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Correlation between Contrast-Enhanced Ultrasound and Microvessel Density via CD31 and CD34 in the rabbit VX2 lung peripheral tumor model

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Abstract

Aim: To evaluate the tumor angiogenesis in lung peripheral VX2 tumor model by contrast-enhanced ultrasound (CEUS) and to determine the correlation between CEUS parameters and microvessel density (MVD) calculated *via* CD31 and CD34 expression. **Materials and methods:** VX2 pulmonary tumors were created in eight Japanese white rabbits by implanting a VX2 sarcoma into the lower portion of the right lung through ultrasound guidance. Tumors were allowed to grow for 14-21 days to achieve a diameter of 7-15 mm, and were examined by CEUS using a SonoVue contrast agent. The results were recorded as digital video images, and the time-intensity curves and hemodynamic parameters were analyzed. Pathological tumor specimens were immediately obtained after the ultrasound examinations. Tumor specimens were stained with hematoxylin and eosin (H&E) and expressed as CD31 and CD34. The different endothelial cell markers were determined by immunohistochemical staining. MVD was calculated *via* CD 31 and CD34, and the relationship between CEUS parameters and MVDs was analyzed. **Results:** Two distinct types of microvessels were identified in lung peripheral VX2 tumors: differentiated (CD34+) and undifferentiated (CD31+) vessels. A significant correlation was found between CEUS parameters and undifferentiated MVD (CD31+ vessels) in lung peripheral VX2 tumors ($p<0.05$). A reverse correlation was observed between different MVDs. **Conclusions:** Two different degrees of differentiation of vascular endothelial cells (CD31 and CD34) exist in the rabbit lung peripheral VX2 tumor model. CD31 MVD can more effectively evaluate tumor angiogenesis compared with CD34 MVD. CEUS, as a non-invasive imaging method, can effectively evaluate tumor angiogenesis in rabbit peripheral lung cancer.

Keywords: contrast-enhanced ultrasonography; VX2 tumor; lung cancer; microvessel density; peak intensity

Introduction

Lung cancer is the most common cause of death among malignant tumors around the world. Data has shown that lung cancer accounted for 19.4% of all cancer deaths in 2012 [1]. Its five-year survival rate after treatment is as low as 10% [2]. Computed tomography (CT) is the most common method for detecting lung cancer. Ultrasound has limitations due to the reflection of the gas and ribs block. However, when lung cancer grows close to the pleura, ultrasound (US) can detect tumor structures and blood flow from the rib space [3]. On the sonogram, most peripheral lung carcinomas are round and polycyclic. These reveal an infiltrating growth or central necrosis. Invasion into adjacent structures of the chest wall is a very reliable sign of malignant growth [4].

Angiogenesis, the formation of new blood vessels from the endothelium of the existing vasculature, is a critical factor for tumor growth and metastasis in many human tumors [5,6]. Various studies have evaluated tumor angiogenesis through noninvasive imaging methods such as contrast-enhanced dynamic CT, contrast-enhanced dynamic magnetic resonance imaging (MRI), non-enhanced magnetic resonance angiography, positron emission tomography, and Doppler ultrasonography [7-

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9]. However, these methods are limited to detecting low flow in small vessels. Contrast-enhanced ultrasonography (CEUS) increases the sensitivity to low flow, offering detailed information on tumor vascularity [10,11].

Microvessel density (MVD) is an independent prognostic indicator, and has been considered the gold standard for evaluating tumor angiogenesis [12]. Different blood vessel makers have been used to reveal the different aspects and characteristics of tumor vasculature. CD31 is usually expressed in both differentiated and undifferentiated endothelial cells, and CD34 is expressed in differentiated endothelial cells [13].

The VX2 tumor model is the most widely used animal model at present. Many studies have discussed the application of CT in the pulmonary tumor model [14-16], but few have dwelled on US and CEUS. The correlation of CEUS parameters with different blood vessel markers have not been discussed in rabbit VX2 lung peripheral tumor models. The object of this study was to assess vascularity measurements *via* noninvasive quantified CEUS, and correlate CEUS parameters with different MVDs through immunohistochemical analysis.

Materials and methods

Animals, tumor model and protocol

The protocol for this study was approved by the research Ethics Committee of the Capital Medical University. Twelve Japanese white rabbits, which were approximately three months old, were implanted with VX2 sarcoma. The average weight of these rabbits was 2.59±0.14 kg. The tumor implantation procedure started with a right intercostal incision between the seventh and eighth ribs. A piece of VX2 tumor tissue (approximately 1 mm^3) was inserted retrograde into the distal portion of an 18-gauge injection needle (BARD, USA). The needle was punctured vertically into the chest wall at a depth of approximately 1 cm from intercostal incision. Then, the tumor was ejected into the pulmonary parenchyma. After the needle was withdrawn, the pinhole was immediately pressed with a gauze to prevent bleeding and pneumothorax. The animals were scanned by ultrasound at 14 days after implantation to monitor tumor growth. The VX2 tumor nodules were allowed to grow for 14-21 days to achieve an approximate size of 7-15 mm in diameter before these rabbits were used for the study. Successful tumor growth was obtained at 66.7% (8 of 12 rabbits) of the implantation sites.

