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Prognostic analysis of hepatocellular carcinoma on the background of liver cirrhosis via contrast-enhanced ultrasound and pathology

YUKAI HE[1], FENGHUA LIU[1], SHAOFENG MOU[2], QINGYAN LI[1] and SIKUI WANG[1]

Departments of [1]Infectious Disease and [2]Otorhinolaryngology, Liaocheng People's Hospital, Liaocheng, Shandong 252000, P.R. China

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Abstract. The aim of this study was to analyze the correlation between the quantitative parameters of contrast-enhancement ultrasound for primary hepatocellular carcinoma (HCC) and biological manifestations of tumor (Ki-67), and to explore the related risk factors of primary hepatocellular carcinoma, so as to provide the theoretical basis for the further study on contrast-enhancement ultrasound manifestations, clinical features and prognosis of HCC. The patients with HCC confirmed by operation or puncture were collected, and those with the background of liver cirrhosis and immunohistochemical staining for tumor sample sections were selected. H&E staining sections of pathological tissues of tumor samples were observed, whether there was any microvessel invasion (MVI) was recorded, the microvessel density (MVD) was counted and the recurrence situations after liver cancer operation was followed up. The change in size of tumor at arterial phase in contrast-enhancement ultrasound, enhancement mode and form at arterial phase, and whether there were tortuous vessels inside or not, and the enhancement intensity, extinction time and extinction intensity at portal phase were observed. The relationship between the parameters of contrast-enhancement ultrasound and Ki-67, AFP, MVD, MVI, tissue differentiation degree of tumor samples and recurrence was analyzed. Under the background of liver cirrhosis, there were significant differences in different enhancement modes and quantification parameters of contrast-enhancement ultrasound for HCC with different expression of Ki-67. Those with obvious tumor enlargement, inhomogeneous enhancement at arterial phase and irregular enhancement form at arterial phase after contrast-enhancement ultrasound had a high incidence of positive Ki-67 and a high early recurrence rate. The inhomogeneous enhancement at arterial phase might predict the proliferative activity and recurrence time of tumor cells; irregular enhancement form at arterial phase might indicate tumor MVI; and the low enhancement of tumor at portal phase may predict a lower degree of tissue differentiation, a higher tumor malignancy and poor prognosis. The incidence of positive Ki-67 under the background of liver cirrhosis is high, indicating poor prognosis. The enhancement mode and parameters of contrast-enhancement ultrasound for HCC may help evaluate the clinical biological manifestations of HCC and predict the postoperative recurrence of HCC.

Correspondence to: Dr Qingyan Li, Department of Infectious Disease, Liaocheng People's Hospital, 67 Dongchang-xi Road, Liaocheng, Shandong 252000, P.R. China
E-mail: qingyanlill@126.com

Key words: HCC, contrast-enhancement ultrasound, pathology, Ki-67, prognosis

Introduction

Primary liver cancer is one of the most common malignant tumors in the world, among which hepatocellular carcinoma (HCC) accounts for approximately 70-85% (1). Surgical operation, local ablation, radiotherapy and chemotherapy provide opportunities to cure or prolong the survival time of patients. However, the 5-year recurrence rate after primary liver cancer operation is as high as 45-60%, seriously affecting the prognosis of patients with liver cancer (2-4). Therefore, searching for the indexes for evaluating the poor prognosis of HCC, such as proliferative activity and early recurrence, is of significance for the tumor staging, selection of treatment plan and prognosis.

Ki-67 is a kind of nuclear antibody Ki-67 protein, mainly existing in the dense fibrillar components of nucleoli and cortices, which is a reliable index of detecting the tumor cell proliferative activity (5). The biological manifestations of tumor are closely related to its proliferative activity. The higher the expression of Ki-67 is, the faster the tumor cell proliferation will be and the higher the probability of metastasis will also be (6,7). However, there are few studies on the correlation between contrast-enhancement ultrasound of HCC and Ki-67 immunohistochemistry and other prognostic factors.

