ORIGINAL RESEARCH

Association between quantitative parameters of CEUS and Sall4/Wnt/β-catenin signaling in patients with hepatocellular carcinoma

Jianjun Wang[1]
Jiaxin Huang[2]
Qianfeng Ma[1]
Guanghui Liu[3]

[1]Department of Ultrasonography, General Hospital of Ningxia Medical University, Ningxia, People's Republic of China; [2]The University of Hong Kong, Hong Kong; [3]Human Anatomy and Histoembryology, School of Physical Education, Wuhan Business University, Wuhan, People's Republic of China

Objectives: In this study, we aim to investigate the correlations of quantitative parameters of contrast-enhanced ultrasonography (CEUS) and Spalt-Like Transcription Factor 4 (Sall4)/Wnt/β-catenin signaling pathway with clinicopathological features and prognosis of patients with hepatocellular carcinoma (HCC).
Methods: The CEUS was performed to detect the liver function and the prognosis of patients. The expression of Sall4, WNT3a and β-catenin was evaluated using immunohistochemical staining. Sall4, WNT3a and β-catenin mRNA expression was measured by SYBR green qPCR assay.
Results: We found that the mRNA and protein expression of Sall4, WNT3a and β-catenin in the HCC tissues were significantly upregulated compared with the adjacent normal tissues. Upregulation of these proteins was associated with tumor differentiation, TNM stage, tumor size, vascular invasion and liver cirrhosis of HCC patients. In addition, we found that decreased time to peak and washout time and increased peak intensity and area under the curve of CEUS in the HCC were also correlated with TNM stage, tumor size and vascular invasion. Moreover, Sall4, WNT3a and β-catenin protein were significantly associated with the TTP, PI, AUC, and WOT.
Conclusion: This study suggests that quantitative parameters of CEUS and Sall4/Wnt/β-catenin signaling may be helpful for early diagnosis and prognosis prediction of HCC patients.
Keywords: hepatocellular carcinoma, Sall4, wnt signaling, contrast-enhanced ultrasonography, early diagnosis

Introduction

Hepatocellular carcinoma (HCC) accounting for 70–85% of the primary tumors of the liver is one of the highest mortality tumors all over the world.[1] The improvement in HCC treatment including liver resection, chemotherapy and transplantation has improved 5-year survival rates.[2] However, survival rates of the HCC patients diagnosed in late stage remain extremely low.[3] Therefore, development of novel and helpful early detection approaches is urgently needed for improving the therapeutic efficiency for HCC.

The Spalt-Like Transcription Factor 4 (Sall4) oncogene plays a central function in embryo-fetal development and is absent in differentiated tissues.[4] Reexpression of Sall4 in HCC tissues involved in demethylation of its CpGs is associated with poor prognosis.[5,6] In addition, 1 week after treatment, higher levels of Sall4 protein in serum were observed in HCC patients, and these patients had poor prognosis evidenced by high tumor recurrence and low overall survival rate.[7] Thus, reexpression of Sall4 is

Correspondence: Guanghui Liu
Human Anatomy and Histoembryology, School of Physical Education, Wuhan Business University, Wuhan, People's Republic of China
Tel +86 278 479 1352
Fax +86 278 479 1352
Email hahliugh@sina.com

relevant to the poor prognosis of HCC patients. Activation of WNT/β-catenin signaling that was positively correlated with Sall4 has been described as a crucial regulator in tumorgenesis.[8] Constitutive expression of Sall4 causes myelodysplastic syndrome)/acute myeloid leukemia through the Wnt/beta-catenin pathway.[9] In esophageal squamous cell carcinoma (ESCC), silencing of Sall4 inhibited tumor formation in vivo via Wnt/β-catenin signaling pathway.[10] And Sall4 promotes intrahepatic cholangiocarcinoma cell proliferation through Wnt/β-catenin signaling and enhancing epithelial–mesenchymal transition (EMT) process.[11] Thus, these studies proposed that Sall4/Wnt/β-catenin signaling may be a potentially promising molecular signaling for guiding HCC diagnosis and treatment.

