Cancer Management and Research

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ORIGINAL RESEARCH

Screening differential circular RNA expression profiles reveal that hsa_circ_0128298 is a biomarker in the diagnosis and prognosis of hepatocellular carcinoma

This article was published in the following Dove Press journal:
Cancer Management and Research

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Aim: The aim of this study was to analyze the diagnostic and prognostic values of the circular RNA (circRNA) hsa_circ_0128298 in hepatocellular carcinoma (HCC).

Patients and methods: The global circRNA expression was measured using circRNA microarray using three pairs of cancer and noncancerous tissues from HCC patients. The microarray analysis revealed that two circRNAs were differentially expressed in the three pairs of cancerous and noncancerous tissues. The higher levels of two representative circRNAs, such as hsa_circ_0128298 and hsa_circ_0091582, were further confirmed by real-time polymerase chain reaction. In addition, the association between the expression level of hsa_circ_0128298 and the clinicopathological features of patients with HCC was further analyzed. The clinical diagnosis value was confirmed by receiver operating characteristic (ROC) curve analysis. Independent prognostic factors of patient outcome were identified using the Cox regression model. The survival data were analyzed by the Kaplan–Meier method, and the differences were evaluated using log-rank tests. Two-sided *P*-values <0.05 were considered statistically significant.

Results: The expression levels of hsa_circ_0128298 in HCC were significantly higher than those of paratumorous tissues ($P<0.001$). Additionally, hsa_circ_0128298 was a diagnostic factor, with the area under the ROC curve of 0.668 (95% CI =0.503–0.794, $P<0.001$). The sensitivity and specificity values were 0.716 and 0.815, respectively. The AFP and hsa_circ_0128298 expression levels were independent prognostic factors. The overall survival of patients with low hsa_circ_0128298 expression was significantly higher than that of patients with high hsa_circ_0128298 expression.

Conclusion: hsa_circ_0128298 may promote proliferation and metastasis and potentially represents a novel diagnostic and prognostic biomarker for HCC patients. However, studies with larger sample size are needed to confirm our conclusion.

Keywords: hepatocellular carcinoma, circular RNA, hsa_circ_0128298, biomarker, diagnosis, prognosis

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world and ranks third in the number of cancer-related deaths.[1,2] Currently, surgical resection remains the primary option for patients with resectable HCC, but the rate of tumor recurrence remains high even after operation due to the refractory property of tumors.[3] Therefore, treatments to combat HCC recurrence and metastasis are prioritized. Some

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http://dx.doi.org/10.2147/CMAR.S166740

strategies have been adopted to suppress HCC metastasis, but they did not show obvious positive effects.[4] Therefore, exploring molecular mechanisms and new therapeutic targets is crucial for treating HCC. Unfortunately, there is no clinical tumor marker that provides both satisfactory sensitivity and specificity in the diagnosis and prognosis of HCC. Therefore, it is necessary to study the molecular mechanisms underlying the occurrence and development of HCC and to explore the tumor markers that can ensure an early diagnosis of liver cancer.

Circular RNA (circRNA) is a type of noncoding RNA that exists widely in mammals and is mainly involved in gene regulation in vivo.[5–7] Most circRNAs are derived from the exon regions of the genes, and a small percentage is spliced by introns.[8,9] They differ from long noncoding RNA (lncRNA) and microRNAs (miRNAs) in that they do not have the 5′ and 3′ end structures but represent covalently closed cyclic structures.[10] CircRNA is widely involved in the regulation of human physiology and pathology by the following three main mechanisms: 1) as an miRNA "sponge" (miRNA sponge), 2) as a protein-binding molecule, and 3) as a template for translation into polypeptides. The mechanisms of miRNA sponge regulation of downstream target genes have been widely reported.[11–13]

Since the global circRNA expression profile in HCC has not been widely studied, in our study, we tested the circRNA expression profile in a cohort of HCC patients. We identified differentially expressed circRNAs in HCC tissues compared with those in paratumorous tissues. hsa_circ_0128298, which is transcribed from ENST00000296695 on chromosome 5, has never been studied before (fold change =127.67, *P*-value =0.006). Therefore, we focused on its association with both the clinicopathological features and prognosis of HCC.