Compared with enhancement MRI/CT, PET and other examinations, contrast-enhancement ultrasound (CEUS) is characterized by the convenient and efficient application, wide indications and no radiation, and it can show the whole process of contrast enhancement for tumor and liver parenchyma in a real-time and dynamic manner, which not only

improves the value of diagnosis and differential diagnosis, but also indirectly reflects some biological manifestations of tumors (8-10). In the field of contrast-enhancement ultrasound, there are some scholars who use special computer software to conduct the quantization parameter analysis for CEUS images of HCC, establish the peak intensity curve, and study the relationship between CEUS parameters on the curve and microvessel density (MVD), microvessel invasion (MVI) and tumor tissue differentiation degree (11-14). These factors are associated with the recurrence of liver cancer and the survival time of liver cancer patients.

This study aimed to evaluate the relationship between the enhancement mode and parameters of CEUS for HCC and the prognostic factors and clinical recurrence, and evaluate the biological behavior and prognosis of liver cancer from the perspective of ultrasonography, so as to provide new non-invasive evaluation indexes of prognosis.

Patients and methods

Subjects. One hundred and eighty patients with HCC confirmed by operation or puncture were collected, and they all had the background of liver cirrhosis. The tumor samples received immunohistochemical Ki-67 and AFP-labeled staining. This study was approved by the Ethics Committee of Qianfoshan Hospital Affiliated to Shandong University (Jinan, China). Signed written informed consents were obtained from all participants before the study.

Pathological examination of Ki-67. Each section was observed via 10 visual fields randomly selected under high-power microscope (x400), and the percentage of brown-stained cell nuclei every 100 cells was the percentage of Ki-67. Ki-67 was divided into Ki-67-positive group (>10%) and the Ki-67-negative group (≤10%) according to the percentage of Ki-67.

Microvessel density, MVD. After the blood vessels in tumor tissues were stained by antigen-antibody reaction, the slide was glanced with low-power microscope (x40-x100) to find the area with the highest blood vessel density. Then the stained blood vessels within the field were counted under high-power microscope (x400). The mean MVD of the patients included was calculated, and they were divided into the high-MVD group (greater than the mean) and low-MVD group (lower than the mean).

Microvessel invasion, MVI. Microvessel infiltration means that multiple or massed cancer cells can be found in venules in para-cancer mesenchyme, obvious endothelial cells can be found around the lumen and red blood cells may appear within the lumen. Patients were divided into the MVI-positive group (MVI was found) and MVI-negative group (no MVI was found).

Contrast-enhancement ultrasound, CEUS. Color Doppler ultrasound was used to record the lesion site and size, internal echo and number, and Doppler blood flow signal; and then the contrast agent was injected under the radiography mode, and the timer and recording were started. The sum of two maximum orthogonal radial lines of lesion on the same section was taken as size of the tumor; according to the changes before and after CEUS, those greater than the mean of all change values were enrolled into the significant change group; otherwise, they were enrolled into the non-significant change group. The image at 10-30 sec after injection of contrast agent was taken as arterial phase. The images with peak intensity were observed; if there were low enhancement or no enhancement area in different sizes in the lesion, the enhancement mode was seen as the inhomogeneous enhancement. The irregular enhancement form of lesion was regarded as the irregular tumor form. Whether there were tortuous blood vessels in the enhancement process at arterial phase were observed. The images at 90-120 sec after injection of contrast agent were selected as the portal phase. In comparison with surrounding liver parenchyma, the extinction time of contrast agent was recorded, the enhancement intensity and regression degree of lesion at portal phase were observed.

Recurrence of liver cancer. If the focal hepatic lesions showed the typical manifestation of HCC or obvious extra hepatic metastasis in imaging examination for the first time, it was the recurrence. According to the first time of discovering recurrent lesion, it was divided into early recurrence (≤1 year) and late recurrence (>1 year).

Statistical analysis. All statistics were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). t-test was performed for the comparison of mean value, Chi-square test for the significant difference between groups. Binary logistic regression analysis was used to evaluate the relationship between the parameters of ultrasound contrast and prognostic factors. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Ki-67/AFP immunohistochemistry. In this study, the clinical characteristics between Ki-67 positive group (Fig. 1) and Ki-67 negative group (Fig. 1), AFP positive group and AFP negative group were compared. There were no statistically significant differences in the age, sex, positive rate of hepatitis B serum marker and liver function Child-Pugh grade between the two groups ($P>0.05$); but the proportion of high-differentiated liver cancer in Ki-67 negative group was higher than that in Ki-67 positive group, and the proportion of low-differentiated liver cancer in Ki-67 positive group was higher than that in Ki-67 negative group ($P<0.05$) (Table I).

