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Hsa_circ_0001649: A circular RNA and potential novel biomarker for hepatocellular carcinoma

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Abstract.

BACKGROUND: It has been shown that circular RNA (circRNA) is associated with human cancers, however, few studies have been reported in hepatocellular carcinoma (HCC).

OBJECTIVE: To estimate clinical values of a circular RNA, Hsa_circ_0001649, in HCC.

METHODS: Expression level of hsa_circ_0001649 was detected in HCC and paired adjacent liver tissues by real-time quantitative reverse transcription-polymerase chain reactions (qRT-PCRs). Differences in expression level of hsa_circ_0001649 were analyzed using the paired t-test. Tests were performed between clinical information and hsa_circ_0001649 expression level by analysis of variance (ANOVA) or welch t-test and a receiver operating characteristics (ROC) curve was established to estimate the value of hsa_circ_0001649 expression as a biomarker in HCC.

RESULTS: hsa_circ_0001649 expression was significantly downregulated in HCC tissues ($p = 0.0014$) based on an analysis of 89 paired samples of HCC and adjacent liver tissues and the area under the ROC curve (AUC) was 0.63. Furthermore, hsa_circ_0001649 expression was correlated with tumor size ($p = 0.045$) and the occurrence of tumor embolus ($p = 0.017$) in HCC.

CONCLUSIONS: We first found hsa_circ_0001649 was significantly downregulated in HCC. Our findings indicate hsa_circ_0001649 might serve as a novel potential biomarker for HCC and may function in tumorigenesis and metastasis of HCC.

Keywords: Circular RNA, hsa_circ_0001649, hsa_circ_001599, hepatocellular carcinoma, biomarker

Abbreviations

CircRNA,	Circular RNA;
HCC,	Hepatocellular carcinoma;
ROC,	Receiver operating characteristics;
ANOVA,	Analysis of variance;
AUC,	Area under the ROC curve;
HDV,	Hepatitis delta virus;

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SHPRH, SNF2 histone linker PHD RING helicase;
MMP, Matrix metalloproteinase;
EMT, Epithelial mesenchymal transition;
RBP, RNA-binding protein;
U2AF65, U2 auxiliary factor 65 kDa subunit;
EIF4A3, Eukaryotic initiation factor 4A-III;
UPF1, Regulator of nonsense transcripts 1.

1. Introduction

Circular RNAs (CircRNAs) are class of RNAs formed by back-splicing events as loops, and are found in all types of organisms. They have been identified in viroidal plant pathogens and the hepatitis delta virus (HDV) [1,2], and described as spliced tRNAs, rRNA introns, and as rRNA processing intermediates in archaea [3,4]. CircRNAs in eukaryotes were initially detected in 1979, and then in mouse in 1993 [5,6].

In the early decades, circRNAs were thought to be expressed at so low levels that they have been generally regarded as byproducts of splicing errors [7], and have not been regarded as biologically active regulators. However, the advent of next generation sequencing has illuminated circRNAs as an entire class of abundant, non-coding RNAs ubiquitous among eukaryotes [8,9] and several circRNAs has been identified and reported to have physiological functions recently. CircRNAs can act as miRNA sponges [8,10] and can function in gene regulation as transcription regulators interacting with the Pol II transcription complex [11] or by competing with linear splicing [12]. Other investigators have reported that circRNAs are involved in the growth of colorectal and ovarian cancers [13], and be associated with DNA damage in breast cancer cells [14].

CircRNAs have recently been described as biomarkers of aging in Drosophila [15] and disease biomarkers in human saliva [16]. Additionally, circRNA has been suggested as a potential novel biomarker for use in diagnosing cancers [17,18]. Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related death world-wide [19], and the majority of HCC patients have a poor prognosis due to the high frequency of disease metastasis and recurrence [20,21]. Therefore, it is critical to find novel biomarkers for improving the prognosis and therapy of HCC. In this study, we focused on a circRNA, hsa_circ_0001649 (ID: hsa_circ_0001649), from database CircBase, because hsa_circ_0001649 has been predicted to be associated with HCC in circ2Traits (ID:hsa_circ_001599 in database circ2Traits) [22]. We first verified hsa_circ_0001649 expression was significantly downregulated in HCC tissues. Our results indicate that hsa_circ_0001649 may serve as a novel potential biomarker for HCC and may play roles in tumorigenesis and metastasis of HCC.