US and CEUS

US imaging was performed using an Acuson Sequoia 512 machine equipped with a 15L8 linear array probe operated at a transmission frequency of 14 MHz. The procedure was operated by a single sonographer with 12 years experience. The rabbits were placed in a supine position and anesthetized by injecting 3% pentobarbital sodium from the ear margin at a dose of 1.0 ml/kg. US parameters including acoustic gain, depth, and focus were optimized for each tumor. The tumors were first scanned by gray scale US, followed by color Doppler US in order to obtain the best imaging plane.

Following standard US the tumors were examined by real-time CEUS after injection of the intravascular tracer SonoVue (Bracco SpA, Milan, Italy), which comprise of phospholipid-stabilized microbubbles of sulfur hexafluoride with a mean diameter of 2.5 μm and a concentration of $1\text{-}5\times10^8$/mL [17]. The microbubble can freely flow through the tissues of tiny capillaries, but cannot enter the tissue space. Prior to injection, 5 mL of saline solution was added to the lyophilized powder under a sulfur hexafluoride atmosphere, and shaken thoroughly. The contrast agent (0.2 ml/kg) was injected *via* an ear margin vein as a bolus within 2-3 seconds, followed by a saline bolus of 2 mL. Continuous scanning was started shortly before injecting the ultrasound contrast agent, which lasted for 90 seconds with a low mechanical index (MI) of 0.16. During the whole process, the operator kept the transducer stable. All CEUS images were recorded as digital video images.

Tumor enhancement was quantified offline using the time–signal intensity curve analysis software installed on the Acuson Sequoia 512. This software can display the signal time-intensity curve in the regions of interest (ROI) during enhancement. Quantitative data were retrospectively analyzed by two investigators who worked independently. The region of interest (ROI) was manually and independently selected by the investigators to highly enhance the tumor regions and avoid the area of tumor necrosis. The size of the ROI was maintained at approximately 2-3 mm diameters as much as possible. For each tumor, three ROIs were selected for plotting the contrast enhancement time-intensity curves, and the results were averaged. The following parameters of the time–intensity curves were noted: arrival time (AT), time to peak (TTP), baseline intensity (BI) and peak intensity (PI).

Immunohistochemistry

After the CEUS examination, rabbits were sacrificed by injecting an overdose of anesthetics, and the tumors were harvested for further histological investigation. The tumor was marked to make sure that it was consistent with the results of the CEUS analysis. Pathological specimens were fixed in 4% paraformaldehyde for 30 minutes, and blocked with 5% donkey serum for 30 minutes. Next, the sections were incubated overnight at 4°C with mouse anti-human CD31 monoclonal antibody and mouse anti-

human CD34 monoclonal antibody, respectively. Then, the sections were colored by diaminobenzidine.

The method for counting the MVD number was based on the study conducted by Weidner et al [18], that is, to first look for high density regions of angiogenesis under the low power field (100× total magnification), and switch to a high power field (200× total magnification) to count the number of microvessels. Five digital images were recorded per high power field. The mean of these five fields was calculated and regarded as the tumor's MVD. The result was reviewed by a pathologist who was blinded to the experiment.

Statistical analysis

Continuous variables were reported as a mean±standard variation. Spearman correlation analysis was used to determine the correlation between PI of CEUS-, and the different MVDs (histological CD31 count or CD34 count). A p value <0.05 was considered statistically significant. Statistics were analyzed using the Statistical Package for the Social Sciences (SPSS 21.0; SPSS Inc., Chicago, IL, USA).

Results

Ultrasonography and CEUS of lung peripheral VX2 tumors

The average size of the tumors was 14±7 mm × 11±4 mm after 14-21 days from implantation of the VX2 sarcoma through ultrasound guidance in the lower portion of the right lung. The gray scale US demonstrated that tumors with heterogeneous structure and low echo had no envelopes, but the boundaries remained clear. In color Doppler imaging, blood flow signals could be observed around the tumor (fig 1) and the arterial blood flow spectrum could be detected. The average peak flow rate was 13.5 cm/s, and the average resistance index (RI) was 0.46.

On CEUS images, lung peripheral VX2 tumors were illustrated as highly enhanced in peripheral tissues, and low or not enhanced in the center tissue. All eight tumor lesions were shown as "fast in" and "slow out" in the time-intensity curve. The mean value of PI of the lung peripheral VX2 tumor was 24.01±7.36 dB (fig 2).

