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Circular RNA Circ_0001971 Aggravates the Progression of Hepatocellular Carcinoma Cells by Activating the miR-186-5p/KLF7

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Abstract

Background: Circ_0001971 has been shown to play an important role in cancer development, yet its mechanism of action in hepatocellular carcinoma (HCC) remains somewhat unknown. **Methods:** RNA sequence analysis was performed on HCC tissues and paired non-tumor tissues to detect differentially expressed Rnas. qPCR was used to detect the expression level of circ_0001971. CCK-8, Transwell, RNA immunoprecipitation experiment, WB detection, Double luciferase reporter gene assay et al. were used to analyze the interaction between circ_0001971 and miR-186-5p/KLF7. **Results:** Circ_0001971 expression was significantly upregulated in HCC cells; circ_0001971 inhibition significantly inhibited proliferation, migration and invasion of HCC cells ($P<0.05$); miR-186-5p was a downstream target of circ_0001971; KLF7 was a target gene of miR-186-5p; circ_0001971 can indirectly positively regulate KLF7. **Conclusions:** Circ_0001971 aggravates HCC progression by activating miR-186-5p/KLF7.

Keywords

Circular RNA, Circ_0001971, hepatocellular carcinoma cells, miR-186-5p/KLF7

1. Introduction

Liver cancer is the third leading cause of death in the world according to global cancer statistics 2020, of these, 75% to 85% are hepatocellular carcinomas (HCC) [1]. Although the development of surgical resection, transplantation and targeted drug therapy have improved the efficacy of HCC, the long-term survival prognosis of patients is still not optimistic due to distant metastasis, drug side effects and drug resistance [2]. Thus, it is urgently necessary to investigate the molecular mechanism of the development of HCC and find more diagnostic markers to improve the therapeutic efficacy of HCC patients.

Circular RNAs (circRNAs), as part of the noncoding RNA family, which is a circular closed-loop structure formed by reverse splicing without 3' poly (A) tail and 5' ends [3]. So far, more and more evidence have shown that circRNAs have been involved in the development and progression of HCC [4]. Circ_0001971 is one of many circRNAs, which serves an important role in multiple cancers [5]. For example, circ_0001971 enhance OSCC cell proliferation of by interacting with miR-194 and miR-204 in vitro and in vivo [5]. However, the role of the circ_0001971 in HCC remains unclear.

The research on the mechanism of competing endogenous RNAs (ceRNAs) has revealed that as miRNA sponge, circRNAs can regulate gene expression through competing with the downstream target gene mRNA to bind

miRNA [6]. MiR-186-5p/KLF7 is a small endogenous non-coding RNA molecule with 22 nucleotides, has been reported to be associated with proliferation, migration, and invasion of numerous cancers, such as gastric cancer [7-9], colon cancer [10, 11] and lung cancer [12]. However, the relationship between circ_0001971 and miR-186-5p in HCC remains unclear. Therefore, the aim of this study is to investigate the role of circ_0001971 in HCC and whether circ_0001971 affects the proliferation, migration and invasion of HCC by regulating the expression of miR-186-5p.

2. Methods

2.1 Clinical Tissue Samples

The HCC tissues (n=30) and adjacent non-tumor tissues (n=30) were collected from patients at our hospital from 2020 to 2022, which were obtained from surgery and were treated with other antineoplastic therapy (including chemotherapy and radiotherapy) before surgery. The clinical specimens were immediately frozen in liquid nitrogen, and then stored at −80°C refrigerator for further experiments. This research was authorized by the Hospital Ethics Committee of our hospital and written informed consent was obtained from all patients before the surgery. All clinical trials were performed in accordance with the provisions of the "Declaration of Helsinki".

2.2 Inclusion and exclusion criteria.

The inclusion criteria are as follows: 40-80 years old; pathological examination confirmed it; patients provided written informed consent; The adjacent nontumor tissues were obtained 2 cm away from the tumor tissues. The exclusion criteria are as follows: patients receive preoperative chemotherapy or radiation therapy.

