Research Square

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New diagnosis for hepatocellular carcinoma via hsa_circ_0097009

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Research Article

Keywords: Hsa_circ_0097009, Hepatocellular carcinoma, Diagnosis, Prognosis, Biomarker

Posted Date: November 4th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1705078/v2

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Abstract

Background

Although circular RNAs take a key part in the regulation of cancer, its role in the diagnosis and prognosis of hepatocellular carcinoma (HCC) still unclear. This study investigated the diagnostic and prognostic usefulness of hsa_circ_0097009 in HCC.

Methods

qRT-PCR assay was performed to detect the presence of hsa_circ_0097009 in plasma collected from 30 HCC patients and 30 healthy controls. Receiver operating characteristic (ROC) curve, Cox regression, and Kaplan–Meier curve analyses were applied to evaluate the clinical diagnostic and prognostic values of hsa_circ_0097009.

Results

The experimental data suggested that hsa_circ_0097009 expression of HCC plasma and cell lines is upregulated. The plasma hsa_circ_0097009 level of HCC patients was linked to tumor diameter ($P = 0.026$) and alpha-fetoprotein (AFP) expression level ($P = 0.019$). In addition, the plasma level of hsa_circ_0097009 decreased markedly after surgery to remove the tumor. Moreover, the level of plasma of hsa_circ_0097009 in HCC patients was increased compared with healthy controls ($P < 0.0001$) and hepatitis B patients. ROC curve reflected that the area under the curve (AUC) of plasma hsa_circ_0097009 to distinguish HCC from healthy controls and hepatitis B patients were 0.9368 (95 percent CI = 0.8874–0.9862) and 0.9067 (95 percent CI = 0.8439–0.9695), respectively, with the same sensitivity 0.9779 and the same specificity 0.6889. Furthermore, the AUC value of hsa_circ_0097009 combined with AFP for distinguishing HCC from healthy controls was 0.9822 (95 percent CI = 0.9682–0.9961), with the sensitivity 0.9889 and specificity 0.667. The plasma hsa_circ_0097009 low expression group had a considerably longer overall survival (OS) than the high expression group.

Conclusion

The study suggests that hsa_circ_0097009 could be used as a new potential biomarker in diagnoses and prognoses of HCC patients.

1 Introduction

Globally, liver cancer is the sixth most prevalent cancer, with the second-highest death rate, and its occurrence is rising year after year [1, 2]. Nearly 75 to 85 percent of liver cancer cases are due to hepatocellular carcinoma (HCC) [3]. Because metastasis is common, most patients do not notice

symptoms until late, and the five-year survival rate is only 10 percent [4]. However, the overall prognosis for HCC still poor, and HCC patients' survival times are often short [5]. The fact that liver cancer is an occult illness with no specific early markers is the main cause for its high morbidity and fatality rate. At present, the most widely used biomarker for HCC detection is alpha-fetoprotein (AFP), but its sensitivity (39–65%) and specificity (76–94%) are insufficient and about 30–40% of HCC patients are AFP-negative clinically rendering it inappropriate for clinical use [6]. As a result, it’s critical to find markers for the specific diagnosis of HCC in the clinic.

Circular RNAs (CircRNAs) are a recently discovered non-coding RNA with covalent closed loop, no 5'-3' polarity, and polyadenylated tails [7–9]. The structure of circRNAs are conservative and stable, which can regulate mammalian gene expression[10, 11]. CircRNAs have been a study focus recently as new potential biomarkers and cancer therapeutic targets [12–14]. CircRNAs have been linked to cancer progression and occurrence in a variety of studies, including non-small cell lung cancer (NSCLC), gastric cancer, head and neck cancer, and HCC [15–18]. Previous research has suggested that hsa_circ_0097009 was highly increased in HCC tissues and cell lines, and knocking it down decreased HCC cells proliferation and invasion [19]. It indicates that hsa_circ_0097009 may play some important roles in HCC. Given the high expression of hsa_circ_0097009 in HCC tissues, this suggests that hsa_circ_0097009 may be useful in the diagnosis of HCC.