Patients and methods

Patients and samples

HCC and paratumor tissues were collected from patients with HCC who underwent surgery at Fudan University Shanghai Cancer Center between January 1, 2011, and December 31, 2015. Informed consent was obtained from the patients before sample collection, in line with the institutional guidelines. The paratumor samples were collected from tissues 3 cm from the edge of the HCC to guarantee the absence of tumor cells. These tissue specimens were immediately placed in RNA fixer reagent and kept at −80°C. Histology was independently assessed by two experienced pathologists who were blinded to the clinical data.

Ethical approval

The study was approved by the Ethics Committee of Shandong Cancer Hospital affiliated with Shandong University. All procedures performed in the studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Written informed consent was obtained from all patients before their participation in this clinical research.

RNA isolation, reverse transcription (RT), and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from all tissues was extracted by using the TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA). All complementary DNAs (cDNAs) were obtained with the GoScriptÔ Reverse Transcription System (Promega Corporation, Fitchburg, WI, USA) following the manufacturer's instructions. The sequences of hsa_circ_0128298 divergent primers were as follows: forward, 5′-TAGACTCAACAGGGCCAAGG-3′; reverse, 5′-TCCATCTTTCTTGAGCAGCA-3′. The primer sequences of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as a control, were 5′-TCGACAGTCAGCCGCATCTTCTTT-3′ and 5′-ACCAAATCCGTTGACTCCGACCTT-3′. These primers were synthesized by the PrimerBank. To determine the reproducibility of the qRT-PCR method for detecting the hsa_circ_0000520 levels in HCC tissue, we performed qRT-PCR three times to measure the differences between experimental batches. The data from qRT-PCR were analyzed by the ΔCt method. All results were expressed as the mean ± SD. All assays were performed in a blinded fashion.

Microarray data analysis

The microarray detection was performed by Boao Bio-Tech (Beijing, China) under the guidance of the experiment work flow. The human 8×15K CIRCRNA Array was manufactured by Arraystar Technologies (Rockville, MD, USA). Overall, 65521 circRNAs were detected. Each circRNA was accurately identified using a specific probe targeting the circRNA-specific junction. Sample labeling, array hybridization, and raw data extraction were done with a high-quality control. Quantile normalization of raw data and subsequent data processing was performed using the R software package (R Version 3.1.2).

Statistical analyses

All statistical analyses were performed by the Statistical Product and Service Solutions (SPSS) 22.0 software package (IBM Corporation, Armonk, NY, USA) and GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). The differences in the levels of hsa_circ_0128298 between HCC and paired adjacent nontumorous tissues were assessed using the Student's *t*-test for paired data. Spearman's rank correlation coefficient was introduced to further calculate bivariate correlations. The receiver operating characteristic (ROC) curve was established to evaluate its diagnostic value. Independent prognostic factors for patient outcome were identified using the Cox regression model. The overall survival (OS) data were analyzed using the Kaplan–Meier method, and the differences were evaluated using log-rank tests. Two-sided *P*-values <0.05 were considered statistically significant.

Results

Overview of circRNA profiles in HCC tissues

Our microarray analysis revealed the circRNA expression profiles, as tested from three paired human HCC and paratumorous tissues. The detailed experimental process is shown in Figure 1, and clinicopathological characteristics are shown in Table 1. The median follow-up time was 37 months. The heat-map is depicted as a direct approach to visualize the distributions of the dataset for the circRNAs profiles (Figure 2A and B). The circRNA expression patterns between HCC and paratumorous tissues were found to be significantly different (Figure 2C). Differentially expressed circRNAs with statistical significance (fold changes ≥2.0 and $P<0.05$) between groups were identified by volcano plot filtering (Figure 2D). After screening the differential circRNA expression profiles, we selected hsa_circ_0128298 and hsa_circ_0091582, the two most-changed circRNAs, for further study. We chose these two circRNAs for the following two reasons: 1) these two circRNAs had not been studied previously and 2) both circRNAs were in the top 10 highly expressed circRNAs.

hsa_circ_0128298 was upregulated in HCC tissues

qRT-PCR was used to detect the hsa_circ_0128298 and hsa_circ_0091582 expression levels in liver tissues from HCC patients. In the initial verification process with 30 pairs of HCC and paratumor tissues, hsa_circ_0091582 and hsa_circ_0128298 were upregulated in HCC tissues (P=0.023 and 0.024, respectively) (Figure 3). In the extended verification

