Circ_0064288 acts as an oncogene of hepatocellular carcinoma cells by inhibiting miR-335-5p expression and promoting ROCK1 expression

Yingying Nie1, Xuedan Zhu2, Nan Bu3, Yang Jiang1, Yue Su3, Keming Pan3 and Shanshan Li3*

Abstract

**Background:** Reportedly, circular RNA (circRNA) is a key modulator in the development of human malignancies. This work is aimed to probe the expression pattern, biological effects and mechanism of circ_0064288 on hepatocellular carcinoma (HCC) progression.

**Methods:** The differentially expressed circRNA was screened by analyzing the expression profiles of circRNAs in HCC tissues and normal tissues. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to examine the expression of circ_0064288, miR-335-5p and Rho associated coiled-coil containing protein kinase 1 (ROCK1) mRNA in HCC specimens. After circ_0064288 was overexpressed or knocked down in HCC cells, cell growth was detected by the CCK-8 experiment, and cell migration was evaluated using Transwell experiment and scratch healing experiment. The targeting relationship between miR-335-5p and circ_0064288 and ROCK1 mRNA was predicted and verified using bioinformatic analysis and dual-luciferase reporter gene experiments, respectively. Western blot was executed to examine ROCK1 protein expression in HCC cells.

**Results:** Circ_0064288 and ROCK1 expression was up-modulated in HCC, while miR-335-5p was down-modulated. High circ_0064288 expression was associated with shorter survival time of HCC patients. It was also revealed that circ_0064288 overexpression remarkably enhanced HCC cell growth and migration, while knockdown of circ_0064288 induced opposite effects. Additionally, circ_0064288 could competitively bind with miR-335-5p thereby up-modulate ROCK1 expression. MiR-335-5p overexpression partly counteracted the effect of circ_0064288 overexpression on HCC cells.

**Conclusion:** Circ_0064288 facilitates HCC cell growth and migration by modulating the miR-335-5p/ROCK1 axis.

**Keywords:** HCC, circ_0064288, miR-335-5p, ROCK1

Introduction

Hepatocellular carcinoma (HCC) is the most common histological subtype of liver cancer, taking up around 90% of primary liver cancer cases [1]. Statistically, HCC is the third major cause of cancer-related death worldwide [2]. Although hepatectomy, liver transplantation, chemotherapy and radiotherapy can extend the survival of HCC patients to some extent, the high aggressiveness and...
recurrence rates of HCC still result in adverse prognosis for most patients [3]. Hence, it is imperative to clarify the molecular mechanisms of HCC.

Circular RNAs (circRNAs) are a class of non-coding RNA transcripts that possess a covalent closed-loop structure, formed by reverse splicing of precursor mRNA (pre-mRNA), and do not possess the 5′ end cap structure and 3′ end polyadenylate tail, and the closed-loop structure makes them resistant to cleavage by ribonucleic acid exonuclease, so they are much more stable than linear RNA [4]. The specific expression characteristics and biological functions of circRNAs in tumors confer their potential as tumor biomarkers and therapeutic targets [5, 6]. For instance, circ_0000517 expression is higher in HCC tissues than in paracancerous tissues, and high circ_0000517 expression is linked to unfavorable prognosis in HCC patients [5]. However, the expression characteristics and biological function of most circRNAs in HCC are still undefined.

MicroRNAs (miRNAs) are highly conserved and single-stranded small non-coding RNAs, and these small molecules influence almost all the aspects of biological processes [7]. A lot of miRNAs are reported to participate in the carcinogenesis and progression of HCC [8]. Recent research unveils that circRNAs can work as molecular sponges of miRNAs, and modulate mRNA translation by decoying miRNAs [9]. For instance, circ_0001955 enhances HCC tumorigenesis by adsorbing miR-516a-5p to promote the expression of TRAF6 and MAPK11 [10].

Circ_0064288, also known as circRNA_103285, was generated from the transcript of autophagy related 7 (ATG7), which is reported to be up-regulated in HCC samples [11]. In this work, two Gene Expression Omnibus (GEO) datasets (GSE94508 and GSE97332) were analyzed to look for the differentially expressed circRNAs in HCC, and it was revealed that circ_0064288 was a potential up-regulated circRNA in HCC. We hypothesized that circ_0064288 was an oncogenic circRNA in HCC. Interestingly, bioinformatics analysis suggested that miR-335-5p was a potential downstream target of circ_0064288, and Rho associated coiled-coil containing protein kinase 1 (ROCK1) was a potential downstream target of miR-335-5p. This study was performed to clarify the expression pattern, biological function and the downstream competitive endogenous RNA (ceRNA) mechanism of circ_0064288 in HCC. Herein, we report that circ_0064288, which is highly expressed in HCC, acts as an oncogene by inhibiting miR-335-5p expression and promoting ROCK1 expression.

