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Circular RNA hsa_circ_0000673 promotes hepatocellular carcinoma malignance by decreasing miR-767-3p targeting SET

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ABSTRACT

The importance of circular RNAs (circRNAs) in human cancers has gradually been acknowledged. In hepatocellular carcinoma (HCC), several circRNAs have been reported to regulate tumor growth and metastasis. However, the role of hsa_circ_0000673 in HCC remains largely unknown. In this study, we found that hsa_circ_0000673 was significantly upregulated in HCC tissues compared to adjacent non-tumor tissues. Moreover, we found that hsa_circ_0000673 knockdown markedly inhibited the proliferation and invasion of HCC cells *in vitro*. Besides, hsa_circ_0000673 silence led to delayed tumor growth in vivo. In terms of mechanism, we showed that hsa_circ_0000673 directly associated with miR-767-3p in HCC cells. Via inhibiting miR-767-3p, hsa_circ_0000673 promoted HCC cell proliferation and invasion. Furthermore, we demonstrated that SET was a downstream effector of hsa_circ_0000673/miR-767-3p signaling. We showed that miR-767-3p could significantly promote SET expression by sponging miR-767-3p in HCC cells. Finally, rescue assays indicated that SET expression was essential for the effects of hsa_circ_0000673/miR-767-3p signaling on HCC cell proliferation and invasion. Taken together, our findings demonstrated that hsa_circ_0000673 promoted HCC malignant behaviors via regulating miR-767-3p/SET pathway.

1. Introduction

As the fifth most common malignancy worldwide, hepatocellular carcinoma (HCC) accounts for 70%–85% of all primary liver cancer [1]. Its high recurrence and heterogeneity lead to more than 750000 deaths each year [2]. Previous studies have identified many aberrantly expressed protein-coding genes and non-coding RNAs in HCC [3–5]. Yet the details of oncogenesis remain elusive, and limited therapeutic approaches have been developed for advanced HCC. There is also no novel molecular marker developed to help diagnose this disease in early stage [6,7]. Thus it is critical to elucidate the mechanism of HCC.

Circular RNAs (circRNAs) are a new type of non-coding RNAs, feactured by cell-type specific and stability [8]. In the first years when they were discovered, circRNAs did not draw too much attention [9]. Yet, with the development of sequencing technology, people gradually realized the essential roles of circRNAs in biological processes, including the regulation of cancer [10–17]. Nowadays, people have found that many circRNAs were deregulated in cancers [14]. However, their roles in carcinogenesis are still not clear.

Mechanically, there are several ways by which crcRNAs function in mammalian cells, such as miRNA binding, interacting with proteins and regulating the splicing machine [18]. Among the functions, circRNAs usually functions through sponging miRNA [19]. CircRNAs contain miRNA binding sites within their sequence [20], and this allows them to interact with and regulate miRNA function [21]. As have been reported, miRNAs usually bind to the 3′ UTR region of target mRNAs to suppress target gene expression [22]. Thus, circRNA binding to miRNA leads to the release of target gene expression, and forms a circRNA-miRNA-mRNA function network.

In this study, we discovered that hsa_circ_0000673 is highly expressed in HCC, and was positively correlated with HCC progression. Mechanically, has_circ_0000673 is highly expressed in HCC tissues, which forms a sponge of miR-767-3p. This sponge inhibits miR-767-3p expression, and thus relieves the inhibitory effect of miR-767-3p on SET expression, thus leads to the progression of HCC.

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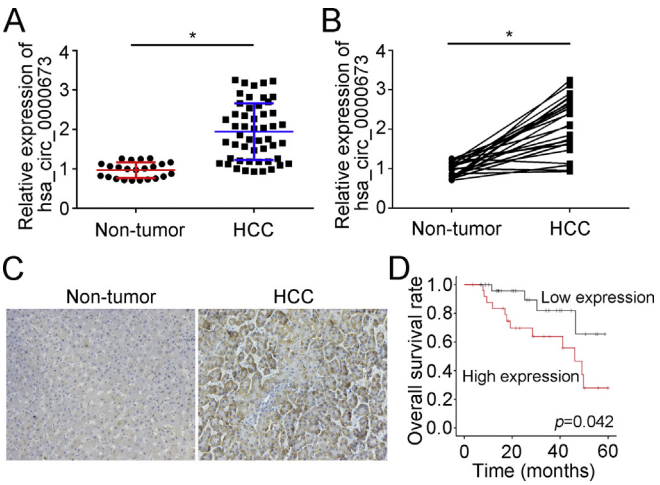

Fig. 1. Hsa_circ_0000673 was highly expressed in HCC tissues. (A) qRT-PCR analysis was used to measure hsa_circ_0000673 expression in HCC tissues (n = 51) and non-tumor tissues (n = 23). (B) qRT-PCR analysis was utilized to determine hsa_circ_0000673 expression in 23 pairs of HCC tissues and adjacent non-tumor tissues. (C) Hsa_circ_0000673 expression levels were analyzed in paired HCC tissues and adjacent non-tumor tissues by *in situ* hybridization (ISH). (D) HCC tissues were divided into high and low expression groups based on the mean value of hsa_circ_0000673 expression. Then overall survival rate was calculated using the Kaplan-Meier analysis. $^{*}p < 0.05$.

