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FULL PAPER

Contrast-enhanced ultrasonography of cervical carcinoma: perfusion pattern and relationship with tumour angiogenesis

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Objective: This study aimed to investigate the use of contrast-enhanced ultrasonography (CEUS) and time–intensity curves to assess angiogenesis in cervical cancer.
Methods: 60 patients who were scheduled to undergo radical surgery for biopsy-proven cervical cancers underwent CEUS. Surgical tissue sections from 32 patients who did not receive neoadjuvant chemotherapy were analyzed with CD34 staining to estimate intratumoral microvessel density (MVD). CEUS images were analyzed for maximum intensity (IMAX), rise time (RT), time to peak (TTP) and mean transit time.
Results: Cervical lesions had a higher IMAX and shorter RT and TTP ($p < 0.001$) than reference regions. There was a linear association between the IMAX of the cervical lesion and the mean intratumoral MVD ($r = 0.624$, $p < 0.001$). There were no significant differences in CEUS variables according to histological type, grade and stage.
Conclusion: Quantitative CEUS variables have potential use for monitoring perfusion changes in tumours after non-surgical therapy for advanced cervical cancer.
Advances in knowledge: The article demonstrates the capability and value of quantitative CEUS as a non-invasive strategy for detecting the perfusion and angiogenic status of cervical cancer. Quantitative CEUS variables have potential use for monitoring tumour response to non-surgical therapy.

INTRODUCTION

Cervical cancer is the third most commonly diagnosed cancer and the fourth leading cause of cancer death among females worldwide.[1] Accurate clinical evaluation and staging of cervical cancers, as well as the assessment of prognostic factors, are of great importance for determining optimal treatment strategies. Recently, angiogenesis has been identified as an essential event for the growth and metastasis of malignant tumours.[2,3] Several studies have demonstrated that tumour angiogenesis is an independent prognostic factor for recurrence and poor outcomes (disease-free survival and overall survival) in cervical cancer, and higher tumour angiogenesis is associated with poorer survival in carcinoma of the cervix.[4–9]

The "gold standard" for the characterization of tumour angiogenesis is immunohistologic analysis of the intratumoral microvessel density (MVD). However, this strategy involves an invasive procedure and may be associated with variable and unreliable results owing to heterogeneity between and within tumours.[10] Therefore, intratumoral MVD counts may not be an ideal tool for all clinical purposes, in particular monitoring tumour response to non-surgical therapy.

Several non-invasive and quantitative image modalities have been developed as an indicator of tumour angiogenesis *in vivo*, including dynamic contrast material-enhanced MRI, CT, positron emission tomography and transvaginal and three-dimensional colour Doppler ultrasonography.[11–14] However, some of these modalities may expose patients to ionizing radiation, while others have poor reproducibility.

Contrast-enhanced ultrasonography (CEUS), a real-time and non-ionizing imaging technique, is a powerful new tool that can assess the spatial and temporal variations in tumour microcirculation.[15–20] CEUS utilizes commercially available encapsulated gas-filled microbubbles as an intravascular agent. These augment the intensity of microvascular flow signals and allow smaller vessels (down to 70 μm in diameter) to be visualized.[17] Evidence suggests

that low-mechanical-index CEUS may provide a non-invasive method to obtain histopathological information in hepatocellular carcinomas.[16,20–22] To date, studies using CEUS to evaluate cervical cancer are limited.

In this study, we used CEUS to explore perfusion patterns in cervical cancer. We quantified CEUS variables and investigated their relationship with intratumoral MVD. Our data demonstrate the capability and value of quantitative CEUS as a non-invasive strategy for detecting the perfusion and angiogenic status of cervical cancer, providing benefits to patients undergoing non-surgical therapy.