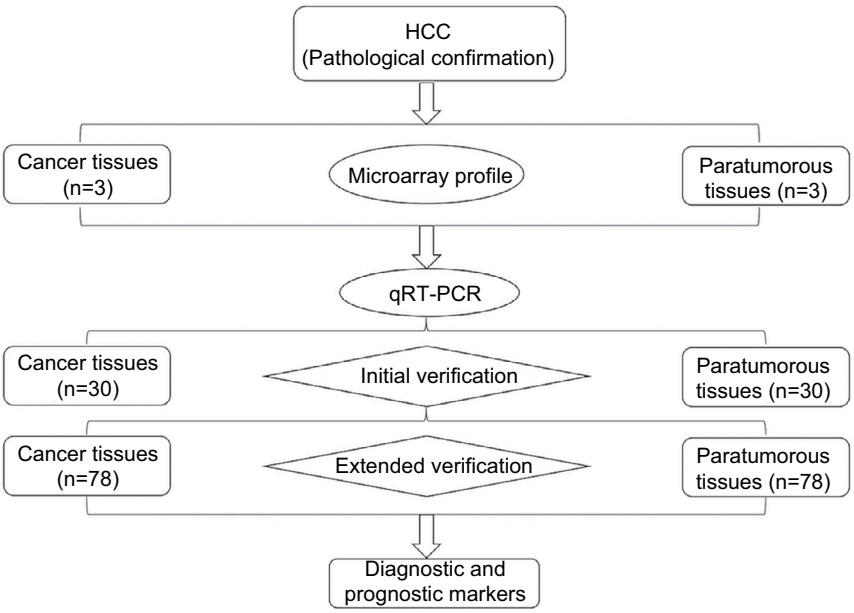

Figure 1 The detailed experimental process.
Notes: circRNA expression profiles were screened in three paired human HCC and paratumorous tissues. Targeted circRNAs were verified by qRT-PCR via initial verification in 30 pairs of samples and extended verification in 78 pairs of samples.
Abbreviations: circRNAs, circular RNAs; HCC, hepatocellular carcinoma; qRT-PCR, quantitative real-time polymerase chain reaction.

Table 1 Clinicopathological characteristics of patient samples and expression of hsa_circ_0128298 in hepatocellular carcinoma

Variable	n
Gender	
M/F	70/8
Age (years)	37 (41)
≥55/<55	
Vascular cancer embolus	
Yes/no	48/29
HBSAG	
Positive/negative	65/13
HBEAB	
Positive/negative	65/13
Cirrhosis	
Yes/no	36/42
AFP	
≥20/<20	52/26
Intrahepatic metastasis	
Yes/no	23/55
LN metastasis	
Yes/no	19/59
Organ metastasis	
Yes/no	7/71
Expression	
Low/high	39/39

Abbreviations: F, female; LN, lymph node; M, male; HBSAG, hepatitis Bs antigen; HBEAG, hepatitis Be antigen.

process with 78 pairs of samples, only hsa_circ_0128298 was upregulated (P=0.006) (Figures 4 and S1).

Upregulation of hsa_circ_0128298 is associated with clinicopathological factors in HCC patients

The data presented earlier demonstrated that the hsa_circ_0128298 expression was significantly upregulated in HCC tissues; hence, we analyzed the association between its expression and clinicopathological factors of patients with HCC.

As shown in Table 2, in HCC tissues, a significant association was observed between the hsa_circ_0128298 expression and vascular cancer embolus (P=0.012), lymphatic metastasis (P=0.018), and organ metastasis (P=0.048). However, no association was found between the hsa_circ_0128298 expression and other clinicopathological factors, including gender (P=0.455), age (P=0.112), hepatitis Bs antigen (P=0.615), hepatitis Be antigen (P=0.761), cirrhosis (P=0.65), AFP (P=0.226), and intrahepatic metastasis (P=0.11). The Spearman analysis in Table 3 indicated that the expression of