2. Materials and methods

2.1. Samples

The 89 samples of HCC and paired adjacent liver tissues were obtained from Zhongshan Hospital (Shanghai, China). All tissue specimens were stored at −80°C until use. Clinical information was obtained under an Institutional Review Board approved study protocol and written informed consent was obtained from each subject.

2.2. Cell culture

Immortalized hepatoma cell lines, HCC-LM3 and MHCC-97L, were from Zhongshan Hospital of Shanghai Medical College, Fudan University [23,24]. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS) and a humidified atmosphere consisting of 5% CO_2 and 95% air at 37°C.

2.3. Total RNA extraction

Total RNA was extracted from each sample of hepatocellular carcinoma and paired paracancerous liver tissue using TRIzol Reagent (Invitrogen; Carlsbad, CA, USA) following the manufacturer's instructions.

2.4. Reverse transcription

The cDNA was synthesized by reverse transcription (RT) using a Primescipt RT reagent kit with gRNA Eraser according to manufacture-provided protocols (TaKaRa) (with random primers).

2.5. Real-time polymerase chain reaction assay

Real-time quantitative reverse transcription-polymerase chain reactions (qRT-PCR) were performed us-

Table 1
qRT-PCR primer sequences

Primer name	Primer FW (5' – 3')	Primer RV (5' – 3')
Hsa_circ_0001649	AATGCTGAAAACTGCTGAGAGAA	TTGAGAAAACGAGTGCTTTGG
β-actin	AGTTGCGTTACACCCTTTCTTG	GCTGTCACCTTCACCGTTCC
SHPRH	ATGAGCAGCCGACGGAAACG	ATCTGAACCTGGGCAGGGCT
MMP9	GCCTTCGCACTGTGGAGC	GGATACCCGTCTCCGTGCTC
MMP10	CACTGGAACCCTGAACCTGAAT	AATAAAAACGGTGTCCCTGCTG
MMP2	ATGCCGTCGTGGACCTGC	TGCTTCCAAACTTCACGCTCTT
MMP13	TCTTTCTTCGGCTTAGAGGTGACT	AAACATTGTATTCACCCACATCAGG
MMP7	CGATGAGGATGAACGCTGGA	AGGAATGTCCCATACCCAAAGAA

ing SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa), following manufacturer's instructions. Divergent primers for hsa_circ_0001649 and primers for β-actin [25], SHPRH, MMP9, MMP10, MMP2, MMP13, as well as MMP7 were synthesized by Sangon Biotech (Shanghai, China). All qRT-PCR primer sequences see Table 1.

2.6. Sanger sequencing

The target fragment was inserted into a T vector for Sanger sequencing to determine its full-length. The following divergent primers were designed for use in experiments to confirm the back-splice junction of hsa_circ_0001649: 5'-CAATGCTGAAAACTGCTG AGAGAAG-3′ (sense) and 5′-CCTGCATTCTTTCTT CTATTGTTGCTTTAA-3′ (antisense). The primers were synthesized by Sangon Biotech (Shanghai, China), and Sanger sequencing was performed by Biosune (Shanghai, China).

2.7. siRNA interference

siRNA of hsa_circ_0001649 was designed and synthesized by GenePharma (Shanghai, China), targeting to the junction region of hsa_circ_0001649 sequence. The siRNA sequences were as follows: 5'-UGGCUGCCCUUCUCUCAGCTT-3′ (sense) and 5′-GCUGAGAGAAGGGCAGCCATT-3' (antisense).