METHODS AND MATERIALS

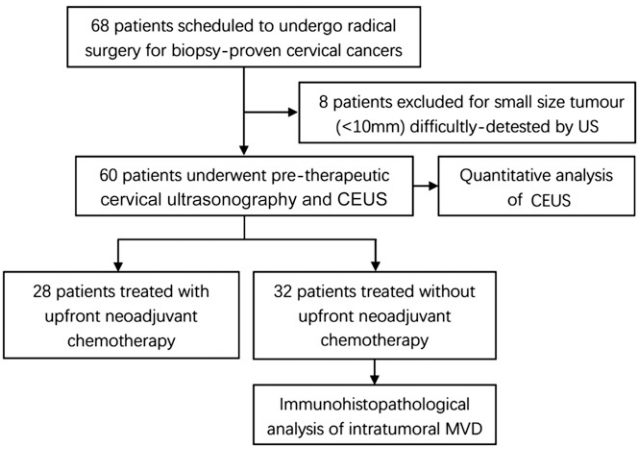
Patients

From December 2009 to May 2014, all patients who were scheduled to undergo radical surgery with or without neoadjuvant chemotherapy for biopsy-proven cervical cancers of International Federation of Gynecology and Obstetrics (FIGO) Stages IB–IIA and referred for pre-therapeutic cervical ultrasonography were considered for recruitment. Patients who had undergone surgical excision or who were unwilling or unable to provide informed consent were excluded. Of 68 malignant masses in 68 patients, 8 masses of <10 mm were excluded because it is difficult for transabdominal ultrasonography to detect lesions of such a small size. Finally, 60 malignant cervical masses [mean (standard deviation) size, 41.6 (8.7) mm; range, 20–56 mm] in 60 patients [mean (standard deviation) age, 48.13 (9.18) years; range, 25–70 years) were included (Figure 1). This study was approved by the institutional ethics committee, and all patients provided written informed consent.

Ultrasonography protocol, contrast agent and injection technique

Within 7 days prior to treatment, sonographic examinations were performed by two experienced radiologists (XQP and WZ, with 14 and 9 years' experience in ultrasonography, respectively, and 8 and 5 years' experience in CEUS, respectively) on all 60 patients. A LOGIQ S8 (GE Healthcare, Milwaukee, WI) equipped with an IC5-9 endovaginal probe (5–9 MHz) was used for baseline ultrasonography and colour Doppler flow imaging (CDFI) to facilitate a more optimal visualization of the cervix. SonoVue® (Bracco, Milan, Italy) was used as the contrast agent. An Acuson Sequoia 512 (Siemens, Mountain View, CA) equipped with a curved 4C1 transducer (1–4 MHz) and built-in cadence contrast pulse sequencing software was used for CEUS to facilitate a representative record of large spatial findings and their relationship to the reference uterine structures in a single image.[23–25]

Figure 1. Flow diagram for the study. CEUS, contrast-enhanced ultrasonography; MVD, microvessel density; US, ultrasound.



A cervical tumour was identified as a mass with heterogeneous echogenicity and irregular border with disruption of the cervical canal. Once a cervical lesion was identified, CDFI was used to assess intratumoral blood flow. Colour Doppler settings were set to achieve maximum sensitivity and detect low-velocity flow without noise (frequency, 5 MHz; power Doppler gain, 0.8; dynamic range, 20–40 dB; edge, 1; persistence, 2; colour map, 5; gate, 2; filter, L1; pulse repetition frequency, 0.6 kHz).

The lipid-based ultrasound contrast agent SonoVue (Bracco) was suspended in 5-ml supplied saline. For each patient, 1.0 ml of SonoVue (Bracco) was administered *via* the antecubital vein in a bolus fashion (within 1–2 s), followed by a flush of 5 ml of 0.9% physiologic saline.[26–28] The bolus injection was performed by a skilled operator with 8 years' experience.