Pathological finding of H&E in lung peripheral VX2 tumors

At low magnification (H&E×10), the manifestation revealed a large tumor cell proliferation and adjacent lung tissue atrophy. At high magnification (H&E×40), tumor cells manifested in different sizes and shapes, and blood vessels were observed between the tumor cells (fig 3 a,b).

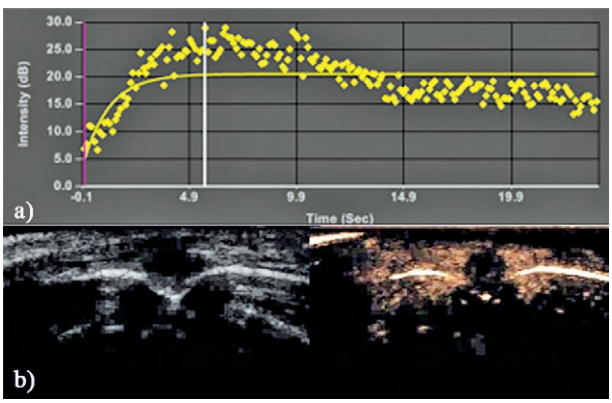

Fig 2. Contrast-enhanced ultrasound examination: a) Time-intensity curve and b) ultrasound aspect.

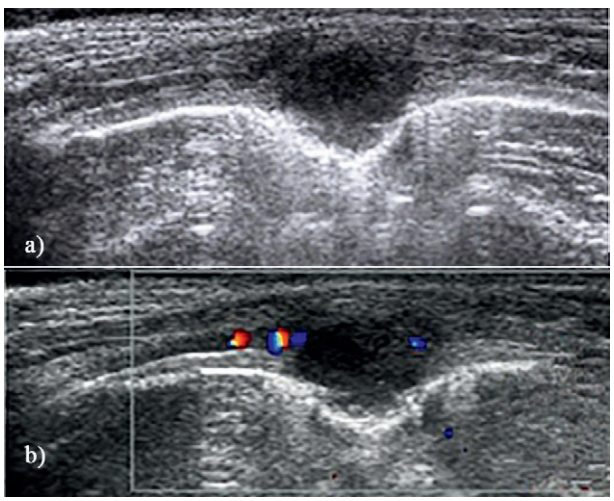

Fig 1. Ultrasound aspect of the lung peripheral VX2 tumor. The lung peripheral VX2 tumor was scanned by gray scale ultrasound (a) and color Doppler ultrasound (b).

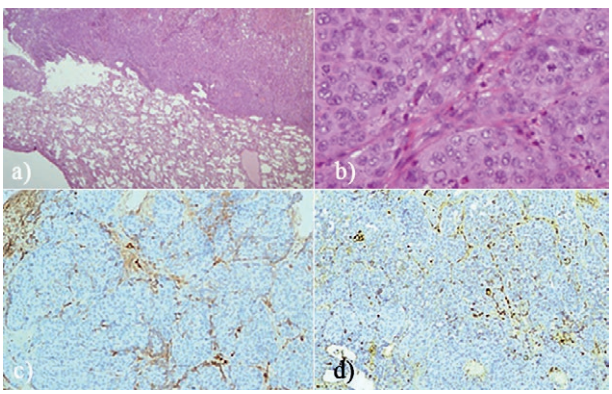

Fig 3. Pathological finding of H&E in lung peripheral VX2 tumors:a) Large tumor cell proliferation and adjacent lung tissue atrophy (H&E×10); b) Tumor cells presented in different sizes and shapes, and blood vessels were seen between these tumor cells (H&E×40). Microvessel immunohistochemistry of lung peripheral VX2 tumors: c) CD31 antibody (original magnification ×200); d) CD34 antibody (original magnification ×200).

Microvessels in lung peripheral VX2 tumors

Anti-CD31 antibody staining and anti-CD34 antibody staining were observed in lung peripheral VX2 tumors (fig 3 c,d).

Correlation between PI and MVD in lung peripheral VX2 tumors

The mean values for AT, TTP, EI, CD31-MVD and CD34-MVD are listed in Table I. The correlations among these parameters are listed in Table II. The positive correlation between PI and CD31 MVD values in lung peripheral VX2 tumors was statistically significant ($r=0.721$, $p<0.05$). However, there was no correlation between PI and CD34 MVD values ($r=-0.564$, $p>0.05$). Furthermore, there was an inverse relationship between CD31 MVD and CD34 MVD ($r=-0.774$, $p<0.05$; fig 4).

Discussions

At present, the technology to determine MVD on biopsy specimens is the immunohistochemical staining of vascular endothelial cells. MVD is an independent prognostic indicator, and has been considered the gold standard for evaluating tumor angiogenesis [12]. Generally, the higher the tumor MVD counts, the higher the degree of malignancy, the worse the prognosis, and the shorter the survival time [13].