2.8. Statistical analysis

All data analyses were performed with R (v 2.15.0, http://www.r-project.org/). Differences in values for hsa_circ_0001649 were based on comparisons of its expression in 89 HCC tissues and their paired paracancerous liver tissues as determined using the paired t-test. Analysis of variance (ANOVA) was used to determine correlations between expression levels of hsa_circ_0001649 and clinical indexes. A receiver operating characteristics (ROC) curve for hsa_circ_0001649 in detecting HCC was drawn using "ROCR" (http://cran.r-project.org/web/packages/ROCR/). P-values < 0.05 were considered statistically significant.

3. Results

3.1. Detection of naturally existing hsa_circ_0001649 in HCC tissues

Hsa_circ_0001649 is produced at the *SHPRH* gene locus containing exon 26–29 (Fig. 1A). Results of RT-PCR performed with divergent primers indicated that hsa_circ_0001649 was expressed in samples of HCC tissues (Fig. 1B), and sequencing of the RT-PCR product of hsa_circ_0001649 showed a full-length sequence of hsa_circ_0001649 that was completely consistent with that in CircBase. Results of the Sanger sequencing experiment confirmed the back-splice junction of hsa_circ_0001649 (Fig. 1C), proving that hsa_circ_0001649 naturally existed as a loop in HCC tissues.

3.2. Downregulation of hsa_circ_0001649 in HCC tissues

We detected the expression levels of hsa_circ_0001649 in 89 samples of HCC tissues and their paired adjacent liver tissues by qRT-PCR, when using β-actin as the internal standard. Our results showed hsa_circ_0001649 expression in HCC tissues was significantly lower compared to its expression in the paired adjacent liver tissues ($n = 89$; $p = 0.0014$) (Fig. 2A). The relative expression level of hsa_circ_0001649 in each sample of HCC and paired adjacent liver tissue is shown in Fig. 2B. The specificity of the qRT-PCR product of hsa_circ_0001649 was analyzed by electrophoresis, and the amplified product yielded a single peak in a melting curve analysis (Supplementary Fig. 1).