Contrast-enhanced ultrasonography (CEUS), a typical nontraumatic examination method providing blood perfusion parameters, has been widely applied for diagnosis of many human cancers, including breast cancer and liver cancer.[12] Abundant new vessel generates around the tumors. CEUS is able to accurately and clearly show the characteristics of angiogenesis and neovessels in liver tumors and parenchyma.[13]

Thus, in this study, we aim to investigate the association between the quantitative parameters of CEUS and Sall4/Wnt/β-catenin pathway in early diagnosis of HCC.

Patients and methods
Clinical patients recruitment

One hundred and sixty-eight patients diagnosed as HCC without diabetes, cardiovascular disease and other tumors from February 2011 to December 2011 in the General Hospital of Ningxia Medical University were included. The age ranged from 20 to 76 years (median is 52 years). The follow-up of all the patients was completed. The clinical information is shown in Table 1. The HCC tissues and adjacent tissues were obtained from surgical section and stored at liquid nitrogen until use. The tumor cells were confirmed by H&E staining. All subjects provided written informed consent. This study was approved by the Ethics Committee of General Hospital of Ningxia Medical University and was conducted in accordance with the Declaration of Helsinki.

Medical records of patients were collected. All patients who underwent tumor resection were followed up. The clinical features and postoperative symptoms were recorded. The CEUS was performed every 3

Table 1 Clinicopathological features of 168 patients with hepatocellular carcinoma

Feature	Case number
Gender (male/female)	108/60
Alcohol history (yes/no)	110/58
Tumor differentiation (high/moderate/low differentiation)	74/52/42
TMN stage (I/II/III/IV)	36/24/47/61
Tumor size (<3 cm/≥3 cm)	75/93
Vascular invasion (yes/no)	97/71
Lymphatic invasion (yes/no)	101/67
Liver cirrhosis (yes/no)	106/62
AFP (<20/≥20 ng/mL)	50/118
HBsAg/HCV (negative/positive)	110/58
HBV-DNA(negative/positive)	70/98

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

months after the surgery to evaluate the liver function. The follow-up started from the end of the surgery and lasted until December 2016. The survival time was defined as from the first day in hospital to the date of death with any reasons. The time of disease-free survival (DFS) was defined as until a tumor recurrence and/or metastatic lesions were observed. The DFS rates (1-, 3- or 5-year) were recorded.

CEUS

The patients were routinely examined with iU22 ultrasonic inspection instrument (Royal Philips Electronics NV, Eindhoven, the Netherlands) according to the standard operating procedure. All CEUS were performed and evaluated by two sonographers with more than 3 years' experience in CEUS. Both low-frequency and high-frequency probe were used for CEUS. All patients received an intravenous injection of 2.4 mL of SonoVue, followed by 5 mL of saline flush. There was an interval of 10 mins between the two examinations in every patient. Four mins after contrast agent administration, CEUS process was recorded for 180 s to observe the lesions. The following CEUS patterns of nodules were considered: extent of enhancement during the arterial phase. The dynamic image was recorded on the hard disk of the sonography machine. Qlab software was used to analyze the microvessel image. The peak intensity (PI), area under the curve (AUC), blood perfusion time–intensity curve, time to peak (TTP) and washout time (WOT) were recorded for analysis.

Immunohistochemical staining assay

Immunohistochemical staining was used to determine the expression of Sall4, WNT3a and β-catenin. Briefly, the tissue sections cut at 4 μm were deparaffinized and hydrated, and then were retrieved with citrate buffer at boiling water for 15 mins. Then, the sections were incubated with primary antibodies (mouse monoclonal anti-Sall4, 1:200, mouse monoclonal anti- WNT3a, 1:300, rabbit polyclonal anti-β-catenin, 1:200, Cell Signal Technology, Danvers, MA, USA) overnight at 4°C. The sections were then incubated with secondary antibody for 60 mins at 37°C. The signaling was visualized by diaminobenzidine (DAB) and counterstained with hematoxylin.

For evaluating the expression of Sall4, WNT3a and β-catenin, the integral optical density (IOD) was obtained by ImageJ (National Institutes of Health, Bethesda, MA, USA). The IOD of Sall4, WNT3a and β-catenin expression was normalized to the average IOD of them in normal tissues.