Microvessel density (MVD). In this study, the mean MVD of patients was 63.40, and they were divided into the high-MVD group (greater than the mean) and low-MVD group (lower than the mean) (Fig. 2). There were no statistically significant differences in the age, sex, hepatitis B serum marker and liver function between high-MVD group and low-MVD group ($P>0.05$).

Microvessel invasion (MVI). In this study, patients were divided into the MVI-positive group (MVI was found in para-cancer mesenchyme) (Fig. 3) and MVI-negative group (no MVI was found in para-cancer mesenchyme) (Fig. 3). There were no statistically significant differences in the age, sex, hepatitis B serum marker and liver function between MVI-positive group and MVI-negative group ($P>0.05$).

Table I. Basic characteristics of Ki-67(+)/Ki-67(-) HCC subjects.

Factors	Ki-67(+)	Ki-67(-)	Test value	P-value
Sex (male %)	90.01%	91.97%	χ^2=0.23	0.57
Age	45.90±8.76	48.86±9.34	t=1.74	0.23
Tumor tissue differentiation degree			χ^2=16.28	NS
High-differentiated	2.63%	15.57%		
Medium-differentiated	81.87%	79.96%		
Low-differentiated	15.50%	4.47%		
Hepatitis B serum marker			χ^2=4.26	0.08
Positive	99	69		
Negative	6	6		
Liver function Child-Pugh grade			χ^2=0.02	0.88
Grade A	96	70		
Grade B	9	5		

HCC, hepatocellular carcinoma; Ki-67(+), Ki-67-positive group; Ki-67(-), Ki-67-negative group. NS, not significant.

Table II. Comparison of CEUS parameters in Ki-67/AFP different HCC groups.

Factors	Ki-67(+)	Ki-67(-)	χ^2 test	P-value	AFP(+)	AFP(-)	χ^2 test	P-value
Change of tumor size			6.15	0.01			2.31	0.10
Conspicuous	46	20			40	26		
Inconspicuous	59	55			54	60		
Enhancement mode at arterial phase			6.57	0.00			0.13	1.13
Inhomogeneous	79	34			59	55		
Homogeneous	26	41			35	31		
Enhancement form at arterial phase			7.34	0.02			0.98	0.56
Irregular	49	21			40	31		
Regular	56	54			54	55		
Tortuous vessels			2.39	0.26			1.13	0.63
Yes	44	24			33	36		
No	61	51			61	50		
Enhancement intensity at portal phase			7.43	0.04			1.77	0.46
Low intensity	81	39			64	56		
High or equal intensity	24	36			30	30		
Extinction speed at portal phase			8.12	0.05			3.12	0.50
Fast	55	24			39	41		
Low	50	51			55	45		
Extinction extent at portal phase			8.64	0.04			0.85	0.67
Obvious	79	37			62	56		
None or slight	26	38			32	30		

HCC, hepatocellular carcinoma; Ki-67(+), Ki-67-positive group; Ki-67(-), Ki-67-negative group.

Relationship between CEUS parameters vs. pathologic histology. The incidence of positive Ki-67 was high for those cases with obvious change in tumor size, inhomogeneous enhancement and irregular enhancement form of tumor at arterial phase, and low enhancement and fast extinction at portal phase after CEUS (P<0.05, Table II). There was no significant correlation between the change in tumor size, enhancement mode and form of tumor at arterial phase and

Table III. Comparison of CEUS parameters in MVD/MVI different HCC groups.

Factors	H-MVD	L-MVD	χ^2 test	P-value	MVI(+)	MVI(-)	χ^2 test	P-value
Change of tumor size			0.75	0.55			7.05	0.04
Conspicuous	39	32			56	15		
Inconspicuous	53	56			74	35		
Enhancement mode at arterial phase			0.34	0.69			3.50	0.16
Inhomogeneous	59	54			87	26		
Homogeneous	33	34			43	24		
Enhancement form at arterial phase			0.72	0.45			5.70	0.01
Irregular	41	33			60	14		
Regular	51	55			70	36		
Tortuous vessels			0.13	0.56			5.70	0.01
Yes	39	35			60	14		
No	53	53			70	36		
Enhancement intensity at portal phase			1.55	0.32			3.12	0.14
Low intensity	65	57			93	29		
High or equal intensity	27	31			37	21		
Extinction speed at portal phase			2.31	0.42			3.54	0.03
Fast	40	45			67	17		
Low	52	43			63	33		
Extinction extent at portal phase			1.75	0.33			3.88	0.14
Obvious	65	57			92	29		
None or slight	27	31			38	21		

HCC, hepatocellular carcinoma; CEUS, contrast-enhancement ultrasound; H-MVD, high microvessel density; L-MVD, low microvessel density; MVI(+), microvessel invasion-positive group; MVI(-), microvessel invasion-negative group.