**Materials & methods**

**Tissue samples**

HCC tissues and paracancerous tissues were collected from 36 patients who received hepatectomy in Jiamusi Central Hospital from May 2012 to April 2014. All subjects did not received radiotherapy or chemotherapy before undergoing tumor resection. The research was endorsed by the Ethics Committee of Jiamusi Central Hospital and written informed consent was acquired from each subject.

**Acquisition of GEO data**

GEO database is a public database that provides access to gene expression datasets. CircRNA microarray datasets of HCC were searched using the following keywords “circRNA” and “liver cancer”, and GSE94508 and GSE97332 were obtained. The corresponding raw data were then downloaded and analyzed with GEO2R online analysis tool. The circRNAs with P < 0.05 and \( |\log_{2}(\text{Fold Change})| > 1 \) in each data set were regarded the differentially expressed circRNAs.

**Cell culture and transfection**

Human HCC cell lines (Huh7, Hep3B, HCCLM3, and MHCC97-L) and human normal liver epithelial cell line (THLE-3) were procured from American Type Culture Collection (ATCC, Manassas, VA, USA) and Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA) and 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C with 5% CO₂. Circ_0064288 overexpression plasmid pcDNA3.1-circ_0064288 (circ_0064288), empty vector (NC), circ_0064288 siRNA (si-circ_0064288#1 and si-circ_0064288#2), scramble siRNA (si-NC), miR-335-5p mimics/miR-335-5p inhibitors and negative controls (mimics NC/inhibitors NC) were procured from Invitrogen (Carlsbad, CA, USA). Transient transfection was performed with Lipofectamine®2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s direction.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

Homogenized HCC tissue and cells were collected, and total RNA was extracted using TRIzol Reagent (Yeasen Biotech, Shanghai, China). cDNA synthesis was implemented using the TaqMan MiRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) for miR-335-5p and employing a PrimeScript RT Master Mix Kit (TaKaRa, Dalian, China) for ROCK1 and circ_0064288, respectively. Then with cDNA as the template, qRT-PCR was implemented to determine the relative expressions of ROCK1 and circ_0064288 with the SYBR® Premix Ex Taq™ II kit (TaKaRa, Dalian, China).
were incubated at 37 °C for 2 h and the absorbance at 450 nm was measured with a microplate reader. Subsequently, the cells were supplemented to each well at the indicated time points (0 h, 24 h, 48 h, 72 h, 96 h). 10 μL of CCK-8 solution (Beyotime, Shanghai, China) was added to each well, and then cultured with serum-free medium. Photographs were taken at 0 h and 24 h after scratch formation and the width of the scratch was recorded.

Western blot

Transfected HCC cells were lysed using RIPA lysis buffer (Beyotime, Shanghai, China), the supernatant of the lysate was harvested to collect the total protein. A BCA protein assay kit (Beyotime, Haimen, China) was used to determine protein concentration. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane. 5% skim milk was used to block the membrane for 1 h at room temperature. Subsequently, anti-ROCK1 antibody (1:1000, ab134181, Cambridge, UK) and anti-GAPDH antibody (1:2000, ab18245, Cambridge, UK) were applied to incubate the PVDF membrane at 4 °C overnight. The next day, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000, Beyotime, Shanghai, China) for 50 min at room temperature. The protein bands were visualized using a Beyotime ECL (electrochemiluminescence) kit (Biosharp, Hefei, China).

Luciferase reporter experiment

StarBase database was utilized to predict the binding sites between miR-335-5p and circ_0064288 / ROCK1 3’UTR, respectively, and the binding fragments of circ_0064288 and ROCK1 3’UTR were amplified by PCR. The amplification products were inserted into the PGL3-promoter plasmid vector (Promega, Madison, WI, USA) to construct the circ_0064288 and ROCK1 wild-type (WT) plasmids; the binding fragment was mutated using gene mutation technology to construct the circ_0064288 and ROCK1 mutant type (MUT) plasmid. The recombinant plasmids were co-transfected with miR-335-5p mimic (or mimic NC) respectively in HEK-293 T cells. After 48 h, the cells were collected. Fluorescence values were measured using a dual-luciferase reporter gene assay system (Promega, Madison, WI, USA).

