detection. The gene-specific primers used in this study were chosen in accordance with those used in a previous report:¹ VEGF forward, 5'-CAG TGA ATT CGA GAT GAG CTT CCT ACA GCA C-3'; VEGF reverse, 5'-CCT GGA ATT CTC ACC GCC TCG GCT TGT CAC-3'; β -actin forward, 5'-CCT TCC TGC GCA TGG AGT CCT GG-3'; and β -actin reverse, 5'-GGA GCA ATG ATC TTG ATC TTC-3'.

Statistical analyses

The Statistical Package for the Social Sciences (SPSS®) v. 13.0 software (IBM Corporation, Armonk, NY; formerly SPSS Inc., Chicago, IL) was used for all statistical analyses. Quantitative variables are expressed as mean $\pm$ standard deviation and categorical variables as frequencies or percentages. A two-sample *t*-test was performed to compare the CT perfusion parameters in different regions, the CEUS parameter changes and the

parameters of the NIC between the two regions during AP and VP. A Pearson's correlation analysis was carried out to calculate the correlation between the VEGF, the FGF2 and the following: the quantitative perfusion CT parameters, the NIC and the CEUS parameters measured for each tumour. A two-tailed *p*-value ≤ 0.05 was considered to indicate a statistically significant difference.

Receiver operating characteristic curves were generated to establish the cut-off point values of these parameters required for the significant differentiation of the TR from the normal liver region on Day 14. Optimal sensitivity and specificity were defined as the maximal sensitivity and maximal specificity values. The null hypothesis tested was that the area under the receiver operating characteristic curve was 0.5; the alternative was that this area was >0.5 .

RESULTS

Tumour implantation and tumour growth

A total of 35 rabbits were implanted with VX2 hepatic tumours, and 3 of them died during or after the surgery owing to air embolus, an overdose of anaesthesia and post-operative infection, respectively. Another two rabbits failed to develop the VX2 tumour successfully. Therefore, the success rate of tumour implantation was 85.7%. After 14 days of implantation, the other 30 rabbits exhibited neoplastic nodules with diameters ranging from 0.2 to 4.0 cm. Abdominal metastasis at the operative incision occurred in two rabbits, whereas metastasis to the greater omentum was observed in two other rabbits. Lung metastasis was proven in one rabbit, and "satellite nodules" in the liver and malignant ascites were found in five and four rabbits, respectively. The remaining rabbits developed a solitary tumour in the liver.

Figure 2a–d illustrates the tumour specimen. VX2 hepatic tumours were round, grey nodules with an intact capsule and necrosis in the centre of the tumour with diameters >1.0 cm (Figure 2a,b). The histological analysis showed that VX2 tumours had a "nest" arrangement, with separated fibres and abundant ascent capillaries (Figure 2c). The tumour cells were large and had a round or irregular morphology. The nucleus of the VX2 cells was hypertrophic and heterogeneous, with a variable size and shape; the phases of nuclear mitosis were observed in VX2 carcinoma cells (Figure 2d).

Spectral CT imaging of a rabbit VX2 tumour model

Figure 3a,b shows the CT perfusion maps of tumours in each group of tumour-bearing rabbits. The values of five perfusion parameters (BF, BV, PS, HAF and HAP), calculated using the CT perfusion maps shown in Table 1, were significantly higher in tumour tissues than those in the normal rabbit liver after 7 and 14 days (all $p < 0.05$). However, the values of two other perfusion parameters (MTT and PVP) decreased in the tumour tissues ($p < 0.05$ for all). In addition, from Day 7 to Day 14, six perfusion CT parameters (BF, PS and HAP of TR) continuously increased along with tumour growth, while the MTT and PVP decreased ($p < 0.05$). The CT perfusion parameters in a normal liver showed no statistically significant difference with tumour growth ($p > 0.05$) (Table 1).

Figure 2. (a) Pathological specimen from a rabbit VX2 liver tumour. (b) VX2 liver tumours with necrosis in the centre. (c, d) Haematoxylin and eosin staining of rabbit VX2 liver tumours with $\times 10$ (c) and $\times 40$ (d) enlargement.

The TDC of the VX2 liver TR and that of a normal liver are shown in Figure 3c. The TDC of the TR has a steep increase and a gradual decrease, while the TDC of a normal liver has a gradual increase and a gradual decrease. Thus, the latency of the peak value in the TR (7.96 ± 0.57 s) was shorter than that in the normal liver (14.4 ± 1.02 s) ($p = 0.00$).

CT spectral enhancement and iodine-based images and spectral curves are shown in Figure 3d–f. The spectral curve revealed that with an increase of keV, the CT values gradually decayed. At Day 7, the NIC of the rim of the tumour and the normal liver region during the AP exhibited a statistically significant difference ($t = 5.97$, $p = 0.00$), whereas there was no statistical significance during the VP ($t = 0.18$, $p = 0.68$). The TR and the normal liver region spectral curves had no intersections (Figure 3f). After 14 days, the NIC of the TR was higher than it had been at 7 days ($t = -2.42$, $p = 0.03$) and also showed a statistically significant difference compared with that in the normal liver region during AP ($t = 3.75$, $p = 0.02$). By contrast, during the VP, the NIC was not significantly different from what it had been at 7 days ($t = -1.86$, $p = 0.77$) and from that in the normal liver region ($t = 0.31$, $p = 0.59$).

