Predictive value of quantitative contrast-enhanced ultrasound in hepatocellular carcinoma recurrence after ablation

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Abstract

AIM: To investigate the relationship between contrast-enhanced ultrasound (CEUS), basic fibroblast growth factor (bFGF), endothelin-1 (ET-1), and hepatocellular carcinoma (HCC) recurrence after ablation.

METHODS: A total of 51 HCC patients (38 males and 13 females) who received radiofrequency ablation in our hospital from June 2012 to July 2014 were enrolled in this study. The patients were divided into two groups: recurrence group and non-recurrence group. Routine abdominal examination was first performed in the horizontal position. Then the patients underwent CEUS and immunohistochemical staining before receiving radiofrequency ablation. All patients were followed-up every three months for one year.
The results of CEUS and serum tumor marker levels were evaluated and combined together to estimate HCC recurrence and metastasis. Patients were divided into two groups: recurrence group and non-recurrence group. Quantitative parameters of CEUS and tumor expression levels of bFGF and ET-1 were compared between the two groups, respectively. Binary logistic regression analysis was used to analyze the relationship between CEUS quantitative parameters, expression levels of ET-1 and bFGF, and HCC recurrence after ablation.

RESULTS: Based on the quantitative parameters of CEUS before patients received radiofrequency ablation, the levels of tumor rise time (tRT), tumor time to peak (tTTP), tumor peak intensity (tPI) and tumor-parenchymal peak intensity (t-pPI) in the recurrence group were significantly lower than those in the non-recurrence group (16.6 ± 6.1 vs 23.2 ± 7.0, P = 0.000; 41.2 ± 10.2 vs 59.6 ± 14.2, P = 0.000; 23.8 ± 6.7 vs 31.4 ± 6.4, P = 0.000; 7.1 ± 3.4 vs 14.6 ± 7.4, P = 0.000; respectively). The expression levels of bFGF in the recurrence group were significantly higher than those in the non-recurrence group (P < 0.05). Levels of tTTP showed a significant inverse correlation with the level of bFGF in tumors (r = -0.312, P = 0.037). The Binary logistic regression analysis results revealed that the levels of tRT, tTTP, tPI and the level of bFGF were associated with HCC recurrence after radiofrequency ablation (P < 0.05).

CONCLUSION: CEUS is a noninvasive and effective method for evaluating the angiogenesis of HCC, and predicting its recurrence and prognosis.

Key words: Contrast-enhanced ultrasonography; Hepatocellular carcinoma; Radiofrequency ablation

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Core tip: This study quantitatively analyzed contrast-enhanced ultrasound (CEUS) images from patients with hepatocellular carcinoma (HCC). The results showed that HCC patients who have low levels of tumor time to peak (tTTP), tumor peak intensity and tumor-parenchymal peak intensity before ablation were more likely to relapse after ablation. Expression levels of basic fibroblast growth factor (bFGF) in the recurrence group were higher than those in the non-recurrence group, and tTTP levels were negatively correlated with bFGF expression levels. This was expected to be a predictable index for HCC recurrence after ablation. The results also revealed that CEUS is a non-invasive and effective method for evaluating HCC angiogenesis, its recurrence and metastasis.
will be helpful in the early prediction of HCC recurrence and metastasis, and in selecting appropriate treatment programs. In addition, it is hoped that the results of this study will show that CEUS is a noninvasive, accurate, and convenient method for evaluating tumor angiogenesis and HCC prognosis.

MATERIALS AND METHODS

Subjects

HCC patients who received radiofrequency ablation in our hospital from June 2012 to July 2014 were enrolled in this study. The inclusion criteria were as follows: pathologically confirmed HCC, single lesions, a maximum tumor diameter < 50 mm; no extrahepatic metastasis; Child class A or B; no serious heart or lung disease. Finally, 51 cases (38 males and 13 females) were enrolled in this study. The average age of the patients was 54.2 ± 7.5 years.