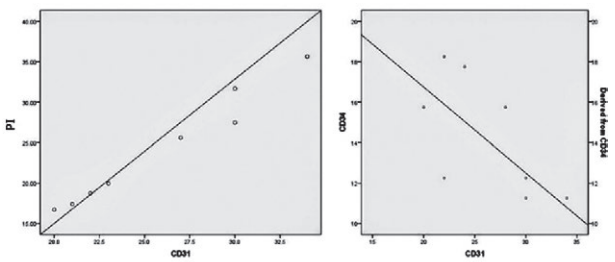

Fig 4. Correlations between PI, CD31 MVD and CD34 MVD of lung peripheral VX2 tumors. The significant positive correlation between PI and CD31 MVD, and the reverse correlation between CD31 MVD and CD34 MVD is shown.

Table I. The mean value of contrast-enhanced parameters and microvessel density

Variable	Mean value
AT (s)	1.41±0.95
TTP (s)	12.85±6.88
PI (dB)	24.15±7.07
CD31	25.63±4.69
CD34	15.38±2.20

AT – arrival time; TTP – time to peak; PI – peak intensity

It has been confirmed that there are two different degrees of endothelial cells (CD31 and CD34) in the presence of renal tumors. CD31 is presented in undifferentiated and differentiated mature endothelial cells, while CD34 is only presented in differentiated mature vascular endothelial cells. Highly-expressed CD31 MVD may suggest a higher degree of malignancy and shorter survival time for patients. In contrast, highly-expressed CD34 MVD may indicate a lower degree of malignancy, but survival time is significantly longer [19-21].

Since the lung of rabbits is similar to humans in anatomical structure, rabbits have been widely used in the experimental study of lung cancer. In the present study, we used rabbits to study the angiogenesis in the peripheral lung cancer model. The US detected the blood flow around the tumor and the arterial blood flow spectrum. The average of RI was 0.46, which was very low. However, this cannot be used to judge the situation of microcirculation perfusion.

CEUS is a new method for evaluating microcirculation perfusion [22]. It can be used to quantitatively, reliably and sensitively measure the blood perfusion of tumors [23], and evaluate the angiogenesis and biological behavior of tumors [24]. CEUS has been widely used in the evaluation of angiogenesis in kidney, breast, prostate, and liver tumors [25-28]. In the present study, CEUS was used to investigate the formation of angiogenesis in lung tumors.

Previous studies have always chosen a tumor endothelial cell marker, either CD31 or CD34, as a single evaluation of angiogenesis, in order to correlate with CEUS quantitative parameters [29-31]. However, these studies did not further explore the degree of differentiation of tumor vascular endothelial cells. The correlation between different MVDs and CEUS in lung cancer needs further studies. In the present study, we selected MVD in two different endothelial markers (CD31 and CD34), and studied the correlation of both markers with the quantitative parameters of CEUS, respectively, as well as the correlation with CD31 and CD34. A significant correlation between CD31 MVD and CEUS parameter PI ($r=0.721$,

Table II. The correlation among contrast-enhanced parameters and microvessel density in lung peripheral VX2 tumors.

Variable		AT (s)	TTP (s)	PI (dB)	CD31	CD34
Correlation	CD31	0.275*	-0.232*	0.721	–	-0.774
(r)	CD34	-0.471*	0.409*	-0.564*	-0.774	–

AT – arrival time; TTP – time to peak; PI – peak intensity; * – $p>0.05$

$p<0.05$) was demonstrated, while there was a non-linear correlation between CD34 MVD and CEUS parameters PI ($r=-0.564$, $p>0.05$). Other CEUS parameters such as AT and TTP had no significant correlation with MVDs. Statistical analysis results indicate that CEUS quantitative parameters can effectively evaluate angiogenesis conditions in rabbit peripheral lung cancer and CD31 MVD better reflect the status of tumor blood vessels. Another result is that CD31 MVD negatively correlated with CD34 MVD ($r=-0.774$, $p<0.05$). This indicates that when differentiated mature endothelial cells dominate (CD34 MVD presents a high expression) in the same tumor tissue, the number of undifferentiated endothelial cells is relatively smaller with a relatively lower degree of malignancy, and vice versa.

The limitation of this experiment was that the VX2 tumors were prone to necrosis, which can lead to the reduction of active tumors. Hence, it is important to choose the timing of the examination. Furthermore, the sample size for the present study was slightly small. A larger sample size is needed for further research. Moreover, the prognosis of the tumor was not discussed in the present study.

Conclusion

CD31 MVD can more effectively evaluate tumor angiogenesis compared with CD34 MVD. CEUS, as a non-invasive imaging method, can effectively evaluate tumor angiogenesis in rabbit peripheral lung cancer.

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Conflict of interest: none

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