Circular RNA Circ-0003006 Promotes Hepatocellular Carcinoma Proliferation and Metastasis Through Sponging miR-542-3p and Regulating HIF-1A

Qiang Tu1,2, Xiaoxiang You1,2, Jun He1,2, Xuguang Hu1,2, Changji Xie1,2, Guohui Xu1,2

1Department of Hepatobiliary Oncology Surgery, Jiangxi Cancer Hospital of Nanchang University, Nanchang, People’s Republic of China; 2Department of Oncology Interventional, Jiangxi Cancer Hospital of Nanchang University, Nanchang, People’s Republic of China

Introduction: Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and the second leading cause of tumor-related death. In recent years, researchers have made significant progress in the treatment of hepatocellular carcinoma (HCC), including liver resection, liver transplantation, radio-frequency ablation, interventional therapy (chemotherapy and embolization), and comprehensive treatment of targeted drugs. Surgical resection is the best treatment of hepatocellular carcinoma (HCC), but because of hepatocellular carcinoma (HCC) onset conceals, early and no apparent symptoms. Most patients failed to do a related inspection, when liver area pain or particular symptoms, often lesions in the...
middle-late stage, thus intrahepatic and extrahepatic distant metastases, lost the optimal timing of surgery, leading to poor prognosis. In addition, due to the atypical imaging of early micrometastatic lesions and the lack of biomarkers for screening early metastatic lesions, the patients with HCC still have a high recurrence rate after surgical resection, so the overall survival rate of HCC patients is still poor. Therefore, it is of great significance for the patients with HCC to search for biomarkers related to early diagnosis, therapeutic intervention, and prognosis and to study the molecular mechanism of HCC occurrence and metastasis.

CircRNA, as an essential part of non-coding RNAs, has received more and more attention in recent years due to their unique structure and potential functions. In recent years, with the development of biological technology, especially bioinformatics and high-throughput sequencing technology, many circRNAs have been discovered. CircRNAs are a class of abundant, diverse, and highly conserved molecules with tissue-specific and developmental stage-specific expression levels. As a large number of RNAs have been studied, our understanding of their functional mechanisms has deepened. Specifically, circRNAs may sponge on miRNAs, thereby blocking the translation of target gene mRNAs, influencing gene expression by regulating splicing sites, transcription, or interaction with RNA-binding proteins. A large number of studies have shown that circRNAs play an essential role in biological processes. Some circRNAs have a specific expression in tumor tissues and play an essential role in the occurrence and development of tumors, such as pancreatic cancer, bladder cancer, cervical cancer, etc. At present, there are research reports on the role of circular RNA in the occurrence and development of hepatocellular carcinoma. For example, circCAMSAP1 is upregulated in vitro and in vivo and can promote the biological function of HCC. At the same time, circCAMSAP1 promotes HCC proliferation, migration, and invasion through the miR-1294/GRAMD1A pathway so that circCAMSAP1 may be a potential prognostic and therapeutic target for liver cancer. Knockdown of circPVT1 can inhibit the proliferation and glycolysis of liver cancer cells and promote cell apoptosis. circPVT1 can bind to miR-377, inhibiting miR-377 can restore the HCC cell effect mediated by circPVT1 knockdown. At the same time, studies have found that hsa_circ_0007456/miR-6852-3p/ICAM-1 axis is an essential signaling pathway in hepatocellular carcinoma immune escape and tumorigenesis. Since circRNAs have a closed ring structure and are not affected by exonuclease or other factors, their expression is relatively stable, and they have the potential to become tumor biomarkers. Therefore, it is of great significance to study the role of circRNA in tumor development and its specific molecular mechanism and to explore whether circRNA can be used as a stable biomarker in early diagnosis, therapeutic intervention, and prognosis analysis of tumors.

In this study, we screened the differentially expressed circRNAs in HCC through bioinformation analysis and focused on the influence of circ-0003006 on the biological process of HCC. Based on the database of Circbank, we found that the features of circ-0003006, namely, host gene symbol is FLNB and the position is chr3:58111307–58112489. Circ-0003006 was significantly elevated in HCC and effectively promoted the development of hepatocellular carcinoma. Downregulation of circ-0003006 inhibited HCC progression by regulating the miR-542-3p/HIF-1A axis. Therefore, it is suggested that circ-0003006/miR-542-3p/HIF-1A axis may be a new target of HCC.