RNA extraction and quantitative PCR analysis

Total RNA was extracted from cells using trizol reagent (Invitrogen, Carlsbad, CA, USA). Sall4, WNT3a and β-catenin mRNA expression was measured by SYBR green qPCR assay (Takara, Dalian, China) according to the recommended protocol. Expression of β-actin was used as an endogenous control. The primers were used as follows: Sall4, sense, CTAGACACGGCTCCCAATGC, antisense, AGACGTGA-ACTGCCCGAATC; WNT3a, sense, CTGGAGCTAGTGT-CTCCTCTCT, antisense, GGAAGAAGCCTCATCCAC-CA; β-catenin, sense, ATAAGAGCTCCTTGTGCGGC, antisense, GGCCATGTCCAACTCCATCA; β-actin, sense, AGGGGCCGGACTCGTCATACT, antisense, GGCGGCA-CCACCATGTACCCT. q-PCR was performed at the condition: 95.0°C for 3 mins and 39 circles of 95.0°C for 10 s and 60°C for 30 s. Data were processed using $2^{-\Delta\Delta CT}$ method.

Statistical analysis

SPSS17.0 statistical software (SPSS Inc. Chicago, IL, USA) was used to analyze the data. The data were presented as mean ± SD. The clinical association between protein expression, quantitative parameters of CEUS and clinicopathological variables in HCC patients was evaluated by Chi-square test. The difference among the groups was analyzed by Student's *t*-test or one-way ANOVA depending on the conditions. And multivariate survival analysis was performed using Cox proportional hazards model. *P*-value <0.05 was considered to be statistically significant.

Results

The expression of Sall4, WNT3a and β-catenin in HCC and adjacent tissues

Sall4 was expressed in the nucleus, and WNT3a and β-catenin were expressed in the cytoplasm. The expressions of Sall4, WNT3a and β-catenin in the HCC tissues evaluated by IHC were significantly upregulated compared with the adjacent tissues (Figure 1). The mRNA levels of Sall4, WNT3a and β-catenin were measured by q-PCR. In line with the findings of immunochemistry, the results revealed that Sall4, WNT3a and β-catenin mRNA levels were higher in the HCC tissues than those in the adjacent tissues (Figure 2, all *P*<0.05).

Alterations of the parameters of CEUS in liver parenchyma and HCC tissues

Angiogenesis in HCC was enhanced compared with the liver parenchyma. We analyzed of the blood perfusion time–intensity curve and found that the TTP and WOT of CEUS in the HCC were significantly decreased, whereas PI and AUC of CEUS in HCC were dramatically increased than in the liver parenchyma (all *P*<0.05, Table 2).

Associations between Sall4, WNT3a and β-catenin expression and clinicopathological features of HCC patients

The expression of Sall4, WNT3a and β-catenin was not associated with age, gender and smoking history (*P*>0.05), but associated with tumor differentiation, TNM stage, tumor size, vascular invasion and liver cirrhosis of HCC patients (*P*<0.05). However, the positive rates of these proteins had no significant difference among viral cirrhosis, alcohol cirrhosis and other types of cirrhosis. In addition, Sall4 expression was significantly associated with HBV, HCV and alcohol history (*P*<0.05), whereas WNT3a and β-catenin expression was not associated with these features (*P*>0.05) (Table 3).
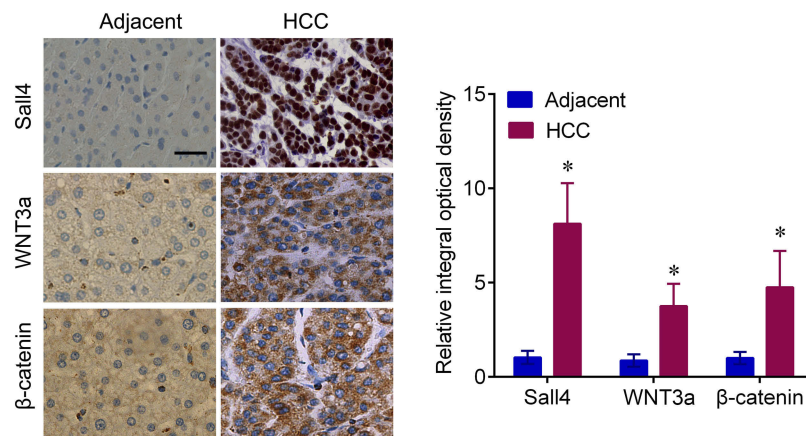
Figure 1 The representative IHC staining of Sall4, WNT3a and β-catenin in the HCC and adjacent normal tissues, and semiquantification. Scale bar, 100 μm. *P<0.05 vs adjacent control.
Abbreviations: HCC, hepatocellular carcinoma; IHC, immunohistochemistry; Sall4, Spalt-Like Transcription Factor 4.
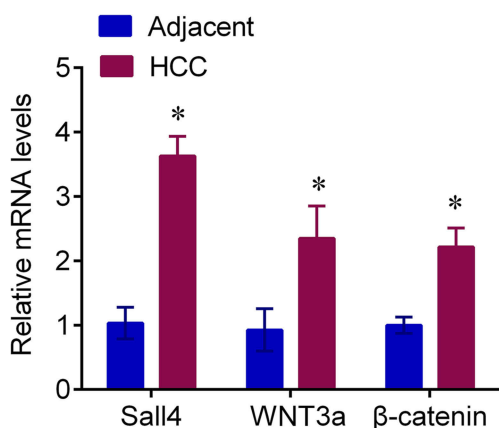