Statistical analysis

All of the experiments were performed at least in triplicate, and at least repeated for 3 times. All data were expressed as “mean ± SD”, and GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA, US) was employed for graphing and

| Table 1 Primer sequences |
|-------------------------|
| Name                    | Primer sequences                             |
| circ_0064288            | Forward: 5'-TTGAACACGACGCAAATGAG-3'          |
|                         | Reverse: 5'-AATACTGGAGCGAACAGC-3'            |
| miR-335-5p              | Forward: 5'-UGUUGUGAAGCUGGUGAACAGC-3'       |
|                         | Reverse: 5'-UUCUCAGAUUGCUAAUUAUCA-3'        |
| ROCK1                   | Forward: 5'-AACATCGGTCTGGATAAACTCGG-3'       |
|                         | Reverse: 5'-TGTATCACATCTGACATGATTG-3'        |
| U6                      | Forward: 5'-CTCCAGTCCGAGCGACA-3'             |
|                         | Reverse: 5'-AACGGTCAGGATTCTGGT-3'            |
| GAPDH                   | Forward: 5'-GGAAAACTGTCGCGTGAATGTTG-3'       |
|                         | Reverse: 5'-GATGGGGTTCGCTGTTGA-3'            |

CCK-8 experiment

Transfected HCC cells were planted in 96-well plates (2 × 10^4 cells/well) containing 100 μL of medium/well. 10 μL of CCK-8 solution (Beyotime, Shanghai, China) was supplemented to each well at the indicated time points (0 h, 24 h, 48 h, 72 h, 96 h). Subsequently, the cells were incubated at 37 °C for 2 h and the absorbance at 450 nm was measured with a microplate reader.

Transwell experiment

Transwell chambers (Corning Life Sciences, Corning, NY, USA) were utilized to detect the migration of HCC cells. The cells of each group in the logarithmic growth period were randomly selected under a microscope, and the cells were stained with 0.1% crystal violet. Five fields of view were taken, trypsinized with 0.25% trypsin and re-suspended in serum-free medium, and the cell concentration was modulated to 1 × 10^5 cell/ml. In the top compartment, 200 μL of cell suspension was supplemented, and 500 μL of medium containing 10% FBS was added into the bottom compartment, and subsequently, the cells were cultured for 24h. Next, methanol-fixed migrated cells were stained with 0.1% crystal violet. Five fields of view were randomly selected under a microscope, and the cells were counted, and the average value was calculated.
statistical analysis. Kolmogorov-Smirnov test was used to examine the normality and equal variance of the data. Student’s t-test was utilized to make the comparison between the two groups. One-way ANOVA and Dunnett’s post-hoc multiple comparisons were employed for comparisons among three or more groups. For data that were skewed distributed, comparisons between two groups were performed by Wilcoxon signed-rank test. Chi-square test was employed to elucidate the correlation between LINC00518 expression and pathological parameters. Kaplan-Meier survival curve and log-rank test were adopted to compare patient’s prognosis in different groups. Gene expression correlations were analyzed by Pearson correlation coefficient. $P < 0.05$ signified statistical significance.

Results

Circ_0064288 is overexpressed in HCC

GSE94508 and GSE97332 were obtained from the GEO database, and circRNAs’ expression levels in HCC tissues and adjacent tissues were analyzed online using GEO2R tool. The volcano plot showed the expression patterns of the circRNAs in the datasets (Fig. 1A-B). 72 circRNAs were up-modulated in both datasets, and circ_0064288 was among them ($\log_2$ fold change $= 1.11$ and $1.14$, respectively) (Fig. 1C, Table 2, Supplementary Table 1, Supplementary Table 2). qRT-PCR was executed to examine circ_0064288 expression in normal hepatic epithelial cell line (THLE-3) and HCC cell lines (Huh7, Hep3B, HCCLM3, and MHCC97-L) was measured by qRT-PCR. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. 

![Fig. 1](image-url)
associated with larger tumor size of the patients ($P = 0.042$, Table 3). Furthermore, a Kaplan-Meier survival curve was plotted, and log-rank test showed that high circ_0064288 expression was remarkably with shorter overall survival time of the HCC patients ($P = 0.0443$, Fig. 1E). Besides, circ_0064288 expression was markedly augmented in HCC cell lines [Huh7 ($P < 0.001$), Hep3B ($P < 0.001$), HCCLM3 ($P < 0.001$), and MHCC97-L ($P < 0.001$)] relative to normal hepatic epithelial cell line (THLE-3) (Fig. 1F).

### Table 2 The up-regulated circRNAs in HCC in both GSE97332 and GSE94508

| Up-regulated circRNAs            |
|----------------------------------|
| circRNA_101441, circRNA_103316,| |
| circRNA_102148, circRNA_101798,| |
| circRNA_101313, circRNA_400087,| |
| circRNA_103348, circRNA_105363,| |
| circRNA_100090, circRNA_103809,| |
| circRNA_102559, circRNA_101051,| |
| circRNA_104268, circRNA_103038,| |
| circRNA_102034, circRNA_104575,| |
| circRNA_104760, circRNA_101195,| |
| circRNA_101144, circRNA_101707,| |
| circRNA_102954, circRNA_104993,| |
| circRNA_000872, circRNA_104017,| |
| circRNA_100571, circRNA_101109,| |
| circRNA_001416, circRNA_104003,| |
| circRNA_103546, circRNA_400071,| |
| circRNA_103910, circRNA_000792,| |
| circRNA_400091, circRNA_101287,| |
| circRNA_104499, circRNA_000996,| |
| circRNA_103385, circRNA_101201,| |
| circRNA_104336, circRNA_102482,| |
| circRNA_104771, circRNA_101777,| |
| circRNA_100445, circRNA_000956,| |
| circRNA_102587, circRNA_100053,| |
| circRNA_103213, circRNA_104475,| |
| circRNA_001069, circRNA_104833,| |
| circRNA_001506, circRNA_400048,| |
| circRNA_103188, circRNA_105027,| |
| circRNA_10367, circRNA_101748.  | |