Ultrasound imaging features of a rabbit VX2 tumour model

As depicted in Figure 4a–d, the lesions were round, isoechoic tumours with acoustic halos nearby and a low-level echo of necrosis in the centre upon 2D ultrasound (Figure 4a). Colour Doppler flow images detected spot-like and strip-like echoes of blood vessels inside the tumour “ring” near the area of central degeneration and no >1.0 cm outside the tumours in seven

nodules (Figure 4b). After injection of the contrast agent, a majority of the tumours gradually displayed round or branching hyperechoic enhancement during the AP; then the enhancement gradually decreased, followed by the enhancement of the adjacent liver parenchyma during the VP (Figure 4c,d).

The time–intensity curve analysis showed that the peak intensity of the rim of the tumour was significantly higher than that of a normal liver at Day 7 ($p < 0.05$), whereas other features (the RT, PT and WT) had no statistical significance ($p > 0.05$). Along with tumour growth, the time–intensity curve parameters (the PI, PT and WT) of the TR showed significant differences compared with those of normal livers at Day 14 (all $p < 0.05$), whereas the RT also had no statistically significant difference between the TR and a normal liver ($p > 0.05$), as described in Figure 5. Furthermore, all time–intensity curve parameters of the TR showed a statistically significant difference between Day 7 and Day 14 (all $p < 0.05$), as shown in Table 2).

Quantitative CT and contrast-enhanced ultrasound image analysis of a rabbit VX2 tumour model

The values for CT perfusion and the CEUS parameters in the rim of the tumour and the normal liver region at different phases are compared in Figures 1 and 2. There were significant differences in the perfusion parameters of CT (BF, BV, MTT, PS and HAF) between the TR and the normal liver region ($p < 0.05$). The peak intensity and NIC of the TR and the normal liver region in the CEUS also had a statistically significant difference ($p < 0.05$). In addition, the perfusion

Figure 3. (a, b) A VX2 tumour showing ring enhancement on arterial phase (a) and venous phase (b). (c) The time–density curve of the rim of the tumour (green line) and normal liver region (blue line). (d) Spectral CT image of a VX2 liver tumour. (e) Iodine-based material-decomposition image of VX2 liver tumour. (f) The energy spectral curve of the rim of the tumour (blue line) and the normal liver region (yellow line). For colour image see online.

parameters BV [area under receiver-operating characteristic curve (AUC) = 0.988], PS (AUC = 0.970) and HAF (AUC = 0.914) proved to be highly accurate and stable in the differential diagnosis of the TR and the normal liver region. However, the BF (AUC = 0.858), MTT (AUC = 0.722), peak intensity (AUC = 0.651) and NIC during

the AP (0.698) had certain diagnostic value. Thus, we used the receiver operating characteristic curves to determine the parameter cut-off point required to optimize both the sensitivity and the specificity for differentiating between the rim of the tumour and the normal liver region at Day 14 as described in Table 3 and Figure 6.

Table 1. Comparison of CT perfusion parameters in different regions ($n = 30$)

Region	Time (day)	BF ($\text{ml min}^{-1} 100 \text{ g}^{-1}$)	BV ($\text{ml } 100 \text{ g}^{-1}$)	MTT (s)	PS ($\text{ml min}^{-1} 100 \text{ g}^{-1}$)	HAF (100%)	HAP ($\text{ml min}^{-1} 100 \text{ g}^{-1}$)	PVP ($\text{ml min}^{-1} 100 \text{ g}^{-1}$)
Rim of the tumour	7	66.01 ± 13.16	21.09 ± 5.49	19.59 ± 4.38	68.05 ± 13.00	0.46 ± 0.25	29.40 ± 17.34	36.62 ± 21.21
	14	79.97 ± 22.85	25.74 ± 6.20	14.98 ± 3.64	78.10 ± 11.38	0.60 ± 0.17	51.44 ± 27.35	28.53 ± 6.67
Normal liver region	7	45.49 ± 8.05	12.27 ± 2.16	39.03 ± 6.23	51.02 ± 16.94	0.16 ± 0.20	7.42 ± 9.96	38.07 ± 10.22
	14	52.01 ± 8.74	12.63 ± 1.48	37.59 ± 4.05	37.78 ± 11.81	0.32 ± 0.13	17.84 ± 9.78	34.18 ± 2.51
p^a		0.00	0.00	0.00	0.00	0.02	0.00	0.00
p^b		0.00	0.00	0.00	0.00	0.00	0.00	0.00
p^c		0.04	0.19	0.01	0.02	0.23	0.00	0.00

BF, blood flow; BV, blood volume; HAF, hepatic arterial fraction; HAP, hepatic arterial perfusion; MTT, mean transit time; PS, permeability surface; PVP, portal venous perfusion.

With exception of p -values, data are expressed in mean $\pm$ standard deviation.

^a p -values for comparisons between the rim of the tumour and the normal liver region at 7 days.

^b p -values for comparisons between the rim of the tumour and the normal liver region at 14 days.

