European Review for Medical and Pharmacological Sciences 2020; 24: 11058-11064

Circular RNA circGFRA1 promotes angiogenesis, cell proliferation and migration of hepatocellular carcinoma by combining with miR-149

Y.-X. YU[1], T.-W. GE[2], P. ZHANG[1]

[1]Department of Hepatobiliary and Pancreatic Surgery, The First Hospital of Jilin University, Changchun, China
[2]Cancer Center, The First Hospital of Jilin University, Changchun, China

Abstract. – OBJECTIVE: We aimed to explore the effect of circGFRA1 on the progression of hepatocellular carcinoma (HCC) and its underlying mechanism.

PATIENTS AND METHODS: First, quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was conducted to detect the level of circGFRA1 in HCC tissues and cells. Survival analysis was applied to detect the effect of highly expressed circGFRA1 on the prognosis of HCC patients. Subsequently, circGFRA1 level was silenced in HCC cells, and proliferative, migration and angiogenesis activity of HCC cells was examined using Cell Counting Kit-8 (CCK-8), transwell test, and angiogenesis experiment. Then, we predicted the binding target of circGFRA1 through the bioinformatics website, and verified it through qRT-PCR and Dual-Luciferase reporter assay. Lastly, the interaction between them was verified through a series of *in vitro* experiments.

RESULTS: qRT-PCR analysis showed that circGFRA1 was abnormally highly expressed in HCC tissues and HCC cells, and the high expression of circGFRA1 may lead to poor prognosis in patients with HCC. After transfecting si-circGFRA1 in HCC cells, CCK-8 and transwell experiments showed that the proliferative ability and migration of HCC cells were inhibited. Moreover, angiogenesis experiments showed that the knockdown of circGFRA1 can inhibit the blood vessels replenishment of HCC cells. The bioinformatics website suggested that miR-149 may be able to bind circGFRA1. MiR-149 was upregulated by the knockdown of circGFRA1 in HCC cells. Pearson analysis suggested that the expression levels of the two genes were negatively correlated. Dual-Luciferase reporter assay further indicated that circGFRA1 can bind to miR-149. Reverse experiment showed that the knockdown of miR-149 can partially restore the inhibited proliferative, migration, and angiogenesis activity of HCC cells caused by circGFRA1 knockdown.

CONCLUSIONS: CircGFRA1 is highly expressed in HCC and its level is negatively correlated with miR-149 expression. CircGFRA1 can promote the proliferative, migration and angiogenic activity of HCC by binding miR-149.

Key Words:
Hepatocellular carcinoma, CircGFRA1, MiR-149, Cell proliferation, Cell migration.

Introduction

Primary liver cancer is the third leading malignant tumor. Among them, hepatocellular carcinoma (HCC) is the most important subtype of liver cancer[1]. HCC is the fifth most common malignant tumor in the world, characterized by high morbidity and high mortality[2]. Currently, surgical resection and liver transplantation are the main treatments for early stage HCC[3]. Although the etiology and pathogenesis of HCC have been extensively studied in recent years, due to its high metastasis rate and high recurrence rate, the 5-year postoperative survival is only 20-30%[4]. Therefore, it is urgent to explore the molecular mechanism of HCC.

Circular RNA (circRNAs) is a type of non-coding RNA[5] that can barely encode proteins. CircRNAs are highly conserved with a covalently closed loop structure. They have neither 5′ nor 3 ′ends, which are RNA transcripts produced by the reverse splicing of a single precursor mRNA[5,6]. In recent years, the role of circRNAs in human malignant tumors has emerged. In particular, hsa-circ-0092306 regulates protein kinase C in MKN-45 cells by targeting miR-197-3p, thus aggravating gastric cancer[7]. Circ-FOXO3 inhibits the development of lung carcinoma through the miR-23a/PTEN axis[8]. CircRNA-cRAPGEF5 inhibits the growth and metastasis of renal cancer *via* the miR-27a-3p/TXNIP axis[9]. However, the molecular mechanism of circGFRA1 in HCC is not fully understood, and further research is still needed.

Corresponding Author: Ping Zhang, MD; e-mail: z_ping@jlu.edu.cn

MicroRNAs (miRNAs) are single-stranded, endogenous non-coding RNAs that can participate in many cellular processes, including cell cycle progression, proliferation, apoptosis, migration, etc.[10-12]. Abnormally expressed miRNAs are involved in the development of HCC. It is reported that miR-342 regulates the proliferative ability and apoptosis of hepatoma cells through the Wnt/β-catenin signaling pathway[13]. MiR-5692a promotes the proliferative ability of HCC cells and inhibits the apoptosis by targeting HOXD8[14]. MiR-200c-5p inhibits the proliferative and migration ability of HCC cells by inhibiting MAD2L1[15].