2.3 Cell culture

Human hepatoma cell lines Hep3B, HepG2, PLC5 and Huh-7 and human stem cell line HL-7702 were obtained from the U.S. Typical Culture Preservation Center (ATCC, USA). Hep3B, HepG2, PLC5, Huh-7 and HL-7702 were implanted in 10%FBS and 1% Hyclone DMEM for culture, respectively.

2.4 Construction of siRNA and Cell Transfection

We have obtained Small inter-fering RNA (siRNA) for circ_0001971 and the siRNA negative control (si-NC) from RiBo Biotech (Guang-Zhou, China).There were subcloned into pcDNA3.1, and then the inhibition vector of circ_0001971 (si-circ_0001971) and its control (si-NC) were generated. We were planted PLC5 or HepG2 cells on 6-well plates to about the confluence of 60% in each well, and then transfectedsi-circ_0001971 or si-NC into the prepared cells by Lipofecta-mine 2000 (Invitrogen, USA) pursuant the protocol. Before using in subsequent experiments, cells were cultured for 24 h. And cells with circ_0001971 inhibition were obtained. Pursuant the protocol, cells were prepared on the plates and then, transfected miR-186-5pmimics, mimics NC, miR-186-5p inhibitor and inhibitor NC into the target cells by Lipofectamine 2000.

2.5 RNA extraction and real-time quantitative PCR

Trizol (Invitrogen, USA) was used to extract total RNA from tissue samples and reverse transcribed into cDNA using TaKaPa kit. cDNA samples were taken for real-time PCR using remix Ex Taq II (Takara, Beijing, China), with GAPDH and U6 as internal references. The relative expression of circ_0001971 was calculated using $2^{-\Delta\Delta^{CT}}$.

2.6 Detection by CCK-8 method

CCK-8 kit was used to detect cell viability. The transfected cells were placed in 96-well plates with the number of $3*10^3$/well. After 24h culture, 10μlCCK-8 reagent was added into each well at 24h, 48h, 72h and 96h, respectively. The temperature was 37℃ and the incubation time was 1h, and the optical density (D) value of each hole was detected by spectrophotometer at 450mm.

2.7 Transwell Assay

Transfected 150μl cell suspension was placed into the upper chamber of Transwell containing 10%FBS, the cell concentration was adjusted to 4*105/ml, and 20%FBS was added into the lower chamber. The incubator temperature was 37°C. After 24h of culture, the chamber was removed and cleaned with PBS for 3 times. The migrated cells were injected with 95% methanol for 10min, fixed with 0.5% crystal violet, rinsed to remove crystal violet after 20min and observed under an optical microscope.

2.8 RNA immunoprecipitation experiment.

Mmagna RIP RNA-binding protein underwear precipitation kit was used to verify the targeting relationship between circ_0001971 and miR-186-5p, and the operation process was conducted in strict accordance with the kit instructions. The cells were lysed in RIP cell lysis buffer and immunoprecipitated magnetic beads were added. 100μl cell lysate and magnetic beads containing human anti-Argonaute2 (Ago2) antibody or negative control IgG antibody were incubated in RIP buffer. The samples were added to the protease K buffer and suspended, incubated with oscillations and obtained RNA precipitates, purified RNA, and analyzed by qPCR.

2.9 WB detection.

Total protein was extracted with protein extraction reagent and detected with BCA protein concentration kit. 40μl protein samples were selected as 5% concentrated glue and 10% separated glue for loading. After electrophoresis, the proteins were transferred to PVDF membrane at a constant current of 200mA for 90min. PVDF membrane was enclosed in skim milk at room temperature for 2 h, primary antibody was added, and incubated at 4°C overnight. Wash three times with TBST. The membrane was incubated with the secondary antibody solution at room temperature for 2h and washed three times with TBST. Proteins are detected in the ECL detection system.