2. Materials and methods

2.1. Human samples

A total of 51 HCC patients, who underwent surgeries at The Second Hospital of Jilin University, were included in this study. The 23 adjacent normal tissues were obtained from 1 cm away from the edge of the HCC; and there were no obvious tumor cells. The diagnosis of HCC was confirmed by histological examination. Patients with HCC who had prior treatment of their tumor or history of other solid tumors were excluded in this study. This study was approved by the Human Research Ethics Committee from The Second Hospital of Jilin University. Informed consent was obtained from all patients.

2.2. Cell culture

Human HCC cell lines (Hep3B and Huh7) were obtained from were obtained from the American Type Culture Collection. All cells were cultured in Dulbecervical cancero's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 μg/ml of streptomycin (Sigma-Aldrich). Cells were cultured in 5% CO2 at 37 °C in incubators at 100% humidity.

2.3. Invasion assays

The cell invasion assay was performed using Matrigel-coated transwell chambers (8.0 μm, Costar, USA). 10^5 cells were plated in the upper chamber in serum-free DMEM medium, while bottom chamber was filled with culture medium containing a chemoattractant. 24 h of incubation, invaded cells were fixed with 4% formaldehyde and stained with crystal violet.

2.4. In situ hybridization

HCC tumor tissues were sectioned and mounted on Surgipath X-tra glass slides (Leica BioSystems, Buffalo grove, IL). Then the slides were baked for 1 h at 60 °C, and then stored at −80 °C. ISH probe (Affymetrix, Santa Clara, CA) was designed against hsa_circ_0000673 transcripts, and ISH was performed using the manual ViewRNA™ platform (Affymetrix, Santa Clara, CA).

2.5. CCK-8 assay

Cells were suspended in DMEM with 10% FBS, and seeded into 96-well plates at a density of 2×10^3 cells per well. 24 h later, 10 μl CCK-8 solution (Dojindo, Japan) was added and incubated in dark for another 2 h. Absorbance was measured at 450 nm at indicated time points with the absorbance value measured on the first day as a control. Each measurement was performed in triplicate, and the experiments were conducted at least three times.

2.6. Colony formation assay

Cells were seeded in 6-well plates at 5×10^2 per well. And then, they were incubated for 2 weeks. After that, cells were washed with PBS, fixed with paraformaldehyde, and stained with crystal violet (Sigma, China). The number of colonies was counted under a microscope (Olympus IX81, Japan).

2.7. Luciferase assays

Cells were transfected with indicated transcripts by Lipofectamine 2000 (Invitrogen) 24 h after transfection, cells were lysed, and luciferase activities were analyzed with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

2.8. qRT-PCR

Total RNA was isolated with Trizol reagent (Thermo Fisher Scientific, USA) and treated with DNase I (Thermo Fisher Scientific, USA) to remove residual genomic DNA Reverse transcription was performed using an RT-PCR Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Gene expression was analyzed with SYBR Green Real-Time PCR Master Mixes (Thermo Fisher Scientific, USA) on ABI 7300 thermal cycler (Applied Biosystems, Foster City, CA, USA), and ACTIN was serviced as the internal control.

2.9. Tumor xenograft assay

BALB/c (nu/nu) mice were randomly divided into groups and maintained under standard conditions according to institutional animal guidelines. 2×10^6 cells were separately injected subcutaneously into the right flank of nude mice, the tumor size was monitored every week. 5 weeks later, the mice were euthanized, and the xenograft tumors were weighted. The animal protocols were approved by the Institutional Animal Care and Use Committees at The Second Hospital of Jilin University.

2.10. Western blotting

Cells were lysed with lysis buffer (50 mM Tris−HCl, 137 mM NaCl, 10% glycerol, 100 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1% Nonidet P-40, and 5 mM protease inhibitor cocktail; pH 7.4), and 50 μg of the whole-cell lysates were separated by 10% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA). The membrane was incubated with primary antibody overnight at 4 °C, and then with a secondary antibody for 1 h at room temperature, the reactive bands were visualized using a Bio-Rad imaging system.

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