^c p -values for comparisons between the rim of tumour at 7 and 14 days.

Figure 4. (a) Two-dimensional ultrasound of a rabbit with VX2 liver tumours. Arrows indicate the iso-echoic tumour with acoustic halo near by. (b) Colour Doppler flow image of a rabbit with VX2 liver tumours. (c) Contrast-enhanced ultrasound (CEUS) of a rabbit with VX2 liver tumours in the arterial phase. Arrow indicates the enhancement ring. (d) CEUS of a rabbit with VX2 liver tumours in the portal phase. Arrow indicates the enhancement ring.

Qualitative CT and contrast-enhanced ultrasound image analysis of a rabbit VX2 tumour model

The qualitative assessment of the TR during the AP and the VP and the enhancement changes between these two phases are shown in Table 4. Homogeneous enhancement was observed in 28 (93%) and 26 (87%) rabbits for spectral CT enhancement, in 27 (90%) and 25 (83%) for CEUS, and in 28 (93%) and 25 (83%) for CT perfusion, respectively, during the AP and the VP. The lesion attenuation greater than that of the aorta during the AP and less than that of the liver parenchyma during the VP was identified in 22 (73%), 24 (80%) and 22 (73%) of rabbits using spectral CT enhancement, CEUS and CT perfusion, respectively. Washout enhancement changes during both the AP and the VP, washout during the AP and expansion during the VP and expansion during the AP and the VP were noted in 17 (57%), 10 (33%) and 3 (10%) rabbits using spectral CT enhancement and CT perfusion, respectively, while CEUS were observed in 21 (70%), 8 (27%) and 1 (3%), respectively.

Compared with the pathological findings after sacrifice, the detection rate of hepatic tumours by CT perfusion was 93% (28/30) and ranged from 0.9 to 3.9 cm. For hepatic tumours with diameters ≤ 3.0 cm, we achieved a sensitivity and a specificity of 92.3% (24/26) and 60% (3/5), respectively. For hepatic tumours < 1.0 cm

in diameter, the sensitivity and the specificity were 50% (2/4) and 96.3% (26/27), respectively. The detection rate of hepatic tumours with spectral CT enhancement was 93% (28/30) with a range of 0.9–3.2 cm. We achieved a sensitivity of 88.5% (23/26) and a specificity of 50% (3/6) for small hepatic tumours with diameters ≤ 3.0 cm. The sensitivity and specificity were 50% (2/4) and 96.3% (26/27), respectively, for small hepatic tumours with a diameter < 1.0 cm. CEUS detected hepatic tumours at a rate of 96.7% (29/30), with a range from 0.7 to 3.5 cm. The sensitivity and specificity for the diagnosis of small hepatic tumours with diameters ≤ 3.0 cm were 96.2% (25/26) and 66.7% (2/3), respectively, while those of small hepatic tumours with diameters < 1.0 cm were 75% (3/4) and 96.2% (25/26), respectively.

Vascular endothelial growth factor messenger RNA and fibroblast growth factor 2 protein expressions
The mRNA expression of VEGF in the TR was higher than that seen in the normal liver tissues after 14 days (all $p < 0.05$). On the seventh day, the expression of VEGF was positively correlated with the CT perfusion parameters and ultrasound examination parameters (BV, PS, PT and WT). At 14 days, this correlation was further strengthened. In addition, the spectral CT parameters' NIC in the TR during the AP was positively correlated with VEGF

Figure 5. Dynamic contrast-enhanced ultrasound time-intensity curve analysis revealed the rise time, peak time and washout time of the rim of the tumour. (a) Time-intensity curve of the rim of the tumour (red line) and the normal liver region (blue line) after 7 days. (b) Time-intensity curve of the rim of the tumour (red line) and the normal liver region (yellow line) on the 14th day. For colour image see online.

expression. However, the other parameters of CT perfusion, ultrasound examination and spectral CT showed no correlation with the expression of VEGF, as depicted in Tables 5 and 6.

The protein expression of FGF2 in the TR was also higher than that in the normal liver tissues both at 7 days and at 14 days (all $p < 0.05$). Furthermore, the expression levels of FGF2 at 14 days were significantly higher than those at 7 days ($p < 0.05$). FGF2 expression was positively correlated with the CT perfusion parameters and the ultrasound examination parameters (BV, PS, HAF, PT and WT) at 7 and 14 days. In addition, the NIC of the TR during the AP according to spectral CT parameters was still positively correlated with VEGF expression. All other parameters of CT perfusion, ultrasound examination and spectral CT showed no correlation with the expression of VEGF, as described in Tables 5 and 6, and Figures 7 and 8.

DISCUSSION

The rabbit VX2 hepatic tumour model was established using a metastatic tumour cell line derived from squamous cell carcinoma in rabbit skin infected using the Shope virus *via* 72 sequential *in vitro* passages. This hepatic tumour model was supplied by the hepatic artery and is easily constructed *in situ*.⁷ So far, the rabbit model bearing VX2 hepatic tumours is the only available model for imaging investigations of hepatic tumours.

