OncoTargets and Therapy

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ORIGINAL RESEARCH

RETRACTED ARTICLE: Correlations of microvascular blood flow of contrast-enhanced ultrasound and HG F/c-Met signaling pathway with clinicopathological features and prognosis of patients with hepatocellular carcinoma

This article was published in the following Dove Press journal:
OncoTargets and Therapy
14 February 2017
Number of times this article has been viewed

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Abstract: The study is designed to explore the correlations of microvascular blood flow of contrast-enhanced ultrasound (CEUS) and hepatocyte growth factor (HGF)/c-Met signaling pathway with clinicopathological features and prognosis of patients with hepatocellular carcinoma (HCC). One hundred and eighteen patients pathologically diagnosed as primary HCC were selected. All HCC patients underwent CEUS examination before operation. HCC tissues and adjacent normal tissue specimens were obtained to detect the protein rates of HGF and c-Met expressions by immunohistochemistry. The mRNA expressions of HGF and c-Met were detected by quantitative real-time polymerase chase reaction assay. The microvessel density (MVD) was tested by CD34 immunohistochemistry. Compared with liver parenchyma, the HCC lesions had higher MVD, preoperative peak intensity (PI), area under the curve (AUC), lower preoperative time to peak, and washout time (WOT). Compared with adjacent normal tissues, the protein and mRNA expressions of HGF were reduced in HCC tissues, but the protein and mRNA expressions of c-Met and MVD were increased. The protein expressions of HGF and c-Met exhibited evident correlations with TNM stage, tumor size, vascular invasion, liver cirrhosis, and hepatitis B virus and hepatitis C virus infection of HCC patients. The tumor size and number, vascular invasion, the protein expressions of HGF and c-Met, and MVD were associated with the TTP, PI, WOT, and AUC of CEUS in HCC patients. The protein expressions of HGF and c-Met, MVD and preoperative PI revealed negative associations with the prognosis of HCC patients. In conclusion, quantitative parameters of CEUS and HGF/c-Met signaling pathway-related proteins may be helpful for early diagnosis and prognosis prediction of HCC patients.

Keywords: hepatocellular carcinoma, hepatocyte growth factor, cellular-mesenchymal to epithelial transition factor, signaling pathway, microvascular blood flow, contrast-enhanced ultrasound, clinicopathological feature

Introduction

Hepatocellular carcinoma (HCC) is claimed to be the third commonest cause of cancer-associated mortality across the world for its rapid recurrence and metastasis. And HCC is commonly diagnosed in most countries with a high incidence of viral hepatitis.[1] HCC takes up 85%–90% of primary liver cancers with 500,000 new cases as well as 250,000 deaths of HCC all over the world every year.[2] The main reasons for the high mortality rate of HCC patients are ascribed to the lack of effective treatments and

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Dovepress
http://dx.doi.org/10.2147/OTT.S113353

the increasing resistance to conventional radiotherapy and chemotherapy.[3] In recent years, improved data of signaling pathways modulating the growth and progression of HCC have resulted in the identification of several new molecular targets. At present, several studies proposed that one of the most promising molecular signaling pathways guiding HCC treatment is the hepatocyte growth factor (HGF)/c-Met signaling.[4,5]

HGF was first discovered as a factor inducing hepatocyte proliferation, characterized as a motility factor for epithelial cells.[6] Cellular mesenchymal to epithelial transition factor (c-Met), as a high-affinity tyrosine kinase receptor of HGF, can be mediated by HGF. Both HGF and c-Met play an important role in the tumor invasion and metastasis.[7] Increasing expressions of HGF and c-Met in circulating tumor cells reveal a correlation with the progression and metastasis of HCC.[8] The signal triggered by the binding of HGF to c-Met is shown to be one of the leading stimuli during the G1-S development and progression in hepatocytes.[2] Interestingly, HGF/c-Met signaling pathway is crucial for normal embryonic development along with adult tissue reparation in mammal animals, with a certain level in human normal tissue.[4] However, improper amplification of the *c-Met* gene and activation of HGF/c-Met signaling, along with eventually elevated protein expression and constitutive kinase activation, may result in growth, invasion, migration, and tumorigenesis.[9]

Recently, the technology of contrast-enhanced ultrasonography (CEUS) has been widely applied in the differential diagnosis of metastasis and survival of many human cancers, including breast cancer, colorectal cancer, gastric cancer, pancreatic cancer, and liver cancer.[10–14] CEUS is a typical nontraumatic examination method providing blood perfusion parameters and enhancing the accuracy in detecting angiogenesis and neovessels in livers.[14] CEUS is able to show the characteristics of liver tumors accurately and clearly, as well as the perfusion differences between residual carcinoma and necrotic tissue after ablation.[15] Also, CEUS can offer details of tumor necrosis subsequent of ultrasound-guided local ablation more sensitively, which may deliver prompt supplementary treatment and better efficacy.[16] Based on previous studies, the objective of this study is to investigate the correlations of quantitative parameters of CEUS and HGF/c-Met signaling pathway with clinicopathological features and prognosis of patients with HCC.

Materials and methods

All subjects provided written informed consent. The collection and application of sample tissue followed ethical guidelines. This study was approved by Ethics Committee of The Hospital of Xi'an Jiao Tong University.

Subjects

A total of 118 patients pathologically diagnosed as HCC from January 2010 to October 2010 in The Hospital of Xi'an Jiao Tong University were included in the follow-up visit. The follow-up and survival data of eligible subjects were complete. All patients were aged between 20 and 76 years, with a median age of 54 years. The clinical information was listed in Table 1. The HCC tissues and adjacent normal tissues (over 5 cm distant from cancer tissue) were obtained and reserved in liquid nitrogen.