Methods

CEUS before ablation: The CEUS (MyLab Twice system, Esaote, Genoa, Italy) procedure was carried out as follows: Routine abdominal examination was first performed in the horizontal position. Each section of the liver tumor was observed; and tumor size, shape, boundary echo and blood flow signals were recorded. The acoustic contrast agent, SonoVue, was then injected into the median cubital vein. Subsequently, a five-minute continuous contrast process was observed, and radiographic contrast images were sent to the software. In the focal area of the tumor and surrounding areas of normal liver parenchyma, a uniform and obvious part of the tumor was chosen as the region of interest (ROI); and the liver parenchyma 2 cm beyond the tumor was set as the control ROI. The time-intensity curve was obtained from the time-intensity curve analysis after motion compensation. Tumor peak intensity (tPI), tumor rise time (tRT), parenchymal rise time (pRT), tumor time to peak (tTTP), parenchymal time to peak (pTTP), parenchymal-tumor time to peak (p-tTTP), and tumor-parenchymal peak intensity (t-pPI) levels of the time-intensity curves were calculated and recorded in Microsoft Excel (Microsoft, Redmond, WA, United States).

Biopsy procedure: Tumor tissue samples were obtained by liver puncture under ultrasound guidance. The puncture target was determined, and the entry point and route were selected. Local anesthesia with lidocaine was administered. Specimens were obtained when the needle punctured and reached the leading edge of the tumor. Samples were sent to pathology for examination.

Radiofrequency ablation: The patients were positioned in the supine or left-lateral position, and puncture positioning was determined under ultrasound guidance. First, the needle was sterilized and draped.

Table 1 Comparison of general data between the recurrence and non-recurrence groups

|                | Non-recurrence group (n = 37) | Recurrence group (n = 14) | χ²/t | P-value |
|----------------|-----------------------------|--------------------------|------|---------|
| Gender         |                             |                          |      |         |
| Male           | 28                          | 10                       | 0.002| 0.756   |
| Female         | 9                           | 4                        |      |         |
| Age (yr)       | 54.1 ± 7.7                  | 54.3 ± 8.2               | 0.453| 0.653   |
| Tumor location |                             |                          |      |         |
| Left lobe of liver | 6                         | 3                        | 0.001| 0.663   |
| Right lobe of liver | 31                       | 11                       |      |         |
| Maximum diameter of tumor (cm) | ≤ 3                  | 28                       | 0.023| 0.828   |
|                | 3-5                         | 9                        |      |         |
| Child grade    |                             |                          |      |         |
| A              | 34                          | 12                       | 0.018| 0.508   |
| B              | 3                           | 2                        |      |         |
| AFP (ug/L)     |                             |                          |      |         |
| > 200          | 6                           | 4                        | 0.356| 0.321   |
| ≤ 200          | 31                          | 10                       |      |         |
| Degree of tumor differentiation | High         | 12                       | 0.672| 0.437   |
|                | Medium                      | 23                       |      |         |
|                | Low                         | 2                        |      |         |

Then, local anesthesia with 1% lidocaine hydrochloride was administered. Patients were asked to hold their breath before implanting the bipolar radiofrequency needle to the pre-set position of the tumor lesions. Ablation therapy began under intravenous anesthesia with propofol. At the end of treatment, the needle tract was solidified during needle withdrawal to avoid bleeding.

Follow-up after ablation: All patients were followed-up every three months for one year. The results of CEUS, enhanced CT/MRI, and serum tumor marker levels were combined to evaluate HCC recurrence and metastasis. For CEUS, no recurrence was defined as a “black hole” on the screen for three periods after ablation. High arterial phase enhancement and low delayed phase enhancement suggested tumor recurrence.

Immunohistochemistry: (1) paraffin sections were cut, deparaffinized and rehydrated, then washed for five minutes with running water and five minutes with distilled water; (2) sections underwent antigen repair; (3) sections were dripped with 30% H₂O₂ at room temperature to deactivate endogenous enzymes, and washed three times with distilled water; (4) sections underwent primary antibody incubation; (5) sections underwent secondary antibody incubation; (6) sections were stained with DAB; and (7) sections were lightly re-stained by hematoxylin, dehydrated and vitrified, then the slices were sealed and examined with a microscope.