We previously detected upregulation of circGFRA1 in HCC tissues and cells, and speculated that circGFRA1 may be an oncogene in HCC. Therefore, this study aims to explore its potential biological functions and molecular mechanisms in HCC development through a series of *in vitro* experiments.

Patients and Methods

Patients and Specimen

A total of 23 pairs of HCC tissues and adjacent tissues were collected. HCC patients with anti-cancer treatment or history of other solid tumors were excluded. All patients signed an informed consent. Tumor staging of HCC was in accordance with international standards of the Union for International Cancer Control (UICC) were implemented. All tissues were stored at -80°C. This investigation was approved by the Ethics Committee of The First Hospital of Jilin University. All experiments in this study conformed to the Declaration of Helsinki.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA in tissues and cells was lysed with TRIzol reagent (Life Technologies, Gaithersburg, MD, USA). According to the product manual, the extracted RNA was reversely transcribed into cDNA using PrimeScript RT kit (TaKaRa, Otsu, Shiga, Japan). qRT-PCR was performed on the ABI 7300 thermal cycler (Applied Biosystems, Foster City, CA, USA) using the SYBR Green real-time PCR kit (Thermo Fisher Scientific, Waltham, MA, USA). U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal parameters. The primer sequences were as follows (5'-3'): circGFRA1-F: CCTCCGGGTTAAGAACAAGC, R: CTGGCTGGCAGTTGGTAAAA. GAPDH-F: ATGGGGAAGGTGAAGGTCG, R: GGGGTCATTGATGGCAACAATA. U6-F: CTCGCTTCGGCAGCACA, R: ACGCTTCACGAATTTGCGT. miR-149-F: TCTGGCTCCGTG, R: CAGTGCGTGTCGTGGAGT.

Cell Culture

Normal hepatocytes (LO2) and HCC cell lines (SK-HEP-1, Huh6, Huh7, HCCLM3) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured using Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin in a cell humidified incubator at 37°C and 5% CO_2.

Transfection

MiR-149 inhibitor, small interfering RNA against circGFRA1 (si-circGFRA1) and its negative control were obtained from GenePharma (Shanghai, China). After the cells adhered to more than 60%, the cells were transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the product instructions.

Cell Counting Kit-8 (CCK-8) Experiment

HCC cells were seeded in 96-well plates (3×10^3 /well). Subsequently, 10 μL of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added to each well at different time points (0, 24, 48, and 72 h). After incubation for 2 hours in the dark, the optical density (OD) value was measured at 450 nm, and the absorbance value measured at 0 hours was used as a control.

Angiogenesis Test

Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was used for the angiogenesis test. HCCLM3 cells or Huh-7 cells transfected with si-circGFRA1 or si-NC were seeded into a 24-well plate (2×10^3 cells/well) coated with Matrigel. After incubation for 8 hours, the cells were observed using an IX-73 inverted microscope (Olympus, Tokyo, Japan), and 5 visual fields were randomly collected, photographed and counted.

Cell Migration Assay

Cells suspended in serum-free medium (4×10^4) were seeded into the upper layer of the Matrigel-containing chamber (Corning, Corning, NY, USA), and complete medium was placed as a chemical attractant in the bottom of the chamber. After cell culture for 48 h, penetrating cells in the bottom were col-

lected, fixed with methanol, and stained with crystal violet for 20 min. After washing with PBS, cells in 10 random fields were counted.

Dual-Luciferase Reporter Assay

First, wild-type (WT) binding sites in the 3'UTR of circGFRA1 and miR-149 were cloned into the pMIR reporter (Promega, Madison, WI, USA), as well as the mutant-type (MUT) one. Next, the Luciferase reporter plasmid circGFRA1-WT or circGFRA1-MUT was co-transfected into HCC cells with miR-149 mimics or negative control. 24 hours after transfection, the Luciferase activity of cells was detected using a Dual-Luciferase reporter detection system from Promega (Madison, WI, USA).

Statistical Analysis

The *t*-test was used for comparison between groups. Pearson's method was used for correlation analysis. Kaplan-Meier analysis method was used for survival analysis. GraphPad Prism 7.0 software (La Jolla, CA, USA) was used for graph drawing, and *p*-value less than 0.05 was considered significantly different. Each experiment was repeated three times independently.

