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Original Article

Evaluation of hepatocellular carcinoma angiogenesis before and after microwave ablation by real-time gray-scale contrast-enhanced ultrasound

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Abstract: This study aims to evaluate focal angiogenesis of hepatocellular carcinoma (HCC) before and after microwave ablation (MWA) by real-time gray-scale contrast-enhanced ultrasound (CEUS). One month before and after MWA, biopsy was performed in all 90 HCC patients. Immunohistochemistry was used to detect vascular endothelial growth factor (VEGF) expression and calculate microvessel density (MVD). CEUS was performed to assess tumor size. Tumor peak intensity (t PI), rise time of peak intensity (RT), tumor time to peak (t TTP), and parenchyma time to peak (p TTP) was recorded to determine its correlation with VEGF expression and MVD. There were 106 lesions detected by CEUS and MWA were successfully performed with CEUS as guidance. No serious complications were detected during the procedure. After 1-year follow-up, complete remission (CR) was found in 74.4% patients, partial remission was observed in 21.1% patients and the rest 4.4% were in stable condition. In the mean time, immunohistochemistry detected high VEGF expression and MVD in tumor tissues. In CR group, t RT, t TTP, t PI and tumor and liver parenchyma peak intensity difference (t-p PI) were significantly lower than that of non-CR group ($P < 0.05$). The t TTP was negatively correlated with VEGF expression with significance ($r = -0.352$, $P = 0.03$); however, there were no correlation between t RT, p TTP, t PI, t-p TTP and MVD, VEGF expression ($P > 0.05$); MVD and VEGF expression level was positively correlated with significance ($r = 0.532$, $P = 0.005$). CEUS can show angiogenesis in HCC before and after treatment. The negative correlation between t-p PI and VEGF expression and MVD might be a non-invasive, reliable and efficient indicator for angiogenesis detection.

Keywords: Hepatocellular carcinoma, CEUS, microwave ablation, vascular endothelial growth factor, microvessel density

Introduction

Hepatocellular carcinoma (HCC) is the most common and most aggressive malignant tumor, which ranks the second place in cancer mortality in China [1]. Surgical resection and liver transplantation is considered the optimal treatment, however, the 5-year survival rate of advanced liver cancer is less than 50% [2]. In recent years, microwave ablation (MWA) has become an important adjuvant therapy for HCC, with conventional ultrasound, CT and MRI as guidance. Conventional ultrasound is dominantly used for MWA due to its real-time monitoring with multi-angles. However, after MWA, conventional ultrasound echo changes are not correlated with necrosis size and shape [3]. There were no quantitative indicators of color Doppler flow imaging, pulsed Doppler or colored angiography for HCC vascular detection. Contrast-enhanced ultrasound (CEUS) can detect tumor perfusion and quantitatively evaluate its blood supply. However, its correlation with angiogenesis factor, including microvessel density (MVD) and vascular endothelial growth factor (VEGF) expression, has seldom been reported. This study is to detect peritumoral and intratumoral blood supply of 90 HCC patients by real-time gray-scale CEUS, and to analyze patients' characteristics, postoperative pathology and VEGF expression and MVD, in order to provide treatment guidance for HCC patients in the future.

Materials and methods

Subjects

Ninety patients from Zhengzhou University Affiliated Luoyang Central Hospital from October

Table 1. Baseline characteristics of all patients

	CR group (n = 62)	Non-CR group (n = 28)
Gender (male/female)	41/21	16/12
Age (years)	51.7 ± 10.3 (21-72)	57.3 ± 14.6 (28-75)
Kps score (100/≥90)	34/28	12/16
Child staging (A/B)	48/14	10/18
Hepatitis (HBV/HCV/others)	31/27/4	11/15/2
BCLC (0/A/B)*	23/31/8	3/8/17
AFP (μg/L)*	216.3 ± 57.5	342.7 ± 97.35
AFP (>200 or ≤200)	39/23	17/11
Lesion size (cm)	3.8 ± 1.7 (1.8-4.7)	4.1 ± 1.8 (2.8-5.0)
Number of tumor (single/multiple)	57/15	24/10

Note: *BCLC: Barcelona Clinic Liver Cancer [4]; *AFP: Alpha-fetoprotein.