We collected plasma from patients at various stages of HCC and also from healthy volunteers for this research to analyze the expression and the significance of plasma has_circ_0097009 level for the diagnosis of HCC. Meanwhile, the link between hsa_circ_0097009 and clinical liver function indicators was analyzed. The goal of this study was to see the clinical significance of has_circ_0097009 value in the early diagnosis, efficacy, and prognosis of HCC.

2 Materials And Methods

2.1 Patients and samples

Plasma samples were collected from the HCC patients (n = 30), hepatitis B patients (n = 30) and healthy volunteers (n = 30) at the Shanghai Pudong New Area Gongli Hospital during the period of April 2015 – April 2016. Inclusion criteria: pathologically confirmed patients; complete clinical data; informed consent of patients.Exclusion criteria: radiotherapy and chemotherapy before surgery; combined with other tumors; intolerance to surgery or perioperative death.A total of 30 healthy subjects in our hospital during the same period were selected as the healthy control group. The clinicopathological characteristics of the patients were listed in Table 1. Fasting venous blood samples (4 mL) were collected from patients who had not received chemotherapy before surgery and centrifuged within 1 h after collection to obtain plasma samples. In addition, a pair of preoperative and postoperative plasma (7 days after surgical resection of the primary tumor) was collected from 6 patients with HCC. All the samples were stored at -80°C for further experiment. Shanghai Pudong New Area Gongli Hospital's Medical Ethics Committee approved this study.

2.2 Cell culture

HCC cell lines SMCC-7721, HeP3B, Huh-7, HepG2 and normal human hepatocytes L02 were purchased from ATCC (Manassas, VA, USA). All cells were cultured in DMEM (BasalMedia, Henan, China) with 10% FBS and 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA) at 37°C in a humidified atmosphere of 5% CO_2.

2.3 qRT-PCR (Real-time quantitative reverse transcription-polymerase chain reaction)

1 mL pre-cooled TRIzol reagent (Invitrogen) was poured to the cells, then blowing evenly. Total RNAs were extracted according to the kit instructions after 5 minutes at room temperature. Concentration of total RNAs was quantified using the Nano-300 Micro-Spectrophotometer (ANNSHENG, Hangzhou, China). Next, the RNAs were reverse transcribed into cDNAs as directed by the instructions of reverse transcription kit. qRT-PCR was performed using a 7500 real-time PCR system (ABI, Thermo Fisher Scientific; USA) and SYBR Green qPCR Master Mix (MedChem Express, Monmouth Junction, NJ, USA) following the manufacturer's instructions. GAPDH was used for normalization. All the primers used are as follows, hsa_circ_0097009-F: 5’-TCAAAGGACTCCACTGATGGAT-3’, hsa_circ_0097009-R: 5’-GTCCCACTGTAAGCACAGGC-3’; GAPDH-F: 5’- GATTTGGTCGTATTGGGCGC-3’, GAPDH-R: 5’-GCCTTCTCCATGGTGGTGAA – 3’.

2.4 CircRNA identification

Ribonuclease R (RNase R; Beyotime, Shanghai, China) was used to identify circular features of hsa_circ_0097009. Briefly, the extracted RNA was treated with RNase R, and then the expression of hsa_circ_0097009 and GAPDH was determined by qRT-PCR. GAPDH is a representative of linear RNA. Then, the cDNA PCR products were taken and subjected to SDS-polyacrylamide gel electrophoresis to verify the products. DNA marker used was DL2000 (Takara, Japan). The bands were examined by UV irradiation.