Results

CircGFRA1 Is Highly Expressed in HCC

We collected 23 cases of HCC tissues and normal para-cancerous tissues. Through qRT-PCR detection, it was found that the level of circGFRA1 was markedly elevated in HCC tissues than that of normal ones (Figure 1A). At the same time, the level of circGFRA1 was also abnormally upregulated in HCC cells, and we selected HCCLM3 and Huh7 cells for subsequent studies (Figure 1B). We then analyzed the effect of circGFRA1 on the prognosis of HCC patients through a database. As shown in Figure 1C, high expression of circGFRA1 may lead to poor prognosis in HCC patients.

Knockdown of CircGFRA1 can Inhibit the Proliferative, Migration and Angiogenesis Ability of HCC Cells

To verify the effect of circGFRA1 on HCC cells, we used small interfering RNA to knock down the expression of circGFRA1 and verify its interference efficiency. As shown in Figure 2A, the expression of circGFRA1 in hepatocytes transfected with si-circGFRA1 #1 or si-circGFRA1 #2 was markedly lower than those transfected with si-NC. Then, CCK-8 assay was performed to verify the effect of circGFRA1 on the proliferative ability of HCC cells. The knockdown of circGFRA1 could inhibit the proliferation ability of HCC cells (Figure 2B, 2C). Next, the vascular regeneration capacity of HCCLM3 and Huh7 cells was markedly inhibited after knockdown of circGFRA1 (Figure 2D). Transwell experiment also showed that the migration ability of HCC cells was also inhibited after knockdown of circGFRA1 (Figure 2E). All these results demonstrated that circGFRA1 may promote the progression of HCC.

CircGFRA1 can Bind MiR-149

A binding site within miR-149 was predicted using the bioinformatic method that paired to circGFRA1 3'UTR (Figure 3A). The knock-

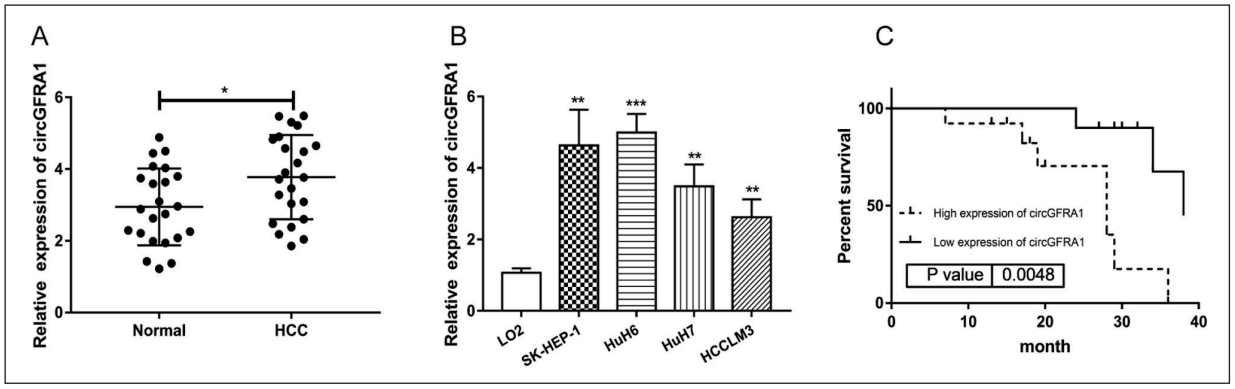

Figure 1. circGFRA1 is highly expressed in HCC. **A**, qRT-PCR analysis of the expression level of circGFRA1 in hepatocellular carcinoma tissue and normal liver tissue. **B**, qRT-PCR analysis of the expression level of circGFRA1 in hepatocellular carcinoma cell lines and normal liver cell lines. **C**, Survival analysis of the survival prognosis of patients with high expression of circGFRA1 and lower expression of circGFRA1. **p*<0.05, ***p*<0.01, ****p*<0.001.