Materials and Methods

Patients and Clinical Samples

A total of 115 patients with HCC admitted to Jiangxi Cancer Hospital from May 2018 to October 2020 were selected for their tumors and corresponding paracancerous tissue samples, which were collected and quickly stored in a refrigerator at −80°C for detection. All patients were biopsies and pathologically confirmed to be HCC. The Institutional Review Committee has approved this study of Jiangxi Cancer Hospital, and all patients have agreed and signed written informed consent. Studies involving patient specimens: all the tumor specimens and para tumor samples were collected with written informed consent in accordance with the Declaration of Helsinki and with the approval of the Ethical Committee of Jiangxi Cancer Hospital (Approval No. CHX-20180136, approve date: July 17, 2019).

Cell Lines and Cell Culture

Human hepatocellular carcinoma cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The corresponding complete growth medium was selected for them needed. The hepatocellular
carcinoma cells were cultured in an incubator at 37°C, 5% CO2, and saturated humidity for routine culture.

**Real-Time Fluorescent Quantitative PCR Detection (qRT-PCR)**

The trizol method was used to extract total cell RNA and detect RNA concentration. Reverse transcription was used to synthesize single-stranded cDNA (Applied Biosystems, Carlsbad, CA, USA), and PCR amplification was performed according to the real-time fluorescent quantitative PCR kit instructions (TaKaRa Biotechnology Co. Ltd., Liaoning China). The PCR primers (Additional File 1: Table 1) were designed by Guangzhou Ruibo Biotechnology Co., Ltd. (Guangzhou, China). The PCR amplification conditions are as follows: 94°C pre-denaturation for 2 minutes, 1 cycle; 94°C denaturation for 30 seconds, 62°C annealing for 30 seconds, 72°C extension for 30 seconds, and 38 cycles. The experiment was repeated three times.

**Luciferase Reporter**

The sequences of circ-0003006 and HIF-1A 3'UTR containing miR-542-3p binding sites were inserted into luciferase vectors pmirGLO to construct wild-type (WT) circ-0003006 and HIF-1A plasmids. The mutant-type (MUT) vectors were acquired by mutating binding sites with miR-542-3p. Further, the cells were co-transfected with WT or MUT plasmids and miR-542-3p mimic or miR-NC. Then, the luciferase activity was examined with the microplate reader System.

**Western Blot Experiment**

The indicated group cell was collected in the ice, and the total protein was extracted and checked the total protein concentration according to the instructions of the BCA kit (Leagene, Beijing, China). The protein samples were boiled and denatured, and 70 μg per well was loaded into the SDS-PAGE gel wells for electrophoresis separation, and then transferred to PVDF membrane. After being blocked with 5% skimmed milk powder for 2 hours, the membrane was reacted with the corresponding primary antibody overnight at 4°C. The secondary antibody labeled with horseradish peroxidase was reacted for 2 hours at room temperature. After exposure with chemiluminescent agent, GAPDH was used as internal reference, and the gel imaging system was used for scanning analysis. The experiment was repeated three times.

**Cell Transfection**

Twenty-four hours before transfection, well-growing cells were seeded in a culture dish with a diameter of 60 mm, and the cells were transfected the next day. The mix LipofectamineTM2000 and siRNA serum-free DMEM medium were incubate at room temperature for 20 minutes. After cell transfection for 6 hours, replaced the serum-free medium with a complete medium containing 10% FBS, and continue culturing for 48 hours. Collect cells to verify the efficiency of silencing. The shRNA-circ-0003006 sequence (shRNA#1: CATCCCCGGTCTCCATGTA, shRNA#2: CACATCCCCGGTCTCCATGTA) was designed by Guangzhou Ruibo Biotechnology Co., Ltd. (Guangzhou, China).