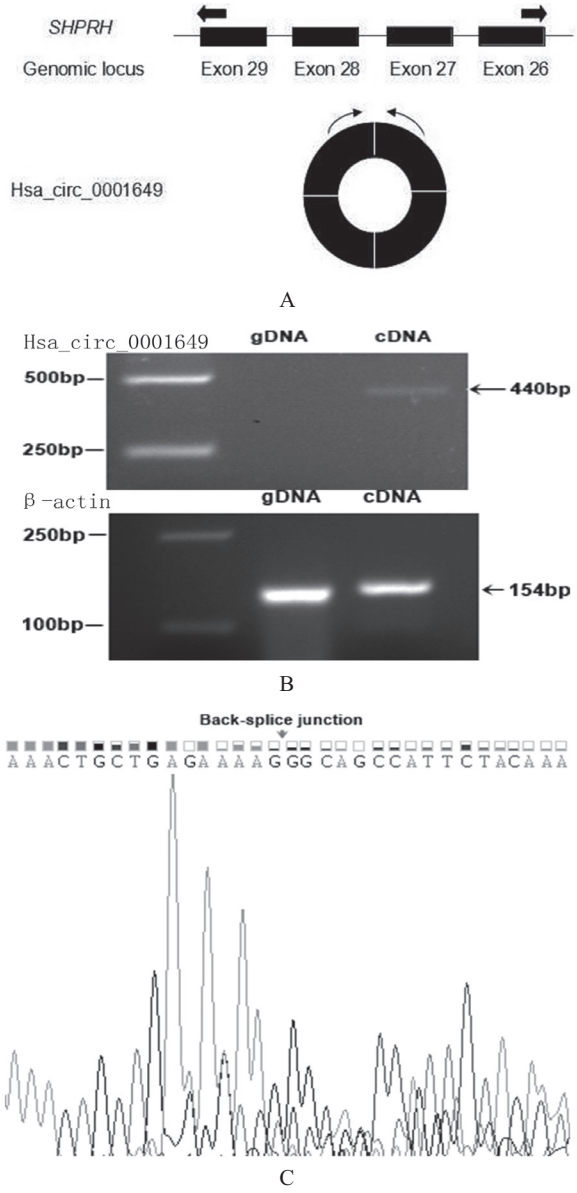

Fig. 1. Identification of hsa_circ_0001649 in samples of HCC tissues. (A) Hsa_circ_0001649 is produced at the *SHPRH* gene locus containing exon 26–29. (B) RT-PCR products with divergent primers of hsa_circ_0001649 in HCC-LM3 cells verified that hsa_circ_0001649 naturally existed in HCC tissues. (C) Sanger sequencing of hsa_circ_0001649 showed the back-splice junction.

3.3. *Potential values of hsa_circ_0001649 as a biomarker for HCC*

Our results showed that hsa_circ_0001649 expression was significantly downregulated in samples of HCC tissue, and we therefore examined our data for potential correlations between hsa_circ_0001649 expression and clinical parameters. We found that tumor size was related to the level of hsa_circ_0001649 expression ($p = 0.045$) (Table 2). Additionally, tumor embolus was significantly related to the level of hsa_circ_0001649 expression by welch t-test ($p = 0.017$) (Fig. 3). The occurrence of tumor embolus reflects invasion status of cancer cells and indicates the metastatic possibility, so we made further research to investigate potential correlations between hsa_circ_0001649 expression and metastasis. And after knockdown of hsa_circ_0001649 with siRNA in

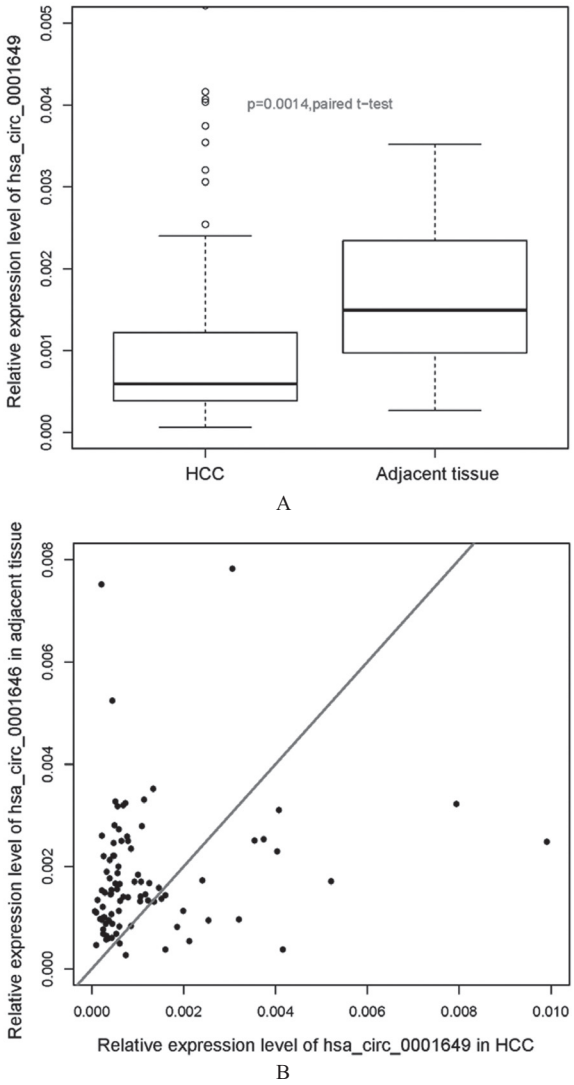

Fig. 2. Downregulation of hsa_circ_0001649 in HCC tissues. (A) Expression differences of hsa_circ_0001649 between HCC and matched adjacent liver tissues ($n = 89$, $p = 0.0014$). (B) The relative expression level of hsa_circ_0001649 in each HCC and paired adjacent liver tissue. Dots above the line are samples with downregulated expression of hsa_circ_0001649.