Statistical analysis

SPSS 18.0 statistical software was used to measure numerical variable data (mean ± SD) and categorical variable data (n). All data were compared using t-test.
or χ² test. Spearman correlation analysis was used to analyze CEUS quantitative parameters and expression levels of ET-1 and bFGF. Binary logistic regression analysis was used to analyze the relationship between CEUS quantitative parameters, expression levels of ET-1 and bFGF, and HCC recurrence after ablation. \( P < 0.05 \) indicates that the differences were statistically significant.

**RESULTS**

**Comparison of general patient data in the recurrence and non-recurrence groups**

All 51 patients were followed-up for one year; wherein,
37 patients had no recurrence and 14 patients were found to have recurrence. Gender, age, tumor size and location, Child grade, and AFP level differences, as well as the degree of tumor differentiation, between the recurrence and non-recurrence groups were not statistically significant ($P > 0.05$) (Table 1).

Figure 2  Time-intensity curve of patients in the recurrence group. A: Time-intensity curve of ROI in the tumor. The green curve was steep, and indicated that the change in intensity was obvious. The Peak was 35.5% and TP was 43.33 s; B: Time-intensity curve of ROI beyond the tumor. The green curve was gentler, compared with the ROI curve in the tumor. The Peak was 33.5% and TP was 57.29 s. ROI: Region of interest.

Differences between time-intensity curves of patients in the recurrence and non-recurrence groups
Differences between time-intensity curves of patients in the recurrence and non-recurrence groups are shown in Figures 1 and 2. The curve was steeper, $t_{RT}$ and $t_{TTP}$ were shorter, and $t_{PI}$ was higher for patients
Multiple factor analysis of the relationship between CEUS quantitative parameters, expression levels of ET-1 and bFGF, and HCC recurrence after ablation

Binary logistic regression analysis was carried out, recurrence was taken as the dependent variable, and both the CEUS quantitative parameters (tRT, pRT, tTTP, pTTP, tPI, t-pTTP, and t-pPI) and expression levels of ET-1 and bFGF were taken as independent variables. The results showed that low levels of tRT and tTTP, as well as high levels of bFGF, were risk factors for HCC recurrence after ablation, as shown in Table 4.

DISCUSSION

This study introduced the use of CEUS, and quantitatively analyzed ultrasonic images of HCC patients using its software. The results showed that HCC patients with low levels of tRT, tTTP, tPI and t-pPI before ablation had a high probability of relapse after ablation. Expression levels of bFGF in patients in the recurrence group were significantly higher than those in the non-recurrence group. Levels of tTTP were negatively correlated with expression levels of bFGF, suggesting that low levels of tTTP can easily cause HCC patients to relapse after ablation. This was expected to be the predictable index for HCC recurrence after ablation. It was also revealed that CEUS is a noninvasive and effective method for evaluating HCC angiogenesis, and its recurrence and metastasis.

Expression levels of bFGF were higher in the recurrence group

This study revealed that bFGF expression levels in patients in the recurrence group were significantly higher than those in the non-recurrence group. A high bFGF expression level is a risk factor for HCC recurrence after ablation; demonstrating that bFGF expression levels are correlated with the prognosis of HCC patients, and that this could predict the recurrence of HCC after ablation. A possible reason for this may be due to the widespread distribution of bFGF in the body, as its expression level increases with ischemia and hypoxia. Previous studies have shown that increasing bFGF expression levels in the liver are