### Table 3 Correlation between circ_0064288 expression and clinicopathological features of the 36 HCC patients

| Characteristics                        | Number | circ_0064288 expression | P value |
|----------------------------------------|--------|-------------------------|---------|
|                                        |        | Low | High |                  |         |
| Age (years)                            |        |     |     |                  |         |
| ≤ 50                                   | 9      | 3   | 6   | 0.441             |         |
| > 50                                   | 27     | 15  | 12  |                    |         |
| Gender                                 |        |     |     |                  |         |
| Male                                   | 22     | 11  | 11  | 1.000             |         |
| Female                                 | 14     | 7   | 7   |                    |         |
| TMN stage                              |        |     |     |                  |         |
| I/II                                   | 13     | 9   | 4   | 0.165             |         |
| III/IV                                 | 23     | 9   | 14  |                    |         |
| Tumor size (cm)                        |        |     |     |                  |         |
| < 5                                    | 15     | 11  | 4   | 0.042*            |         |
| ≥ 5                                    | 21     | 7   | 14  |                    |         |
| Vascular invasion                      |        |     |     |                  |         |
| Yes                                    | 9      | 2   | 7   | 0.124             |         |
| No                                     | 27     | 16  | 11  |                    |         |
| AFP (μg/L)                             |        |     |     |                  |         |
| ≤ 400                                  | 11     | 6   | 5   | 1.000             |         |
| > 400                                  | 25     | 12  | 13  |                    |         |
| Aetiology                              |        |     |     |                  |         |
| HBV infection                         | 26     | 13  | 13  | 0.435             |         |
| Alcoholic liver disease                | 4      | 3   | 1   |                    |         |
| Fatty liver disease & others          | 6      | 2   | 4   |                    |         |

P value was calculated by Fisher's exact test. * denotes $p$ values less than 0.05

Circ_0064288 contributes to the multiplication and migration of HCC cells

Among the four cell lines, the expression of circ_0064288 was the lowest in Huh7 cells (fold change was about 1.8, v.s. THLE-3 cells), but the highest in Hep3B cells (fold change was about 3, v.s. THLE-3 cells), so circ_0064288 was overexpressed in Huh7 cells, and it was knocked down in Hep3B cell (Fig. 1E). circ_0064288 overexpression plasmid and circ_0064288 siRNA (si-circ_0064288#1 and si-circ_0064288#2) and the corresponding controls (NC or si-NC) were transfected into Huh7 and Hep3B cells, respectively, to construct circ_0064288 overexpression and circ_0064288 knockdown cell models (Fig. 2A). si-circ_0064288#1 knockdown efficiency was higher (knockdown efficiency of about 80% in si-circ_0064288#1 group, knockdown efficiency of about 70% in si-circ_0064288#1 group), so si-circ_0064288#1 was selected for subsequent experiments (Fig. 2A). The data of CCK-8 experiment revealed that the cell growth in circ_0064288 overexpression group was notably promoted than that of the control group ($P < 0.001$) (Fig. 2B). Moreover, in Transwell assay, after circ_0064288 was overexpressed, the number of Huh7 cells which passed the filter was increased (fold change $\approx 1.6$, $P < 0.01$, v.s. the control group); in wound-healing assay, the speed of scratch healing in circ_0064288 overexpression group is also higher (fold change $\approx 1.6$, $P < 0.01$, v.s. the control group) (Fig. 2C-D). These experiments suggested that circ_0064288 worked as an oncogenic factor in HCC.