Table 2. VEGF and MVD grading in tissue

Grading	VEGF (case)	MVD (case)
1	9	12
2	17	14
3	20	19
4	29	24
5	15	31
Total	90	90

2013 to October 2015 were recruited based on the following inclusion criteria: 1) diagnosed by biopsy, pathology and laboratory tests, or by 2 types of radiology; 2) max tumor diameter ≤ 5 cm or number of tumors ≤ 3; 3) no intrahepatic vascular thrombosis or adjacent organ involvement; 4) liver function of Child A and B. The clinical data of patients were shown in **Table 1**. Of the included HCC patients, 66 cases were diagnosed by pathology and 24 cases were diagnosed combined with medical history, laboratory tests and radiology. In total, there were 106 lesions detected by CEUS. The follow-up was 1 year, and remission was evaluated based on CEUS, enhanced CT and MRI. All specimens were obtained by biopsy, which were preserved by formalin. All patients were above 90 of Karnofsky scoring. Prior written and informed consent were obtained from every patient and the study was approved by the ethics review board of Zhengzhou University Affiliated Luoyang Central Hospital.

Procedures

CEUS was conducted using color Doppler ultrasound (Pihilps iU 22, Amsterdam, Netherland) with pulse subtraction harmony synthesis, probe frequency of 4-9 MHz and mechanical index of 0.07-0.10. The contrast agent Sono Vue (59 mg, BRACCO company, Italy) was used, diluted with 5 ml saline solution and injected into elbow vein. CEUS was used to real-time detect tumor shape, location, size and infiltration, echo, blood flow and resistance. Ultrasound scanned the liver with high mechanical index and "Silhouette" corresponding grays-scale image was obtained. Time intensity curve and dynamic vascular pattern curve was drawn and their reliability was evaluated based on curve fitting. Tumor peak intensity (t PI), rise time (RT), tumor time to peak (t TTP), and parenchyma time to peak (p TTP) were recorded at each intensity curve.

Ultrasound guided MWA (ECO-100, Nanjing Yigao CO., Ltd) was performed with cold run microwave knife (ECO-100, 2.0 mm) and power of 60-80 w. Patients were in supine or lateral position, and conventional ultrasound and CEUS were used to detect tumor size, shape, location, echo and surrounding tissue. After measuring its maximum diameter and identifying the best puncture direction and angle, ultrasound-guided MWA needle punctured predetermined portion of the tumor and treatment started. With real-time ultrasound monitoring, the lesion echo significantly increased, and treatment stopped after complete coverage or exceeding of the tumor for 300 seconds. ECG, blood pressure and oxygen saturation were monitored during the procedure. MWA were performed by two experienced physicians.

Immunohistochemistry

The specimens were restored in incubator at 60°C overnight and paraffin was removed by repetitive ethanol bathing. Then the endogenous peroxidase was inactivated for 10 min by 3% hydrogen peroxide. Then the specimens were microwave retrieved at 98°C for 10 min and cool slowly at room temperature. The secondary antibody was added and incubated