Following tumour growth, necrosis appeared at the centre of the tumour and had morphological features similar to those of human hepatic cell carcinoma.^{2,3} Blood vessels began to form in the tumour 2–3 weeks after implantation, reflecting the early stage of the tumour. During this early stage, hepatic function was normal and no metastasis occurred.⁸ In this study, after 3 weeks of implantation, the rabbits became anxious and depressed. The

Table 2. Comparison of real-time contrast-enhanced dynamic time and intensity curve analysis in different regions ($n = 30$)

Region	Time (day)	Peak intensity (dB)	Rise time (s)	Peak time (s)	Washout time (s)
Rim of the tumour	7	15.53 $\pm$ 1.98	8.24 $\pm$ 0.11	18.35 $\pm$ 0.81	29.15 $\pm$ 3.01
	14	25.52 $\pm$ 1.17	9.89 $\pm$ 1.05	21.89 $\pm$ 4.24	36.90 $\pm$ 5.82
Normal liver region	7	14.17 $\pm$ 1.99	8.24 $\pm$ 0.45	18.62 $\pm$ 7.07	29.95 $\pm$ 6.37
	14	10.72 $\pm$ 1.95	10.12 $\pm$ 1.80	22.60 $\pm$ 6.97	41.76 $\pm$ 8.61
p^a		0.17	0.89	0.91	0.74
p^b		0.00	0.81	0.42	0.26
p^c		0.00	0.00	0.02	0.00

With exception of p -values, data are expressed in mean $\pm$ standard deviation.

^a p -values for comparisons between the rim of the tumour and the normal liver region at 7 days.

^b p -values for comparisons between the rim of the tumour and the normal liver region at 14 days.

^c p -values for comparisons between the rim of the tumour at 7 and 14 days.

tumours were clearly visible on plain CT images, as were their blood supply vessels. Tumour vascularization is not only important for tumour growth but also for accelerating the process of metastasis.⁹ In the early stages of vascularization, the growth of tumour vessels was slow and linear. As the tumour develops its own blood-vascular system, the growth of the tumour vessels displays a rapidly increasing index in the middle stage of vascularization. The immature vessel wall leads to the high permeability of tumour vessels, further evoking metastasis.¹⁰

A spectral CT scanning mode that is based on the rapid switching between high- and low-energy data sets from view to view was recently introduced. This scanning mode has enabled the precise registration of data sets for the creation of accurate material-decomposition images and monochromatic spectral images at energy levels ranging from 40 to 140 keV. For medical diagnostic imaging, water and iodine are often selected as the basic pair for material-decomposition image presentation because their atomic numbers span the range of atomic numbers for materials generally found in medical imaging and approximate those of soft tissue and iodinated contrast material,

resulting in material-attenuation images that are intuitive to interpret.¹¹

The iodine concentration in the lesions was used to evaluate the iodine-based material-decomposition images. According to our study results, the NIC was higher in the rim of the tumour than in a normal liver region during the AP. This finding could have been due to high level of angiogenesis and perfusion in the TR that results from the high iodine concentration in this region. However, there was no statistically significant difference in iodine concentration during the VP between the TR and the normal liver region, which could be a result of inhomogeneous enhancement in the TR during this phase due to haemorrhaging and necrosis.

Multislice CT perfusion was able to reflect the microcosmic functions of new tumour vessels *via* macroscopic perfusion parameters.^{12,13} In this study, 3 weeks after tumour implantation, the TR showed an abundance of blood vessels, and 30–50% of the centre of the tumour was necrotic, which may have been due to a lack of nutrition. The four increased perfusion

Table 3. The cut-off points, sensitivities and specificities for distinguishing the rim of the tumour and the normal liver region at 14 days

Parameter	Cut-off point	Sensitivity (%) ^a	Specificity (%) ^b
BF	68.12	76.9 (23)	100 (30)
BV	16.36	93.3 (28)	100 (30)
MTT	37.04	70.0 (21)	76.7 (23)
PS	51.05	93.3 (28)	83.3 (25)
HAF	0.56	76.7 (23)	100 (30)
HAP	38.17	76.7 (23)	100 (30)
PVP	32.41	76.7 (23)	93.3 (28)
PI	20.88	83.3 (25)	70.0 (21)
NIC AP	0.46	100 (30)	60.0 (18)

BF, blood flow; BV, blood volume; HAF, hepatic arterial fraction; HAP, hepatic arterial perfusion; MTT, mean transit time; NIC AP, normalized iodine concentration during arterial phase; PI, peak intensity; PS, permeability surface; PVP, portal venous perfusion.

^aSensitivity values are cited as percentages. Data in parentheses are numbers of rim of the tumour ($n = 30$) used to calculate percentages.

^bSpecificity values are cited as percentages. Data in parentheses are numbers of normal liver region ($n = 30$) used to calculate percentages.