CEUS was performed to obtain a longitudinal transabdominal view of the cervix. A full bladder was required. The target lesion was zoomed in and placed at the centre of the screen and the probe was kept in a stable position. The imaging mode was changed to CEUS with low mechanical index (between 0.17 and 0.19). Focus was kept at the bottom of the lesion during the examination. A timer was started at the time of SonoVue (Bracco) administration. Imaging was continuously recorded on cine clips for a period of 80 s immediately after contrast agent injection, without any change in the machine settings. After 80 s, the whole cervical lesion was intermittently scanned before loss of contrast. The timing of the CEUS phases were: arterial phase (<30 s) and venous phase (31–120 s).[28,29]

Quantitative analysis of contrast-enhanced ultrasonography (time–intensity curve)

SonoLiver software (TomTec Imaging Systems, Unterschleissheim, Germany) was used for the quantitative analysis of CEUS. CEUS cine clips were downloaded in digital imaging and communications in medicine format for offline processing to achieve a time–intensity curve (TIC) for each cervical lesion.

An analysis region of interest (ROI) was identified, located inside the margin of the cervical lesion, away from necrotic regions, and defined according to the enhanced area in the artery phase by one of the investigators (WZ, with 5 years' experience in quantitative analysis of CEUS) who was blind to the patients' histopathology information. A reference ROI at a matched depth in the region of the myometrium, avoiding the endometrium and myomas, was also selected by the same investigator. The mean video intensity for each ROI measurement was automatically calculated and expressed as a percentage, assuming the peak intensity of the reference ROI to be 100%. For each imaging protocol, the TIC of the analysis and reference ROIs were

plotted and fitted with a mathematic equation model for bolus kinetics. A modified log-normal distribution was used for best-fit optimization.[30]

Perfusion variables calculated from the fitted model included: maximum intensity (IMAX), defined as the maximum increase in signal intensity produced by injection of the contrast agent and calculated as the ratio of the peak intensity of the analysis ROI and the reference ROI (100%); rise time (RT), defined as the interval during which the intensity reached 10–90% of IMAX; time to peak (TTP), defined as the interval from the beginning of enhancement to the peak of the fitted curve; and the quality of fit, which was used to test the fit between the raw data and the fitted mathematic model (Figure 2).[16,20]

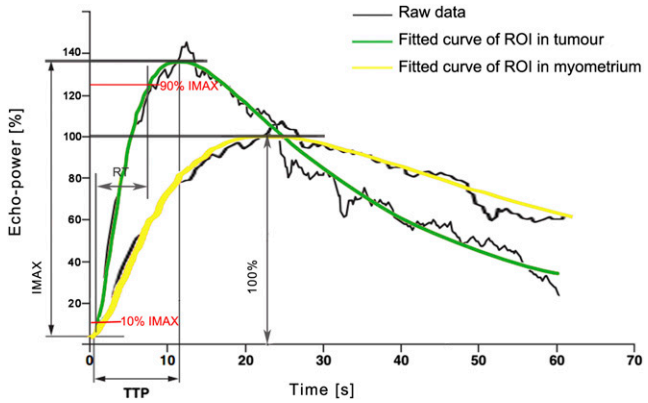
Pathology analysis

Pathology was performed in all 60 patients. Immunohistopathological analysis was studied in the 32 patients in whom radical surgeries were performed. The other 28 patients, who underwent cervical biopsy before neoadjuvant chemotherapy and surgery, were excluded from immunohistopathological analysis. These biopsy specimens were too small to represent the entire tumour, and specimens obtained during surgery may have been tumour-free following chemotherapy.

Several studies have suggested that CD34 is an appropriate immunohistochemical marker for quantifying angiogenesis of cervical cancer. The association of intratumoral MVD with pathoanatomical features may be a prognostic indicator.[9,31,32] Therefore, we applied CD34 staining to estimate intratumoral MVD.

Formalin-fixed, paraffin-embedded tumour tissues retrieved during surgery were sectioned into 5-μm-thick specimens and processed for haematoxylin–eosin and immunohistochemistry staining. Following incubation overnight at 4 °C with a mouse monoclonal CD34 antibody (ZSGB-BIO, Beijing, China) (1 : 100 dilution), immunostaining was performed using the Envision System with diaminobenzidine (Dako, Glostrup, Denmark).