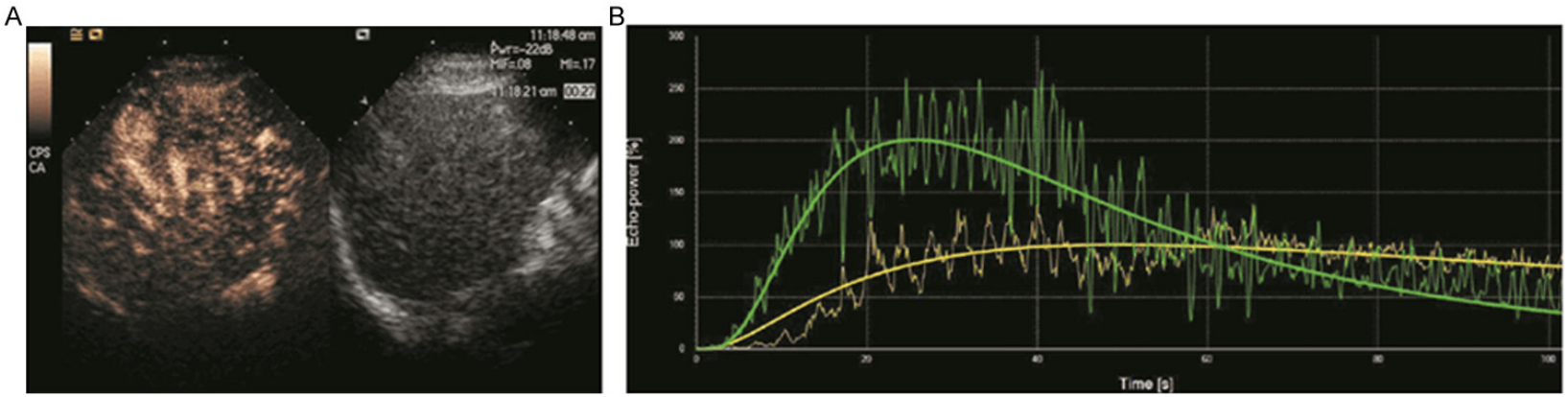

Figure 1. Conventional ultrasound and CEUS (A) and time intensity curve (B).

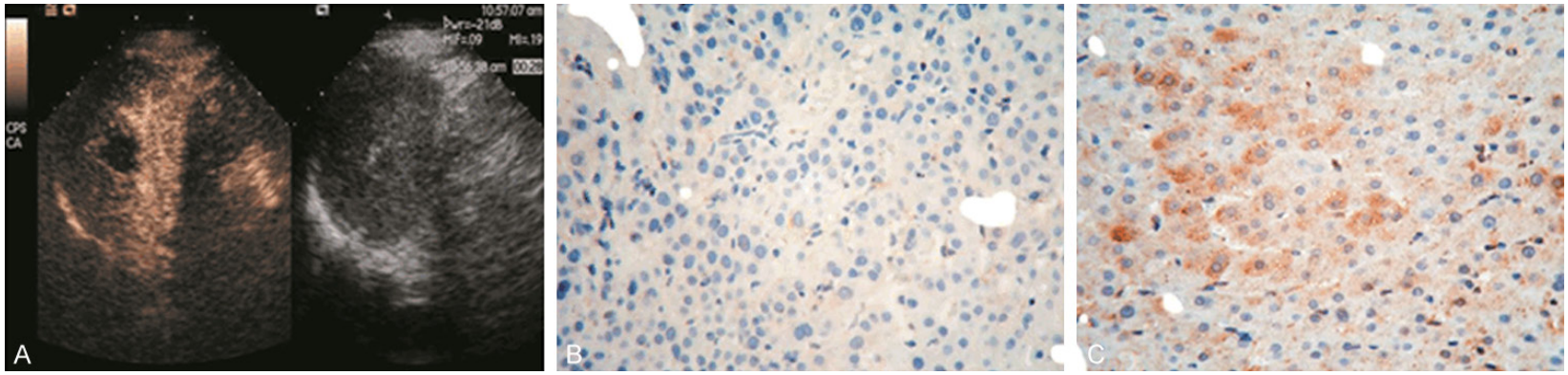

Figure 2. Postoperative conventional ultrasound and CEUS (A), VEGF expression (B) and MVD expression (C).

Table 3. CEUS comparison between CR group and non-CR group

	CR group (n = 62)	Non-CR group (n = 28)	P
t RT (s)	15.2 ± 5.7	9.3 ± 2.6	0.01*
p RT (s)	23.8 ± 8.6	15.9 ± 7.1	0.36
t TTP (s)	17.7 ± 6.8	11.5 ± 6.3	0.01*
p TTP (s)	34.8 ± 12.5	29.8 ± 14.6	0.43
t PI (s)	208.7 ± 69.4	143.4 ± 54.6	0.00*
p-t TTP (s)	-17.2 ± 7.5	-23.8 ± 10.8	0.59
t-p PI (s)	113.8 ± 38.4	75.6 ± 29.7	0.00*

Note: *P < 0.05.

overnight at 2-8°C and DAB was added. Then hematoxylin was added for counterstaining.