CEUS

After fasting for 12 h, the patients were examined with iU22 ultrasonic inspection instrument (Royal Philips electronics NV, Eindhoven, the Netherlands) L9-3 wideband probe; pulse inversion harmonic contrast imaging; mechanical index at 0.07. By applying Color Doppler, the strongest flow signal area was frozen and stored as images. Intravenous bolus injection of 0.02 mL/kg fluorocarbon developed by Nanfang Hospital was injected. After the injection, an observation by CEUS lasted for at least 3 min. The real-time dynamic video of CEUS in the whole process was recorded and stored. The patients were inquired of allergic history. No allergic reaction occurred in the experiment.

According to the blood flow pattern and amount and distribution presented by Doppler, the blood flow signal was scaled as follows: Grade 0 refers to no blood signal in the tissue and its surrounding; Grade 1 refers to a few punctiform

Table 1 Clinicopathological features of 118 patients with hepatocellular carcinoma

Feature	Case number
Gender (male/female)	75/43
Smoking history (yes/no)	68/50
Alcohol history (yes/no)	79/39
Tumor differentiation (high/moderate/low differentiation)	46/41/31
TMN stage (I/II/III/IV)	20/21/36/41
Tumor size (<3 cm/≥3 cm)	56/62
Tumor number (<3/≥3)	53/65
Vascular invasion (yes/no)	67/51
Lymphatic invasion (yes/no)	71/47
Liver cirrhosis (yes/no)	75/43
AFP (<20/≥20 ng/mL)	35/83
HBsAg/HCV (negative/positive)	87/31
HBV-DNA (negative/positive)	42/76

Abbreviations: TNM, tumor, node, and metastasis; AFP, alpha-fetoprotein; HBsAg/HCV, hepatitis B surface antigen/hepatitis C virus; HBV-DNA, hepatitis B virus-DNA.

blood flow signals in the lesion and a small quantity of blood flow signals in the tissue that surrounds the lesion; Grade 2 refers to a medium amount of blood flow in the tissue, along with a main vessel stretching branches from surrounding to center; Grade 3 refers to abundant blood flow with over 2 main vessels stretching into lesion tissues or being connected with each other. The microvessel image was analyzed with Qlab software (Qlab 9.0; Philips Medical System, Andover, MA, USA) for observing the trace of microbubble in the microvessel. The targeted area of abundant blood vessel was sampled for 3 times and then analyzed to obtain blood perfusion time–intensity curve, time to peak (TTP), peak intensity (PI), area under the curve (AUC), and washout time (WOT).

Immunohistochemistry

The HCC tissues and adjacent nontumor tissues were obtained and fixed with 4% formalin. After gradient alcohol dehydration, the tissues were embedded with paraffin and then sliced to serial tissue sections of 5-μm thick. After routine dewaxing and antigen reparation, the tissues were sealed with blocking buffer. Then the slices were added with HGF or c-Met antibody (dilution at 1:500, 100 μL/slice; Abcam Co., Cambridge, MA, USA) and incubated at 4°C overnight. After w[ashing] by phosphate buffer solution Tween-20 (PBST), the bio[tiny]-lated secondary antibody (dilution at 1:500; A[bcam] Co.) [was] added and the tissues were incubated at 37[°C] for 2[h]. Avidi[n]-Biotin-Enzyme Complex (ABC) were a[dded] for i[ncub]ation at 37°C for 1 h. After diaminobenzidi[ne] (DA[B] c]olor reaction, the slices were blocked, obser[ved] and photog[raph]ed under optical microscope (Olymp[us], Sou[thb]orough, MA, USA). The immunohistochemi[stry] kit was pu[rch]ased from Roche (Hoffmann-La Roch[e] Ltd, Ma[nn]heim, Ger[many]). Under the same light intensity, the HG[F] and c-Met in HCC tissues and adjacent non[tum]or tis[sue]s were [exa]mined, with a random selection [of] 4 field[s] assigned [to] each slice. The percentage of positiv[e] cells dis[cove]red in the immunohistochemical staining under the [high] power lens of ×400 was counted as an index. The positive r[ate] = the number of positive cells/the number of cells in the fields ×100%.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The RNA was extracted with TRIzol (Invitrogen Biotechnology Co., Shanghai, People's Republic of China) and examined of its concentration and purity with NanoDrop2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). The extracted RNA was reserved at −80°C for further usage. According to the published gene sequence by Genebank database, PCR primer sequence (Table 2) was designed with Primer 5.0 software (Shanghai GenePharm Co., Ltd., Shanghai, People's Republic of China). The PCR system was 12.5 μL of SYBR Premix Ex Taq II (Takara Bio Inc., Dalian, People's Republic of China); 1 μL of forward and 1 μL of reverse primers; 2 μL of DNA template; 8.5 μL of sterile water; internal reference gene, β-actin. The PCR condition was initial denaturation at 95°C for 30 s; denaturation at 95°C for 5 s, annealing at 51°C for 25 s, and elongation at 65°C for 10 s, 40 cycles of these three procedures in total. The PCR results were evaluated b[y] [dis]solution curve. The threshold cycle (CT) was obtai[ned] at the [in]flection point of amplification curve. ΔCt = C[T] (target gene) − CT (β-actin), ΔΔCt = ΔCt (HCC tissue) − ΔCt (a[dja]cent tiss[ue]). The relative expression of target [gene] was calcul[ated] as $2^{-\Delta\Delta Ct}$.[17]

CD34 immunohistochemistry

The micro[ves]sel density (MVD) was detected through immun[ohis]toch[em]istry (the immunohistochemistry kit was [pur]chased fro[m] Hoffmann-La Roche Ltd) using CD34 [mo]noclonal antibody. The slices of HCC tissues and adjacent [non]tumor tis[sue]s were taken for routine dewaxing, antigen [re]trieval, and [se]aling. After adding CD34 monoclonal antibody [(di]l[uti]on at 1:100; Abcam Co.) of 100 μL into each [sl]ice, the tissues were incubated at 4°C overnight. Then, the slices were washed with PBST and added with the biotin-labelled secondary antibody (dilution at 1:500; Abcam Co.) for a 2-hour incubation at 37°C. The streptavidin avidin biotin complex was added, and the slices were incubated at 37°C for 1 h. After DAB color reaction, hematoxylin was applied for a 2-min counterstaining. And the slices were sealed with neutral resin and observed under optical microscope (Olympus) in a low power (×100) for selection of areas with most new vessels. Cell counting was performed in a high power (×400). Each group was assigned with 6 fields, the average of which was calculated as the MVD. The endothelial cells or cell clusters that presented brown color in immunohistochemistry staining and displayed a distinct border with adjacent microvessels and tumor cells in tumor tissues were all new tumor vessels.