Table 2
Correlation between hsa_circ_0001649 expression and clinical parameters in HCC

Characteristics	Patient number	Mean ± SD	P value
Gender			0.88202
Female	15	0.00113 ± 0.00149	
Male	74	0.00120 ± 0.00162	
Age			0.96582
⩽ 60	58	0.00118 ± 0.00178	
> 60	31	0.00119 ± 0.00119	
Cirhosis_history			0.78937
Negative	15	0.00109 ± 0.0014	
Positive	75	0.00121 ± 0.00164	
HCC_history			0.51943
Negative	80	0.00122 ± 0.00167	
Positive	9	0.00086 ± 0.00045	
HBsAg			0.81243
Negative	23	0.00112 ± 0.00108	
Positive	66	0.00121 ± 0.00174	
HBcAb			0.4407
Negative	11	0.00153 ± 0.00138	
Positive	78	0.00114 ± 0.00162	
Cirhosis_nodes			0.98257
’1	21	0.00118 ± 0.00208	
> 1	68	0.00119 ± 0.00143	
Membrane			0.38813
Negative	41	0.00103 ± 0.00123	
Positive	48	0.00132 ± 0.00185	
Events			0.1144
Negative	40	0.00148 ± 0.00208	
Positive	49	0.00094 ± 0.00099	
Recurrence			0.62813
Negative	44	0.00127 ± 0.00175	
Positive	45	0.0011 ± 0.00143	
Embolus			0.05239
Negative	58	0.00142 ± 0.00185	
Positive	31	0.00074 ± 8e-04	
Tumor size			**0.04512***
⩽ 5 cm	50	0.00148 ± 0.00198	
> 5 cm	39	8e-04 ± 0.00074	
Tumor number			0.45038
1	73	0.00112 ± 0.00135	
> 1	16	0.00146 ± 0.00244	
AFP1			0.55783
Negative	32	0.00132 ± 0.00164	
Positive	57	0.00111 ± 0.00158	
AFP2			0.29426
Negative	54	0.00133 ± 0.00184	
Positive	35	0.00096 ± 0.0011	
GGT			0.27857
Negative	35	0.00141 ± 0.00218	
Positive	54	0.00104 ± 0.00105	
ALT			0.80302
⩽ 54	76	0.00117 ± 0.00167	
> 54	13	0.00129 ± 0.0011	
Differentiation			0.30002
I + II	71	0.00127 ± 0.00172	
III + IV	19	0.00083 ± 0.00095	
TNM			0.57101
Early	51	0.00127 ± 0.00149	
Late	38	0.00107 ± 0.00173	
BCLC			0.13539
0 + A	19	0.00167 ± 0.00208	
B + C + D	70	0.00105 ± 0.00142	

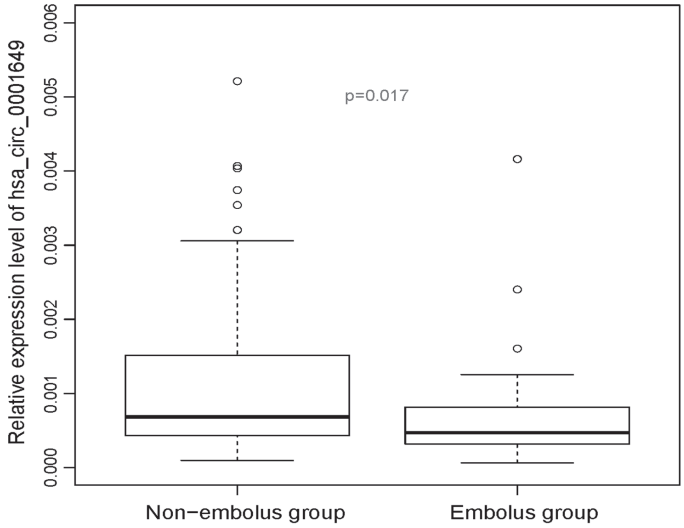

Fig. 3. Hsa_circ_0001649 expression was correlated with tumor embolus in HCC ($p = 0.017$). The expression level of has_circ0001649 is significantly lower in the samples with embolus ($n = 31$) than those without embolus ($n = 58$) according to welch t-test. The occurrence of tumor embolus reflects invasion status of cancer cells.