2.5 Statistical analyses

We used IBM SPSS 22.0 software (Version X; Armonk, NY, USA) and GraphPad Prism 9.3.1 (Version X; La Jolla, CA, USA) to analyze statistical data. We analyzed the differences in hsa_circ_0097009 levels using Student's *t*-tests and one-way ANOVA. An analysis of the link between hsa_circ_0097009 expression level and clinicopathological factors was carried out by the Student's t-test and X2 test. The diagnostic value of hsa_circ_0097009 was evaluated using a ROC curve. The OS was analyzed with Kaplan-Meier analysis and log-rank statistics. A P value of < 0.05 was considered statistically significant.

3 Results

3.1 Hsa_circ_0097009 was up-regulated in HCC plasma and HCC cell lines

qRT-PCR was performed to confirm the expression of hsa_circ_0097009 in 30 paired human HCC plasma and HCC cell lines. The level of hsa_circ_0097009 in the HCC group was higher than in the healthy control group ($P < 0.001$), according to qRT-PCR analysis (Fig. 1A). The same was true in SMCC-7721, HeP3B, Huh-7, and HepG2 cells ($P < 0.001$) (Fig. 1B). qRT-PCR was performed to detect the level of hsa_circ_0097009 in Huh7 and HepG2 cell lines, respectively. RNase R had no effect on hsa_circ_0097009, according to the results. (Fig. 1C, D). Electrophoresis results show only one band, which can be used to confirm the specificity of products (Fig. 1E).

3.2 Relationship between the expression level of hsa_circ_0097009 and clinicopathological factors in HCC.

By comparing the clinicopathological factors of HCC patients and healthy people, the level of hsa_circ_0097009 in HCC patients plasma has no significant association with the patient's gender, age, tumor number, TNM stage, metastasis, Alanine aminotransferase (ALT) expression as well as Aspartate aminotransferase (AST) expression ($P > 0.05$), but correlated with alpha-fetoprotein (AFP) expression level and tumor diameter ($P = 0.026$) ($P = 0.019$) as shown in Table 1.

Table 1
The relationship between hsa_circ_0097009 expression levels and clinical characteristics of HCC patient

Characteristics	Patient number (total = 30)	has_circ_0097009 expression High	Low	x^2	*P* value
Gender	16	9	7	0.117	1.000
Male	14	7	7		
Female					
Age(year)	6	4	2	0.536	0.657
≤30	24	12	12		
> 30					
Tumor size (cm)	14	4	10	**6.467**	**0.026**
≤4.0	16	12	4		
>4.0					
Tumor number Solitary	21	12	9	0.408	0.694
Multiple	9	4	5		
TNM	14	5	9	3.274	0.074
I + II	16	11	5		
III + IV					
Metastasis	18	10	8	0.089	1.000
Yes	12	6	6		
NO					
AFP					
≥ 400ng/ml	20	14	6	**6.696**	**0.019**
400ng/ml	10	2	8		
ALT					
≥ 50U/L	9	5	4	0.026	1.000
50U/L	21	11	10		
AST					
≥ 40U/L	11	6	5	0.010	1.000
40U/L	19	10	9		

3.3 Diagnostic values of plasma hsa_circ_0097009

Plasma samples of HCC patients were collected before and after surgery and the ΔCt of hsa_circ_0097009 was calculated (Fig. 2A). The results showed that the plasma levels of hsa_circ_0097009 decreased significantly after surgery to remove the tumor ($P < 0.001$). HCC patients had higher plasma levels of hsa_circ_0097009 than hepatitis B patients or healthy people. ($P < 0.001$) (Fig. 2B).

The ROC curve was carried out to detect the diagnostic value of plasma hsa_circ_0097009. ROC curves of HCC patients distinguishing from hepatitis B patients (Fig. 2C) and healthy people (Fig. 2D) were drawn respectively, and the AUC were 0.9067 and 0.9368, with the same sensitivity 0.9779 and the same specificity 0.6889. In addition, the AUC value of hsa_circ_0097009 and AFP used to distinguish HCC from healthy patients was 0.9822. (Fig. 2E). The sensitivity and specificity were 0.9889 and 0.667.