Table 2 The primers for quantitative real-time polymerase chase reaction

Gene	Primer sequence (5′-3′)
HGF	Forward: GAATGACACTGATGTTCCTTTGG
	Reverse: GGATACTGAGAATCCCAACGC
c-Met	Forward: GCAGGTTGTGGTTTCTCG
	Reverse: TGCAGCCCAAGCCATTCA
β-Actin	Forward: CGGGAAATCGTGCGTGAC
	Reverse: AGGCAGCTCGTAGCTCTTCT

Follow-up

After the collection of basic information of patients, the follow-up was conducted for all patients, who underwent tumor resection. The clinical information and postoperative symptoms were recorded and completed. The reexaminations were performed for these patients at 1 week, 1 month, 3 months, and every 3 months after the surgery. At the same time, the CEUS was applied to detect the liver function and the prognosis of patients. The follow-up visit spanned for 37 months on average. The survival time for the patient was from the first day in hospital to the date of death. Whether the patient died of other diseases, accident, or was alive at the end of the follow-up visit, the censored value was regarded as his survival time. The follow-up visit started from the end of the surgery and lasted until November 2015. The survival time should be monthly, and the time of disease-free survival (DFS) was counted until a tumor recurrence or metastatic lesions was observed with imageological examination, or until the death of patient apart from the tumor recurrence. The recurrence was first defined as the intrahepatic manifestation of HCC or obvious extrahepatic metastasis with imageological examination. The 1-, 3-, and 5-year DFS rates were recorded. And the correlations of clinical characteristics and contrast parameters with prognosis of patients were analyzed.

A nude mice model of HCC

The human HCC cell line HepG2 was purchased from cell bank of Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, People's Republic of China). The SU11274, a c-Met kinase inhibitor, was from Sigma-Aldrich Chemical Company (St Louis, MO, USA). The BALB/C (n/n) male nude 3-week-old mice were bought from Laboratory Animal Center of Chinese Academy of Sciences. The mice were stored in feeding boxes of polymethyl methacrylate in an ultraclean laminar-flow cabinet and fed in the conditions of a constant temperature, constant humidity, and specific pathogen free. Free access to sterile water and food was provided for these mice. After trypsinization of HepG2 cells at logarithmic phase of growth, the concentration of HepG2 cells was adjusted to 1×10^7 cells per mL with phosphate-buffered saline. Each mouse was given subcutaneous injection with 0.5 mL prepared solution. After ~10 days, the average volume of tumor in mice could be 100 mm^3, mice with tumor volume much larger or smaller than which were excluded. Then, 30 HCC mice of the rest were randomly selected and divided into three groups with 10 mice in each group, including experimental group (injection of SU11274, a c-Met inhibitor, 0.09 mg/kg), negative control group (injection of normal saline), and blank group (no injection). The administration of intraperitoneal injection lasted for 30 days, once daily. The growth of HCC in mice was observed each day since the first day of injection. The width and length of the tumor in HCC mice were measured and the tumor volume was calculated using the formula $V = ½ab^2$ (a, length; b, width). Finally, a tumor growth curve was obtained.

On the 24th hour after the last administration, the CEUS examination was applied in HCC mice. The HCC mice were fixed after intraperitoneal anesthesia with certain 10% chloral hydrate on the basis of their weight (0.1 mL/20 g). The time–intensity curve was obtained with the enhancing region served as a region of interest. The AUC, PI, TTP, and WOT of CEUS were obtained. The HCC mice were killed after CEUS examination and tumor weights were obtained after complete tumor resection. The inhibition rate of tumor (%) = [1 − (average weight of tumors)$_{\text{the experiment group}}$/(average weight of tumors)$_{\text{the blank group}}$] ×100%. The MVD of HCC tissues was measured by CD34 immunohistochemistry.

Statistical analysis

Data were analyzed with the statistical package for the social sciences (SPSS) version 20.0 (SPSS Inc.; Chicago, IL, USA). *T*-test was applied to analyze the differences between two groups, where continuous data were presented as mean ± standard deviation. Differences among multiple groups were compared by one-way analysis of variance. Categorical data were expressed as ratio and percentage and examined with chi-square test. Univariate survival analysis was based upon Kaplan–Meier analysis, and multivariate survival analysis was performed using Cox proportional hazards model. The level of $P<0.05$ was considered as significant.

Results

HGF and c-Met protein expressions in HCC and adjacent normal tissues

The positive expression of HGF was signaled by brown particles in nucleus and cytoplasm and the positive expression of c-Met was implied by brown particles in both cytoplasm and cell membrane. The results of immunochemistry indicated that positive expressions of HGF in the HCC tissues were significantly reduced while positive expressions of c-Met in HCC tissues were elevated in comparison with the adjacent normal tissues (Figure 1). The PCR detection revealed that HGF mRNA expressions were lower and c-met mRNA expressions were higher in the HCC tissues than these in the adjacent normal tissues, which were consistent with the findings of immunochemistry (all $P<0.05$).