Circ_0064288 functions as ceRNA and sponges miR-335-5p

Increasing research has reported that circRNAs act as ceRNA to regulate miRNAs in cancer biology [9, 12]. Candidate miRNA targets of circ_0064288 were analyzed online using two bioinformatics tools (StarBase and
Circular RNA Interactome), and three common miRNAs (miR-335-5p, miR-498, miR-574-5p) were predicted by both of two bioinformatics tools (Fig. 3A). qRT-PCR revealed that circ_0064288 was mainly distributed in the cytoplasm, suggesting it could probably function as a ceRNA (Fig. 3B). Dual-luciferase reporter gene experiment demonstrated that miR-335-5p repressed the luciferase activity of circ_0064288-WT reporter (inhibition efficiency is about 70%, \( P < 0.001 \)), but exerted no remarkable effect on luciferase activity of circ_0064288-MUT reporter (\( P > 0.05 \)) (Fig. 3C). Further, miR-335-5p expression was remarkably weakened in HCC cell lines [Huh7 (\( P < 0.001 \)), Hep3B (\( P < 0.001 \)), HCCLM3 (\( P < 0.001 \)), and MHCC97-L (\( P < 0.001 \))] relative to normal hepatic epithelial cell line (Fig. 3E). In addition, miR-335-5p expression was negatively correlated with circ_0064288 expression in HCC tissues (\( R^2 = 0.5730, P < 0.001 \)) (Fig. 3F). Collectively, there was a binding relationship between miR-335-5p and circ_0064288, and there could probably be a regulatory relationship between them in HCC cells.

**MiR-335-5p counteracts the promoting effects of circ_0064288 on HCC progression**

To probe the role of circ_0064288 and miR-335-5p in modulating the growth and migration of HCC cells, Huh7
cells overexpressing circ_0064288 were co-transfected with miR-335-5p mimics (Fig. 4A). The data of the rescue assay showed that the effect of circ_0064288 overexpression in facilitating the proliferation ($P < 0.001$) and migration ($P < 0.05$ in Transwell assay, $P < 0.01$ in wound healing assay) of Huh7 cells was counteracted by miR-335-5p (Fig. 4B-D). The above results indicated that the effect of circ_0064288 in facilitating the malignant phenotypes of Huh7 cells was probably dependent on miR-335-5p.

**Circ_0064288 augmented ROCK1 expression in HCC cells by targeting miR-335-5p**

With three bioinformatics algorithms including miRDIP, StarBase and Targetscan, we found that 160 potential target genes were predicted, including ROCK1 (Fig. 5A, Supplementary Table 3). Next, Gene Set Enrichment Analysis (GSEA) was performed, and results demonstrated that high ROCK expression is positively associated with the multiple signal pathways / biological processes, including DNA replication, cell cycle, Notch signal pathway and mTOR signal pathway ($P < 0.001$, and FDR < 0.25) (Supplementary Fig. 1A-D). Next, the targeting relationship between ROCK1 and miR-335-5p was confirmed by dual-luciferase reporter gene experiment: miR-335-5p repressed the luciferase activity of ROCK1-WT reporter (inhibition efficiency is about 70%, $P < 0.001$), but exerted no remarkable effect on luciferase activity of ROCK1-MUT reporter ($P > 0.05$) (Fig. 5B). StarBase database showed that ROCK1 expression was remarkably higher in HCC tissues than in paracancerous tissues.

![Fig. 3 Circ_0064288 functions as ceRNA and sponges miR-335-5p. A. Bioinformatics databases (StarBase and Circular RNA Interactome) were used to predict the binding site of circ_0064288 to miR-335-5p. B. qRT-PCR was employed to detect circ_0064288 expression in the cytoplasm and nucleus of Huh7 and Hep3B cells. C. Dual-luciferase reporter gene experiment showed that the predicted binding sequence between circ_0064288 and miR-335-5p was functional. D. qRT-PCR was employed to detect miR-335-5p expression in HCC tissues and paracancerous tissues. E. qRT-PCR was used to detect miR-335-5p expression in normal hepatic epithelial cell line (THLE-3) and HCC cell lines (Huh7, Hep3B, HCCLM3, and MHCC97-L). F. The correlation between circ_0064288 expression and miR-335-5p expression in HCC tissues was evaluated by Pearson's correlation coefficient. ***$P < 0.001$, ns, means no statistical significance. ***$P < 0.001$, ns, means no statistical significance.](image-url)
tissues (fold change = 1.39, $P = 0.0039$) (Fig. 5C). qRT-PCR and Western blot experiments showed that transfection of miR-335-5p mimics remarkably repressed ROCK1 expression in Hep3B cells at protein level and mRNA level (fold change < 0.5, $P < 0.001$); conversely, transfection of miR-335-5p inhibitors in Huh7 cells markedly augmented ROCK1 expression at protein level and mRNA level (fold change > 2.5, $P < 0.001$) (Fig. 5D). Circ_0064288 overexpression induced an augmentation in ROCK1 expression at protein level and mRNA level (fold change ≈ 3, $P < 0.001$) (Fig. 5D). Circ_0064288 overexpression induced an augmentation in ROCK1 expression at protein level and mRNA level (fold change < 0.5, $P < 0.001$) (Fig. 5E). Besides, circ_0064288 overexpression induced an increase in ROCK1 expression at protein level and mRNA level (fold change > 2.5, $P < 0.001$), and that could be counteracted by the co-transfection of miR-335-5p mimics at protein level and mRNA level (fold change ≈ 0.6, $P < 0.001$) (Fig. 5F). The above results implied that ROCK1 was a target of miR-335-5p and circ_0064288 elevated ROCK1 expression by targeting miR-335-5p in HCC cells.