Figure 2. Time–intensity curve (TIC) after bolus injection of contrast agent: the TIC of the analysis region of interest (ROI) (in green) and reference ROI (in yellow) are plotted according to video intensity and fitted with a mathematic equation model for bolus kinetics. The variables are calculated from the fitted model. IMAX, maximum intensity; RT, rise time; TTP, time to peak. For colour image see online.



Intratumoral MVD was quantified by counting the CD34-stained vessels under light microscopy. Assessments were performed independently by two experienced observers who were blinded to the tumour treatment and ultrasonography findings, according to an established method by Weidner et al.[33] After hot spots were identified under ×200 magnification, three fields were randomly chosen and the number of brown-stained cells was counted. The average of the two observer results was used for statistical analysis.

Statistical analysis

All statistical analyses were performed using SPSS® v. 13.0 (IBM Corp., New York, NY; formerly SPSS Inc., Chicago, IL). Data are described as mean ± standard error. A paired *t*-test was used to compare quantitative variables in the analysis and reference ROIs. The Kolmogorov–Smirnov test was used to assess the normal distribution of continuous variables, and the Levene's test was used for the evaluation of homogeneity of variance. The independent samples Student's *t*-test or one-way analysis of variance with the least significant difference *t*-test was used to compare quantitative variables according to different tumour features. Spearman correlation analysis was used to analyze the correlation between quantitative variables and intratumoral MVD. $p < 0.05$ was considered statistically significant. The correlation coefficient value <0.20 showed virtually no correlation; 0.21–0.40, weak correlation; 0.41–0.60, moderate correlation; 0.61–0.80, high correlation; and 0.81, greater or very high correlation.

RESULTS

Characteristics of baseline ultrasonography and contrast-enhanced ultrasonography

In each case, conventional baseline ultrasonography showed a mass with heterogeneous echogenicity, irregular shape and poorly defined borders with or without disruption of the cervical canal. CDFI showed spotted intratumoral blood flow signals (Figure 3).

In CEUS, all cervical lesions appeared to be well-defined hyperenhanced masses during the arterial phase and hypoenhanced masses [45/60 (75%) cases] or isoenhanced masses [(25%) 15/60 cases] during the venous phase compared with the reference region (Figure 4).

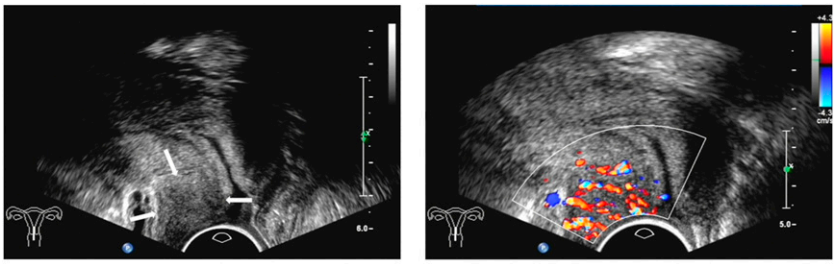
Quantitative analysis of contrast-enhanced ultrasonography (time–intensity curve)

The quality of fit (QOF) of the analysis and reference ROIs was >80%. All cervical lesions showed rapid enhancement in the arterial phase. The lesions had higher IMAX and shorter RT and TTP ($p < 0.001$) than the reference regions (Figure 5). Table 1 shows the quantitative variables in the analysis and reference ROIs.

Pathological and immunohistochemical results

All patients were pathologically confirmed as having squamous carcinoma [54 (90%) patients] or other types of carcinoma

Figure 3. Cervical carcinoma in a 41-year-old female. (a) In the greyscale sonography image with an endovaginal probe, ultrasonography showed a hypoechoic lesion (arrows) in the cervix. (b) Colour Doppler flow imaging showed spotted intratumoral blood flow signals.