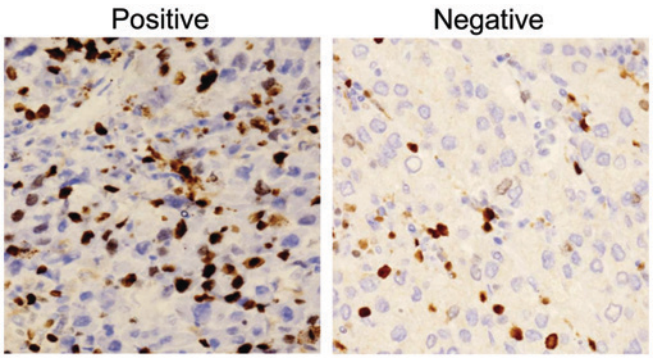

Figure 1. Different pathological expression levels of Ki-67 (ELPS staining, x400).

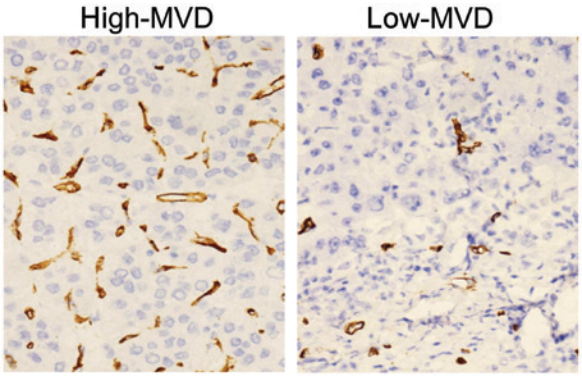

Figure 2. Different pathological expression levels of MVD (ELPS staining, x400).

other parameters after CEUS and the immunohistochemical AFP (P>0.05, Table II).

There was no significant correlation between the change in tumor size, enhancement mode and form of tumor at arterial phase and other parameters after CEUS or the MVD level (P>0.05, Table III). The incidence of positive MVI was high for those cases with obvious change in tumor size, irregular enhancement form of tumor at arterial phase, tortuous vessels inside and fast extinction at portal phase after CEUS (P<0.05, Table III).

The tissue differentiation degree of tumor samples was lower for those cases with inhomogeneous enhancement and irregular enhancement form of tumor at arterial phase, tortuous vessels inside, low enhancement and fast extinction at portal phase after CEUS (P<0.05, Table IV).

CEUS parameters vs. recurrence of liver cancer. The recurrence time was shorter for those cases with inhomogeneous enhancement of tumor at arterial phase in CEUS (P<0.05, Table V and Fig. 4).

Table IV. Comparison of CEUS parameters in tumor tissue differentiation HCC groups.

Factors	Low/Medium-differentiated	Highly-differentiated	χ^2 test	P-value
Change of tumor size			4.34	0.07
Conspicuous	64	8		
Inconspicuous	96	22		
Enhancement mode at arterial phase			6.75	0.01
Inhomogeneous	105	12		
Homogeneous	45	18		
Enhancement form at arterial phase			5.12	0.04
Irregular	66	8		
Regular	84	22		
Tortuous vessels			7.13	0.03
Yes	64	7		
No	86	23		
Enhancement intensity at portal phase			6.45	0.01
Low intensity	102	13		
High or equal intensity	48	17		
Extinction speed at portal phase			6.83	0.00
Fast	106	12		
Low	44	18		
Extinction extent at portal phase			4.27	0.04
Obvious	68	9		
None or slight	82	21		

HCC, hepatocellular carcinoma; CEUS, contrast-enhancement ultrasound.