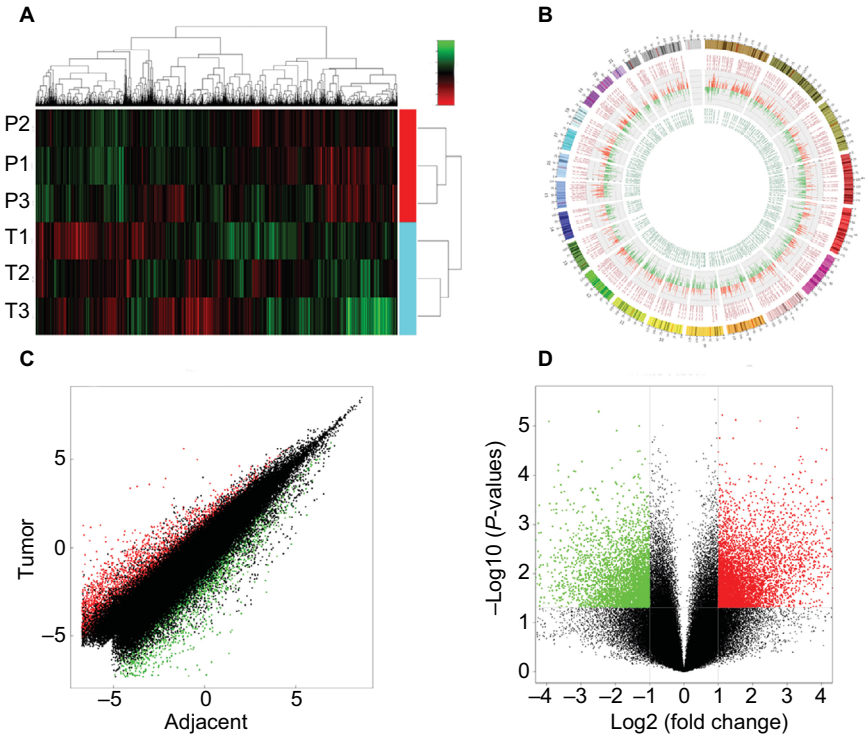

Figure 2 Overview of the microarray signatures.
Notes: (**A**) Unsupervised clustering analysis clearly distinguished the tumors and their corresponding nontumorous samples. (**B**) Illustration of the human genome showing the overall expression profile of the samples. (**C**) The whole transcriptome profiles of the tumor and their adjacent paratumor tissues were highly correlated. (**D**) Volcano plot showing the significantly deregulated genes in tumor samples.

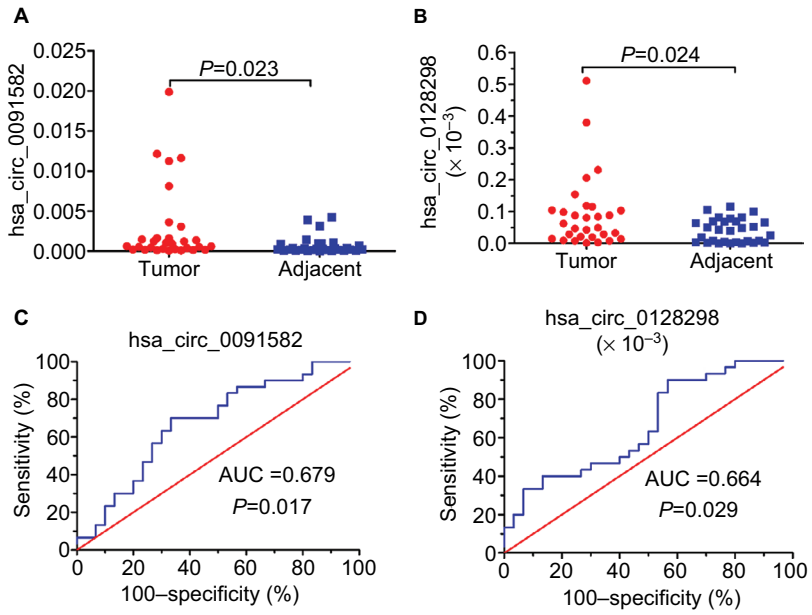

Figure 3 Target circRNAs from the initial verification from 30 pairs of samples.
Notes: (**A** and **B**) Increased expression of hsa_circ_0091582 and hsa_circ_0128298 in HCC tissues. RT-PCR was used to determine the expression levels. The ΔCt values were determined by subtracting the Ct value for GAPDH from the Ct values for circRNAs. Larger ΔCt values indicate higher expression (n=30, *P*=0.023 and n=30, *P*=0.024, respectively). (**C** and **D**) The ROC curve for using hsa_circ_0091582 and hsa_circ_0128298 as biomarkers (*P*=0.017 and 0.029, respectively).
Abbreviations: circRNAs, circular RNAs; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; ROC, receiver operating characteristics; RT-PCR, real-time polymerase chain reaction; AUC, area under the curve.