Table 2  Relationship of contrast-enhanced ultrasound quantitative parameters between the two groups

| Parameters | Non-recurrence group (37) | Recurrence group (14) | t | P-value |
|------------|---------------------------|-----------------------|---|---------|
| tRT (s)    | 23.2 ± 7.0                | 13.6 ± 6.1            | 4.517 | 0.000   |
| pRT (s)    | 20.7 ± 10.2               | 18.5 ± 9.9            | 0.693 | 0.492   |
| tTTP (s)   | 59.6 ± 14.2               | 41.2 ± 10.2           | 4.423 | 0.000   |
| pTTP (s)   | 66.4 ± 17.3               | 56.8 ± 13.2           | 1.876 | 0.067   |
| tPI (%)    | 31.4 ± 6.4                | 23.8 ± 6.7            | 3.737 | 0.000   |
| p-tTTP (%) | -6.3 ± 10.7               | -12.3 ± 10.2          | 1.809 | 0.077   |
| t-pPI (%)  | 14.6 ± 7.4                | 7.1 ± 3.4             | 3.632 | 0.000   |

tRT: Tumor rise time; pRT: Parenchymal rise time; tTTP: Tumor time to peak; pTTP: Parenchymal time to peak; tPI: Tumor peak intensity; p-tTTP: Parenchymal-tumor time to peak; t-pPI: Tumor-parenchymal peak intensity.

Table 3  Correlation analysis of contrast-enhanced ultrasound quantitative parameters and expression levels of endothelin-1 and basic fibroblast growth factor

| Parameters | ET-1 | bFGF |
|------------|------|------|
| tRT        | 0.211| -0.317|
| tTTP       | 0.133| -0.175|
| pTTP       | 0.132| -0.132|
| tPI        | -0.412| 0.067|
| p-tTTP     | -0.005| 0.000|

ET-1: Endothelin-1; bFGF: Basic fibroblast growth factor; tRT: Tumor rise time; tTTP: Tumor time to peak; pTTP: Parenchymal time to peak; tPI: Tumor peak intensity; p-tTTP: Parenchymal-tumor time to peak.

Figure 3: Endothelin-1 and basic fibroblast growth factor expression levels in the non-recurrence and recurrence groups. ET-1: Endothelin-1; bFGF: Basic fibroblast growth factor.
closely related to HCC invasion and metastasis\textsuperscript{[12-13]}. Basic research has indicated that bFGF can activate the Ras-Raf-MARK system in angiogenesis\textsuperscript{[14,15]}. The cooperation of bFGF with VEGF promotes vascular endothelial cell generation, and provides nutrition for the invasion and metastasis of tumor cells. Therefore, bFGF could be used to predict HCC metastasis and recurrence. However, this study found that ET-1 expression levels in the recurrence and non-recurrence groups were not significantly different, which did not conform to previous studies\textsuperscript{[16,17]}. Vasoactive substances such as ET-1 have an important regulatory role in the angiogenesis, proliferation, invasion and metastasis of malignant tumors\textsuperscript{[19-24]}. It was found that ET-1 did not only reflect tumor angiogenesis, but also determined tumor proliferation and metastasis\textsuperscript{[11,21]}. In this study, no differences in ET-1 expression levels were found, which was probably because ET-1 expression levels are related to the degree of malignancy. ET-1 expression levels were low in tumors that had high differentiation and low malignancy, while expression levels were only high in tumors that had low differentiation and high malignancy\textsuperscript{[25-28]}. In this study, only three patients showed low differentiation, causing the overall ET-1 expression level to be low. Hence, the difference between the recurrence and non-recurrence groups was not statistically significant.

**Levels of tTTP were negatively correlated with expression levels of bFGF**

This study revealed that tTTP levels were negatively correlated with bFGF expression levels, suggesting that tTTP may predict HCC recurrence. In principle, tTTP is mainly determined through hepatic artery and portal vein blood flow\textsuperscript{[29]}. The more blood is supplied to the hepatic artery, the earlier the tTTP level is reached. Conversely, the more blood is supplied to the portal vein, the tTTP level is delayed. These results indicate that tTTP may reflect the tumor blood supply component. Angiogenesis was prevalent in tumors with a rich arterial blood supply, causing HCC invasion and metastasis. Tumor angiogenesis was closely related to bFGF expression levels\textsuperscript{[26]}. Therefore, we believe that as bFGF expression levels increase in HCC patients, tumor angiogenesis becomes more prevalent and arterial blood supply increases; which is followed by a decrease in tTTP levels. On the other hand, when bFGF expression levels were low in tumors, angiogenesis and arterial blood supply were slow, and tTTP levels increased.