**Discussion**

CircRNA can affect gene expression through different mechanisms [13, 14]. In the nucleus, because circRNAs have repetitive sequences with parental DNA, when the accumulation of circRNAs in the nucleus increases, the circRNAs that remain in the nucleus will directly block the transcription process by forming RNA-DNA heterodimeric chains with parental DNA in a “negative feedback” manner, thus altering gene expression [15]. In the cytoplasm, circRNAs can modulate downstream genes’ expression by working as the molecular sponge for miRNAs [16]. Many circRNAs are identified as ceRNAs in cancer research. For instance, circ_0003998 may work as a ceRNA for miR-143-3p to attenuate the inhibitory effect of miR-143-3p on the epithelial-mesenchymal transition (EMT)-related regulator, FOS like 2, AP-1 transcription factor subunit [17]. In this work, circ_0064288 expression
was revealed to be remarkably up-modulated in HCC. High circ_0064288 expression implied shorter overall survival time of HCC patients. Additionally, circ_0064288 could regulate the growth and metastatic potential of HCC cells. Considering circRNA has more stability than linear non-coding RNA, and it can be detected in not only tumor tissues, but also body fluids such as urine and blood, it is regarded as promising biomarkers for human diseases [18]. Our study suggest that circ_0064288 is a potential biomarker to evaluate the prognosis of HCC patients.

**MiRNAs are key regulators of gene expression [19].** The aberrant expression of miRNAs is closely related to the progression of human malignancies. For example, miR-218 represses the EMT process of HCC cells by targeting serpin mRNA binding protein 1 [20]. MiR-517a enhances the Warburg effect and growth of HCC cells by targeting fructose-1,6-bisphosphatase [21]. Reportedly, miR-335-5p is under-expressed and exerts tumor-suppressive effects in different human cancers. For instance, miR-335-5p impedes the growth, migration and invasion of colorectal cancer cells through down-modulation of lactic dehydrogenase B [22]. MiR-335-5p is also reported to remarkably restrain HCC progression in a previous study [23]. In this work, miR-335-5p was unveiled to be under-expressed in HCC tissues, miR-335-5p was validated to be a target of circ_0064288, and miR-335-5p counteracted the promoting effect of circ_0064288 on the malignancy of HCC cells.

Rho-associated protein kinase (ROCK), which is composed of ROCK1 and ROCK2, is implicated in modulating the movement of cells by functioning on the cytoskeleton...
Reportedly, ROCK1 is also an essential player in regulating cell viability, motility and angiogenesis [26]. ROCK1 overexpression / activation is commonly linked to a more highly metastatic and aggressive phenotype of human cancers. ROCK1 overexpression is reported in many different types of cancer, including glioblastoma [27], melanoma [28], osteosarcoma [29], prostate cancer [30] and HCC [31]. Importantly, ROCK1 works as an oncogene in HCC, and it promotes HCC progression [31, 32]. In this work, ROCK1 was identified as a target of miR-335-5p, circ_0064288 augmented ROCK1 expression through competitive binding with miR-335-5p. Our data suggest that circ_0064288 / miR-335-5p axis is crucial to regulate ROCK1 expression in HCC, and for the first time, a ceRNA network consisting of circ_0064288, miR-335-5p, ROCK1 is presented.

There are some limitations in the present work. First of all, the bioinformatics analysis identified 72 up-regulated circRNAs in HCC, and only circ_0064288 was investigated in this study. The other 71 up-regulated circRNAs and the down-regulated circRNAs may also participate in HCC progression; factually, due to the different platform of microarray and the heterogeneity of HCC samples, different studies usually obtain different differentially expressed circRNAs, and there is a long way to fully clarify the role of circRNAs in HCC progression. Secondly, miR-335-5p has multiple potential downstream genes, not only ROCK1, implying that circ_0064288 / miR-335-5p axis can probably exert its biological function via other downstream mechanisms, which remain to be clarified in the following work. Last but not least, only in vitro experiments were designed in the present work, and in vivo models can further consolidate our conclusion.

**Conclusion**

In conclusion, this work reports that circ_0064288 expression is up-modulated in HCC and miR-335-5p expression was down-modulated in HCC. Circ_0064288 elevates ROCK1 expression by working as a sponge for miR-335-5p, thereby enhancing the malignant phenotype of HCC cells (Fig. 6), which provides a novel idea for the diagnosis and therapy of HCC.