Figure 2 The Sall4, WNT3a and β-catenin mRNA expressions in HCC and adjacent normal tissues; *P<0.05 vs adjacent control.
Abbreviations: HCC, hepatocellular carcinoma; Sall4, Spalt-Like Transcription Factor 4.

Association between the parameters of CEUS and clinical features of HCC patients

Based on positive expressions of Sall4, WNT3a and β-catenin protein in HCC tissues, the patients with HCC were divided into positive and negative groups. The results revealed that no significant association was

observed between the patients' age, gender and tumor differentiation and the TTP, PI, AUC and WOT (P>0.05), whereas Sall4, WNT3a and β-catenin expression, tumor size, vascular invasion and TNM stage were significantly associated with the TTP, PI, AUC and WOT (P<0.05, Table 4).

Association between Sall4, WNT3a and β-catenin expression, and the parameters of CEUS in DFS of HCC patients

The HCC patients were divided into 2 groups based on the median of TTP, PI, AUC and WOT (22 s, 60.3 dB, 593.4 dB*s and 72 s, respectively). We found that the DFS rates were not associated with age and gender, but associated with TNM stage, tumor size, tumor differentiation and vascular invasion, as well as Sall4, WNT3a and β-catenin expression (P<0.05). And the results showed that the HCC patients with positive Sall4, WNT3a and β-catenin expression had significantly low survival rates. In addition, the preoperative TTP, PI, AUC, and WOT were also associated with the DFS rates, suggesting reduced survival rates in patients with short TTP, strong PI, large AUC or short WOT

Table 2 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	23.45±4.31*	68.76±13.23*	1,562.50±211.20*	42.76±7.43*
Liver parenchyma	35.02±5.41	46.42±5.76	776.67±77.86	84.54±18.41

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

Table 3 Association of Sall4, WNT3a and β-catenin protein expressions with clinicopathological features of HCC patients

Feature	Sall4 expression (%)	P-value	WNT3a expression (%)	P-value	β-catenin expression (%)	P-value
Age (years) <50 ≥50	 76.33 83.45	0.467	 56.38 63.41	0.643	 66.76 70.34	0.874
Gender Male Female	 78.32 80.21	0.753	 58.51 62.35	0.864	 67.39 72.57	0.647
Tumor differentiation Low Moderate High	 72.75 76.56 86.21	0.043	 62.82 67.35 70.31	0.042	 58.56 65.42 73.11	0.031
TNM stage I+II III+IV	 65.34 89.75	0.005	 55.87 69.47	0.037	 56.42 75.46	0.015
Tumor size <3 cm ≥3 cm	 68.72 87.34	0.013	 54.36 70.26	0.025	 58.23 74.43	0.024
Vascular invasion Yes No	 88.12 63.31	0.001	 69.64 56.18	0.031	 76.26 53.18	0.006
Liver cirrhosis Yes No	 79.84 70.53	0.042	 68.79 52.64	0.046	 69.44 57.58	0.038
HCV infection Positive Negative	 45.63 28.74	0.034	 49.42 32.37	0.572	 44.37 38.71	0.635
HBV infection Positive Negative	 48.76 23.57	0.028	 60.21 54.85	0.092	 68.35 59.46	0.078
Alcohol history Yes No	 90.42 50.43	0.0001	 65.72 56.84	0.061	 70.25 64.93	0.078
Smoking history Yes No	 81.32 75.46	0.093	 62.96 58.73	0.243	 71.22 68.57	0.237