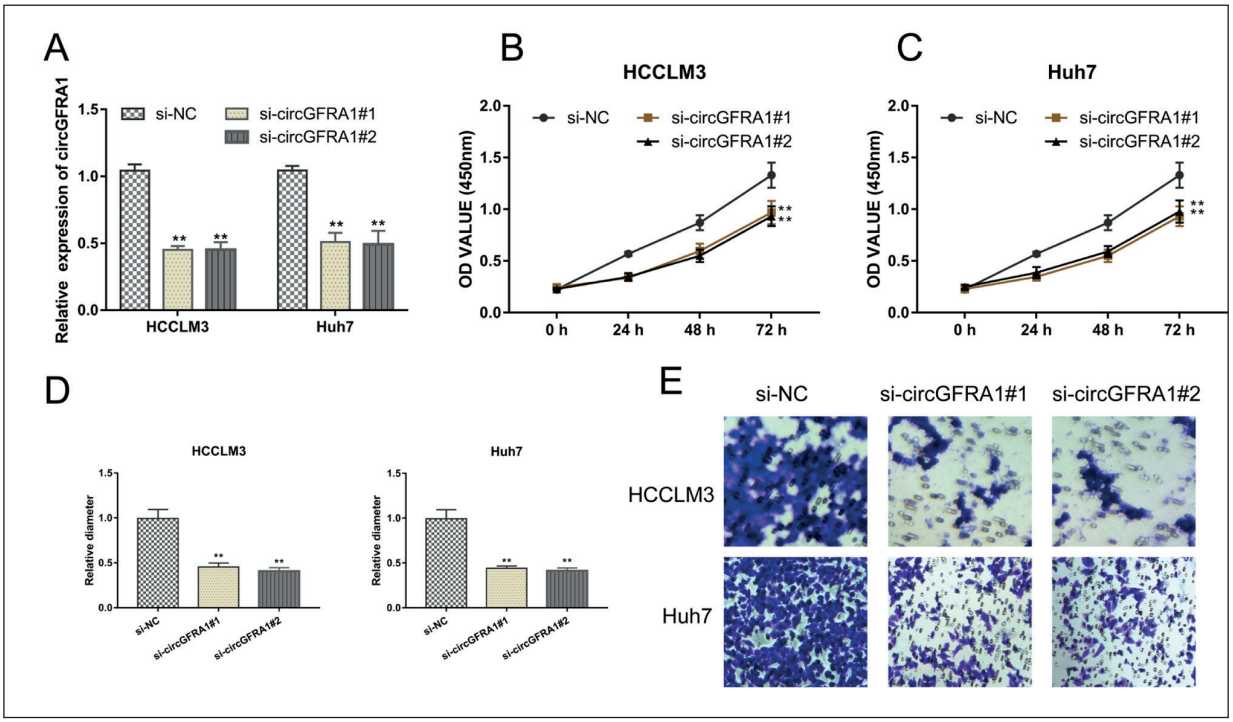

Figure 2. Knocking down circGFRA1 can inhibit the proliferative, migration and angiogenesis ability of HCC cells. **A**, qRT-PCR analysis of the expression level of circGFRA1 in HCCLM3 and Huh7 cell lines transfected with si-circGFRA1. **B-C**, The proliferation ability of HCCLM3(B) and Huh7(C) after knocking down circGFRA1 was detected by CCK-8 experiment. **D**, Angiogenesis experiments was applied to detect the vascular regeneration capacity of HCCLM3 and Huh7 cell lines after knocking down circGFRA1. E, Transwell experimental was used to estimate the migration ability of HCCLM3 and Huh7 cell lines after knocking down circGFRA1 (magnification: 40×). $^{**}p<0.01$.

down of circGFRA1 could lead to upregulation of miR-149 in HCC cells (Figure 3B). Pearson analysis found that the relative expressions of circGFRA1 and miR-149 were in negative correlation (Figure 3C). Then, we conducted a Dual-Luciferase report assay to further explore the binding relationship between the them. As shown in Figure 3D and 3E, miR-149 could bind to the wild-type circGFRA1, rather than the mutant-type one, indicating that circGFRA1 can bind to miR-149.

Knockdown of MiR-149 Can Partially Reverse the Inhibitory Effect of Silenced CircGFRA1 on Proliferation, Migration and Angiogenesis of HCC Cells

To further verify the co-regulation of circGFRA1 and miR-149 in HCC development, co-transfection of miR-149 inhibitor and si-circGFRA1 was conducted. In comparison with HCC cells with solely knockdown of circGFRA1, those with co-silence of miR-149 and circGFRA1 had a higher viability (Figure 4A, B). Similarly, as shown in Figure 4C, the knockdown of miR-149 could partially reverse the inhibited angiogenesis activity caused by the knockdown of circGFRA1 on HCC cells. As shown in Figure 4D, the inhibited migratory ability of HCC cells with circGFRA1 knockdown was partially reversed after the knockdown of miR-149.