**Abbreviations**

ATCC: American Type Culture Collection; ATG7: Autophagy related 7; ceRNA: Competitive endogenous RNA; CCK-8: Cell counting kit-8; circRNA: Circular RNA; Circ_0064288: Circular RNA_0064288; EMT: Epithelial-mesenchymal transition; FBS: Fetal bovine serum; GEO: Gene Expression Omnibus; HCC: Hepatocellular carcinoma; miRNA: MicroRNA; miR-335-5p: MicroRNA-335-5p; MUT: Mutant type; PVDF: Polyvinylidene fluoride; qRT-PCR: Quantitative real-time polymerase chain reaction; ROCK1: Rho associated coiled-coil containing protein kinase 1; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; WT: Wild type.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s12885-022-09323-8.

**Additional file 1.** Supplementary Table 1.

**Additional file 2.** Supplementary Table 2.

**Additional file 3.** Supplementary Table 3.

**Additional file 4.** Supplementary Figures.

**Acknowledgements**

Not applicable.

**Authors' contributions**

Conceived and designed the experiments: Shanshan Li; Performed the experiments: Yingying Nie, Xuedan Zhu; Analyzed the data: Nan Bu, Yang Jiang, Yue Su, Keming Pan; Wrote the paper: Yingying Nie, Xuedan Zhu, Nan Bu. All authors read and approved the final manuscript.
Funding
Not applicable.

Availability of data and materials
The datasets generated or/and analysed during the current study are available in the GEO datasets repository, [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE94508, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE97332].

Declarations

Ethics approval and consent to participate
The present study was approved by the Ethics Review Board of Jiamusi Central Hospital and performed according to the Declaration of Helsinki, and all participants provided written informed consents.

Consent for publication
Consent for publication has been obtained from all patients.

Competing interests
The authors declare that they have no competing interests.

Author details
1. Department of Gastroenterology, Jiamusi Central Hospital, Jiamusi 154002, Heilongjiang, China. 2. Department of Gastroenterology, The First Affiliated Hospital of Harbin Medical University, Harbin 150000, Heilongjiang, China. 3. Department of Gastroenterology, Jiamusi Hospital of Traditional Chinese Medicine, No.526 Jiefang Road, Jiamusi 154002, Heilongjiang, China.

Received: 23 March 2021 Accepted: 28 January 2022
Published online: 14 March 2022