Notes: *P*-value was calculated by Chi-square test.
Abbreviations: Sall4, Sall4, Spalt-Like Transcription Factor 4; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HBV, hepatitis B virus.

(Table 5). Furthermore, we selected the factors including tumor differentiation, TNM stage, tumor size, vascular invasion, Sall4, WNT3a and β-catenin expression, the parameters of CEUS, including TTP, PI, AUC and WOT for multivariate survival analysis. We found that tumor differentiation, TNM stage, tumor size, tumor number, vascular invasion, Sall4, WNT3a and β-catenin expressions, and preoperative TTP and PI (Table 6) were significantly correlated with HCC, indicating that Sall4, WNT3a and β-catenin expressions and preoperative TTP

Table 4 Association 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	21.52±4.33	60.81±8.44	600.18±48.77	72.23±12.68
≥50	22.68±5.23	60.62±9.74	596.40±33.88	76.65±11.82
Gender				
Male	22.44±4.50	60.74±7.65	600.43±54.15	73.42±12.20
Female	22.53±5.23	60.62±9.84	592.58±49.22	74.88±10.72
Tumor differentiation				
Low	22.32±5.12	60.47±7.18	588.45±45.62	71.63±12.76
Moderate	21.86±3.61	59.49±9.48	590.85±49.28	72.75±13.19
High	22.88±4.65	60.74±8.57	585.75±45.47	72.46±9.07
TNM stage				
I+II	24.79±3.48	57.53±9.61	586.66±38.57	71.38±11.46
III+IV	21.02±3.12*	62.55±8.42*	600.85±41.22*	74.64±12.31*
Tumor size				
<3 cm	24.26±4.04	56.62±9.84	578.79±41.07	75.31±12.28
≥3 cm	20.53±4.15*	63.73±6.73*	603.24±41.84*	65.23±10.37*
Vascular invasion				
Yes	21.34±3.68	64.57±8.82	606.51±47.79	66.52±11.75
No	24.55±5.44*	55.32±8.58*	575.84±46.28*	74.11±10.41*
Sall4 expression				
Positive	21.48±5.32	65.00±7.29	597.45±30.26	64.56±11.37
Negative	25.25±3.34*	53.72±8.27*	573.88±43.73*	74.35±10.46*
WNT3a expression				
Positive	20.18±5.61	63.21±8.37	601.34±45.76	66.35±11.42
Negative	24.31±4.23*	54.19±6.98*	576.25±51.28*	75.26±9.62*
β-catenin expression				
Positive	20.88±4.63	63.48±7.60	604.92±42.23	67.32±11.67
Negative	24.77±4.22*	54.52±8.24*	578.48±38.26*	73.89±9.18*

Note: *$P<0.05$ of comparison within group.
Abbreviations: Sall4, Spalt-Like Transcription Factor 4; HCC, hepatocellular carcinoma.

and PI are the independent prognostic factors for HCC patients.

Discussion

In this study, we found that the mRNA and protein expression of Sall4, WNT3a and β-catenin in the HCC tissues were significantly upregulated compared with the adjacent tissues. Upregulation of these proteins was associated with tumor differentiation, TNM stage, tumor size, vascular invasion and liver cirrhosis of HCC patients. In addition, we found that decreased TTP and WOT and increased PI and AUC of CEUS in the HCC were also correlated with TNM stage, tumor size and vascular invasion. In addition, Sall4, WNT3a and β-catenin protein were significantly associated

with the TTP, PI, AUC and WOT. Collectively, our study supported that combination of Sall4/Wnt/β-catenin signaling with CEUS may become a useful supplementary method for HCC patients early diagnosis.