Discussion

Every year, more than 700,000 people are diagnosed with HCC[16]. HCC has the characteristics of high incidence, low detective rate, and poor prognosis. Despite the continuous improvement of medical standards, due to the above reasons, the diagnosis and treatment of HCC are still very difficult. Hence, we urgently need to discover new therapeutic targets for HCC. Here, we found that circGFRA1 was abnormally highly expressed in HCC tissues and cells. We initially found that high expression of circGFRA1 may lead to poor prognosis in HCC patients. We then explored the effect of circGFRA1 on HCC through a series of *in vitro* experiments.

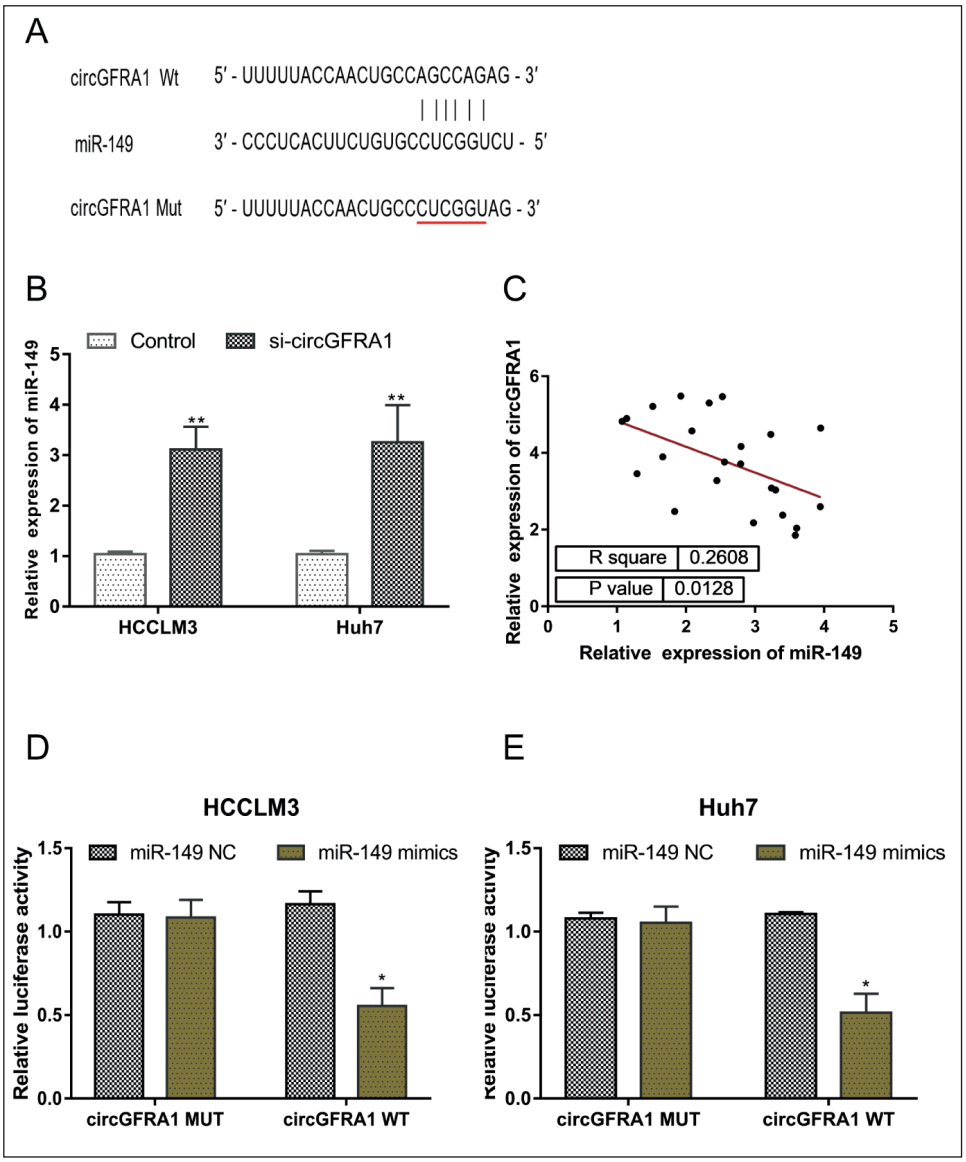

Figure 3. circGFRA1 can combine with miR-149. **A**, Bioinformatics technology predicted that the 3'UTR region of circGFRA1 had a binding site with miR-149. **B**, Analysis of the gene expression of miR-149 expression in hepatocellular carcinoma cells after knocking down circGFRA1 by q-PCR. **C**, Pearson algorithm correlation analysis was used to show the correlation between circGFRA1 and miR-149 in 23 cases of hepatocellular carcinoma tissues. **D**-**E**, The Dual-Luciferase reporter gene experiment was performed to determine the binding relationship between circGFRA1 and miR-149 in hepatocellular carcinoma cells. **p*<0.05, ***p*<0.01.