3.4 The prognostic value of plasma hsa_circ_0097009

Based on our datas on HCC patients collected in 2015–2016, HCC plasma hsa_circ_0097009 was sorted into two groups: high and low expression. After five years of follow-up, we found that the low expression group had a considerably longer OS time than the high expression group, according to Kaplan-Meier analysis. ($P = 0.0048$) (Fig. 3).

4 Discussion

HCC is currently a globally recognized liver disease with strong metastases and poor diagnosis. In China, HCC is the second most common malignancy after stomach cancer[3]. Early radical resection is the most effective method of treating HCC, so early diagnosis becomes the key to early treatment. At present, the combination of AFP detection and imaging examination is used to make an early diagnosis of HCC. AFP is the first choice for HCC tumor markers, but there are certain limitations, about 30%-40% of HCC patients have negative AFP test results[20]. It makes AFP not an ideal indicator for early HCC screening. Therefore, early tumor markers for HCC are urgently needed.

CircRNAs are participated in the process of tumor diseases and the regulation of molecular mechanisms[21]. CircRNAs are newly discovered non-coding RNAs with a circular structure and strong stability. It can regulate gene expression or combine with RNA-related proteins to perform biological functions[9, 22]. Studies have shown that circRNAs are not affected by repeated freezing and thawing and degradation of RNA, and is biologically stable[7, 23]. CircRNAs may be a diagnostic marker for tumors.

In recent years, some research reports revealed that some circRNA can be used as markers for early tumor screening and prognostic evaluation[24, 25]. For example, Chen et al. [26]sequenced prostate cancer tissue and adjacent tissue, and found that 7232 circRNAs were associated with prostate cancer. They

further verified that circCSNK1G3 accelerates the growth and proliferation of cancer cells through combining with miRNA181, and it is expected to be used as a marker for early screening of prostate cancer. Yin et al.[27] determined that 22 circRNAs were decreased and 19 circRNAs were creased by comparing the circRNA sequencing data of breast cancer and healthy people, and they speculated that hsa_circRNA_0001785 might be a biological marker of breast cancer by drawing the expression map of breast cancer. Although, the study of circRNAs in HCC remains in its initial stage, and studies have found that circ0001649 has a low level in HCC and inhibits the progression of HCC through multiple small RNA sponges[28]. circDYNC1H1 inhibits metastasis and proliferation of HCC cells via regulating the level of miR-140-5p [29]. In addition, circ_0005705 adsorbs miR-335 through sponge and further regulates MAPK1 expression, ultimately promoting HCC progression[30]. Circ_103809 inhibited proliferation and invasion of hepatoma cells via suppressing the level of miR-620[31]. Literature reported that CircRNA SMARCA5 can be used as a novel HCC biomarker[32]. Recently, basic experiments have confirmed that downregulation of hsa_circ_0097009 can inhibit HCC cells proliferation and invasion [19], but there are few clinical reports on the relationship between hsa_circ_0097009 and HCC. Therefore, this study included 30 pathologically diagnosed patients with HCC and 30 healthy people, detected plasma hsa_circ_0097009 expression of these subjects and explored its clinical significance in the diagnosis, treatment, prognosis of HCC. It's found that the level of hsa_circ_0097009 in the plasma of HCC patients was markedly higher than that of healthy people, and its level was significantly correlated with tumor size and AFP. At the same time, we found that the level of hsa_circ_0097009 in the plasma of HCC patients was markedly lower than the preoperative level. Further ROC curves showed that the plasma hsa_circ_0097009 level could diagnose HCC with hepatitis B and healthy people. And it has good diagnostic value, including high specificity and moderate sensitivity. In addition, after 5-year follow-up of HCC patients, we also found that the OS time of HCC patients with high plasma hsa_circ_0097009 level was markedly shorter than that of patients with low level. This also suggests that hsa_circ_0097009 can be a biomarker for early diagnosis, treatment and prognosis evaluation of HCC.