Sall4 is highly expressed in various cancers, including gastric cancer, breast cancer, lung cancer, colorectal cancer and HCC.[14–16] Sall4 silencing by RNA interference or Sall4 peptide inhibitor treatment led to impaired lung cancer cell growth through EGFR and IGF1R signaling pathways.[17] Sall4 exhibits its oncogenic roles in gastric cancer progression through directly activating CD44 expression.[18] High Sall4 expression levels were associated with low overall survival, event-free survival and the presence of metastasis in hepatoblastoma, suggesting Sall4 is an independent

Table 5 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	66.53	54.38	30.12
>50	64.74	55.47	31.34
Gender			
Male	65.50	52.32	31.53
Female	65.42	56.52	30.28
Tumor differentiation			
Low	53.24*	33.13*	10.42*
Moderate	65.56*	46.16*	21.16*
High	86.35	63.35	48.85
TNM stage			
I+II	88.63	80.28	41.24
III+IV	56.32*	31.06*	18.21*
Tumor size			
<3 cm	87.53	64.63	38.27
>3 cm	54.75*	37.64*	19.42*
Vascular invasion			
Yes	54.32	32.19	20.14
No	77.43*	65.21*	37.26*
SALL4 expression			
Positive	51.08	30.21	16.43
Negative	83.21*	68.74*	40.89*
WNT3a expression			
Positive	56.45	40.07	22.13
Negative	82.34*	63.52*	33.54*
β-catenin expression			
Positive	57.32	41.23	23.15
Negative	81.21*	58.94*	31.72*
Time to peak			
Short	54.22	41.36	21.17
Long	79.45*	60.29*	34.34*
Peak intensity			
Strong	54.22	41.36	21.17
Weak	79.45*	60.29*	34.34*
Area under the curve			
Small	79.45	60.29	34.34
Large	54.22*	41.36*	21.17*
Washout time			
Long	79.45	60.29	34.34
Short	54.22*	41.36*	21.17*

Note: *$P<0.05$ of comparison within group.
Abbreviations: DFS, disease-free survival; Sall4, Spalt-Like Transcription Factor 4; HCC, hepatocellular carcinoma.

Table 6 Multivariate survival analysis with Cox proportional hazards model of risk factors for the prognosis of HCC patients

Factor	Risk	95% CI	P-value
Tumor differentiation	0.118	0.123–0.225	<0.001
TNM stage	20.668	8.632–30.746	<0.001
Tumor size	6.821	3.546–13.126	<0.001
Vascular invasion	4.623	2.673–6.341	0.001
Sall4 expression	9.568	7.352–20.874	0.002
WNT3a expression	5.243	2.365–10.168	0.014
β-catenin expression	3.032	1.625–6.832	0.032
Time to peak	2.064	1.435–2.341	0.033
Peak intensity	1.658	1.372–3.212	0.042
Area under the curve	0.832	0.767–1.431	0.074
Washout time	0.878	0.885–1.352	0.083

Abbreviations: Sall4, Sall4, Spalt-Like Transcription Factor 4; HCC, hepatocellular carcinoma.

prognostic predictor for overall survival.[19] In line with our results in this study, Hao et al found that Sall4 expression positively correlated with lymph node metastasis and TNM stages in colorectal cancer; and β-catenin was expressed significantly higher in colorectal cancer than normal tissue. The patients with colorectal cancer of co-expression of these two molecules showed advanced lymph node metastasis and TNM stage.[20] Co-location of Sall4 and β-catenin was found in the nucleus and cytoplasm, which indicated that the function of Sall4 in promoting lymph node metastasis and advanced clinical stage might partly be due to the interaction with β-catenin.[20] Sall4 modulates the stemness of ESCC cells via Wnt/β-catenin signaling pathway and in EMT.[10] And Sall4 promotes intrahepatic cholangiocarcinoma cell proliferation by activating Wnt/β-catenin signaling.[11]