Figure 6. Receiver operating characteristic curves for perfusion parameters (BV, BF, MTT, PS, HAF, and PI) and NIC during the arterial phase in differentiating the rim of the tumour from the normal liver region on the 14th day. BF, blood flow; BV, blood volume; HAF, hepatic arterial fraction; HAP, hepatic arterial perfusion; MTT, mean transit time; NIC, normalized iodine concentration during arterial phase; PI, peak intensity; PS, permeability surface; PVP, portal venous perfusion.

parameters of CT (BF, BV, PS and HAF) in the rim of the tumour revealed a high amount of perfusion in the tumour region, suggesting an abundance of blood vessels. The tumour rim was highly permeable and showed a rim-enhancing result

from the immature basement membrane of the blood vessels.¹⁴ During the early period of capillary generation, newborn blood vessels in the basement membrane of the endothelial cells are affected by various factors; therefore, some perfusion parameters

Table 4. Qualitative assessment of the rim of the tumour during arterial phase (AP) and venous phase (VP) and enhancement changes between phases at 14 days

Parameter	Spectrum CT enhancement (<i>n</i> = 30)		CEUS (<i>n</i> = 30)		CT perfusion (<i>n</i> = 30)	
	AP	VP	AP	VP	AP	VP
Enhancement pattern						
Homogeneous diffusion	28 (93)	26 (87)	27 (90)	25 (83)	28 (93)	25 (83)
Heterogeneous diffusion	2 (7)	4 (13)	3 (10)	5 (17)	2 (7)	5 (17)
Attenuation of enhancement						
Greater than aorta during AP, less than liver parenchyma during VP	22 (73)		24 (80)		22 (73)	
Less than aorta during AP, greater than liver parenchyma during VP	6 (20)		3 (10)		6 (20)	
Less than aorta during AP, less than liver parenchyma during VP	2 (7)		3 (10)		2 (7)	
Enhancement change between AP and VP						
Washout during AP and VP	17 (57)		21 (70)		17 (57)	
Washout during AP, expansion during VP	10 (33)		8 (27)		10 (33)	
Expansion during AP and VP	3 (10)		1 (3)		3 (10)	

CEUS, contrast-enhanced ultrasound.

Data are numbers of lesions with percentages in parentheses.

Table 5. Correlation between CT parameters and molecular biological index at 7 and 14 days

Group	BF		BV		MTT		PS		HAF		NIC AP	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
VEGF												
7 days	0.24	0.54	0.99	0.00 ^a	0.14	0.72	0.72	0.03 ^a	0.65	0.06	0.73	0.03 ^a
14 days	0.94	0.00 ^a	0.93	0.00 ^a	0.53	0.05	0.90	0.00 ^a	0.95	0.00 ^a	0.64	0.03 ^a
FGF2												
7 days	0.28	0.46	0.87	0.00 ^a	0.13	0.73	0.78	0.01 ^a	0.70	0.04 ^a	0.81	0.01 ^a
14 days	0.99	0.00 ^a	0.99	0.00 ^a	0.23	0.45	0.55	0.05 ^a	0.98	0.00 ^a	0.99	0.00 ^a

BF, blood flow; BV, blood volume; FGF2, fibroblast growth factor 2; HAF, hepatic arterial fraction; MTT, mean transit time; NIC AP, normalized iodine concentration during arterial phase; PS, permeability surface; VEGF, vascular endothelial growth factor.

^aStatistically significant.

of CT (BV, MTT, HAF and PS) were not stable from Day 7 to Day 14. Thus, CT enhancement had difficulty directly displaying the infiltration surrounding the tumours before morphological changes.⁶ CT perfusion therefore offered a benefit for clinical decisions, treatment and prognosis by reflecting the most accurate degree of infiltration.

The peak value of the TDC was highly correlated with the injection flow rate, the injection dose, the contrast medium concentration and tissue compositions, while the peak time was related to the initial time and the local circulation state.^{6,15} In this study, the contrast medium was injected through the auricular vein into the right atrium and then circulated to the abdominal aorta. Thus, in the early stage of circulation, a small amount of contrast media already existed in the vessels, even if portal venous return had reached its peak value. Owing to the prolonged duration of elevated blood drug levels in the portal vein, the TDC showed both low and moderate curves with a steep increase and a slow decrease. By contrast, the TDC in normal liver parenchyma exhibited a slow increase and a slow decrease as a result of non-vessel components. By contrast, the TDC of the VX2 TR showed a steep increase and a slow decrease, with an earlier peak value.

CEUS reflected the status of BF by injecting contrast agents into the cardiovascular system and organs. It has a high accuracy for

diagnosing solid masses in the liver, even for microhepatic tumours with diameters <1.0 cm.^{16,17} The contrast medium in ultrasound is composed of sulphur hexafluoride microvesicles with phospholipid shells, which can enhance the liver and spleen. The microvesicles may prevent hepatic reticuloendothelial cells from performing phagocytosis.¹⁸ It was possible to adequately increase the 2D greyscale gain before injection to achieve a satisfactory developing effect. Mechanical indices <0.08 are low, so low-mechanical index CEUS utilizes ultrasonic waves with less energy than that used to blast microvesicles and control non-linear echo signals.^{19,20} VX2 hepatic lesions with an arterial blood supply exhibited a typical malignant tumour contrast dynamic-change process. CEUS therefore was able to diagnose liver tumours by reflecting the state of vascularization and haemodynamics in tumours.^{21,22} It improved the accuracy of CEUS in the differential diagnosis. In this study, time-intensity curve analysis showed that peak intensity of the TR was significantly higher than that of a normal liver region at Day 14; the time-intensity curve parameters of the rim of the region also demonstrated significant differences from Day 7 to Day 14, which indicated that the typical arterial blood supply of a malignant tumour exhibits dynamic changes.