**CCK-8 Method to Detect Cell Viability**

The cells were seeded in a 96-well cell culture plate at a density of 3000 cells/well, and cultured in a constant temperature incubator at 37 °C and a CO2 volume fraction of 5% for overnight. At 24, 48, 72 and 96 h (set 5 plates respectively, 6 replicate wells for each experimental group), test the OD450 in each well according to the operation procedure provided by the CCK-8 kit (KeyGe, Nanjing, China) nm value. Discard the culture medium and change it to 100 μL/well serum-free medium, add 10 μL/well CCK-8 reagent, incubate in a cell culture incubator for 2 hours, then measure and record the data with a microplate reader, and use the OD value for graphical analysis.

**Transwell Invasion Test**

Firstly, taken 100μL of Matrigel gel diluted 1:15~1:20 in the upper chamber, and place it in a 37 °C cell incubator for 2 hours to allow it to solidify; then added 150 L of non-woven material containing 50,000 cells in the upper chamber. Serum culture medium, add serum-containing culture medium to the lower chamber and place it in a 37°C cell incubator for 12–24 hours. Remove the upper chamber for washing, fixing, staining, observation under a microscope, and taking pictures. Three replicate holes are set for each experiment.

**Immunohistochemistry**

The collected tumors and the corresponding adjacent tissues were made into paraffin sections and deparaffinized, put into the repair box and immersed in the citric acid antigen retrieval buffer, heated the antigen retrieval in
a microwave oven, and blocked the interior with 3% hydrogen peroxide. Source peroxidase, add the primary antibody after the serum is blocked, add the corresponding secondary antibody after rewarming, incubate at room temperature for 50 min. The slices were incubated with the streptavidin-peroxidase solution at room temperature for 10 min. Then the samples were observed under a microscope after about 10 minutes in the DAB dye solution.

**Fluorescence in situ Hybridization (FISH)**

The cell was fixed in the cell slide (cover glass) with 4% paraformaldehyde. After rinsing with PBS to remove the fixative, proceed as follows: glycine treatment for 5 min, PBS rinse; 0.4% Triton-X-100 for 15 min, PBS rinse, proteinase K treatment Rinse with PBS for 15 minutes; fix with 4% paraformaldehyde and rinse with PBS; treat with 0.25% acetic anhydride for 10 minutes, rinse with PBS and dry. Place the cover glass on the glass slide and drip the hybridization solution for pretreatment for 1 h. Add biotin-labeled pLncRNA-1 probe hybridization solution and incubate for 16 hours, and then add alkaline phosphatase-labeled anti-biotin antibody (1:1000 dilution) to continue hybridization for 16 hours. Then, the cell was incubated with fluorescently labeled secondary antibody (diluted at 1:2000), stain the nucleus with DAPI and incubate at room temperature for 50 min. The slices were incubated with the streptavidin-peroxidase solution at room temperature for 50 min. The slices were incubated with fluorescently labeled secondary antibody (diluted at 1:2000), stain the nucleus with DAPI and observe under a fluorescent microscope.

**Subcutaneous Tumor Formation Experiment in Nude Mice**

After transfection, cells in logarithmic growth phase were taken, washed with serum-free medium and resuspended to make a single cell suspension (5×10^6/mL), and injected subcutaneously into the left armpit of nude mice for 6 weeks. The mice were sacrificed by pentobarbital sodium (150mg/kg), which was injected intravenously (i.v.). Then the mice were sacrificed and the subcutaneous transplanted tumor was taken out, washed with normal saline and weighed. Animal experiments are performed in accordance with the principles of the Ethics Committee of Laboratory Animals in Jiangxi Cancer Hospital (Approval No. 202007009, approve date: July 12, 2020). Animal experiments took place in Experimental Animal Center of Jiangxi Cancer Hospital.

**Statistical Processing**

SPSS 22.0 statistical software was used to perform statistical analysis on the data of this study. Measurement data were expressed as mean ±standard deviation (x±s). Differences between groups were compared by t-test statistical method. P<0.05 indicated that the differences were statistically significant.