Interestingly, Sall4, WNT3a and β-catenin protein were significantly associated with the TTP, PI, AUC and WOT evaluated by CEUS. CEUS can be used to observe the neovessels in tumors. The parameters of CEUS reflect the proportions between the portal veins and hepatic arteries, and the vascular structures in tumors.[21] PI and AUC of CEUS can estimate enhancement intensity, and the TTP and WOT of CEUS are used to evaluate enhancement time.[22] PI and AUC of CEUS are applied to reveal the intratumoral vascularity of ovarian tumors as noninvasive parameters.[23] Tian and Wang showed that the PI and AUC of poor-differentiated HCC lesions were significantly lower than those of well-differentiated HCC lesions.[24] Our findings showed that decreased TTP and WOT and increased PI and AUC of CEUS in HCC lesions were correlated with TNM stage, tumor size and vascular

invasion. Besides, the preoperative TTP, PI, AUC and WOT were associated with the DFS rates, suggesting reduced survival rates in patients with short TTP, strong PI, large AUC or short WOT. Additionally, this study first time found that Sall4/Wnt/β-catenin expression was associated with TTP, PI, AUC and WOT in patients with HCC. Thus, combination CEUS and Sall4/Wnt/β-catenin signaling can more accurately evaluate the intratumoral neovascular generation and prognosis of HCC patients.

Conclusion

This study suggests that combination quantitative parameters of CEUS and Sall4/Wnt/β-catenin signaling may be a useful supplementary method for early screening and diagnosis in HCC patients. And a larger cohort is needed in the future to confirm our results study and provide strong evidence for predicting prognosis of patients with HCC.

Ethics approval and informed consent

This study was approved by Ethics Committee of General Hospital of Ningxia Medical University.

Author contributions

Jianjun Wang and Guanghui Liu contributed to study design, data analysis and wrote this paper. Jiaxin Huang, Qianfeng Ma performed clinical sample collection. Jiaxin Huang and Jianjun Wang performed qPCR. Qianfeng Ma performed immunohistochemical staining assay. All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Sia D, Villanueva A, Friedman SL, Llovet JM. Liver cancer cell of origin, molecular class, and effects on patient prognosis. *Gastroenterology*. 2017;152(4):745–761. doi:10.1053/j.gastro.2016. 11.048
2. Villanueva A, Llovet JM. Liver cancer in 2013: mutational landscape of HCC–the end of the beginning. *Nat Rev Clin Oncol*. 2014;11 (2):73–74. doi:10.1038/nrclinonc.2013.243
3. Zucman-Rossi J, Villanueva A, Nault JC, Llovet JM. Genetic landscape and biomarkers of hepatocellular carcinoma. *Gastroenterology*. 2015;149(5):1226–1239. doi:10.1053/j.gastro.2015.05.061
4. Jones B. Liver cancer: SALL4–a cancer marker and target. *Nat Rev Clin Oncol*. 2013;10(8):426. doi:10.1038/nrclinonc.2013.112