Of course, this study still has limitations. First, the number of clinical samples was small, and studies with larger sample sizes are still needed to verify the function of plasma hsa_circ_0097009 as a novel diagnostic biomarker for HCC, and we will continue to enrich the data later. In addition, the specificity of this diagnostic model is low, and other molecules or detection methods will be combined to improve the specificity in the future. Due to the evolution of cancer, tumor heterogeneity and cell population heterogeneity in human samples are far beyond our imaginations, single-cell analysis methods are becoming increasingly important in elucidating the complexity of biology. No research on hsa_circ_0097009 in other human tissues has been found so far, and we will continue to explore hsa_circ_0097009 at the single-cell level to further reveal its role in HCC and other diseases. According to bioinformatics analysis, miR-1261, miR-873, miR-769-5p, miR-636, miR-568 and miR-182 may be potential targets of hsa_circ_0097009 (Fig. 4). This is consistent with the results of Ning et al. In addition, it was shown that hsa_circ_0097009 is involved in process of liver cancer via the circ0097009/miR-1261/SLC7A11 axis regulating ferroptosis[19]. These findings imply that hsa_circ_0097009 takes a key part in HCC by targeting miRNAs, which may provide new ideas and more in-depth research clues for

studying the molecular regulatory role of hsa_circ_0097009 in HCC. However, the specific molecular mechanism remains unclear. We will further study its molecular mechanism in the future.

In conclusion, the hsa_circ_0097009 level is upregulated in the plasma of HCC patients and can be correlated with the occurrence, development and prognosis of HCC. This makes the hsa_circ_0097009 it to be a potential biomarker for diagnosing HCC and assessing its prognosis.

Declarations

Ethics approval and consent to participate

Ethics Committee approval was obtained from the Institutional Ethics Committee of shanghai gongli hospital to the commencement of the study. The data involved in this study have obtained informed consent from patients.

Consent for publication

This manuscript does not contain any individual person’s data in any form. The data involved in this study have obtained informed consent from patients.

Availability of data and materials

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Competing interests

The authors declare that they have no conflict of interest.

Funding

the Clinical Research Project of Health Industry of Shanghai Municipal Health Commission(Grant No.202140407), the Outstanding Leaders Training Program of Pudong Health Bureau of Shanghai (Grant No. PWR12018-05) the Shanghai Pudong New Area Gongli Hospital(Grant No. 2020YQNJJ-03)

The correspondent author Liu Xinghui is responsible for the coordination of the whole article,organizing the ideas,agreeing to final publication,and being responsible for the publication costs.

Authors' contributions

WX, LX and XL proposed and designed the experiments. PT, LB and WYF carried out the experiments. WX and LQ drafted the article and interpreted the data. LX, LZ and XL revised the article. All the authors approved the final version of this article.