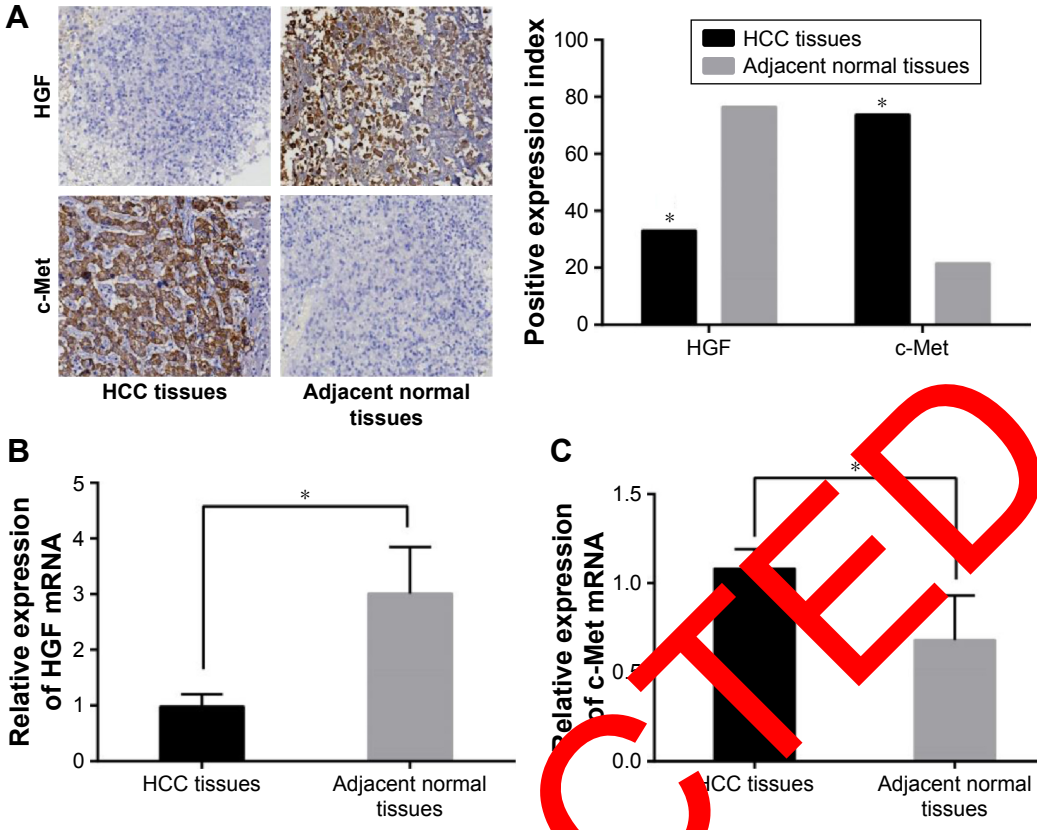

Figure 1 The protein and mRNA expressions of HGF and c-Met expression in the HCC and adjacent normal tissues (×200). (**A**) The protein expressions of HGF and c-Met in HCC and adjacent normal tissues; (**B**) the HGF mRNA expressions in HCC and adjacent normal tissues; (**C**) the c-Met mRNA expressions in HCC and adjacent normal tissues; **P*<0.05 compared with the adjacent normal tissues.
Abbreviations: HGF, hepatocyte growth factor; c-Met, cellular mesenchymal to epithelial transition factor; HCC, hepatocellular carcinoma.

Comparison of MVD between HCC and adjacent normal tissues

The CD34 immunochemistry found no positive expression of CD34 staining in the adjacent normal tissues, while positive expressions of CD34 were abundant in the HCC tissues (Figure 2A). Additionally, MVD in HCC tissues was significantly higher than that in the adjacent normal tissues (Figure 2B).

Comparisons of quantitative parameters of CEUS between liver parenchyma and HCC lesions

The number of blood vessels in HCC lesions was increased in comparison with that in the liver parenchyma. The analysis of the blood perfusion time–intensity curve found that the TTP and WOT of CEUS in the HCC lesions were decreased, while PI and AUC of CEUS in HCC lesions were

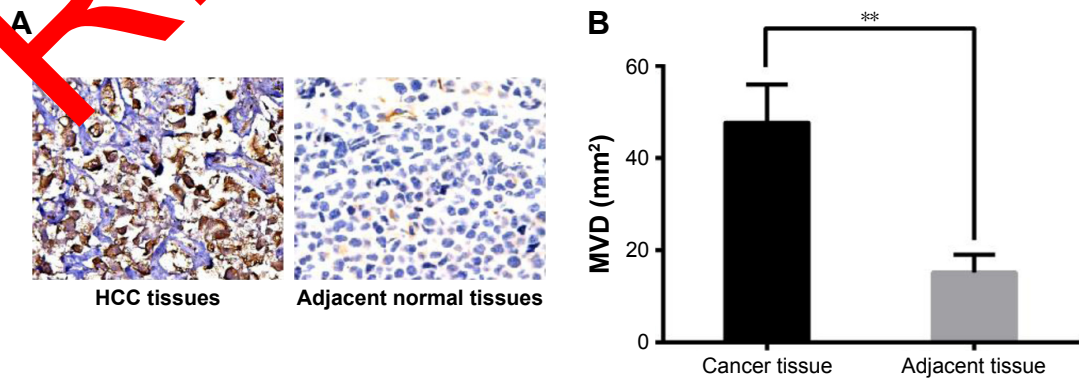

Figure 2 Comparison of microvessel density in the HCC and adjacent normal tissues. (**A**) The CD34 expression in HCC tissues and adjacent normal tissues (×400); (**B**) Comparison of MVD in the HCC and adjacent normal tissues; ***P*<0.01 compared with the adjacent normal tissues.
Abbreviations: MVD, micro-vessel density; HCC, hepatocellular carcinoma.