2.10 Double luciferase reporter gene assay

Double luciferase reporter assay was used to confirm the direct relationship between circ_0001971 and miR-186-5p and miR-186-5p and KLF7. The cells were prepared in a 48-well plate with a cell density of 4.5*104 cells per well and cultured for 12h. The cells were transfected with luciferase plasmid, and the indicated wild and mutant sequences were transfected with Lipofectamine 2000, and then transfected into the indicated cells. After 48h, the luciferase activity was analyzed using a double luciferase reporting system.

2.11 Statistical analysis

SPSS21.0 statistical software and GraphPad Prism 6.0 were used for data processing. Chi-square test was used for counting data, and T-test was used for comparison among groups. SNK method was used for post hoc analysis. 0.05 indicates a statistically significant difference.

3. Results

3.1 circ_0001971 was highly expressed in HCC tissues.

The expression of circRNA in 3 pairs of tumor tissues and non-tumor tissues was analyzed by RT-PCR technology, and the top 10 differentially expressed circrna were screened, and the heat map was drawn. circ_0001971 expression was detected by RTPCR in 30 pairs of HCC tissues and 30 pairs of adjacent normal tissues, and the detection results showed that circ_0001971 was significantly increased in 30 pairs of HCC tissues. According to the expression level of circ_0001971, 30 pairs of HCC tissues were divided into high circ_0001971 expression group and low circ_0001971 expression group. Clinical pathological analysis showed that, There were significant differences in tumor size, distant metastasis and TNM staging between the high circ_0001971 expression level group and the low circ_0001971 expression level group (P<0.05), see Table 1.

Table 1. Comparison of clinicopathological features of HCC patients with different circ_0001971 levels

determinant		n（n=30）	High circ_0001971 expression group（n=15）	Low circ_0001971 expression group（n=15）	X^2/P
gender	male	16	6	10	2.143/0.143
	female	14	9	5	
age	≥60	12	8	4	2.222/0.136
	< 60	18	7	11	
diameter of tumor	≥5cm	17	11	6	5.129/0.023
	< 5cm	13	3	10	
distant metastasis	yes	16	12	4	6.467/0.011
	no	14	4	10	
TNM-staging	I-IIperiod	15	3	12	10.800/0.001
	III-IVperiod	15	12	3	

3.2 circ_0001971 inhibition inhibits HCC cell proliferation, migration and invasion.

After transfection of si-circ_0001971 into HCC cells, the expression of circ_0001971 was significantly inhibited. The results of CCK-8 assay showed that compared with the control group, the cell viability was significantly inhibited after circ_0001971 inhibition for 72h and 96h (P< 0.05). Transwell assay showed that compared with the control group, the number of migrating and invading cells decreased significantly after inhibition of circ_0001971 for 24h ($P< 0.05$ or $P<0.01$).

3.3 miR-186-5p was decreased in HCC tissues.

In order to further explore circ_0001971 role in HCC mechanism, through CircInteractome website (https://circinteractome.nia.nih.gov/) forecast, there was a binding site between miR-186-5p and circ_0001971. The results of double luciferase reporter assay showed that miR-186-5p and circ_0001971 were highly enriched in the Ago2 antibody group, but did not show significant enrichment in the negative control group. In addition, circ_0001971 inhibition significantly upregulated the expression of miR-186-5p in HCC cells.

3.4 Up-regulation of miR-186-5p can inhibit the proliferation and migration of HCC cells.

miR-186-5p and its analog NC were transfected into HCC cells, and miR-186-5p was overexpressed in HCC cells. However, CCK-8 analysis showed that overexpression of miR-186-5p significantly inhibited HCC cell viability compared with miR-186-5p mimics group. Transwell assay showed that the overexpression of miR-186-5p significantly inhibited the migration ability of HCC cells.