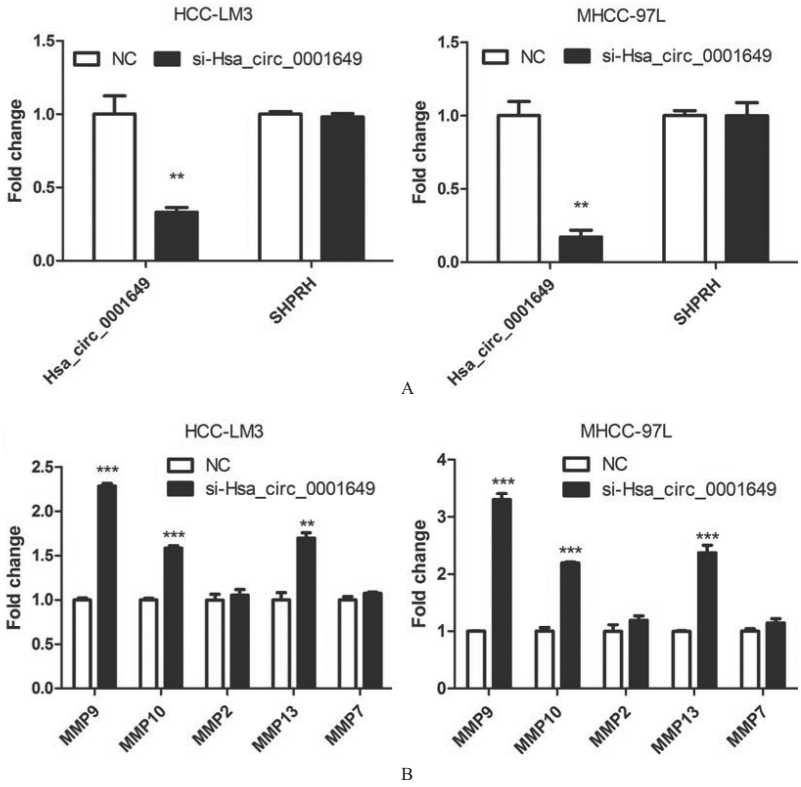

Fig. 4. Hsa_circ_0001649 expression is associated with metastasis in HCC. (A) Effective knockdown of hsa_circ_0001649 with siRNA. It avoided interfering with the expression of linear mRNA SHPRH, both in HCC-LM3 cells and MHCC-97L cells. The siRNA targeted the junction sequences of hsa_circ_0001649. (B) Increase in mRNA levels of MMP9, MMP10, and MMP13 after knockdown of hsa_circ_0001649 with siRNA in HCC-LM3 cells and MHCC-97L cells (P-values < 0.01).