Table 3 Comparisons of quantitative parameters of CEUS between liver parenchyma and HCC lesions

Tissues	Time to peak (s)	Peak intensity (dB)	Area under the curve (dB*s)	Washout time (s)
HCC lesions	21.81±5.13#	58.70±10.10#	1,162.50±141.20#	32.60±6.40#
Liver parenchyma	30.22±7.51	43.20±8.50	572.80±41.70	67.34±11.16

Note: #$P<0.05$ compared with the adjacent normal tissues.
Abbreviations: CEUS, contrast-enhanced ultrasound; HCC, hepatocellular carcinoma.

elevated compared with those in the liver parenchyma (all $P<0.05$, Table 3).

Correlations of HGF and c-Met protein expressions and MVD with clinicopathological features of HCC patients

HGF and c-Met proteins expressions and MVD exhibited no correlation with age, gender, tumor differentiation, tumor number, and vascular invasion (all $P>0.05$), but evident correlations with TNM stage, tumor size, and vascular invasion of HCC patients (all $P>0.05$). Besides, HGF and c-Met proteins expressions were also significantly associated with liver cirrhosis, hepatitis B virus (HBV) and hepatitis C virus (HCV) infection of HCC patients (all $P<0.05$), while MVD was not associated with these of HCC patients (all $P>0.05$) (Table 4).

Correlations of quantitative parameters of CEUS with clinicopathological features of HCC patients

On the basis of positive expressions of HGF and c-Met protein in HCC tissues, the HCC patients were classified into four groups: the HGF-positive group, the HGF-negative

Table 4 Correlations of HGF and c-Met protein expressions and MVD with clinicopathological features of HCC patients

Feature	HGF expression (%)	*P*-value	c-Met expression (%	*P*-value	MVD	*P*-value
Age (years)		0.938		0.367		0.888
<50	32.65		69.3		47.49±7.13	
≥50	33.33		76		47.71±9.09	
Gender				0.573		0.185
Male	37.33		00		48.39±8.42	
Female	25.58		74		46.28±8.00	
Tumor differentiation		0.2		0.239		0.592
Low	22.58		70.97		48.94±9.40	
Moderate	36.59		82.93		47.19±7.12	
High	36.96		67.39		47.12±8.57	
TNM stage		0.025		0.006		0.02
I + II	46.3		58.54		45.19±8.62	
III + IV	97		81.82		48.91±7.88	
Tumor size		0.003		0.027		0.002
<3 cm	4		64.29		45.20±8.80	
≥3 cm	20.9		82.26		49.81±7.21	
Tumor number		0.56		0.219		0.273
<3	35.85		79.25		46.69±7.71	
≥3	30.		69.23		48.38±8.74	
Vascular invasion		0.042		0.005		0.002
Yes	25.37		83.58		49.66±7.14	
No	43.14		60.78		44.94±9.01	
Liver cirrhosis		0.025		0.042		0.157
Yes	25.33					
No	46.51					
HCV infection		0.026		0.046		0.780
Positive	16.13		48.39		47.51±8.82	
Negative	39.08		27.59		47.94±6.76	
HBV infection		0.015		0.024		0.596
Positive	25.00		40.79		47.92±8.15	
Negative	47.62		19.0		47.07±8.63	

Abbreviations: HGF, hepatocyte growth factor; c-Met, cellular mesenchymal to epithelial transition factor; HCC, hepatocellular carcinoma; TNM, tumor, node, and metastasis; MVD, microvessel density; HCV, hepatitis C virus; HBV, hepatitis B virus.

group, the c-Met-positive group, and the c-Met-negative group. And the subgroups of MVD of HCC patients were the high-value group and the low-value group based on the median of MVD (M =48). The data revealed that the patients' age and gender exerted no significant influence on the TTP, PI, AUC, and WOT (all $P>0.05$), while tumor size, tumor number, vascular invasion, HGF and c-Met protein expressions, and MVD were significantly related to the TTP, PI, AUC, and WOT (all $P<0.05$, Table 5).

Correlations of HG/c-Met protein expressions, MVD, and quantitative parameters of CEUS with prognosis of HCC patients

According to the median of TTP, PI, AUC, and WOT (22 s, 58.7 dB, 572.8 dB*s, and 67 s, respectively), each parameter of CEUS of HCC patients was also classified into 2 groups. The univariate survival analysis implied that the patients' age and gender had no significant influence on the survival prognosis and the 1-, 3-, and 5-year DFS rates. Tumor differentiation, TNM stage, tumor size, tumor number, vascular invasion, HGF and c-Met protein expressions, and MVD exerted remarkable influence on the 1-, 3-, and 5-year DFS rates (all $P<0.05$). Moreover, the preoperative TTP, PI, AUC, and WOT were also associated with the 1-, 3-, and 5-year DFS rates (all $P<0.05$, Table 6).

The factors related to recurrence of HCC were obtained with the univariate survival analysis including tumor differentiation, TNM stage, tumor size, tumor number, vascular invasion, HGF and c-Met protein expressions, MVD, and quantitative parameters of CEUS. Further multivariate survival analysis using Cox's proportional hazards model indicated that the recurrence of HCC was significantly correlated with tumor differentiation, TNM stage, tumor size, tumor

Table 5 Correlations of quantitative parameters of CEUS with clinicopathological features of HCC patients