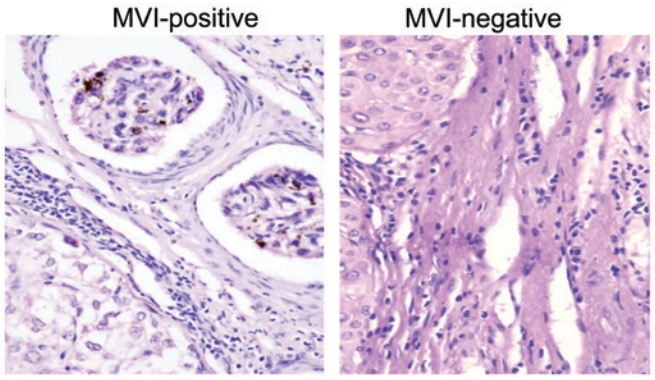

Figure 3. Different pathological expression levels of MVI (H&E staining, x400).

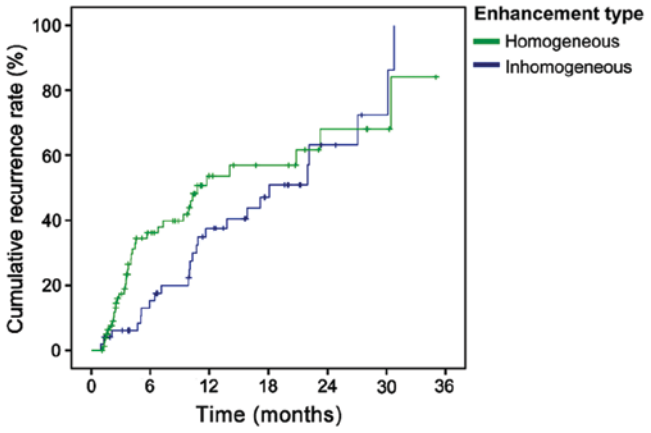

Figure 4. Recurrence rate of HCC under different enhancement at arterial phase.

Logistic regression for prognostic factors of HCC. Logistic regression showed that the inhomogeneous enhancement inside at the arterial phase in CEUS was more helpful to suggest positive Ki-67 and early recurrence; irregular enhancement form at arterial phase suggested a higher risk of MVI; the low enhancement at portal phase might be a parameter suggesting low tissue differentiation degree (P<0.05, Table VI).

Discussion

Approximately 740,000 people die from primary HCC around the world every year, and the patients in China account for more than 50% of the global total (2). The high postoperative recurrence rate of liver cancer is the main factor influencing the survival rate of patients with liver cancer. Early prediction of related risk factors to postoperative recurrence of liver cancer is of great clinical significance, and examining the postoperative recurrence as soon as possible and taking treatment measures timely can effectively improve the prognosis of patients with liver cancer (4).

The prognosis of HCC patients is determined by a variety of factors. Immunohistochemical Ki-67 is a rapid, simple and

Table V. Comparison of CEUS parameters in different recurrence conditions of HCC groups.

Factors	Early recurrence	Late recurrence	No recurrence	χ^2 test	P-value
Change of tumor size				1.90	0.43
Conspicuous	26	8	38		
Inconspicuous	35	17	56		
Enhancement mode at arterial phase				7.84	0.04
Inhomogeneous	40	10	59		
Homogeneous	21	15	35		
Enhancement form at arterial phase				5.87	0.07
Irregular	28	7	42		
Regular	33	18	52		
Tortuous vessels				4.30	0.38
Yes	29	9	35		
No	32	16	59		
Enhancement intensity at portal phase				4.12	0.30
Low intensity	40	12	61		
High or equal intensity	21	13	33		
Extinction speed at portal phase				5.13	0.24
Fast	30	8	41		
Low	31	17	53		
Extinction extent at portal phase				3.07	0.36
Obvious	40	12	59		
None or slight	21	13	35		

HCC, hepatocellular carcinoma; CEUS, contrast-enhancement ultrasound.

Table VI. Logistic regression of HCC prognosis factors.

Factors	r-value	Wald value	P-value	OR
Ki-67				
Inhomogeneous enhancement mode at arterial phase	1.15	26.39	NS	2.78
MVI				
Irregular enhancement form at arterial phase	1.26	17.35	NS	3.10
Tumor tissue differentiation				
Inhomogeneous enhancement mode at arterial phase	1.20	7.03	0.02	3.25
Low intensity enhancement at portal phase	1.55	12.39	NS	4.23

HCC, hepatocellular carcinoma; MVI, microvessel invasion; OR, odds ratio. NS, not significant.

sensitive detection technique for proliferative activity of liver cancer cells, and its overexpression (>10%) can predict the recurrence of liver cancer after surgical treatment, which is associated with the mortality of HCC patients (5-7). In this study, the change in lesion size, the enhancement mode and form inside lesion at arterial phase, and the enhancement intensity, extinction time and degree at portal phase were associated with the incidence of positive Ki-67; and the logistic regression analysis showed that the inhomogeneous enhancement inside the lesion at arterial phase might be the best parameter to predict the Ki-67 overexpression.