(a) (b)

(adenosquamous carcinoma, small-cell carcinoma and large-cell carcinoma) [6 (10%) patients] and graded as well and moderately differentiated [24 (40%) patients] and poorly differentiated [36 (60%) patients]. 30 (50%) patients had early-stage tumours (Stage IB 1) and 30 (50%) patients had advanced stage tumours (10 patients with Stage IB2; 14 patients with Stage IIA1; and 6 patients with Stage IIA2). There were no significant differences in CEUS variables according to histological type, grade and stage (Table 2).

Intratumoral MVD was determined by immunohistochemical evaluation of CD34 endothelial cells in 32 cervical tumours. The mean counts per high power field were 109.54 ± 39.91 (Figure 6). There were no significant differences in intratumoral MVD according to pathological results and clinical stages ($p = 0.970$). The IMAX of cervical lesions was correlated to the mean intratumoral MVD ($r = 0.624$; $p < 0.001$) (Figure 7). There were no significant correlations between RT ($r = -0.022$; $p = 0.903$), TTP ($r = -0.052$; $p = 0.776$) and mean transit time (mTT) ($r = 0.071$; $p = 0.700$) of cervical lesions and the mean intratumoral MVD (Table 3).

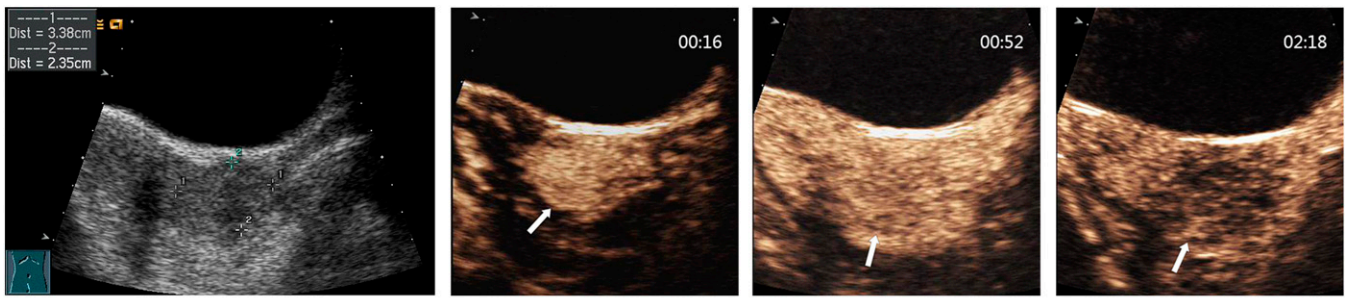
DISCUSSION

In this study, we used CEUS to explore the perfusion patterns of cervical cancer. We quantitatively analyzed CEUS variables to investigate the perfusion and angiogenic status of cervical tumours. In all cases, the CEUS TIC demonstrated classic enhancement patterns, characterized by earlier hyperenhancement in the arterial phase and hypoenhancement or isoenhancement in the venous phase at lesions in the uterine cervix compared with reference regions. These data are in accordance with observations in many other hypervascular tumours.[27–29,34,35]

Angiogenesis is a dynamic process that is defined as the growth and development of new microvessels required for solid tumours to expand in size beyond 1.0–2.0 mm in diameter. Angiogenesis is thought to be an independent prognostic factor for recurrence and poor outcomes in invasive cancer of the uterine cervix.[4–9] To date, there is no validated method for measuring the complex process of tumour angiogenesis other than histologically determining the intratumoral MVD.[36] Although data suggest a strong correlation between intratumoral MVD and aggressiveness of cervical carcinoma, this intratumoral MVD assessment is based on samples from a cervical biopsy or surgery and does not represent the entire tumour. Therefore, imaging of tumour angiogenesis has potential as a more promising indicator.[11–14]