5. Yin F, Han X, Yao SK, Wang XL, Yang HC. Importance of SALL4 in the development and prognosis of hepatocellular carcinoma. *World J Gastroenterol*. 2016;22(9):2837–2843. doi:10.3748/wjg.v22.i9.2837
6. Fan H, Cui Z, Zhang H, et al. DNA demethylation induces SALL4 gene re-expression in subgroups of hepatocellular carcinoma associated with hepatitis B or C virus infection. *Oncogene*. 2016;36 (17):2435–2445. doi:10.1038/onc.2016.399
7. Han SX, Wang JL, Guo XJ, et al. Serum SALL4 is a novel prognosis biomarker with tumor recurrence and poor survival of patients in hepatocellular carcinoma. *J Immunol Res*. 2014;2014:262385. doi:10.1155/2014/ 394127
8. Shuai X, Zhou D, Shen T, et al. Overexpression of the novel oncogene SALL4 and activation of the Wnt/beta-catenin pathway in myelodysplastic syndromes. *Cancer Genet Cytogenet*. 2009;194(2):119–124. doi:10.1016/j.cancergencyto.2009.06.006
9. Ma Y, Cui W, Yang J, et al. SALL4, a novel oncogene, is constitutively expressed in human acute myeloid leukemia (AML) and induces AML in transgenic mice. *Blood*. 2006;108(8):2726–2735. doi:10.1182/blood-2006-02-001594
10. He J, Zhou M, Chen X, et al. Inhibition of SALL4 reduces tumorigenicity involving epithelial-mesenchymal transition via Wnt/beta-catenin pathway in esophageal squamous cell carcinoma. *J Exp Clin Cancer Res*. 2016;35(1):98. doi:10.1186/ s13046-016-0444-6
11. Zhu L, Huang F, Deng G, et al. Knockdown of Sall4 inhibits intrahepatic cholangiocarcinoma cell migration and invasion in ICC-9810 cells. *Onco Targets Ther*. 2016;9:5297–5305. doi:10.2147/OTT. S107214
12. Bo XW, Xu HX, Wang D, et al. Fusion imaging of contrast-enhanced ultrasound and contrast-enhanced CT or MRI before radiofrequency ablation for liver cancers. *Br J Radiol*. 2016;89(1067):20160379. doi:10.1259/bjr.20160379
13. Takada H, Tsuchiya K, Yasui Y, et al. Irregular vascular pattern by contrast-enhanced ultrasonography and high serum Lens culinaris agglutinin-reactive fraction of alpha-fetoprotein level predict poor outcome after successful radiofrequency ablation in patients with early-stage hepatocellular carcinoma. *Cancer Med*. 2016;5(11):3111–3120. doi:10.1002/cam4.932
14. Liu W, Xiao P, Wu H, et al. MicroRNA-98 plays a suppressive role in non-small cell lung cancer through inhibition of SALL4 protein expression. *Oncol Res*. 2016;25(6):975–988. doi:10.3727/ 096504016X14791726591124
15. Tian Q, Xiao Y, Wu Y, et al. MicroRNA-33b suppresses the proliferation and metastasis of hepatocellular carcinoma cells through the inhibition of Sal-like protein 4 expression. *Int J Mol Med*. 2016;38 (5):1587–1595. doi:10.3892/ijmm.2016.2754
16. Dirican E, Akkiprik M. Functional and clinical significance of SALL4 in breast cancer. *Tumour Biol*. 2016;37(9):11701–11709. doi:10.1007/s13277-016-5150-7
17. Yong KJ, Li A, Ou WB, et al. Targeting SALL4 by entinostat in lung cancer. *Oncotarget*. 2016;7(46):75425–75440. doi:10.18632/ oncotarget.12251
18. Yuan X, Zhang X, Zhang W, et al. SALL4 promotes gastric cancer progression through activating CD44 expression. *Oncogenesis*. 2016;5(11):e268. doi:10.1038/oncsis.2016.69
19. Zhou S, Venkatramani R, Gomulia E, Shillingford N, Wang L. The diagnostic and prognostic value of SALL4 in hepatoblastoma. *Histopathology*. 2016;69(5):822–830. doi:10.1111/his.13005
20. Hao L, Zhao Y, Wang Z, et al. Expression and clinical significance of SALL4 and beta-catenin in colorectal cancer. *J Mol Histol*. 2016;47 (2):117–128. doi:10.1007/s10735-016-9656-5
21. Liu Y, Xu Y, Cheng W, Liu X. Quantitative contrast-enhanced ultrasonography for the differential diagnosis of endometrial hyperplasia and endometrial neoplasms. *Oncol Lett*. 2016;12(5):3763–3770. doi:10.3892/ol.2016.5206

22. Zheng W, Xiong YH, Han J, et al. Contrast-enhanced ultrasonography of cervical carcinoma: perfusion pattern and relationship with tumour angiogenesis. *Br J Radiol*. 2016;89(1065):20150887. doi:10.1259/bjr.20150887

23. Wang J, Lv F, Fei X, et al. Study on the characteristics of contrast-enhanced ultrasound and its utility in assessing the microvessel density in ovarian tumors or tumor-like lesions. *Int J Biol Sci*. 2011;7 (5):600–606.

24. Tian H, Wang Q. Quantitative analysis of microcirculation blood perfusion in patients with hepatocellular carcinoma before and after transcatheter arterial chemoembolisation using contrast-enhanced ultrasound. *Eur J Cancer*. 2016;68:82–89. doi:10.1016/j.ejca.2016.08.016