Feature	Time to peak (s)	Peak intensity (dB)	Area under the curve (dB*s)	Washout time (s)
Age (years)				
<50	21.71±6.23	58.81±10.94	576.18±43.77	68.33±11.68
≥50	21.88±4.23	58.62±9.54	570.42±43.78	66.63±10.81
Gender				
Male	22.04±5.50	58.74±9.65	573.53±43.25	66.40±11.10
Female	21.41±4.45	58.62±10.95	571.52±39.32	68.98±11.22
Tumor differentiation				
Low	21.69±4.82	59.42±9.77	572.88±44.60	66.73±12.76
Medium	21.94±4.89	57.36±10.47	569.88±39.48	67.17±10.19
High	21.78±5.63	59±10.47	575.35±42.37	67.90±11.07
TNM stage				
I + II	25.18±4.75	56.73±9.11	576.65±44.77	66.68±12.56
III + IV	20.02±4.09#	59.75±9.42	570.75±40.12	67.69±10.41
Tumor size				
<3 cm	21.56±6.04	55.02±10.84	563.39±45.97	70.71±11.18
≥3 cm	21.91±3.69	62.03±8.13#	583.21±33.84#	63.60±9.97#
Tumor number				
<3	23.30±4.71	54.76±9.83	561.50±39.94	69.93±10.88
≥3	20.60±5.50#	61.92±9.20#	586.65±39.92#	64.15±10.76#
Vascular invasion				
Yes	20.41±5.27	62.87±9.82	595.61±37.39	63.57±12.24
No	23.65±4.34#	53.22±7.58#	555.44±36.27#	70.21±9.38#
HGF expression				
Positive	23.18±6.22	54.00±8.49	567.35±40.86	68.90±10.68
Negative	21.14±4.38#	61.02±10.07#	583.84±41.71#	64.17±11.58#
c-Met expression				
Positive	20.67±4.44	60.41±9.97	591.74±37.66	65.30±12.03
Negative	25.01±5.63#	53.89±8.98#	566.05±41.18#	68.06±10.82#
MVD				
High	20.04±3.73	62.38±9.60	584.62±40.13	65.36±12.13
Low	23.58±5.72#	55.02±9.28#	560.98±40.16#	69.31±9.82#

Note: #$P<0.05$ of comparison within group.
Abbreviations: HGF, hepatocyte growth factor; c-Met, cellular mesenchymal to epithelial transition factor; TNM, tumor, node, and metastasis; MVD, microvessel density.

Table 6 Univariate survival analysis of risk factors for the prognosis of HCC patients

Factor	1-year DFS rate (%)	3-year DFS rate (%)	5-year DFS rate (%)
Age (years)			
<50	71.43	59.18	40.82
≥50	72.46	56.52	37.68
Gender			
Male	76.00	60.00	41.33
Female	65.12	53.49	34.88
Tumor differentiation			
Low	51.61#	32.26#	12.90#
Medium	63.41#	53.66#	34.15#
High	93.48	78.26	60.87
TNM stage			
I + II	92.68	92.68	68.29
III + IV	61.04#	38.96#	23.38#
Tumor size			
<3 cm	94.64	73.21	53.57
≥3 cm	51.61#	43.55#	25.81#
Tumor number			
<3	83.02	71.70	56.60
≥3	63.08#	46.15#	24.62#
Vascular invasion			
Yes	61.19	41.79	25.37
No	86.27#	78.43#	56.86#
HGF expression			
Positive	87.18	79.49	66.67
Negative	64.56#	46.84#	25.32#
c-Met expression			
Positive	66.67	50.57	33.33
Negative	87.10#	77.42#	54.84#
MVD			
High	52.54	32.20	
Low	91.53#	83.05#	61.02
Time to peak			
Short	61.02	44.07	23
Long	83.05#	71.19#	4.24#
Peak intensity			
Strong	61.02	07	23.
Weak	83.05#	71.19#	54.24#
Area under the curve			
Small	83.05	71.19	54.24
Large	61.02#	07#	23.73#
Washout time			
Long	83.	71.19	54.24
Short	.02#	44.	23.73#

Note: #$P<0.05$ comparison within group.
Abbreviations: DFS, dis ; TNM, tumor, node, and metastasis; HGF, hepatocyte gr ctor; c-Met, cellular mesenchymal to epithelial transition factor; MVD, microves ensity.

number, vascular invasion, HGF and c-Met expressions, MVD, and preoperative PI (all $P<0.05$, Table 7).

Effects of c-Met inhibitor on the growth and MVD of transplanted tumors in nude mice

At each time point after the administration of c-Met inhibitor, the volumes of transplanted tumors in nude mice of the

Table 7 Multivariate survival analysis with Cox's proportional hazards model of risk factors for the prognosis of HCC patients

Factor	Risk	95% CI	Regression coefficient	*P*-value
Tumor differentiation	0.219	0.143–0.335	−1.521	<0.001
TNM stage	40.668	14.671–112.735	3.705	<0.001
Tumor size	5.701	2.701–12.036	1.741	<0.001
Tumor number	2.938	1.594–5.416	1.078	0.001
Vascular invasion	2.731	1.436–5.195	1.005	0.002
HGF expression	0.448	0.220–0.916	−0.802	0.028
c-Met expression	4.164	1.562–11.098	1.427	0.004
MVD	1.032	1.000–1.065	0.031	0.049
Time to peak	1.094	0.985–1.214		0.093
Peak intensity	1.048	1.001–1.09	0.047	0.044
Area under the curve	0.989	0.977–1	−0.011	0.084
Washout time	0.964	0.92 –1.00	−0.037	0.073

Abbreviations: TNM, tumor, nod and metastasis; GF, h atocyte growth factor; c-Met, cellular mesenchym to epithelia ansition MVD, microvessel density; CI, confidence interval.

experimental group ere smaller han these of the blank and negative control group all $P<0.05$), and no significant difference of tumor volume was observed between the blank gro and the neg tive control group ($P>0.05$) (Figure 3A). Thus SU11274 (a -Met inhibitor) injection can suppress the growth transplanted tumors in nude mice. The final tumor w ht of the experimental group, the blank group, and the negative control group were 1.05±0.11 g, 1.87±0.15 g, and 1.89±0.18 g, respectively. Compared with the blank and negative control groups, the tumor weight of the experimental group was much lower (both $P>0.05$), and no significant difference of tumor weight was observed between the blank group and the negative control group ($P>0.05$) (Figure 3B). The inhibition rate of tumor of the experimental group (43.73%) indicated that SU11274 can suppress the growth of HCC.