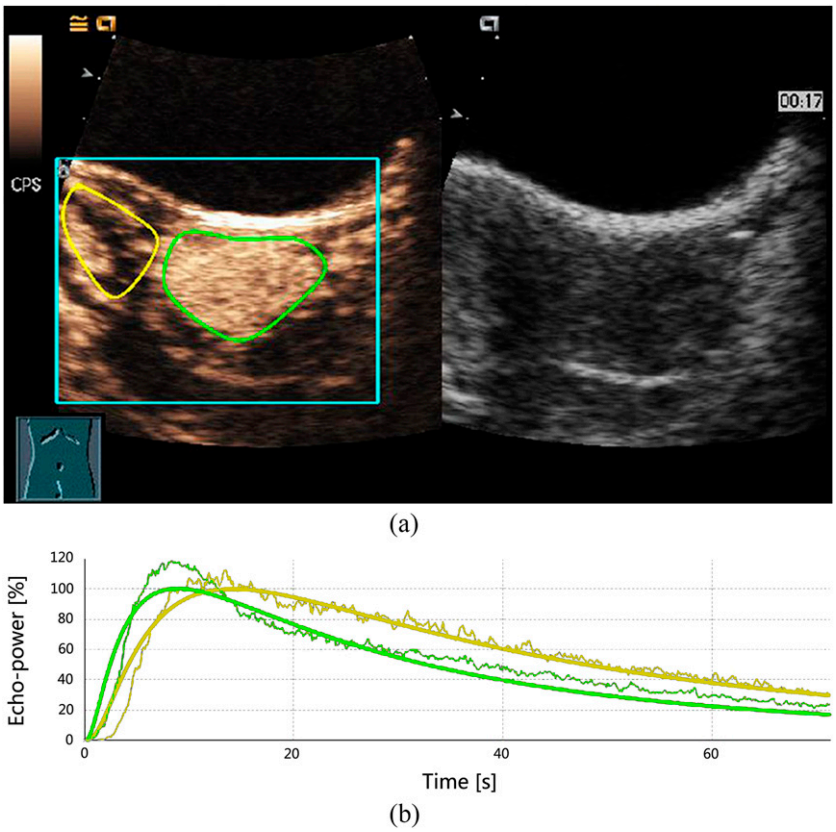
In previous studies, functional imaging modalities such as dynamic contrast-enhanced CT and MRI were evaluated to determine their usefulness in quantifying tumour perfusion, which

Figure 4. Cervical carcinoma in a 58-year-old female. (a) In the greyscale sonography image, ultrasonography showed a 3.38 × 2.35-cm isoechoic lesion in the cervix. (b–d) Serial contrast-enhanced ultrasonography (CEUS) images of the same lesion (arrows) obtained at the same level. Cervical carcinoma showed hyperenhancement at 16 s in the arterial phase (b) hypoenhancement in the earlier and later venous phases (c, d). Dist. distance.



(a) (b) (c) (d)

Figure 5. Screenshot of SonoLiver (TomTec Imaging Systems, Unterschleissheim, Germany) contrast-enhanced ultrasonography (CEUS) in the arterial phase and time–intensity curves (TIC) of a cervical carcinoma fitted by the bolus perfusion model. (a) Native sequence of CEUS image. Three regions of interest (ROIs) were drawn: a square ROI delimiting the region where motion compensation was applied, a reference ROI (left oval) and an analysis ROI (right oval). (b) Output TIC for cervical cancer (in green) and myometrium (in yellow). CPS, contrast pulse sequencing. For colour image see online.



is related to angiogenesis. Although these are practical modalities for assessing tissue perfusion,[11] the perfusion variables do not always correlate with histomorphometric features.[37,38] In fact, the most commonly used contrast agents in CT and MRI are "low-molecular-weight" (<1 kDa) hyperosmolar agents, which diffuse between the intravascular and extravascular extracellular spaces. Tracer kinetics analyses suggest that neither tool is appropriate to assess the intravascular blood volume and blood flow. Indeed, intravascular blood volume was overestimated by MRI.[39] Our previous and present study showed that quantitative CEUS may be an alternative approach.[16,20] In CEUS, the ultrasound contrast agent consists of encapsulated gas-filled microbubbles and is regarded as a purely intravascular substance that can optimally define the functional intratumoral microcirculation.