VEGF and MVD grading

Yellow, yellow brown or brown granules in cytoplasm of tumor cells were considered positive. Under microscope with low magnification, five regions with the most concentrated yellow and brown granules were selected as "hot spot". With high magnification, the number and ratio of VEGF positive cells were identified in each "hot spot", and their average ratio was considered as VEGF positive rate. The VEGF positive rate (n) of each specimen was graded [4]: 0% ≤ n ≤ 15% as grade 1, 15% < n ≤ 5% as grade 2, 25% < n ≤ 50% as grade 3, 50 % < n ≤ 75% as grade 4 and 75% < n ≤ 100% as grade 5.

All yellow, yellow brown or brown endothelial cell clusters with clear boundaries that were adjacent to capillaries and tumor cells were considered microvessel. MVD was graded as Weidner's method [5] based on the average MVD in five selected "hot spot" under high magnification: 0 ≤ n ≤ 40 as grade 1, 40 < n ≤ 60 as grade 2, 60 < n ≤ 80 as grade 3, 80 < n ≤ 100 as grade 4 and n > 100 as grade 5. Each slice was determined by two experienced pathologists.

Evaluation

Evaluation criteria and postoperative follow-up were in accordance with International Union for International Cancer Control (UICC) [6]. CEUS, CT or MRI, routine blood test and liver function was administered preoperative, postoperative 1 week, 1 month, 3 month, 6 month and 1 year to detect tumor size and liver function. Complete lesion disappear was considered as complete remission (CR); lesion size < 50% of that before treatment was considered as partial response (PR); lesion size > 50% or < 125% of that before treatment was considered as stable disease (SD); one or more lesion > 125% of that before treatment was considered as progressive disease (PD). All patients were divided into CR group and non-CR group.

Statistical analysis

SPSS 17.0 was used for statistical analysis. Quantitative data was expressed as mean ± standard deviation and group comparisons used t-test. Non-normal or nonparametric distribution used Spearman correlation analysis. P < 0.05 was considered statistically significant.

Results

VEGF and MVD grading before treatment

To determine the VEGF and MVD grading before treatment, their expression was detected. VEGF expression and MVD were shown in all lesion specimens, and their grading is shown in **Table 2**. The demand for nutrition and oxygen was more in malignant tumors than that of normal tissues, therefore the MVD/VEGF was increased for the nutrition supply for tumor blood vessel induction. The above results indicate that the VEGF and MVD expression and grading would guide clinical treatment and efficacy.

MWA efficacy

To determine MWA efficacy, CEUS was performed. Preoperative CEUS showed that 106 lesions were all enhanced. Postoperative 1 month, CEUS showed no enhancement in 84 lesions of 67 patients, indicating 74.4% (67/90) patients with CR; partial enhancement in 20 lesions of 19 patients, indicating 21.1% (19/90) patients with PR; enhancement in 4 lesions of 4 patients, indicating 4.4% (4/90) SD; no 25% or more lesion increase (PD) was found in all patients after 3 months' follow up. As shown in **Figures 1** and **2**, there was evident lesion necrosis with no significant surrounding blood supply. The "fast forward and fast backward" blood flow of tumor lesions can help differentiation from other tumors, such as hepatic hemangioma. The above results indicate that the effi-

Table 4. Correlation between CEUS and VEGF expression and MVD

		t RT	t TTP	p TTP	t PI	t-p TTP	VEGF
MVD	r	0.286	0.156	0.236	-0.635	-0.563	0.532
	P	0.243	0.637	0.682	0.262	0.864	0.005*
VEGF	r	-0.463	-0.352	-0.652	-0.059	-0.076	
	P	0.246	0.030*	0.063	0..071	0.068	

Note: *P < 0.05.

cacy of MWA was satisfactory and CEUS was of high evaluation value.