References
1. Chen J, Zhou J, Fu H, Ni X, Shan Y. Upregulation of oxidative stress-responsive 1 (OXSR1) predicts poor prognosis and promotes hepatocellular carcinoma progression. Bioengineered. 2020;11(1):958–71.
2. Chen VL, Sharma P. Role of biomarkers and biopsy in hepatocellular carcinoma. Clin Liver Dis. 2020;24(4):577–90.
3. Li J, Zhu Y. Recent advances in liver cancer stem cells: non-coding RNAs, oncogenes and oncoproteins. Front Cell Dev Biol. 2020;8:548335.
4. You X, Vlatkovic I, Babic A, Will T, Epstein I, Tushev G, et al. Neural circular RNAs are derived from synaptic genes and regulated by development and plasticity. Nat Neurosci. 2015;18(4):603–10.
5. Wang X, Wang L, Li W, Zhang Q, Chen J, Chen T. Up-regulation of hsa_circ_0003517 predicts adverse prognosis of hepatocellular carcinoma. Front Oncol. 2019;9:1105.
6. Yan K, Cheng W, Xu X, Cao G, Ji Z, Li Y. Circularizing RNAs, circ_{4911} and circ_{4302}, are novel regulators of endothelial cell function under a hepatocellular carcinoma microenvironment. Oncol Rep. 2020;44(4):1727–35.
7. Lin Z, Chen Y, Lin Y, Lin H, Li H, Su X, et al. Potential miRNA biomarkers for the diagnosis and prognosis of esophageal cancer detected by a novel absolute quantitative RT-qPCR method. Sci Rep. 2020;10(1):20665.
8. Wen Y, Han J, Chen J, Dong J, Xia Y, Liu J, et al. Plasma miRNAs as early biomarkers for detecting hepatocellular carcinoma. Int J Cancer. 2015;137(7):1679–90.
9. Han D, Li J, Wang H, Xu S, Hou J, Gu Y, et al. Circular RNA circMT01 acts as the sponge of microRNA-9 to suppress hepatocellular carcinoma progression. Hepatology (Baltimore, Md). 2017;66(4):1151–64.
10. Yao Z, Xu R, Yuan L, Xu M, Zhuang H, Li Y, et al. Circ_{0001955} facilitates hepatocellular carcinoma (HCC) tumorigenesis by sponging miR-516a-3p to release TRAF6 and MAPK11. Cell Death Dis. 2019;10(12):945.
11. Ou Y, He J, Li Y. MiR-490-3p inhibits autophagy via targeting ATG7 in hepatocellular carcinoma. IUBMB Life. 2018;70(6):468–78.
12. Zhang G, Li S, Liu J, Ge Y, Wang Q, Ma G, et al. LncRNA-MT1JIP functions as a ceRNA in regulating FBXW7 through competitively binding to miR-92a-3p in gastric cancer. Mol Cancer. 2018;17(1):87.
13. Zhang HD, Jiang L, Sun DW, Hou JC, Ji ZL. CircRNA: a novel type of biomarker for cancer. Breast Cancer (Tokyo, Japan). 2018;25(1):1–7.
14. Zhang M, Xin Y. Circular RNAs: a new frontier for cancer diagnosis and therapy. J Hematol Oncol. 2018;11(1):21.
15. Conn VM, Hugouvieux V, Nayak A, Conos SA, Capovilla G, Clidir G, et al. A circRNA from SEPALATA3 regulates splicing of its cognate mRNA through R-loop formation. Nat P lants. 2017;3:17053.
16. Qi X, Zhang DH, Wu N, Xiao JH, Wang X, Ma W. ceRNA in cancer: possible functions and clinical implications. J Med Genet. 2015;52(10):710–8.
17. Song LN, Qiao GL, Yu J, Yang CM, Chen Y, Deng ZF, et al. Hsa_circ_{0003998} promotes epithelial to mesenchymal transition of hepatocellular carcinoma by sponging miR-143-5p and PCBP1. J Exp Clinical Cancer Res. 2020;39(1):114.
18. Zhou Q, Ju LL, Ji X, Cao YL, Shao JG, Chen L. Plasma circRNAs as biomarkers in cancer. Cancer Manag Res. 2021;13:7532–37.
19. Guo J, Nam K, Collins G. Effect of miR-21 on renal fibrosis induced by Nano-SiO_{2} in diabetic nephropathy rats via PTEN/AKT pathway. J Nanosci Nanotechnol. 2021;21(2):1070–84.
20. Wang T, Xu L, Jia R, Wei J. MiR-218 suppresses the metastasis and EMT of HCC cells via targeting SERBP1. Acta Biochim Biophys Sin Shanghai. 2017;49(5):383–91.
21. Zhang D, Li Z, Lu T, Luo D, Feng X, Liu Y, et al. miR-S17a promotes Warburg effect in HCC by directly targeting FBP1. OncoTargets Ther. 2018;11:8025–32.
22. Zhang D, Yang N. MiR-353-5p inhibits cell proliferation, migration and invasion in colorectal cancer through downregulating LDLR. J BUON. 2019;24(3):1128–36.
23. Li HW, Liu J. Circ_{0009910} promotes proliferation and metastasis of hepatocellular carcinoma cells through miR-335-5p/ROCK1 axis. Eur Rev Med Pharmacol Sci. 2020;24(4):1725–35.
24. Hu C, Zhou H, Liu Y, Huang J, Liu W, Zhang Q, et al. ROCK1 promotes migration and invasion of non-small-cell lung cancer cells through the PTEN/PIK3/FAK pathway. Int J Oncol. 2019;55(4):833–44.
25. Sheng J, Wang L, Han Y, Chen W, Liu H, Zhang M, et al. Dual roles of protein as a template and a sulfur provider: a general approach to metal sulfides for efficient photothermal therapy of cancer. Small (Weinheim an der Bergstrasse, Germany). 2016;12:16010.
26. Patel RA, Liu Y, Wang B, Li R, Sebi SM. Identification of novel ROCK inhibitors with anti-migratory and anti-invasive activities. Oncogene. 2014;33(5):550–55.
27. Xu S, Guo X, Gao X, Xue H, Zhang J, Guo X, et al. Macrophage migration inhibitory factor enhances autophagy by regulating ROCK1 activity and contributes to the escape of dendritic cell surveillance in glioblastoma. Int J Oncol. 2016;49(5):2105–15.
28. Huang GX, Wang Y, Su J, Zhou P, Li B, Yin LJ, et al. Up-regulation of rho-associated kinase 1/2 by glucocorticoids promotes migration, invasion and metastasis of melanoma. Cancer Lett. 2017;410:1–11.
29. Wang Z, Wang Z, Liu J, Yang H. Long non-coding RNA SNHG5 sponges miR-26a to promote the tumorigeneisis of osteosarcoma by targeting ROCK1. Biomed Pharmacother. 2018;107:598–605.
30. Steurer S, Hager B, Buscheck H, Hofmayer D, Tsourlakis MC, Minder S, et al. Upregulation of rho-associated coiled-coil containing kinase 1 (ROCK1) is associated with genetic instability and poor prognosis in prostate cancer. Aging. 2019;11(18):7859–79.
31. Zhan Y, Zheng N, Feng F, Bao L, Liu F, Zhang M, et al. MiR-199a/b-5p inhibits hepatocellular carcinoma progression by post-transcriptionally suppressing ROCK1. Oncotarget. 2017;8(40):67169–80.
32. Chen K, Zhang L. LINC00339 regulates ROCK1 by miR-152 to promote cell proliferation and migration in hepatocellular carcinoma. J Cell Biochem. 2019;120(9):14431–43.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.