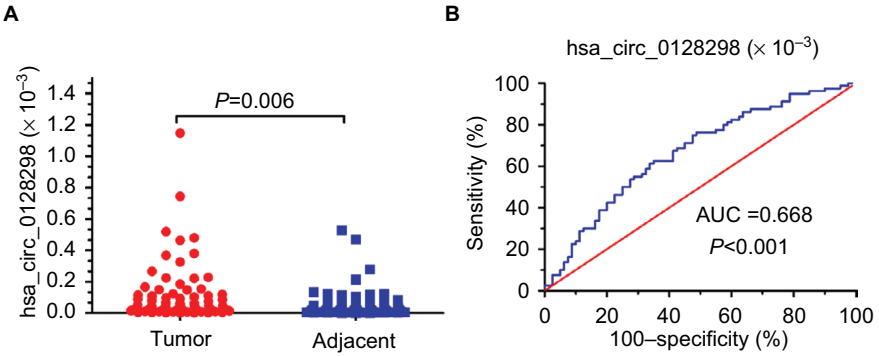

Figure 4 The expression levels and diagnostic value of hsa_circ_0128298.
Notes: (**A**) Increased expression of hsa_circ_0128298 in HCC tissues (*P*=0.006). (**B**) The ROC curve of using hsa_circ_0128298 as a biomarker (*P*<0.001, AUC =0.668).
Abbreviations: AUC, area under the curve; ROC, receiver operating characteristics.

hsa_circ_0128298 was correlated with intrahepatic metastasis (*P*=0.021), lymphatic invasion (*P*=0.004), and organ metastasis (*P*=0.021).

Potential diagnostic values of hsa_circ_0128298

The ROC curve was used to investigate the diagnostic value of hsa_circ_0128298 in distinguishing HCC tissues from paratumorous tissues. In the initial verification, we found that the proportion of hsa_circ_0091582 and hsa_circ_0128298 under the ROC curve (area under the curve [AUC]) was 0.679 and 0.664 (*P*=0.017 and 0.029, respectively) (Figure 3). In the extended verification, only hsa_circ_0128298 was confirmed to have the diagnostic value of AUC =0.668 (*P*<0.001) (Figure 4A and B). The sensitivity and specificity were 0.674 and 0.805, respectively.

Potential prognostic values of hsa_circ_0128298

We analyzed all clinicopathological characteristics and hsa_circ_0128298 expression levels in the prognosis of HCC. Cox regression analysis was used to determine whether hsa_circ_0128298 could serve as a prognostic factor. The univariate analysis showed that the gender (*P*=0.018), AFP (*P*=0.004), intrahepatic metastasis (*P*=0.003), organ metastasis (*P*=0.005), and hsa_circ_0128298 expression (*P*=0.009) were independent

Table 2 Correlation between hsa_circ_0128298 expression and clinicopathological characteristics of hepatocellular carcinoma patients

Characteristics	High expression	Low expression	*P*-value
Gender			
M/F	34/5	36/3	0.455
Age (years)			
≥55/<55	22/17	15/24	0.112
Vascular cancer embolus			
Yes/no	29/9	19/20	0.012
HBSAG			
Positive/negative	29/10	27/12	0.615
HBEAB			
Positive/negative	32/7	33/6	0.761
Cirrhosis			
Yes/no	19/20	17/22	0.650
AFP			
≥20/<20	43343	26/13	0.226
Intrahepatic metastasis			
Yes/no	15/24	8/29	0.110
LN metastasis			
Yes/no	14/25	5/34	0.018
Organ metastasis			
Yes/no	6/33	1/38	0.048

Abbreviations: F, female; LN, lymph node; M, male; HBSAG, hepatitis Bs antigen; HBEAG, hepatitis Be antigen.