Several previous studies have described the correlation between increased signal intensity on CEUS and intratumoral MVD in angiogenesis; however, cervical cancer has not been investigated. McCarville et al[40] found a significant correlation between the rate of increase in signal intensity on CEUS and the mean intratumoral endothelial cell and pericyte density as assessed by CD34 staining in a murine model. Wang et al[41] found a significant correlation between peak intensity and intratumoral MVD in hepatocellular carcinoma ($r = 0.886$; $p < 0.05$). Our present data showed a significant correlation between the IMAX of

Table 1. Contrast-enhanced ultrasonography variables in the region of interest (ROI) of the cervical lesions and reference tissues

ROI	IMAX (%)	RT (s)	TTP (s)	mTT (s)	QOF
Cervical cancer	143.24 ± 54.54	9.36 ± 2.84	9.86 ± 3.00	100.95 ± 79.48	80.02 ± 12.05
Reference	100	17.49 ± 6.90	19.21 ± 7.97	121.12 ± 91.13	87.16 ± 7.42
t	6.141	−11.129	−10.929	−1.885	−4.617
p-value	0.000	0.000	0.000	0.064	0.000

IMAX, maximum of intensity; mTT, mean transit time; QOF, quality of fit; RT, rise time; *t*, *t*-statistic; TTP, time to peak.

Table 2. Correlations of the contrast-enhanced ultrasonography (CEUS) variables and tumour characteristics

Tumour characteristics	Quantitative variables of CEUS			
	IMAX (%)	RT-d (s)	TTP-d (s)	*p*-value
Tumour size				
<4 cm	142.08 (83.78)	8.36 (5.85)	9.57 (6.97)	0.787, 0.600, 0.676
≥4 cm	146.43 (77.07)	7.49 (5.61)	8.75 (8.40)	
Histologic grade				
G1–G2	133.82 (85.07)	6.89 (3.72)	7.81 (4.03)	0.278, 0.167, 0.098
G3	149.52 (71.81)	8.96 (6.34)	10.38 (8.49)	
Tumour stage				
IB1	146.95 (86.78)	9.06 (6.57)	10.31 (7.23)	0.832, 0.644, 0.728
IB2	149.34 (71.07)	7.58 (6.17)	8.71 (10.25)	
IIA1	131.64 (52.87)	6.87 (3.59)	7.99 (3.18)	
IIA2	141.58 (89.16)	7.32 (7.79)	8.82 (7.40)	

G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated; IMAX, maximum of intensity; RT-d, rise time of difference between cervical lesion and reference tissue; TTP-d, time to peak of difference between cervical lesion and reference tissue.
All data are expressed as median; the interquartile range is given in parentheses.

cervical lesions and the mean intratumoral MVD. The index IMAX derived from the CEUS TIC was defined as the ratio of the peak intensity of the analysis ROI and the reference ROI; this excludes intersubject variability. The IMAX value is also related to the maximum concentration of the contrast agent in each image pixel. If the contrast agent remains within the vasculature during dynamic contrast imaging, blood volume in the tissue is directly proportional to the amount of contrast agent perfusing the tissue;[11] therefore, IMAX can reflect blood volume in the microcirculation. Measuring differences in blood volume as a way to monitor the efficacy of non-surgical therapy has potential value. Our study suggests that quantitative variables derived from CEUS may describe the angiogenic status of a cervical tumour. This approach can improve our understanding of cervical tumour angiogenesis and provide predictive information to assist therapy selection and monitor conventional and new treatment regimens, including antiangiogenic therapy.