Acknowledgements

Not applicable

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Figures

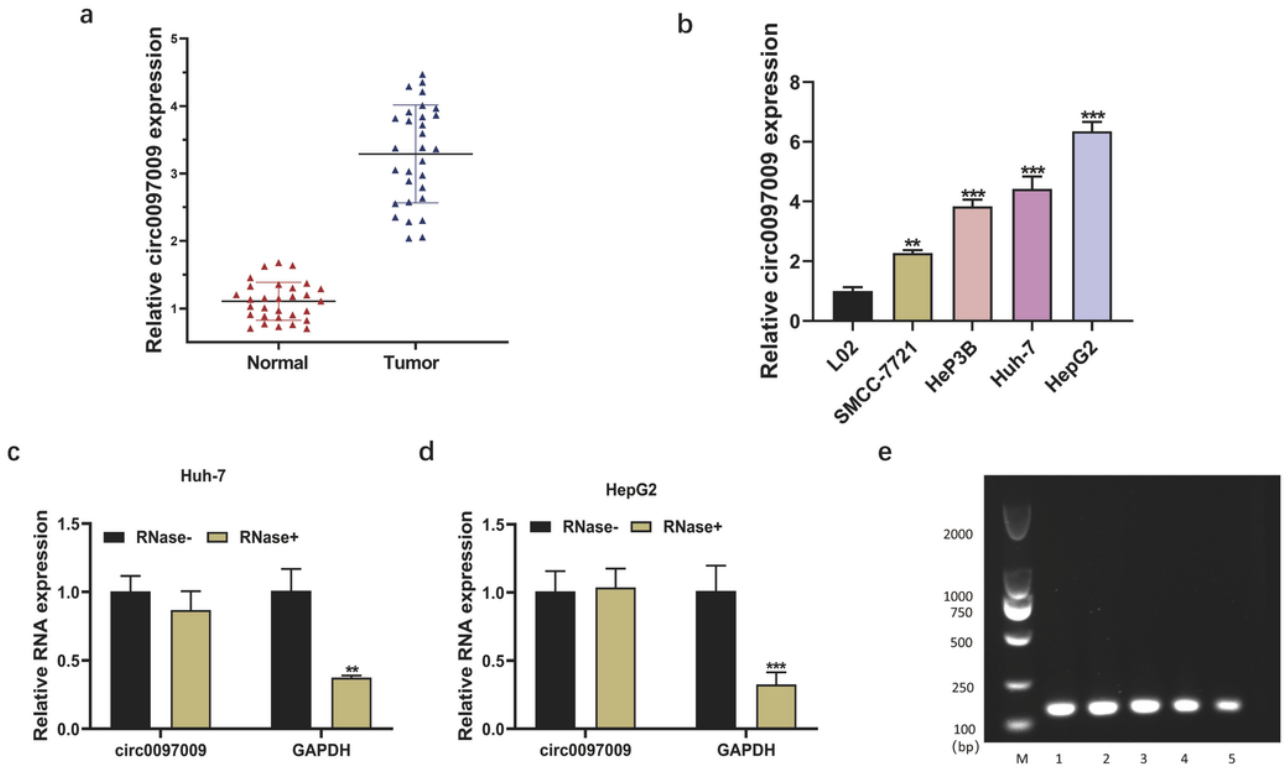


Figure 1

The expression of hsa_circ_0097009 in HCC plasma and cells

a hsa_circ_0097009 in HCC plasma (n=30, *P*<0.001); **b** hsa_circ_0097009 in HCC cell lines (SMCC-7721, HeP3B, Huh-7, HepG2, ***P*<0.01, ****P*<0.001); **c, d** In Huh-7 and HepG2 cells, RNase to verify the circRNA products; **e** PCR product electrophoresis. The DL2000 Marker is in Lane M, while the products are in Lanes 1–5. *HCC* hepatocellular carcinoma, *PCR* Polymerase Chain Reaction

Note: P<0.05 is shown by bolded values.