The MVD in the experimental group (29.24±5.36) was much lower than that in the blank group (51.12±9.08) and the negative control group (49.42±8.75) (both $P<0.05$), while no significant difference of MVD was observed between the blank group and the negative control group ($P>0.05$) (Figure 4).

Comparisons of quantitative parameters of CEUS of transplanted tumors in nude mice among the three groups

Compared with the blank and negative control groups (Figure 5), the PI and AUC of CEUS in the experimental group were evidently decreased (all $P<0.05$), and the TTP and WOT of CEUS in the experimental group were

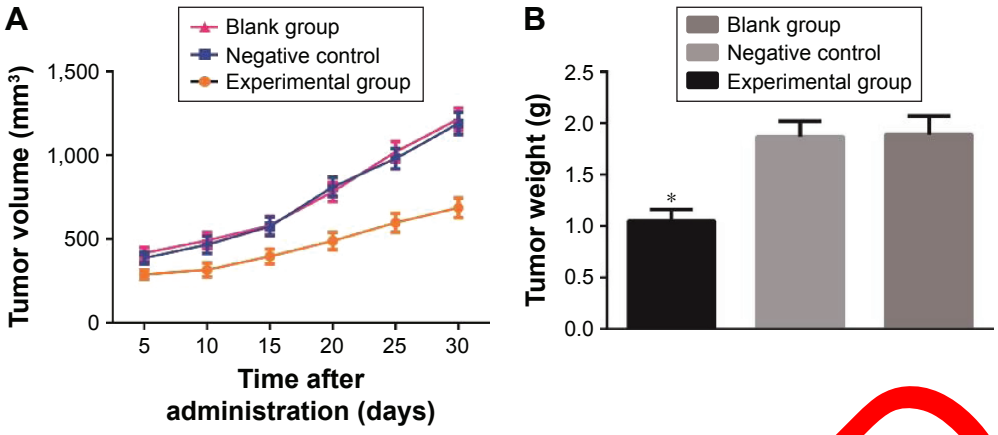

Figure 3 Effects of c-Met inhibitor on the growth of transplanted tumors in nude mice. (**A**) The growth of transplanted tumors in nude mice of three groups; (**B**) comparison of weights of transplanted tumors in nude mice of three groups; *$P<0.05$ compared with blank group.
Abbreviation: c-Met, cellular-mesenchymal to epithelial transition factor.

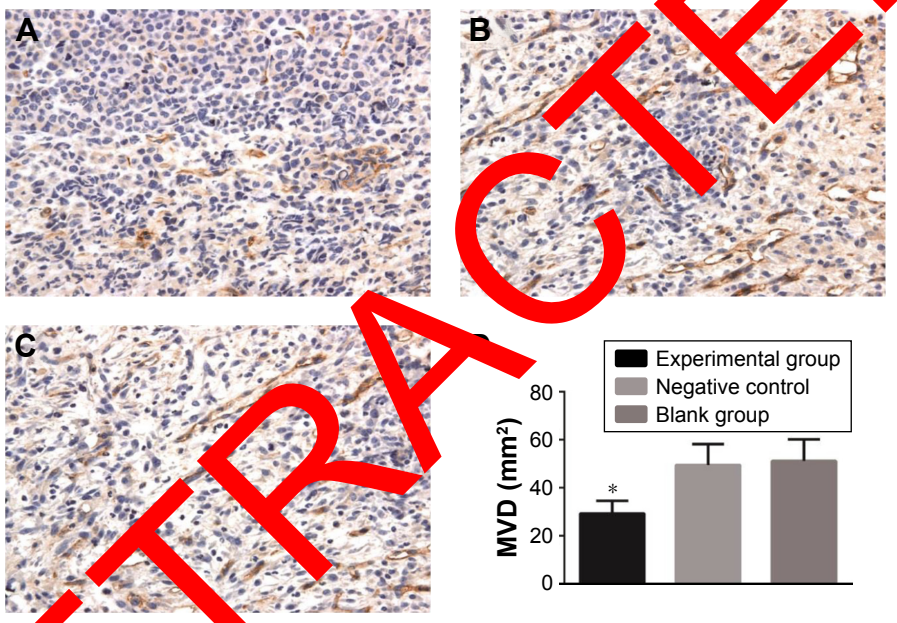

Figure 4 Effects of c-Met inhibitor on MVD of transplanted tumors in nude mice. (**A**) The MVD of transplanted tumors in nude mice of the experimental group, ×400; (**B**) the MVD of transplanted tumors in nude mice of the negative control group, ×400; (**C**) the MVD of transplanted tumors in nude mice of the blank group, ×400; (**D**) comparison of MVD among three groups; $P<0.05$ compared with blank group.
Abbreviations: c-Met, cellular-mesenchymal to epithelial transition factor; MVD, microvessel density.

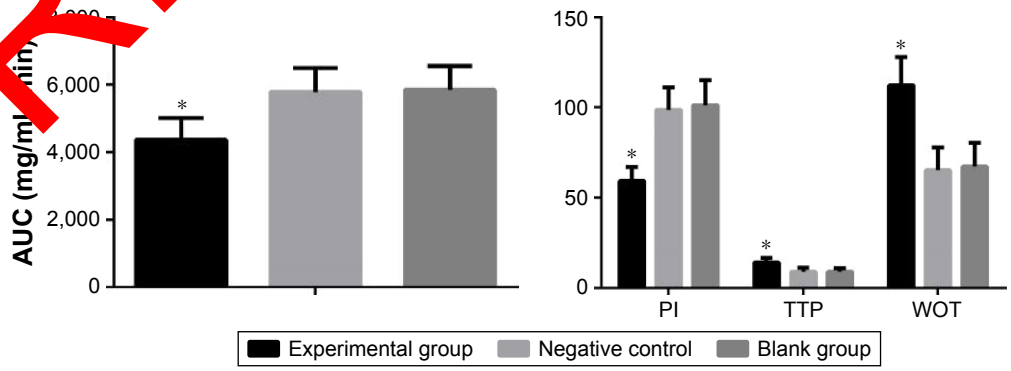

Figure 5 Comparisons of quantitative parameters of CEUS of transplanted tumors in nude mice among three groups. *$P<0.05$ compared with blank group.
Abbreviations: AUC, area under the curve; CEUS, contrast-enhanced ultrasound; PI, peak intensity; TTP, time to peak; WOT, washout time.

evidently raised (all $P<0.05$). These four parameters showed no significant difference between the blank group and the negative control group ($P>0.05$).