**Results**

circ-0003006 is Highly Expressed in Hepatocellular Carcinoma

Based on the GEO database (GSE97332), we analyzed and screened the differently expressed circRNAs in HCC. We found that circ-0003006 was significantly upregulated in the tumor tissue (Figure 1A, n=7). Then, we examined the circ-0003006 expression in our hospital HCC patient’s tissues; the result consisted of the bio-information analysis result that circ-0003006 was upregulated in the HCC tissue (Figure 1B). In addition, qRT-PCR results showed higher expression of circ-0003006 in SUN-449, HCCLM3, HepG2, and SMMC-7721 than normal liver cell HL7702 (Figure 1C). Moreover, we analyzed the clinicopathological parameters of 115 patients. We found that the level of circ-0003006 expression was associated with tumor size (P=0.003), histological grade (P=0.015), TNM stage (P=0.039), lymph node metastasis (P= 0.043), and not related to other clinicopathological features, such as age, gender (Additional File 2: Table 2). Further, the RNA nucleoplasmic separation and FISH assay demonstrated that circ-0003006 was almost located in the cytoplasm (Figure 1D and E). Therefore, circ-0003006 is suggested to be correlated with the progress of hepatocellular carcinoma.

ZEB1 Induced circ-0003006 in Hepatocellular Carcinoma Cell

To clarify the mechanism of circ-0003006 upregulated in the HCC tissue, we hypothesis that circ-0003006 was induced in transcription level. Then, we prediction the upstream transcription factors by information tool TRCirc (http://www.lncpathway.net/). The prediction result indicated that A total of 23 transcription factors might bind to the promoter region of circ-0003006 (Figure 2A, Additional File 3: Table 3). Then, we selected three transcription factors (ZEB1, ERG1, NF-KB) known as the oncogene in the HCC for further verification. After knocking down ZEB1, ERG1, NF-KB, respectively, we found that circ-0003006 was significantly downregulated in the ZEB1 knockdown HCC cells, whereas the circ-0003006 expression has no difference in the ERG1, NF-KB knockdown groups (Figure 2B). Hence, circ-0003006 was probably induced by ZEB1 at the transcriptional level.
Silencing of circ-0003006 Hindered Hepatocellular Carcinoma Cell Proliferation and Metastasis in vitro

To clarify the biological role of circ-0003006 in HCC progression, the loss-of-function experiments were carried out using two random sh-RNAs against circ-0003006 (sh-circ#1, sh-circ#2). In contrast to the negative control, the transfection of the two shRNAs prominently reduced circ-0003006 expression in both SMMC7721 and HepG2 cell lines (Figure 3A). Notably, sh-circ#1 caused the most significant down-regulation in circ-0003006 expression, so we selected it for function assay analyses. The cck-8 assay indicated that
circ-0003006 knockdown significantly inhibited cell viability (Figure 3B). The transwell assay revealed that circ-0003006 knockdown significantly inhibited the cell migration and invasion ability in HCC cells (Figure 3C and D). The above results indicated that circ-0003006 knockdown inhibited the growth and metastasis of HCC cells.

**Circ-0003006 Silencing Suppresses the Growth of HCC Cells in vivo**

To further confirm the role of circ-0003006 in the progression of HCC, stable lentiviral-mediated circ-0003006-silenced or NC transfected HepG2 cells were injected into the nude mice for the xenografts of tumors assay. Circ-0003006 knockdown led to decreases in tumor volume and weight compared to the NC group in vivo (Figure 4A–C). IHC analysis confirmed that KI-67 was downregulated in the xenograft tumor tissues of the circ-0003006 knockdown group (Figure 4D). circ-0003006 knockdown clearly inhibited HCC growth in vivo, which was consistent with the results in vitro.

**Circ-0003006 Functions as a Sponge of miR-542-3p**

To validate the downstream miRNA involved in the function of circ-0003006, we predicted the miRNAs that probably bind with circ-0003006 via Starbase (http://starbase.sysu.edu.cn/starbase2). Next, to select whether these candidate miRNAs can directly bind with circ-0003006, pull-down assays were performed with a specific biotin-labeled circ-0003006 probe. Among the four candidate miRNAs, miR-542-3p was found to be the most abundantly pulled down by the circ-0003006 probe (Figure 5A). As the miRNA and circRNA were usually combined with the AGO2 protein, the RIP assay was performed using an AGO2 antibody. The result indicated that circ-0003006 and miR-542-3p were precipitated by the AGO2 antibody compared with the control group (Figure 5B). Online bioinformatic prediction Starbase (http://starbase.sysu.edu.cn/) indicated that miR-542-3p functioned as the target of circ-0003006 with complementary binding sites (Figure 5C). Luciferase reporter assay indicated that circ-0003006 closely combined with miR-542-3p (Figure 5D). Then, the q-RT-PCR assay demonstrated that miR-542-3p was significantly inhibited in circ-0003006 knockdown HCC cells (Figure 5E). And we further detected the miR-542-3p expression in the HCC tissue and corresponding normal tissue; the result indicated that miR-542-3p was downregulated in HCC tissues (Figure 5F). In addition, q-RT-PCR analysis illustrated that circ-0003006 was negatively correlated with miR-542-3p (Figure 5G).