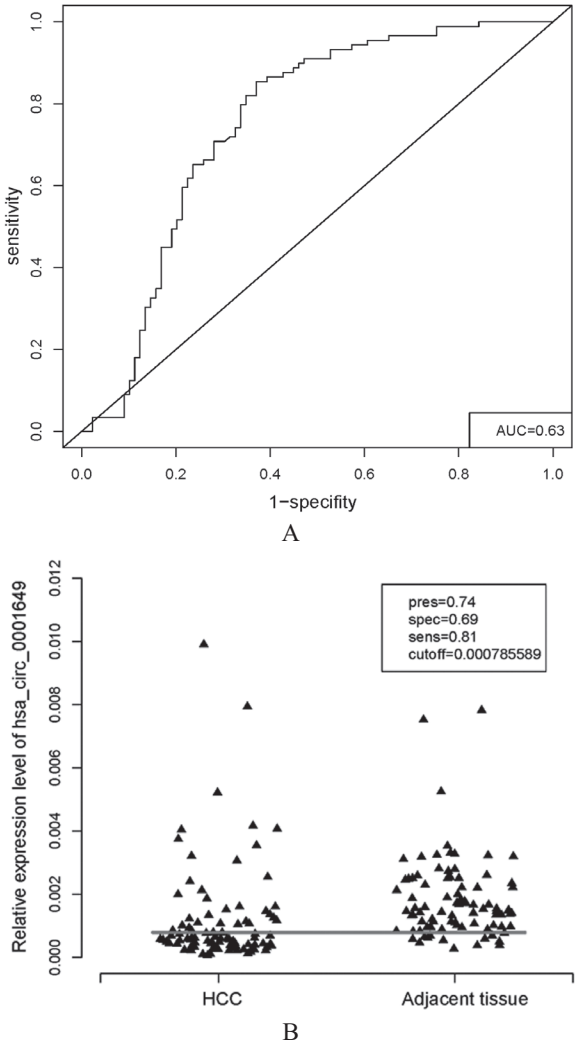

Fig. 5. Hsa_circ_0001649 might serve as a biomarker for HCC. (A) A larger AUC by ROC analysis for hsa_circ_0001649 in HCC indicates greater potential as a biomarker (AUC = 0.63). (B) The cutoff value, sensitivity, and specificity were 0.0007855, 0.81, and 0.69, respectively. Dots above the line predict areas of adjacent non-cancerous tissue and dots below the line predict cancerous tissue.

immortalized hepatoma cell lines, HCC-LM3 and MHCC-97L (Fig. 4A), we tested the expression levels of several matrix metallopeptidases (MMPs) 9, 10, 2, 13, and 7, which had been reported to have key roles in promoting the metastasis of HCC [26–29]. We found significant increase in mRNA levels of MMP9, MMP10, and MMP13 (Fig. 4B) which indicated that low level of hsa_circ_0001649 expression was positively correlated with the metastasis of HCC.

We next investigated whether hsa_circ_0001649 expression could serve as a biomarker for distinguishing cancerous tissue from adjacent non-cancerous liver tissue. When expression levels of hsa_circ_0001649 were examined for this purpose, the area under the ROC curve (AUC) was 0.63. (Fig. 5A), and the cutoff value, sensitivity, and specificity were 0.0007855, 0.81, and 0.69, respectively (Fig. 5B).

4. Discussion

We have shown in this study that the expression level of hsa_circ_0001649 is downregulated in HCC tissues when compared to the matched adjacent liver tissues. The hsa_circ_0001649 expression is related to tumor size ($p = 0.045$). Interestingly, HCC tumors with larger size show lower level of hsa_circ_0001649 expression, suggesting a role for hsa_circ_0001649 in tumor growth. Furthermore, we found expression levels of hsa_circ_0001649 were related to tumor embolus. Moreover, mRNA levels of MMP 9, 10, and 13 were significant increased after knockdown of hsa_circ_0001649 with siRNA in HCC cells. The occurrence of tumor embolus reflects invasion status of cancer cells and MMPs have key roles in promoting the metastasis of HCC. These results indicated that low level of hsa_circ_0001649 expression was positively correlated with the metastasis of HCC and it may function in metastasis of HCC, however, detailed molecular mechanisms of which hsa_circ_0001649 involved in HCC invasion and metastasis need to be revealed. Our results further suggest downregulation of hsa_circ_0001649 is associated with poor prognosis of HCC and ROC analyses show that hsa_circ_0001649 may be used as a novel biomarker for HCC with high degrees of accuracy, specificity, and sensitivity.

It has been proposed that circRNAs may act as competing endogenous RNAs to bind proteins (protein sponges) [30,31]. Then, we focused on the sequence of hsa_circ_0001649 to find its potential RNA-binding proteins (RBPs) binding sites (starBase v2.0) [32], and we found that hsa_circ_0001649 had 6 potential protein binding sites for U2 auxiliary factor 65 kDa subunit (U2AF65), 5 potential protein binding sites for Eukaryotic initiation factor 4A-III (EIF4A3), and 1 potential protein binding site for Regulator of nonsense transcripts 1 (UPF1). Those analysis results imply that hsa_circ_0001649 may function as a protein sponge or a transcription regulator to participate in development and progression of HCC.