CircRNAs were first observed by electron microscopy nearly 40 years ago. With the continuous advancement of high-throughput sequencing and bioinformatics, abundant circRNAs have been gradually recognized over the past decade[17,18]. Biological effects of circRNA have aroused widespread concern, especially in the occurrence and development of cancer[19]. In terms of HCC, various circRNAs have been found to play important biological functions. Of note, circRNA hsa-ucrc-u0056836 serves as an oncogene in HCC by regulating the miR-766-3p/FOSL2 axis[20]. Besides, the down-regulation of circDYNC1H1 inhibits the proliferative and migration ability of HCC cells by binding miR-140-5p[21]. The knockdown of circPVT1 inhibits HCC cell proliferation by regulating the miR-3666/Sirtuin 7 axis[22]. It can be seen that circRNAs, as a new type of non-coding RNA, play important roles in tumorigenesis by acting as microRNA target genes and regulating gene expressions. Our results showed that the knockdown of circGFRA1 could inhibit proliferation, migration, and angiogenesis of HCC cells.

In the past ten years, miRNAs have been implicated in tumorigenesis, progression, migration, drug resistance, and other aspects[23]. MiR-149 inhibits the proliferative and migration ability of non-small cell lung cancer cells by inhibiting the FOXM1/cyclin D1/MMP2