**Circ-0003006 Sequestered miR-542-3p to Regulate HIF-1A in HCC**

Subsequently, we used miRbase, miRTarBase, and Targetscan to predict an oncogene, HIF-1A, as the potential target gene of miR-542-3p. Furthermore, the q-RT-PCR

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*Figure 3* Silencing of circ-0003006 hindered hepatocellular carcinoma cell proliferation and metastasis in vitro. (A) HepG2 and SMMC-7721 cell lines are transfected with NC or sh-circ-0003006 (sh-circ#1, sh-circ#2) and qRT-PCR analysis demonstrates that the transfection is successful. (B) CCK8 experimental results for the growth of hepatocellular carcinoma upon knockdown of sh-circ-0003006. (C) and (D) Transwell assay determined the migration and invasion of hepatocellular carcinoma cells transfected with sh-circ-0003006. Data indicate the means ± SEM of three experiments, *p < 0.05, **p < 0.01.
assay demonstrated that HIF-1A was significantly reduced in the miR-542-3p mimic group HCC cells (Figure 6A). To confirmed whether miR-542-3p directly targeted HIF-1A, we constructed two luciferase reporter plasmids containing the binding site’s wide or mutation type with miR-570-3p (Figure 6B). Then, the luciferase reporter assay indicated that the relative luciferase activity was reduced in the miR-542-3p mimic group (Figure 6C and D). Furthermore, the q-RT-PCR and western-blots assay indicated that HIF-1A was reduced in the miR-542-3p and reversed by circ-0003006 overexpressed (Figure 6E and F). In addition, the HIF-1A was upregulated in the HCC tissues (Figure 6G and H). And the HIF-1A expression was negatively correlated with miR-542-3p (Figure 6I).

Circ-0003006 Mediates HIF-1A Expression to Promote HCC Progression via miR-542-3p

Subsequently, we investigated the role of circ-0003006/ miR-542-3p/ HIF-1A axis in HCC progression. The HepG2 and SMMC7721 cells were transfected with Sh-NC, Sh-circ-0003006, Sh-circ-0003006 + OE-NC, Sh-circ-0003006 + OE-HIF-1A into HepG2 and SMMC7721 cells. The expression of circ-0003006 and HIF-1A were measured (Figure 7A). CCK-8 and Transwell assays showed that the inhibitory effects of downregulated circ-0003006 on HepG2 and SMMC7721 cells were rescued by HIF-1A overexpression (Figure 7B–F), suggested that circ-0003006 regulated HIF-1A expression to promote cervical cancer progression via sponging miR-542-3p.

Discussion

The American Cancer Society points out that the incidence and mortality of malignant tumors, except liver cancer, have decreased globally. However, the incidence and mortality of liver cancer are still increasing year by year. Therefore, it is essential for the clinical treatment of liver cancer to further explore the molecular mechanism of liver cancer. In this study, we firstly screened the differently expressed circRNA to examined the function in proliferation and metastasis. The circ-0003006 was found that highly expressed in the HCC tissues and cells exerted a promoting malignant role in cell growth and metastasis. CircRNA is regarded as a very good biomarker because of its loop-like closure and anti-RNase characteristics. It is abundant in exosomes secreted
by certain cells and plays a role in cell-to-cell communication. Although circRNA can play a volatile biological role in the various tumor diseases remains to be studied, it still has research value as a potential biological marker.