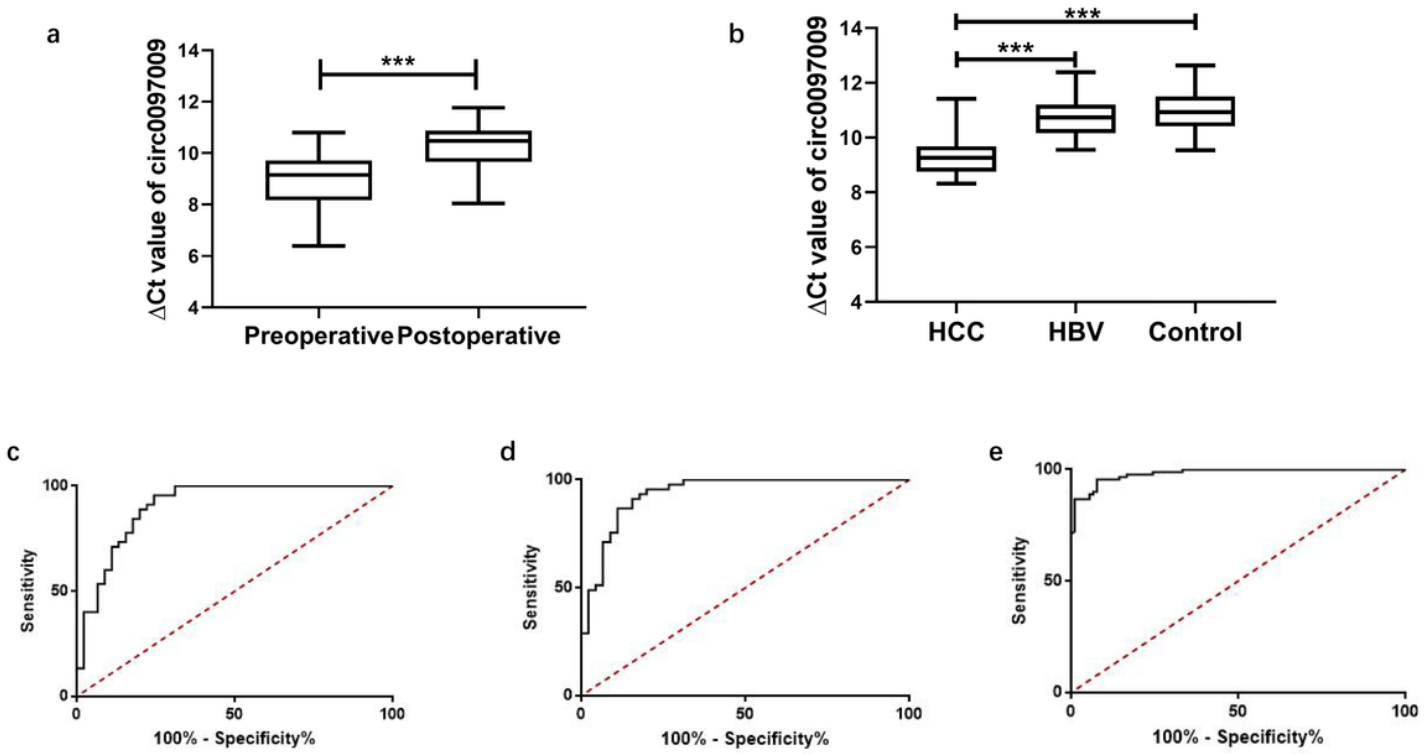


Figure 2

Diagnostic value for hsa_circ_0097009

a Preoperative and postoperative plasma levels of hsa_circ_0097009 (P<0.001); b Plasma hsa_circ_0097009 expression levels in HCC, hepatitis B patients and healthy individuals (P<0.001); c ROC curves of plasma hsa_circ_0097009 for the diagnosis of HCC and hepatitis B patients (P<0.001); d ROC curve of plasma hsa_circ_0097009 used to diagnose HCC and healthy controls (P<0.001); e ROC curves of hsa_circ_0097009 and AFP combined to diagnose HCC and healthy controls (P<0.001). AFP alpha-fetoprotein, HCC hepatocellular carcinoma, ROC receiver operating characteristic. **P<0.01, ***P<0.001.