Small hepatic tumours (diameter <3.0 cm) were round and isoechoic with acoustic halos nearby and a low-level echo of necrosis in the centre upon 2D ultrasound owing to a lack of

Table 6. Correlation between contrast-enhanced ultrasound parameters and molecular biological index at 7 and 14 days

Group	Peak intensity		Rise time		Peak time		Washout time	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
VEGF								
7 days	0.27	0.48	0.39	0.30	0.84	0.00 ^a	0.87	0.00 ^a
14 days	0.55	0.04 ^a	0.18	0.56	0.30	0.31	0.07	0.80
FGF2								
7 days	0.09	0.82	0.44	0.24	0.74	0.02 ^a	0.70	0.04 ^a
14 days	0.71	0.01 ^a	0.18	0.55	0.27	0.37	0.09	0.77

FGF2, fibroblast growth factor 2; VEGF, vascular endothelial growth factor.

^aStatistically significant.

Figure 7. The reverse transcriptase polymerase chain reaction results revealed the vascular endothelial growth factor (VEGF) messenger RNA expression level in the rim of the tumour at 7 days (#2), the rim of the tumour after 14 days (#3) and a normal liver region (#1). The electrophoresis bands represent VEGF (144 bp) and β -actin (258 bp), respectively.

nutrition; a typical structure resulting from hepatic lobules also disappeared. It was difficult to definitively visualize blood vessels in small tumours by colour Doppler flow imaging owing to the slow BF. Xu et al.²³ reported that the sensitivity of detection of small hepatic tumours (diameter <2.0 cm) could be increased to >90% by CEUS. The sensitivity and specificity for the diagnosis of small hepatic tumours with diameters $\leq$ 3.0 cm were 96.2% and 66.7% in our study, respectively, which were similar to the findings of Xu et al.²³ An isolated hepatic tumour (diameter <1.0 cm) without portal vein infiltration and liver metastasis with a negative serology examination could easily be missed by MRI, CT, digital subtraction angiography and 2D ultrasound. In our study, the sensitivity of CEUS for small hepatic tumours with diameters <1.0 cm was far higher than that of 2D ultrasound. There were no significant acoustic impedance and image frequency differences between small hepatic tumours and normal livers, so acoustic halos and image contrast resolution could not be clearly displayed.²⁴

Receiver operating characteristic analysis in our study revealed that three perfusion parameters of CT (BV, PS and HAF) had a high accuracy and stability in the differential diagnosis of the TR and the normal liver region. However, the peak intensity showed a certain diagnostic value. The BV, PS and HAF reflected more blood perfusion information of the microinformation than peak intensity, with a high sensitivity and specificity. Although our statistical analysis revealed no significant difference in the areas under the receiver operating characteristic curve between the quantitative and qualitative analyses, this finding was probably due to the small sample size used in this study. The values for the quantitative parameters represented improvements over the qualitative research data acquired by Lv et al,¹¹ who found that qualitative evaluations of the degree and/or pattern of enhancement were specific but not sensitive. An optimal cut-off point for these parameters should be derived to maximize both sensitivity and specificity.

Angiogenesis is the process of generating new blood vessels from existing blood capillaries or nascent capillary vessels.^{25,26} This process has three stages of vascularization to supply tissues with

enough oxygen and nutrient substances and also to discard wastes. Tumours often secrete a series of materials that accelerate the speed of vascularization. Owing to the incomplete structure and deficient function of hastily constructed blood vessels, tumour cells may permeate blood vessels into the circulation, resulting in metastasis.²⁷ Therefore, high VEGF and FGF2 protein expressions might be a factor used to predict hepatic vein infiltration, which is caused by an acceleration of the proliferation of endothelial cells and an induction of the growth of blood vessels to increase the perfusion of tumours.^{28,29} Suzuki et al.³⁰ reported that VEGF expression in the necrotic tissues of liver tumours was higher than that in the normal livers, and the VEGF expression level was highly correlated with the growth of the liver tumour. Because the genes that encode the FGF protein are similar to those of VEGF, the similarity of the homologous sequence was as high as 53%. The FGF2 protein promotes angiogenesis and cell proliferation. In this study, the expression of VEGF mRNA and FGF2 proteins in the rim of the tumour was much higher than that in a normal liver region. These results revealed a significant difference in angiogenesis between the TR and a normal liver region. Consequently, the degree of abundance of new vessel formation in the TR region might be higher than that in a normal liver region. At Day 14, there were positive correlations between VEGF mRNA, FGF2 protein expression, the data of the NIC and the peak intensity in the rim of the tumour region. In addition, abundant new vessels formed within the tumour and exhibited strong echo and iodine concentrations of the TR. There were remarkable correlations between VEGF, FGF2 protein expression and the data of perfusion parameters (BV, BE, PS and HAF) of the TR in our study, which is consistent with the concept that extensive hepatic tumour vessels lead to high perfusion of the TR at Day 14. The artery, vein, venous sinus and blood capillaries all could impact the MTT value at Day 14, while unstable and incomplete angiogenesis on Day 7 would result in no significant difference in vascularization.