3.5 Elevated KLF7 levels are the direct target of miR-186-5p in HCC cells.

By Targetscan (http://www.targetscan.org/vert_72/) predict the database found that KLF7 is miR - 186-5 p one of potential target genes. Dual luciferase reporter gene experiments show that the transfection miR - 186-5 p mimic can significantly inhibitory KLF7 - wild luciferase activities of groups of cells ($P< 0.01$) and had no significant effects on luciferase activity of KLF7-Mutant group.

3.6 Circ_0001971 promotes proliferation and migration of human hepatoma cells through interaction with miR-186-5p/KLF7 axis

In order to explore the role of Circ_0001971 in the miR-186-5p/KLF7 axis, si-circ_0001971 was transfected into HCC cells, and then miR-186-5p inhibitor was transfected into HCC cells. The experimental results showed that after transfection of si-circ_0001971, circ_0001971 was significantly inhibited, and miR-1236-3p was increased; However, circ_0001971 was elevated, and miR-186-5p was inhibited after co-transfection with miR-186-5p inhibitors in HCC cells. CCK-8 assay results showed that the reduction of cell viability in si-circ_0001971 was saved after co-transfection with miR-186-5p inhibitor in HCC cells. Transwell analysis showed that the inhibited migration and invasion ability of si-circ_0001971 cells was saved after co-transfection with miR-186-5p inhibitor in HCC cells. WB analysis showed that the protein levels of KLF7, Cyclin D1, Cycin E and N-cadherin in si-circ_0001971 decreased after transfection with miR-186-5p inhibitor. However, the accelerated E-cadherin level decreased in si-circ_0001971 after co-transfection with miR-186-5p inhibitor.

4. Discussion

The etiology of HCC is complex [13], and obesity, excessive alcohol intake, aflatoxin exposure and chronic HBV/HCV infection are common risk factors for HCC. However, the specific mechanism of HCC development is still controversial, and most scholars believe that its development is highly correlated with circRNA [14]. circRNA is a special non-coding RNA [15], mainly involved in gene expression regulation at the transcriptional and post-transcriptional levels. By regulating the expression of downstream target genes, mirnas and proteins, CircrNA can affect the proliferation [16], migration, invasion and apoptosis of tumor cells, etc., thus affecting the occurrence and development of HCC. In this study, it was found that circ_0001971 can significantly affect the proliferation, migration and invasion of HCC cells, indicating that circ_0001971 is expected to be a new diagnostic marker and molecular therapeutic target for HCC.

miRNA is a class of non-coding micrnas, which can act on different target genes and thus play an obvious regulatory role in the proliferation, migration, invasion, apoptosis and other aspects of tumor cells [17]. Recent studies on miR-186-5p have shown that it inhibits HCC cell proliferation and migration through its inhibitory effect on YAP1 protein [18]. In this study, through the analysis of circ_0001971 potential downstream miRNA, it was found that miR-186-5p may be one of the potential downstream targets of circ_0001971, and the overexpression of miR-186-5p significantly inhibited the viability and migration ability of HCC cells, and effectively inhibited the

evil biological behavior of HCC cells.

KLFs is a class of transcription factors, which plays an important role in the regulation of cell proliferation, differentiation and embryonic development [19]. Currently, some studies have proved that KLF7 is highly correlated with the occurrence and development of tumor cells [20], and it can be used as a molecular marker for some cancers with adverse effects after rain. The level of KLF7 was significantly increased in HCC cells and was negatively correlated with miR-186-5p. However, the experimental results of this study showed that after transfection with miR-186-5p inhibitors, the reduced cell viability and inhibited migration and invasion ability of HCC cells were improved. This suggests that circ_0001971 can promote the proliferation, migration and invasion of HCC cells by activating miR-186-5p/ KLF7.

In conclusion, in this study, we found that circ_0001971 promoted the proliferation, migration, invasion and other evil biological behaviors of HCC cells by activating the miR-186-5p/ KLF7 axis, suggesting that circ_0001971 could be used as a new molecular marker to predict the progression of HCC disease. This provides new insights into the diagnosis and treatment of HCC.

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