AFP is an important biological marker of liver cancer, whose quantification and immunohistochemistry is associated with the occurrence and development of liver cancer. In recent years, the studies show that AFP lacks sufficient sensitivity and specificity in the effective diagnosis and monitoring of liver cancer. There are few studies about the CEUS parameters and immunohistochemical level of AFP. The results of this study suggested that there was no obvious relationship between the immunohistochemistry AFP and CEUS parameters.

Tumor growth and metastasis depend on the growth of tumor angiogenesis, and MVD, as a sign of tumor angiogenesis

degree, also suggests that it is closely related to the occurrence, development and metastasis of tumors (11,12). Compared with liver cancer patients with low MVD, patients with high MVD tend to suffer from early postoperative recurrence. Some scholars studied the correlation between time intensity curve and MVD, and found that the peak intensity, intensity enhancement and area under the curve were positively correlated to the MVD count (13), but in this study, the change in tumor size, enhancement form at arterial phase and other parameters after CEUS had no significant relationship with MVD count.

MVI of liver cancer means that the cancer cells have the potential of intrahepatic metastasis and spread to the circulatory system (14,15). In this study, it can be seen that the change in lesion size, enhancement form at arterial phase, tortuous vessels inside and extinction time at portal phase were related to MVI. Logistic regression analysis showed that irregular form at arterial phase was the most closely related to MVI. The irregular enhancement form of lesion at arterial phase is associated with the high incidence of MVI from both univariate and multivariate regression analysis, which may be the most valuable independent factor of predicting MVI.

Typical CEUS of HCC is characterized by high enhancement of lesion at arterial phase and low enhancement at portal phase and delayed phase, namely the so-called 'fast in and fast out' of contrast agent. However, the high-differentiated HCC may present the atypical imaging manifestations, such as the 'fast in and slow out' or 'slow in and slow out' of contrast agent (8-10). In this study, the enhancement mode and form of lesions at arterial phase, tortuous vessels, and the enhancement intensity, extinction time and degree at portal phase were correlated with the pathological tissue differentiation degree. Logistic regression analysis showed that the inhomogeneous enhancement inside lesions at arterial phase and low enhancement at portal phase were risk factors of low tissue differentiation degree. Compared with the inhomogeneous enhancement at arterial phase, low enhancement at portal phase is more dangerous. The study results showed that the proportion of high or equal enhancement at portal phase in high-differentiated HCC group was significantly higher than that in moderate- and low-differentiated group, and the reason may be that: i) high-differentiated HCC causes the retention of contrast agent due to the presence of neat bone trabecula-type cellular bundles and a large number of hepatic sinusoids; ii) with the increase of malignancy degree, the degree of differentiation becomes lower and the abnormal arteries are formed derived from the increase of abnormal arterial blood supply. Normal blood supply in hepatic artery and portal vein decreases, the duration of enhancement at portal phase declines and the enhancement intensity is lower; iii) there are many abnormal new vessels at the center or around the tumor, as well as abundant arteriovenous fistulas, thus shortening the duration of enhancement.

To sum up, this study investigated the relationship between the enhancement pattern and parameters of CEUS for HCC and the prognostic factors and clinical recurrence, and evaluated the biological behavior and prognosis of liver cancer from the perspective of pathology and ultrasonography, so as to provide a new prognosis evaluation index.

On the background of liver cirrhosis, there were significant differences in different enhancement modes and quantification parameters of contrast-enhancement ultrasound for HCC with different expression of Ki-67. The high incidence of positive Ki-67 may suggest poor prognosis. CEUS is helpful to predict the prognosis of HCC. The inhomogeneous enhancement at arterial phase may predict the proliferative activity and recurrence time of tumor cells; irregular enhancement form at arterial phase may indicate tumor MVI; and the low enhancement of tumor at portal phase may predict a lower degree of tissue differentiation and poor prognosis.

Competing interests

The authors declare that they have no competing interests.

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