**Levels of tRT, tTP, tPI and t-pPI in patients were correlated with HCC recurrence after ablation**

The results showed that tRT, tTP, tPI and t-pPI levels in the recurrence group were significantly lower than those in the non-recurrence group. Low levels of tRT, tPI and t-pPI were risk factors of HCC recurrence after ablation, suggesting that tRT, tPI and t-pPI levels before ablation could be used to predict HCC recurrence after treatment. Levels of tPI decreased in patients in the recurrence group due to liver parenchyma changes close to the tumor tissue. Levels of tRT and tTP were low, because tRT and tTP reflected the tumor’s blood supply component. These were determined by the hepatic artery blood supply and tumor portal vein component ratio\textsuperscript{[27]}. The larger the proportion of the hepatic artery blood supply, the faster the tumor’s rise time, and the earlier the time to peak is reached. Conversely, the larger the proportion of the portal vein blood supply, the slower the tumor rise time, and the time to peak is delayed. In CEUS, HCC was closely related to tumor hemodynamic characteristics\textsuperscript{[28]}. There was more blood supply for HCC than in benign lesions. Filling time was shorter, and the rate of increase of peak intensity per unit-time was higher. HCC lesions had a rich blood supply and the hepatic artery was the main blood supply. The contrast agent completely filled the early and middle artery phase. Peak time was short, showing a “fast-forward” movement\textsuperscript{[29]}. The lower the levels of tRT, tPI and tTP, the larger the proportion of the hepatic artery, and the more dangerous the recurrence would be after ablation.

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**Table 4 Multiple factor analysis of the relationship between contrast-enhanced ultrasound quantitative parameters, expression levels of endothelin-1 and basic fibroblast growth factor, and hepatocellular carcinoma recurrence after ablation**

| Parameters                  | β     | SE    | Wald  | P-value | OR    | 95%CI Lower Limit | 95%CI Upper Limit |
|-----------------------------|-------|-------|-------|---------|-------|-------------------|-------------------|
| Low level of tRT            | -0.207| 0.062 | 5.326 | 0.037   | 0.813 | 0.720             | 0.918             |
| Low level of tPI            | -0.076| 0.007 | 7.392 | 0.006   | 0.927 | 0.914             | 0.940             |
| Low level of tRTP           | -0.312| 0.008 | 5.892 | 0.019   | 0.732 | 0.721             | 0.744             |
| High level of bFGF          | 0.071 | 0.029 | 4.872 | 0.011   | 1.074 | 1.015             | 1.137             |
| Low level of pRT            | -0.091| 0.091 | 5.322 | 0.137   | 0.913 | 0.764             | 1.091             |
| Low level of t-pTP          | -0.057| 0.032 | 4.352 | 0.086   | 0.945 | 0.888             | 1.006             |
| Low level of p-pPI          | -0.086| 0.042 | 4.892 | 0.136   | 0.936 | 0.862             | 1.016             |
| Low level of t-pPI          | -0.013| 0.032 | 3.842 | 0.187   | 0.987 | 0.927             | 1.051             |
| High level of ET-1          | 0.031 | 0.032 | 5.762 | 0.089   | 1.032 | 0.969             | 1.099             |

ET-1: Endothelin-1; bFGF: Basic fibroblast growth factor; tRT: Tumor rise time; tTTP: Tumor time to peak; pTTP: Parenchymal time to peak; tPI: Tumor peak intensity; p-TTP: Parenchymal-tumor time to peak.
Limitations
There are some limitations in this research. First, the sample size was small, and a larger sample size is needed to validate our conclusions. Secondly, the relationship between the CEUS index and molecular biological index needs to be explored further. Other or more detailed biological indices are needed, combined with clinical practice.

In conclusion, CEUS is a simple and noninvasive method for tumor angiogenesis assessment. Quantitative parameters analyzed by CEUS software were more objective; where ITTP was found to be a special predictor of HCC recurrence and is worthy of further study. CEUS is important for determining tumor tissue hemodynamics in detail, and can be used to evaluate tumor angiogenesis and predict tumor recurrence.

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