Studies have shown that circRNA participates in the regulation of cell function in a variety of ways. In the cytoplasm, the expression of circRNA is also involved in the regulation of cell genes. Many studies have confirmed that competitive endogenous RNA (ceRNA) can regulate the expression of downstream genes in the cytoplasm by acting as a miRNA sponge and competitively binding to miRNA. The circRNA in the cytoplasm can also interact with different proteins to form a specific circRNA, which in turn affects the way the combined proteins work, such as circMbl, circFOXO3, circANRIL, circRNA-100338 can also drive the translation of circRNA into peptides, but the translation efficiency is still limited. Many studies in recent years have shown that the unbalanced expression of non-coding RNA, especially circRNA, can affect the occurrence and development of HCC, and circRNA plays a role in promoting or suppressing cancer in the development of HCC. Zheng et al. found that has_circ_0079929 is under-expressed in HCC. When has_circ_0079929 is overexpressed, cell growth is inhibited and cell cycle progression is also blocked. Further studies show that has_circ_0079929 is through PI3K/AKT/mTOR signal transduction pathway Inhibited cell proliferation. In addition, some circRNAs act as cancer-promoting factors in HCC. Huang et al. found that circRNA-100338 is highly expressed in HCC tissues, and the 5-year survival rate of hepatitis B-related HCC patients is lower, and the risk of recurrence and
metastasis is higher. The study by Wang et al. showed that circRHOT1 is upregulated in HCC tissues and is associated with a poor prognosis. In-body and out-of-body studies have shown that circRHOT1 triggers the expression of NR2F6 to promote tumor growth and metastasis. The above research shows that circRNA participates in various biological behaviors of HCC, and circRNA has potential research value as biological markers and therapeutic targets. Based on the function characters of circRNA which is located in the cytoplasm, we hypothesized that circ-0003006 acts as a sponge for miRNA to regulate the target gene. Therefore, we predicted and examined the downstream miRNAs and target genes, the miR-542 and HIF-1A were probably the target of circ-0003006. Then the function assay also demonstrated that circ-0003006/miR-542/HIF-1A axis could as a promoted regulation network in the HCC progress.

Hypoxia-inducible factor 1 (HIF-1) is one of the key regulatory factors for cells to respond to the hypoxic microenvironment. Studies have shown that under hypoxic conditions, activated HIF-1A binds to the hypoxia response element (HRE) in the promoter region of the downstream target gene to promote transcriptional activation of the downstream target gene, including the glucose transporter GLUTs, Hexokinase (HKs), Phosphoglycerate Kinase (PGK), etc. Studies in the past two years have shown that in addition to protein-coding genes, HIF-1A can also promote the metabolic reprogramming of tumor cells by regulating the expression of non-coding RNA.

**Conclusion**

This study found that circ-0003006 was overexpressed in HCC tissues and correlated with poor prognosis of HCC.
Figure 7 Circ-0003006 mediates HIF-1A expression to promote HCC progression via miR-542-3p. (A) q-RT-PCR analysis of the circ-0003006 and HIF-1A expression in HepG2 and SMMC7721 cell transfected with Sh-NC, Sh-circ-0003006, Sh-circ-0003006 + OE-NC, Sh-circ-0003006 + OE-HIF-1A. (B) CCK8 analysis the proliferation in indicated groups of HepG2 and SMMC7721 cell. (C–F) Transwell assay analysis the proliferation in indicated groups of HepG2 and SMMC7721 cell. Data indicate the means ± SEM of three experiments, *p < 0.05, **p < 0.01.
patients. circ0003006 upregulates the expression level of HIF-1A through sponge adsorption of miR-542-3p, thereby promoting the proliferation, invasion and metastasis of hepatocellular carcinoma cells, providing a meaningful experimental basis for exploring biomarkers for the diagnosis and treatment of liver cancer.

**Highlights**

1. circ0003006 is highly expressed in HCC.
2. circ0003006 was induced by ZEB1 in HCC cells.
3. circ0003006 affects the proliferation, migration and invasion of HCC cells.
4. circ0003006 functions as a sponge of miR-542-3p to upregulate HIF-1A in HCC cells.

**Data Sharing Statement**

The data used to support the findings of this study are available from the corresponding author upon request.

**Ethics Statement**

Our study was approved by the Ethics Review Board of the Jiangxi Cancer Hospital of Nanchang University.

**Acknowledgments**

We appreciate all participants who provide technical support for our research.

**Disclosure**

The authors declare that they have no competing interests.

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