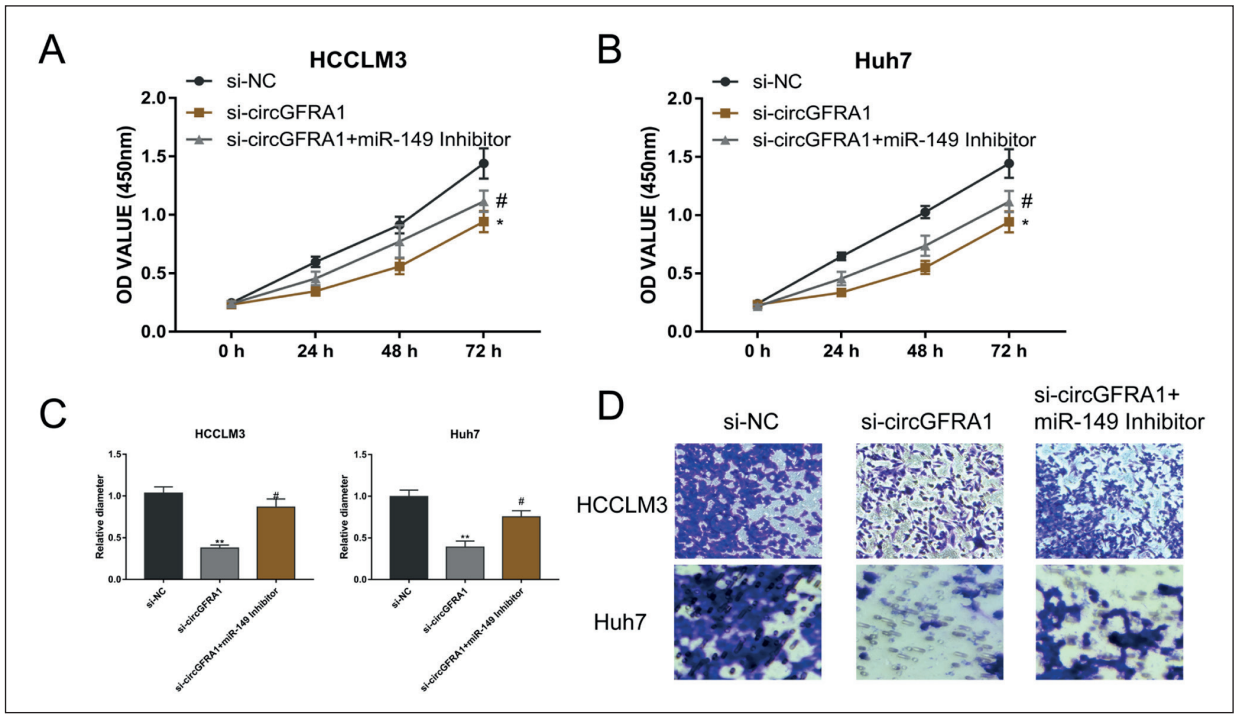

Figure 4. Knocking down miR-149 can partially reverse the inhibitory effect of low-expression circGFRA1 on proliferation, migration and angiogenesis of HCC cells. **A**-**B**, CCK-8 experiment was used to detect the proliferation activity of HCCLM3 and Huh7 cells after co-transfection of circGFRA1 knockdown vector and miR-149 inhibitor. **C**, Angiogenesis experiments was applied to detect the vascular regeneration capacity of HCCLM3 and Huh7 cells after co-transfection of circGFRA1 knockdown vector and miR-149 inhibitor. **D**, Transwell experiments was used to detect the cell scratch experiments of HCCLM3 and Huh7 cells after co-transfection of circGFRA1 knockdown vector and miR-149 inhibitor (magnification: 40×). *p<0.05, **p<0.01, p<0.05.

axis[24]. Moreover, miR-149 has also been found to have a certain biological function in HCC. MiR-149 inhibits the migration of HCC by targeting the actin regulatory protein PPM1[25]. We found through the bioinformatics website that miR-149 can bind to circGFRA1 and verified this result through a series of experiments. Pearson analysis found that the expression levels of circGFRA1 and miR-149 were negatively correlated. The knockdown of miR-149 can partially reverse the inhibitory effect of silenced circGFRA1 on the proliferation, migration and angiogenesis of HCC cells. Taken together, this study for the first time clarified the oncogenic role of circGFRA1 in HCC development by mediating proliferation, migration and angiogenesis. Its prognostic potential in HCC has emerged as well. We believed that due to the unique structure of circRNAs and their stable expression in body fluids, circRNAs have promising biomarkers for diagnosis and treatment of HCC.

Conclusions

Briefly, the above data demonstrated that circGFRA1 is highly expressed in HCC and plays a role as an oncogene. The circGFRA1/miR-149 regulatory axis may be a potential target for clinical diagnosis and treatment of HCC.

Conflict of Interests

The authors declare that they have no conflict of interests.

References

1) Fu Y, Cai L, Lei X, Wang D. Circular RNA ABCB10 promotes hepatocellular carcinoma progression by increasing HMG20A expression by sponging miR-670-3p. Cancer Cell Int 2019; 19: 338.
2) Mittal S, El-Serag HB. Epidemiology of hepatocellular carcinoma: consider the population. J Clin Gastroenterol 2013; 47 Suppl: S2-S6.