Table 3 Spearman analysis of correlation between hsa_circ_0128298 and clinicopathological characteristics

Variables	hsa_circ_0128298 expression level	
	Spearman correlation	*P*-value
Gender	–0.101	0.07
Age	0.093	0.418
Vascular cancer embolus	0.143	0.216
HBSAG	0.149	0.165
HBEAB	0.055	0.632
Cirrhosis	–0.084	0.466
AFP	0.058	0.617
Intrahepatic metastasis	–0.244	0.034
LN metastasis	–0.323	0.004
Organ metastasis	–0.264	0.019

Abbreviations: LN, lymph node; HBSAG, hepatitis Bs antigen; HBEAG, hepatitis Be antigen.

factors (Table 4). Multivariate Cox regression analysis found that the AFP level (P=0.018) and hsa_circ_0128298 expression level (P=0.014) were the prognostic factors predicting poor survival among HCC patients (Table 4).

We further examined whether the hsa_circ_0128298 expression level was correlated with the outcome of HCC patients after hepatectomy. Kaplan–Meier's survival curves were used to compare the low (n=39) and high (n=39) hsa_circ_0128298 subgroups, and the results are presented in Figure 5. Patients with higher hsa_circ_0128298 expression levels had a statistically significant difference in the OS compared to the low expression group (P=0.003).

Discussion

In recent years, there have been few advances in the treatment of HCC. Clinically, surgery and systemic chemotherapy are the standard treatments for HCC.[3] However, only 13–15% of patients with HCC are suitable for surgery. Most patients are diagnosed with an advanced HCC, losing the precious opportunity for surgical operation. In addition, patients with HCC are prone to be multidrug resistant.[3] There are several challenges in the diagnosis and treatment of HCC. First, there are difficulties in early diagnosis. Among the clinicopathological characteristics of HCC is its lack of specific early symptoms. Furthermore, the resection rate is only 15%, but ~50% patients are found to have distant metastases during treatment.[14] Second, the heterogeneity of HCC further increases the difficulty. The genome-wide analysis of HCC has shown that genetic changes exist in >10 core signaling pathways. It has been reported that the alterations of multiple genes could possibly lead to ineffective treatments, resulting in poor prognosis.[15] Therefore, the diagnosis and treatment of HCC should rely on early detection and diagnosis. The detection of HCC-related molecular markers predicting the early occurrence of HCC could be a more pragmatic way to diagnose and treat HCC.

Table 4 Univariate and multivariate analyses of various prognostic parameters in patients with HCC (Cox regression analysis)

Variables	Univariate analysis			Multivariate analysis		
	P-value	Hazard ratio	95% CI	*P*-value	Hazard ratio	95% CI
Gender	0.018	3.268	1.220–8.750			
Age	0.944	1.001	0.972–1.031			
Vascular cancer embolus	0.08	0.513	0.243–1.084			
HBSAG	0.055	2.415	0.920–5.207			
HBEAB	0.312	1.511	0.679–3.364			
Cirrhosis	0.454	0.773	0.394–1.518			
AFP	0.004	4.25	1.420–4.329	0.018	1.251	1.032–3.989
Intrahepatic metastasis	0.003	1.343	1.144–7.056			
LN metastasis	0.387	0.703	0.316–1.562			
Organ metastasis	0.005	0.244	0.091–0.657			
hsa_circ_0128298 expression	0.009	1.978	1.341–3.024	0.014	6.661	2.661–8.418

Abbreviations: LN, lymph node; HBSAG, hepatitis Bs antigen; HBEAG, hepatitis Be antigen.

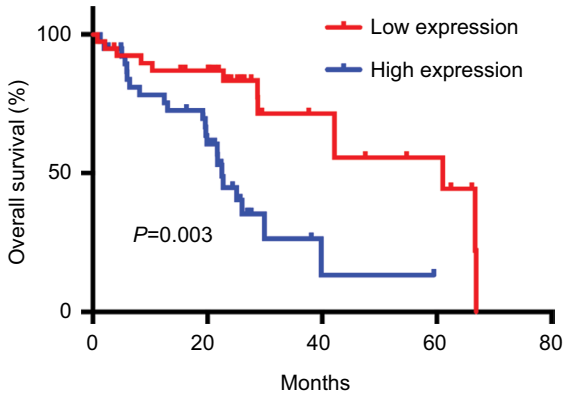

Figure 5 hsa_circ_0128298 can be an independent prognostic factor to predict OS.
Note: Kaplan–Meier's analyses of correlations between the hsa_circ_0128298 expression levels and OS of 78 HCC patients are shown.
Abbreviations: HCC, hepatocellular carcinoma; OS, overall survival.