Two circRNAs have been verified as being actual miRNA sponges in mammals so far [10]. One specific circRNA, (ciRS-7, also known as CDR1as), has

$>$ 70 binding sites for miR-7, and it has been shown that circRNA impairs the regulatory effect of miR-7 in vivo. The second circRNA is the testis-specific transcript of the male sex-determining gene *Sry*, which contains 16 binding sites for miR-138. Many of the predicted miRNA binding sites in circRNAs are functional [33]. An additional proposed function of circRNAs is the transport of miRNAs [34]. We found hsa_circ_0001649 had potential for harboring miRNA binding sites (http://www.mirdb.org/miRDB/custom.html) and It possessed potential binding sites for hsa-miR-1283, hsa-miR-4310, hsa-miR-182-3p, hsa-miR-888-3p, hsa-miR-4502, hsa-miR-6811-5p, hsa-miR-6511b-5p, and hsa-miR-1972. Those analysis results suggest that hsa_circ_0001649 may play roles in HCC by interacting with miRNAs.

In summary, we first identified hsa_circ_0001649 expression was significantly downregulated in HCC tissues and it might serve as a novel potential biomarker for HCC. Furthermore, our results indicated that hsa_circ_0001649 might be involved in tumorigenesis and metastasis of HCC.

Acknowledgments

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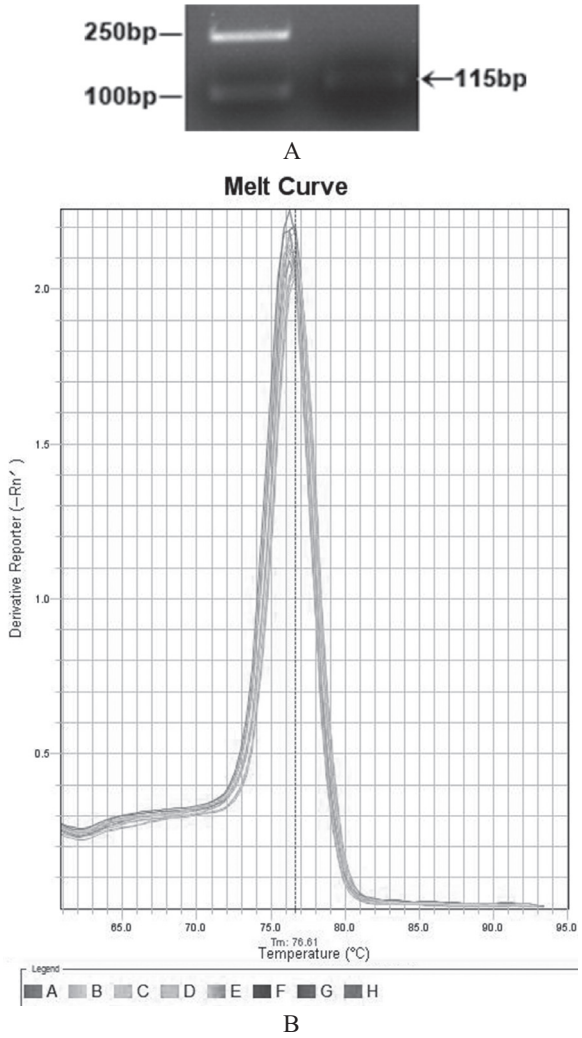
Supplementary material



Supplementary Fig. 1. The specificity of qRT-PCR product of hsa_circ_0001649 with divergent primers. (A) Electrophoresis of qRT-PCR product of hsa_circ_0001649. (B) Melting curve analysis of qRT-PCR product of hsa_circ_0001649.