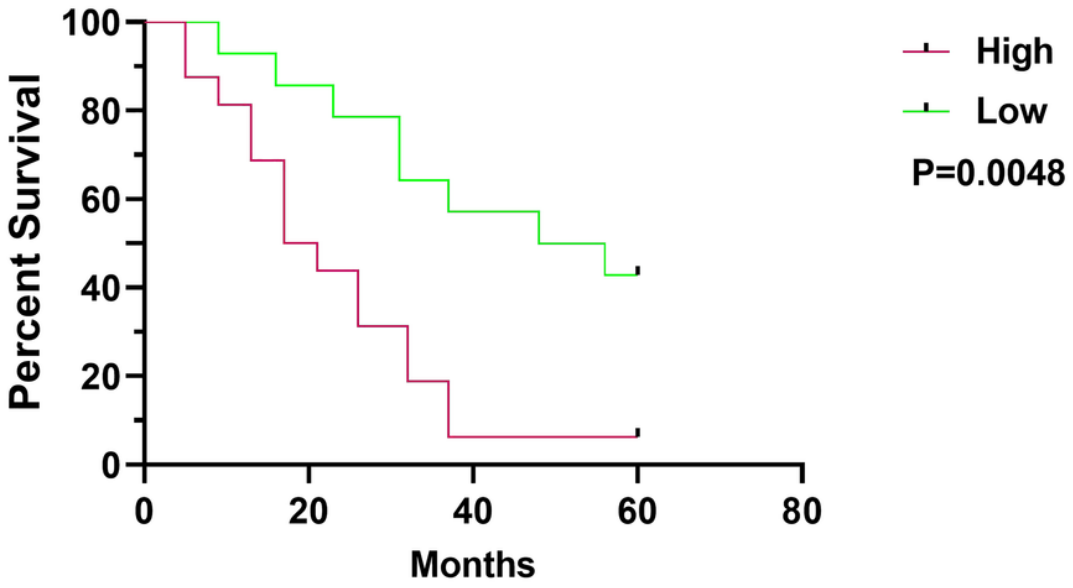


Figure 3

Kaplan-Meier analysis of overall survival in HCC patients (n=15, P=0.0048). *HCC* hepatocellular carcinoma

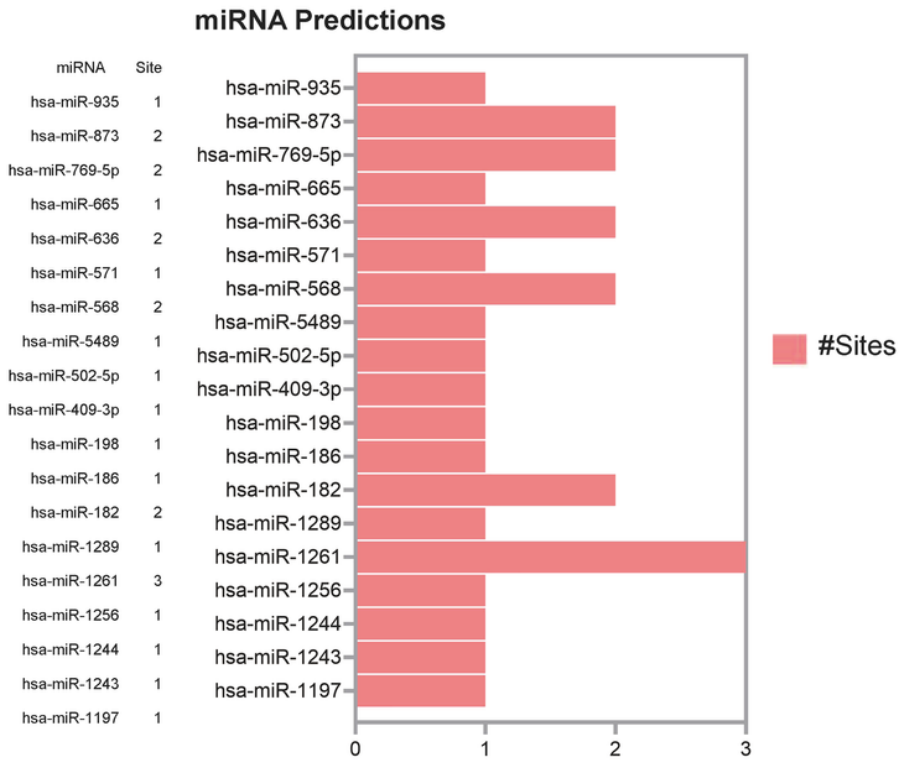


Figure 4

TargetScan miRNA predictions. *miRNA* MicroRNA