Increasing numbers of studies have shown that noncoding RNAs play an important role in the development of cancer.[5–9,11] However, there are few studies on circRNA. circRNA is a type of special noncoding RNA and is the latest research hotspot in the RNA field. Unlike traditional linear RNAs (including 5′ and 3′ ends), circRNAs are closed-loop structures that are not affected by RNA exo-enzymes and are more stable and less degradable.[7] Functionally, recent studies have shown that circRNAs are enriched at the miRNA-binding sites and play the role of miRNA sponges in cells, thus releasing the inhibitory effect of miRNAs on their target genes and increasing the target gene expression, which is known as the competitive endogenous RNA (ceRNA) mechanism.[16,17] In addition, circRNAs play an important regulatory role in cancer via their interaction with cancer-associated miRNAs.[18]

Here, we identified a new circRNA (hsa_circ_0128298) that was significantly upregulated in HCC tissues. The expression of hsa_circ_0128298 was significantly associated with vascular cancer embolus, lymph node (LN), and organ metastasis ($P<0.05$). The Spearman analysis of correlation between hsa_circ_0128298 and various clinicopathological factors indicated that the expression of hsa_circ_0128298 was correlated with intrahepatic metastasis, LN metastasis, and organ metastasis ($P<0.05$). Moreover, we found that HCC patients with lower expression levels of hsa_circ_0128298 have prolonged OS compared to patients with higher hsa_circ_0128298 expression levels. The ROC analysis proved that hsa_circ_0128298 could be recognized as an HCC biomarker with favorable sensitivity and specificity. Note, in the Spearman analysis, hsa_circ_0128298 was negatively correlated with the three metastatic indicators, which is inconsistent with other results. Considering that hsa_circ_0128298 was an indicator of prognosis in HCC, we speculated that hsa_circ_0128298 did not affect the patients' prognosis by promoting metastasis. It might affect the prognosis of patients through other phenotypes, such as promoting vascular proliferation/immune escape. This also provides a certain direction for future research on the functional experiments of hsa_circ_0128298.

However, only 78 pairs of HCC tissues were analyzed in this study because of the limited number of available HCC samples. In addition, the tumor staging, T staging, and N staging were not included in our study due to a failure to acquire this information from some HCC patients. A larger number of samples should be tested at multiple centers to further confirm our conclusion. Furthermore, we did not test the molecular biological effects of hsa_circ_0128298 on HCC in vivo and in vitro.

Conclusion

Our data indicate that hsa_circ_0128298 expression is significantly upregulated in HCC samples and is a predictor of HCC occurrence and prognosis. Therefore, hsa_circ_0128298 could serve as a potential biomarker in the diagnosis of HCC and as a prognostic marker for the survival of HCC patients.

Acknowledgments

We thank the Innovation Project of Shandong Academy of Medical Science for support. This study was supported jointly by the National Natural Science Foundation of China (No 81603348), the China Postdoctoral fund (No 21300075311104), and the Shandong Postdoctoral Innovation special fund (No 201602012). The authors are grateful to the recruited patients and their kin.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

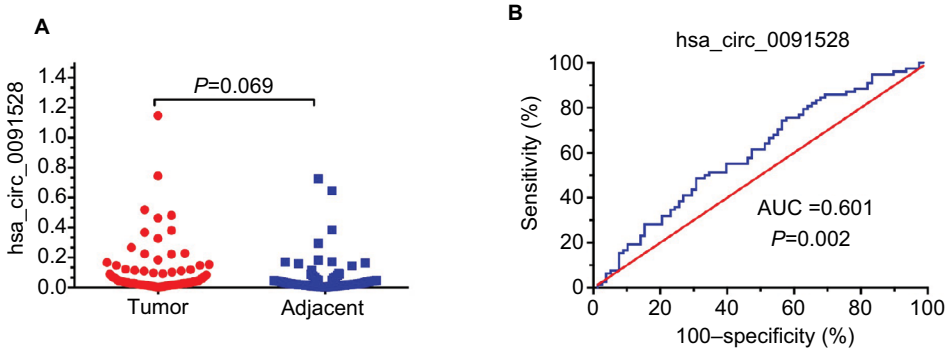

Figure S1 The expression levels and diagnostic value of hsa_circ_0091528.
Abbreviation: AUC, area under the curve.