In our study, the correlation coefficients of peak intensity at Day 14 were not very high, probably because CEUS detected the tumour lesions using the BF signals within the tumour, reflecting a dynamic and real-time observation of the

Figure 8. Western blot results revealed the expression level of fibroblast growth factor 2 (FGF2) in the rim of the tumour (#1) and a normal liver region (#2) after 7 days and in the rim of the tumour (#3) and a normal liver region (#4) on the 14th day.

microvasculature of the enhancement of the more mature blood vessels, which were the functional blood vessels. However, the VEGF and FGF2 protein detections were all based upon the number of tumour endothelial cells. The peak intensity was connected with an enhanced start time, contrast agent measurement, section depth and other factors.²⁷ However, the quantification of CT perfusion at the capillary level reflected the BV, capillary permeability and other information, accurately indicating tumour angiogenesis and a high spatial resolution for the detection and quantification of angiogenesis using physiological changes.

Several shortcomings should be mentioned in our study, such as the fact that the CEUS operator would display subjective deviation depending upon his or her operational skills and experience. During every CEUS examination, only the tumours in the probe-detected section could be acquired after injection, which would exclude the overall information from the entire liver. Gas intervention in the rabbit gastrointestinal tract and rabbit respiratory frequency disturbances were the two main uncontrollable factors during CT and ultrasound examinations.

Owing to rabbit anaesthesia tolerance and the small sample size, any dynamic observation of tumour growth and changes would be limited.

CONCLUSION

Angiogenesis and haemodynamic information of hepatic tumours can be evaluated using CT perfusion and CEUS parameters. The perfusion parameters of CT (BV, PS and HAF) have a high accuracy and stability in the differential diagnosis of the TR and the normal liver region. As a quick, convenient and accurate method that also does not expose patients to iodine or radiation, CEUS was superior to other imaging techniques in detecting microhepatic tumours.

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REFERENCES

- Ozaki NK, Beharry KD, Nishihara KC, Akmal Y, Ang JG, Sheikh R. Regulation of retinal vascular endothelial growth factor and receptors in rabbits exposed to hyperoxia. *Invest Ophthalmol Vis Sci* 2002; **43**: 1546–57.
- Kuszyk BS, Boitnott JK, Choti MA, Bluemke DA, Sheth S, Magee CA, et al. Local tumor recurrence following hepatic cryoablation: radiologic-histopathologic correlation in a rabbit model. *Radiology* 2000; **217**: 477–86. doi: [10.1148/radiology.217.2.r00nv41477](https://doi.org/10.1148/radiology.217.2.r00nv41477)
- Shao GL, Zhou KR, Wang JH, Yan ZP, Liu R. Improvement in making rabbit model bearing VX2 liver tumor for interventional experimental study and CT evaluation of tumor. *J Clin Radiol* 2000; **19**: 653–4.
- Holmes DR 3rd, Fletcher JG, Apel A, Huprich JE, Siddiki H, Hough DM, et al. Evaluation of non-linear blending in dual-energy computed tomography. *Eur J Radiol* 2008; **68**: 409–13. doi: [10.1016/j.ejrad.2008.09.017](https://doi.org/10.1016/j.ejrad.2008.09.017)
- Graser A, Johnson TR, Hecht EM, Becker CR, Leidecker C, Staehler M, et al. Dual-energy CT in patients suspected of having renal masses: can virtual nonenhanced images replace true nonenhanced images? *Radiology* 2009; **252**: 433–40. doi: [10.1148/radiol.2522080557](https://doi.org/10.1148/radiol.2522080557)
- Jiang HJ, Xu K, Zhang HB, Liu BL, Shu SJ, Huang YH. *In vivo* assessment of neovascularization of rabbit VX2 liver tumor by multi-slice CT perfusion. *Chin J Med Imaging Technol* 2007; **23**: 967–70.
- Zheng LF, Li YJ, Wang H, Zhao JL, Wang XF, Hu YS, et al. Combination of vascular endothelial growth factor antisense oligonucleotide therapy and radiotherapy increases the curative effects against maxillofacial VX2 tumors in rabbits. *Eur J Radiol* 2011; **78**: 272–6.
- Zhao JG, Feng GS, Kong XQ, Li X, Li MH, Cheng YS. Changes of tumor microcirculation after transcatheter arterial chemoembolization: first pass perfusion MR imaging and Chinese ink casting in a rabbit model. *World J Gastroenterol* 2004; **10**: 1415–20.
- Lei Z, Ma H, Xu N, Xi H. The evaluation of anti-angiogenic treatment effects for implanted rabbit VX2 breast tumors using functional multi-slice spiral computed tomography (f-MSCT). *Eur J Radiol* 2011; **78**: 277–81.
- Kambadakone AR, Sahani DV. Body perfusion CT: technique, clinical applications, and advances. *Radiol Clin North Am* 2009; **47**: 161–78.
- Lv P, Lin XZ, Li J, Li W, Chen K. Differentiation of small hepatic hemangioma from small hepatocellular carcinoma: recently introduced spectral CT method[J]. *Radiology* 2011; **259**: 720–9. doi: [10.1148/radiol.11101425](https://doi.org/10.1148/radiol.11101425)
- Choi SH, Chung JW, Kim HC, Baek JH, Park CM, Jun S, et al. The role of perfusion CT as a follow-up modality after transcatheter arterial chemoembolization: an experimental study in a rabbit model. *Invest Radiol* 2010; **45**: 427–36.
- Bellomi M, Viotti S, Preda L, D'Andrea G, Bonello L, Petralia G. Perfusion CT in solid body-tumors. Part II: clinical applications and future development. *Radiol Med* 2010; **115**: 858–74.
- Jiang HJ, Zhang ZR, Shen BZ, Wan Y, Guo H, Li JP. Quantification of angiogenesis by CT perfusion imaging in liver tumor of rabbit. *Hepatobiliary Pancreat Dis Int* 2009; **8**: 168–73.
- Wu H, Exner AA, Shi H, Bear J, Haaga JR. Dynamic evolutionary changes in blood flow measured by MDCT in a hepatic VX2 tumor implant over an extended 28-day growth period: time-density curve analysis. *Acad Radiol* 2009; **16**: 1483–92.
- Jung EM, Schreyer AG, Schacherer D, Menzel C, Farkas S, Loss M, et al. New real-time image fusion technique for characterization of tumor vascularisation and tumor perfusion of liver tumors with contrast-enhanced ultrasound, spiral CT or MRI: first results. *Clin Hemorheol Microcirc* 2009; **43**: 57–69.
- Weskott HP. Emerging roles for contrast-enhanced ultrasound. *Clin Hemorheol Microcirc* 2008; **40**: 51–71.
- Zuber-Jerger I, Schacherer D, Woenckhaus M, Jung EM, Schölmerich J, Klebl F. Contrast-enhanced ultrasound in diagnosing liver malignancy. *Clin Hemorheol Microcirc* 2009; **43**: 109–18. doi: [10.3233/CH-2009-1225](https://doi.org/10.3233/CH-2009-1225)
- Kim TK, Jang HJ, Wilson SR. Benign liver masses: imaging with microbubble contrast agents. *Ultrasound Q* 2006; **22**: 31–9.
- Dai YI, Chen MH, Yin SS, Yan K, Fan ZH, Wu W, et al. Focal liver lesions: can