Discussion

The findings of this study revealed that HGF protein expression was reduced, and c-Met protein expression was elevated in the HCC tissues in comparison with these in the adjacent normal tissues. MVD value in the HCC tissues was significantly higher than that in the adjacent normal tissues. Further analysis reveals that HGF and c-Met expressions and MVD were related to TNM stage, tumor size, and vascular invasion. Moreover, HGF and c-Met protein expressions, MVD, and preoperative PI exerted significant correlations with the prognosis of patients with HCC. Collectively, this study supported that HGF/c-Met signaling pathway combined with CEUS might be related to the clinicopathological features and prognosis of patients with HCC.

Compared with the adjacent normal tissues, the mRNA and protein expressions of HGF were reduced, and mRNA and protein expressions of c-Met level were increased in the HCC tissues, both of which were closely correlated with clinicopathological features and prognosis of HCC patients. The HGF/c-Met signaling pathway is involved in a wide variety of solid tumors and hematopoietic derived malignancies, which plays a crucial role in malignant transformation through promotion of tumor cell migration, invasion, and epithelial to mesenchymal transition. Goyal et al demonstrate that HGF expression is decreased in HCC tissues in comparison with adjacent normal tissue; on the other hand, c-MET transcription is elevated in 30%–100% of cancer tissue relative to adjacent normal tissue.[18] Consistent with this finding, the results of this study indicated lower protein and mRNA expressions of HGF, and higher protein and mRNA expressions of c-Met in HCC tissues than these in the adjacent normal tissues. Furthermore, Li et al suggest that HGF/c-Met signaling pathway is able to promote cell migration and invasion through STAT3 activation.[4] Marquardt et al report that overexpression of MET is indicated to be significantly related with vascular invasion, neoangiogenesis, and poor outcome in patients with liver cancer.[19] The present study further proved that the protein expressions of HGF/c-Met were associated with the clinicopathological features of HCC patients such as TNM stage, tumor size, vascular invasion, liver cirrhosis, and HBV and HCV infections. It has been reported that aberrant expression of HGF/c-Met signaling is involved in aggressive liver tumors and may cause poor prognosis.[20] Bozkaya et al identified that the activation of HGF/c-Met signaling is correlated with aggressive phenotype and poor prognosis in HCC.[2] Kondo et al have proposed that c-Met may be a useful predictive marker of recurrence in resected HCC patients.[21] Therefore, it is assumed that HGF/c-Met signaling may play an important role in the recurrence and prognosis of HCC. With univariate survival analysis, the protein expressions of HGF/c-Met were closely related to the DFS rates of HCC; moreover, the tumor differentiation, tumor size, tumor number, TNM stages, and vascular invasion were also associated with the DFS rates of HCC.

Additionally, the study revealed that quantitative parameters of CEUS as well as MVD were also correlated with clinicopathological features and prognosis of HCC patients. Nowadays, quantitative determination of the MVD is a commonly used method for both evaluating angiogenesis in tumor tissues and identifying the biological characteristics and prognosis of patients with HCC.[22] MVD is high in prostate cancer tissues and an association of high MVD with the prognosis and progression of prostate cancer is observed in the meta-analyses, disease-specific survival as well as stage of disease.[23] The technology of CEUS has been developed to reflect the neovessels in tumors.[15] Different CEUS parameters are correlated with the different proportions between the portal vein and hepatic arteries, together with the different vascular structures in tumors.[14] As main parameters of CEUS, PI and AUC are used to estimate enhancement intensity, and TTP and AUC are associated with enhancement time. PI and AUC of CEUS are applied to evaluate tumor vascularity in ovarian tumors as noninvasive parameters.[24] In compliance with the above points, positive expressions of CD34 were abundant and MVD was high in the HCC tissues. The TTP, PI, AUC, and WOT were significantly associated with tumor size, tumor number, vascular invasion, and MVD. These parameters were also correlated with 1-, 3-, and 5-year DFS rates. PI of CEUS was also demonstrated to be associated with the recurrence of HCC. Additionally, this study first time found that TTP, PI, AUC, and WOT were associated with the HGF and c-Met protein expressions in patients with HCC. Therefore, CEUS might directly reflect the intratumoral HGF and c-Met protein expressions and neovascular generation as well as prognosis of patients with HCC.

To conclude, the present study demonstrated that HGF and c-Met protein expressions and quantitative parameters of CEUS were correlated with the clinicopathological features of HCC patients. Further evidences indicated that HGF and c-Met protein expressions, MVD, and preoperative PI exert significant influence on the prognosis of HCC patients. Collectively, this study supports that quantitative parameters

of CEUS and HGF/c-Met signaling pathway-related proteins may be helpful for early diagnosis and prognosis prediction of HCC patients. Nevertheless, the small sample size might have a small influence on the conclusion so that a larger sample size is needed in the future study. And more specific mechanism of this finding should be supplemented later so that strong evidence could be provided for predicting prognosis of patients with HCC.

Acknowledgments

We thank the reviewers for critical comments.

Author contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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