CEUS between CR group and non-CR group

To determine the lesion differences between CR group and non-CR group, CEUS was performed. Time intensity curve was drawn based on CEUS. There were significant differences of t RT, t TTP, t PI and t-p PI between CR group and non-CR group. The t RT, t TTP, t PI and t-p PI was significantly lower in non-CR group than that of CR group ($P < 0.05$). However, there were no differences of p RT, p TTP and t-p TTP between the two groups ($P > 0.05$), as shown in **Table 3**. The above results indicate that CEUS can reflect the lesion blood supply.

Correlation of CEUS and VEGF expression and MVD

To determine correlation of CEUS and VEGF expression and MVD, Spearman correlation analysis was used. As shown in **Table 4**, the t TP and VEGF expression was negatively correlated with statistical significance ($r = -0.352$, $P = 0.03$). VEGF expression and MVD were positively correlated with significance ($r = 0.532$, $P = 0.005$). However, there were no correlation between t RT, p TTP, t PI, t-p TTP and VEGF expression and MVD ($P > 0.05$). The above results indicate that CEUS can be used to evaluate tumor growth as well as treatment efficacy.

Discussion

HCC is the most common type of malignant tumors that ranks the second highest prevalence in China [7]. Currently its optimal treatment is surgical resection, but its 5-year recurrence rate is as high as 45.2% to 60% [8]. Postoperative intrahepatic recurrence is common even for HCC with diameter less than 5 cm [8]. In recent years, HCC MWA is rapidly developing with satisfactory clinical efficacy [9]. However, recurrence still remains, and early detection is of great importance for prognosis and survival. After MWA, real-time gray-scale CEUS with low mechanical index can real-time monitor lesion perfusion that correlated lesion size and immunohistochemistry grading [10]. Angiogenesis is vital in tumor growth and recurrence thus early detection of microvessel would guide HCC treatment and its prognosis [11, 12]. Therefore, correlation analysis between CEUS and VEGF expression and MVD is critical.

In this study, CEUS was used to build time intensity curve and showed "fast forward and fast backward" blood flow of tumor lesions. Immunohistochemistry showed strong positive or positive VEGF expression and MVD. Postoperative CEUS and other radiology showed CR of lesions, and laboratory tests showed AFP and ALT decreased. VEGF expression and MVD were mostly concentrated in grade 3, 4 and 5, indicating high VEGF expression and MVD in tumor lesion. VEGF expression and MVD are closely related with HCC growth, invasion, metastasis and prognosis [13]. Therefore, VEGF expression and MVD can be used as important indicators of HCC target therapy and prediction of its metastasis and recurrence [14].

In this study, angiography used liver parenchyma as reference to exclude peak intensity influencing factors and to accurately reflect tumor size and blood supply of surrounding tissue. Compared with CR group, CEUS showed that t RT, t TTP, t PI and t-p PI was significantly lower in non-CR group ($P < 0.05$). This could be resulted from the increased ratio of hepatic artery, leading to fast perfusion categorized as "fast forward". In addition, in this study, it is found that t TTP was negatively correlated with VEGF expression, and VEGF expression was positively correlated with MVD. However, there was no correlation between t RT, p TTP, t PI, t-p TTP and VEGF expression and MVD. The above may result from the positive correlation between tumor angiogenesis and VEGF, thus, high VEGF expression indicated tumor angiogenesis, increased blood supply and increased t TTP. Therefore, the correlation between t TTP and VEGF expression and MVD is expected to be HCC recurrence indicator.

In summary, CEUS has been widely accepted in clinical practice due to its minimal invasiveness, real-time monitoring and cost-effectiveness. The correlation between CEUS and VEGF expression and MVD may provide important information for evaluating tumor angiogenesis and HCC prognosis. However, this study is still preliminary. Future study with large sample and multi-center randomized controlled clinical trials is needed.

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Disclosure of conflict of interest

None.

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