The other indexes measured in this study, such as RT, TTP and mTT, are related to the velocity of blood flow in the uterus. The basic haemodynamics of individual uteri varies and is influenced by cardiopulmonary resuscitation and physical condition. This could explain why we found no correlation between RT, TTP, mTT and mean intratumoral MVD. However, hypervascular tumours, including cervical cancers with high intratumoral MVD and dilated blood vessels, always have an abnormal blood flow, which can be depicted by CEUS. Therefore, the time

Figure 6. Representative sections of cervical cancer immunohistochemically stained for CD34 (×200) (arrows).

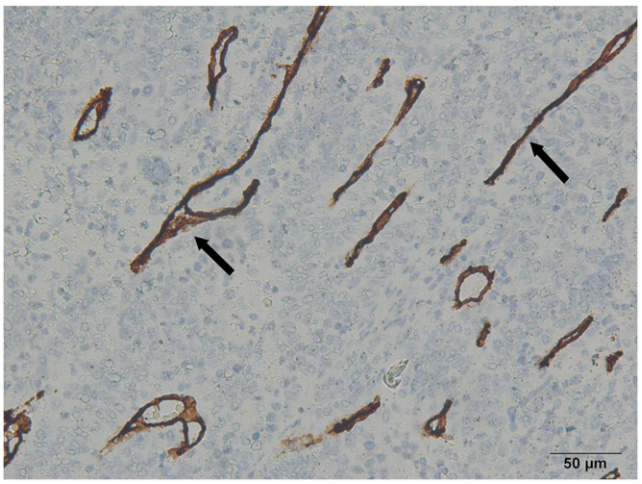


Figure 7. Scatter plot showing the relationship between maximum intensity (IMAX) and mean intratumoral microvessel density (MVD) in cervical lesions. IMAX increases as mean intratumoral MVD increases ($r = 0.624$, $p < 0.001$).

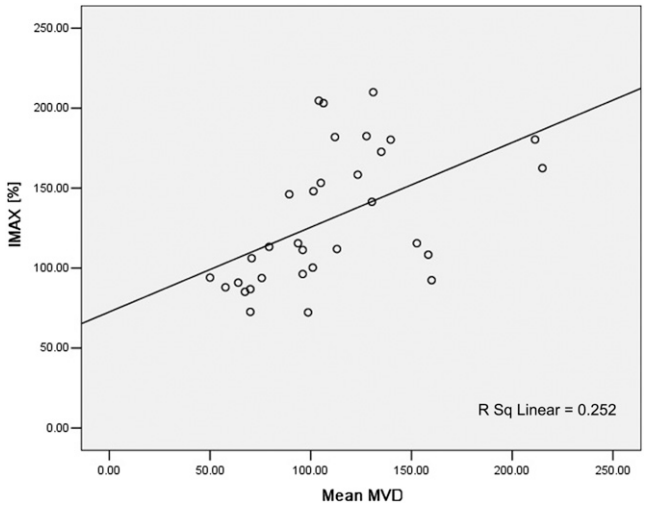


Table 3. Correlation between mean intratumoral microvessel density (MVD) and contrast-enhanced ultrasonography variables

Statistic	MVD and IMAX	MVD and RT (s)	MVD and TTP (s)	MVD and mTT (s)
r	0.624	−0.022	−0.052	0.071
p-value	0.000	0.903	0.776	0.700

IMAX, maximum intensity; mTT, mean transit time; *r*, correlation coefficient value; RT, rise time; TTP, time to peak.

indexes may have potential for assessing microvascular changes before and after non-surgical therapy.

There were some limitations associated with our study. Firstly, mismatch between the imaging plane and the histologic section could have occurred, which may affect the correlation of quantitative variables and neoangiogenesis. Second, the variables are based on the digital imaging and communications in medicine format (non-linearly compressed image), which could result in distorted perfusion variables. Recording kinetic data as uncompressed signals will provide a more accurate evaluation of intratumoral blood flow.

CONCLUSION

In summary, our study suggests that quantitative CEUS is a promising non-invasive technique for evaluating the angiogenic activity of an entire cervical tumour.

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