3) Johnson PJ, Berhane S, Kagebayashi C, Satomura S, Teng M, Reeves HL, O'Beirne J, Fox R, Skowronska A, Palmer D, Yeo W, Mo F, Lai P, Inarrairaegui M, Chan SL, Sangro B, Miksad R, Tada T, Kumada T, Toyoda H. Assessment of liver function in patients with hepatocellular carcinoma: a new evidence-based approach-the ALBI grade. J Clin Oncol 2015; 33: 550-558.
4) Yan X, Zhu Z, Xu S, Yang LN, Liao XH, Zheng M, Yang D, Wang J, Chen D, Wang L, Liu X, Liu J, Chen RH, Zhou XZ, Lu KP, Liu H. MicroRNA-140-5p inhibits hepatocellular carcinoma by directly targeting the unique isomerase Pin1 to block multiple cancer-driving pathways. Sci Rep 2017; 7: 45915.
5) Memczak S, Jens M, Elefsinioti A, Torti F, Krueger J, Rybak A, Maier L, Mackowiak SD, Gregersen LH, Munschauer M, Loewer A, Ziebold U, Landthaler M, Kocks C, le Noble F, Rajewsky N. Circular RNAs are a large class of animal RNAs with regulatory potency. Nature 2013; 495: 333-338.
6) Jeck WR, Sharpless NE. Detecting and characterizing circular RNAs. Nat Biotechnol 2014; 32: 453-461.
7) Chen Z, Ju H, Zhao T, Yu S, Li P, Jia J, Li N, Jing X, Tan B, Li Y. hsa_circ_0092306 targeting miR-197-3p promotes gastric cancer development by regulating PRKCB in MKN-45 cells. Mol Ther Nucleic Acids 2019; 18: 617-626.
8) Xing Y, Zha WJ, Li XM, Li H, Gao F, Ye T, Du WQ, Liu YC. Circular RNA circ-Foxo3 inhibits esophageal squamous cell cancer progression via the miR-23a/PTEN axis. J Cell Biochem 2020; 121: 2595-2605.
9) Chen Q, Liu T, Bao Y, Zhao T, Wang J, Wang H, Wang A, Gan X, Wu Z, Wang L. CircRNA cRAPGEF5 inhibits the growth and metastasis of renal cell carcinoma via the miR-27a-3p/TXNIP pathway. Cancer Lett 2020; 469: 68-77.
10) Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009; 136: 215-233.
11) Babski J, Maier LK, Heyer R, Jaschinski K, Prasse D, Jager D, Randau L, Schmitz RA, Marchfelder A, Soppa J. Small regulatory RNAs in Archaea. RNA Biol 2014; 11: 484-493.
12) Gong J, He XX, Tian A. Emerging role of microRNA in hepatocellular carcinoma (Review). Oncol Lett 2015; 9: 1027-1033.
13) Lu C, Jia S, Zhao S, Shao X. MiR-342 regulates cell proliferation and apoptosis in hepatocellular carcinoma through Wnt/beta-catenin signaling pathway. Cancer Biomark 2019; 25: 115-126.
14) Sun S, Wang N, Sun Z, Wang X, Cui H. MiR-5692a promotes proliferation and inhibits apoptosis by targeting HOXD8 in hepatocellular carcinoma. J BUON 2019; 24: 178-186.
15) Li Y, Bai W, Zhang J. MiR-200c-5p suppresses proliferation and metastasis of human hepatocellular carcinoma (HCC) via suppressing MAD2L1. Biomed Pharmacother 2017; 92: 1038-1044.
16) Bruix J, Gores GJ, Mazzaferro V. Hepatocellular carcinoma: clinical frontiers and perspectives. Gut 2014; 63: 844-855.
17) Bruix J, Reig M, Sherman M. Evidence-based diagnosis, staging, and treatment of patients with hepatocellular carcinoma. Gastroenterology 2016; 150: 835-853.
18) Hsu MT, Coca-Prados M. Electron microscopic evidence for the circular form of RNA in the cytoplasm of eukaryotic cells. Nature 1979; 280: 339-340.
19) He Q, Zhao L, Liu Y, Liu X, Zheng J, Yu H, Cai H, Ma J, Liu L, Wang P, Li Z, Xue Y. Circ-SHKBP1 regulates the angiogenesis of u87 glioma-exposed endothelial cells through miR-544a/FOXP1 and miR-379/FOXP2 pathways. Mol Ther Nucleic Acids 2018; 10: 331-348.
20) Li Z, Liu Y, Yan J, Zeng Q, Hu Y, Wang H, Li H, Li J, Yu Z. Circular RNA hsa_circ_0056836 functions an oncogenic gene in hepatocellular carcinoma through modulating miR-766-3p/FOSL2 axis. Aging (Albany NY) 2020; 12: 2485-2497.
21) Wang ZY, Zhu Z, Wang HF, Qin B, Liu J, Yao XH, Li WC, Chen KS. Downregulation of circDYNC1H1 exhibits inhibitor effect on cell proliferation and migration in hepatocellular carcinoma through miR-140-5p. J Cell Physiol 2019; 234: 17775-17785.
22) Li Y, Shi H, Yuan J, Qiao L, Dong L, Wang Y. Downregulation of circular RNA circPVT1 restricts cell growth of hepatocellular carcinoma through downregulation of Sirtuin 7 via microRNA-3666. Clin Exp Pharmacol Physiol 2020; 47: 1291-1300.
23) Melo SA, Esteller M. Dysregulation of microRNAs in cancer: playing with fire. FEBS Lett 2011; 585: 2087-2099.
24) Zhao L, Liu L, Dong Z, Xiong J. MiR-149 suppresses human non-small cell lung cancer growth and metastasis by inhibiting the FOXM1/cyclin D1/MMP2 axis. Oncol Rep 2017; 38: 3522-3530.
25) Qian B, Zhao L, Wang X, Xu J, Teng F, Gao L, Shen Luo G, Chao YL, Tang B, Li BS, Xiao YF, Xie R, Wang SM, Wu YY, Dong H, Liu XD, Yang SM. miR-149 represses metastasis of hepatocellular carcinoma by targeting actin-regulatory proteins PPM1F. Oncotarget 2015; 6: 37808-37823.