- sonovue-enhanced ultrasound be used to differentiate malignant from benign lesions? *Invest Radiol* 2007; **42**: 596–603. doi: [10.1097/RLI.0b013e318050ab29](https://doi.org/10.1097/RLI.0b013e318050ab29)
21. Watanabe R, Matsumura M, Chen CJ, Kaneda Y, Fujimaki M. Characterization of tumor imaging with microbubble-based ultrasound contrast agent, sonazoid, in rabbit liver. *Biol Pharm Bull* 2005; **28**: 972–7. doi: [10.1248/bpb.28.972](https://doi.org/10.1248/bpb.28.972)
 22. Wilson SR, Burns PN. Liver mass evaluation with ultrasound: the impact of microbubble contrast agents and pulse inversion imaging. *Semin Liver Dis* 2001; **21**: 147–59.
 23. Xu HX, Xie XY, Lu MD, Liu GJ, Xu ZF, ZhengYL, et al. Contrast-enhanced sonography in the diagnosis of small hepatocellular carcinoma ≤ 2 cm. *J Clin Ultrasound* 2008; **36**: 257–66.
 24. Yang YR, Zhang GX, Xu XY, Jiang LY, Li Y, Zhao J. Dynamic US scanning and their biological study of micro hepatocellular carcinoma (MH-CC). *J Ultrasound Clin Med* 2006; **8**: 129–32.
 25. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 2000; **407**: 249–57. doi: [10.1038/35025220](https://doi.org/10.1038/35025220)
 26. Purdie TG, Henderson E, Lee TY. Functional CT imaging of angiogenesis in rabbit VX2 soft-tissue tumor. *Phys Medicine Biology* 2001; **46**: 3161–75. doi: [10.1088/0031-9155/46/12/307](https://doi.org/10.1088/0031-9155/46/12/307)
 27. Jiang HJ, Lu HB, Zhang ZR, Wang YM, Huang Q, Huang YH, et al. Experimental study on angiogenesis in a rabbit VX2 early liver tumour by perfusion computed tomography. *J Int Med Res* 2010; **38**: 929–39.
 28. Zachary I, Glikli G. Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. *Cardiovasc Res* 2001; **49**: 568–81. doi: [10.1016/S0008-6363\(00\)00268-6](https://doi.org/10.1016/S0008-6363(00)00268-6)
 29. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996; **86**: 353–64. doi: [10.1016/S0092-8674\(00\)80108-7](https://doi.org/10.1016/S0092-8674(00)80108-7)
 30. Suzuki K, Hayashi N, Miyamoto Y, Yamamoto M, Ohkawa K, Ito Y, et al. Expression of vascular permeability factor/vascular endothelial growth factor in human hepatocellular carcinoma. *Cancer Res* 1996; **56**: 3004–9.