The circHIPK3/miR-524-5p/Nr4A2 axis regulates colon cancer cell proliferation, migration, invasion, and apoptosis

Xiang Li  
The First Hospital of China Medical University

Zitao Li  
Hongqi Hospital, Mudanjiang Medical University

Caijuan Li  
Hongqi Hospital, Mudanjiang Medical University

Gaosen Zhang  
The First Hospital of China Medical University

Donglin Bian  
The First Hospital of China Medical University

Zhen Zhang (zhangzhen@cmu.edu.cn)  
The First Hospital of China Medical University

Research Article

Keywords: circRNA, miRNA, Nr4A2, colon cancer, circHIPK3

Posted Date: October 13th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2151178/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Purpose

Circular RNAs (circRNAs) are shown to play various key roles in cancer development and progression. This study sought to identify the role of one circRNA, circHIPK3, in colon cancer and determine the mechanism by which it impacts this disease.

Methods

CircHIPK3, miR-524-5p, and Nr4A2 expression were measured using qRT-PCR. Colon cancer cell proliferation, migration, and invasion were determined using CCK-8 and transwell assays, and apoptosis was assessed by TUNEL assay. Western blotting was used to measure the expression of related proteins. The correlation between miR-524-5p and circHIPK3 or Nr4A2 was confirmed using dual-luciferase reporter gene analysis. Finally, animal experiments were used to evaluate the effect of circHIPK3 silencing in vivo.

Results

CircHIPK3 is highly expressed in colon cancer cells. Silencing of this gene inhibits colon cancer cell proliferation, migration, and invasion and promotes apoptosis. In addition, miR-524-5p, which interacts with Nr4A2, was shown to be a circHIPK3 target gene. Knockdown of miR-524-5p reversed the effect of circHIPK3 silencing on colon cancer cells. Meanwhile, Nr4A2 overexpression reversed the effects of miR-524-5p overexpression on colon cancer cells.

Conclusion

CircHIPK3 uses the miR-524-5p/Nr4A2 axis to inhibit colon cancer cell proliferation, migration, and invasion and to promote apoptosis. These findings suggest that circHIPK3 could serve as a potential target for colon cancer treatment.

Introduction

Colon cancer is a serious threat to human health and is the third most diagnosed cancer worldwide[1]. Based on 2022 epidemiological data, colon cancer morbidity and mortality have been trending towards a younger demographic in recent years[2]. Even with improvements in surgical treatments and chemoradiotherapy, and the rapid development of cancer screening methods, many patients are still diagnosed with advanced stage disease[3]. Thus, it is critical to explore effective therapeutic targets for colon cancer patients.
Circular RNAs (circRNAs) play a key role in cancer development and progression using a variety of mechanisms. As a result, circRNAs have a strong potential to serve as diagnostic, prognostic, and predictive biomarkers[4]. Recent studies have shown that circRNA homeodomain-interacting protein kinase 3 (circHIPK3) is closely related to the occurrence and progression of various malignant tumors, including colon cancer[5–8]. CircHIPK3 expression in colorectal cancer (CRC) is significantly higher than in normal tissues, and circHIPK3 downregulation inhibits the malignant behavior of CRC cells. CircHIPK3 is shown to sponge miR-7 in CRC cell lines and upregulate the expression of various oncogenes that impact the proliferation, invasion, migration, and apoptosis of cancer cells[9].

MicroRNAs (miRNAs) are a class of noncoding RNAs, 17–22 nucleotides in length, with key functions in post-transcriptional gene regulation[10]. One miRNA, miRNA-524-5p, is shown to have an inhibitory effect on various cancers, including liver cancer, lung cancer, and glioma[11–13]. Our previous study also found that miRNA-524-5p can inhibit angiogenesis in colon cancer cells[14]. Thus, the impact of circHIPK3’s role as a miRNA-524-5p sponge on the development and progression of colon cancer requires further study.

Nr4A2 promotes cancer cell proliferation, migration, and invasion[15], and serves as a prognostic and predictive marker of colon cancer. High expression of Nr4A2 in CRC cells confers chemotherapeutic resistance in colon cancer patients[16]. Yao et al. [17] found that IncRNA MSC-AS1 aggravated NPC progression by sponging miR-524-5p and increasing Nr4A2 expression. The current study sought to explore the role of the circHIPK3/miR-524-5p/Nr4A2 axis in regulating the biological functions of colon cancer.

Materials And Methods

Culture Of Colon Cancer Cell Lines

HCoEpiC, SW480, Caco-2, HT-29, HCT116 and RKO cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HCoEpiC, HT-29, HCT116, and RKO cells were maintained in RPMI 1640 medium (HyClone, GE Healthcare, UK) containing 10% fetal bovine serum (FBS, HyClone). Caco-2 and SW480 cells were maintained in DMEM medium containing 10% fetal bovine serum. All cells were cultured at 37°C in 5% CO₂.

Transfection

Specific small interfering RNA (siRNA) against circHIPK3 (si circHIPK3) and its siRNA control (si-NC), miR-524-5p mimic and its negative control (control mimic), and miR-524-5p inhibitor and its negative control (control inhibitor) were purchased from RiboBio (Guangzhou, China). Overexpressing Nr4A2 (Nr4A2 OE) and its negative control (Nr4A2 NC) were purchased from OriGene (WuXi, China). HT-29 and Caco-2 cells were transfected with Lipofecamine3000 (Invitrogen, Grand Island, NY) according to the manufacturer’s instructions. The sequences were as follows: si-circHIPK3: 5'‐CUACAGGUAUGGCCUCACA‐3', si-NC: 5'-UUCUCCGAACGUGUCACGUTT-3', control mimic: 5'-CAAAAAACCGAAGCACUUUC-3', miR-524-5p mimic:
Dual-luciferase Reporter Assay

The miR-524-5p binding sites on circHIPK3 were predicted using the bioinformatics database, starBase v2.0 (http://starbase.sysu.edu.cn/). The miR-524-5p and Nr4A2 binding sites were predicted using the TargetScan bioinformatics website (http://www.targetscan.org/). Wild-type (WT) or mutant (MUT) reporter plasmids (RiboBio) of Nr4A2 and circHIPK3 were constructed and named circHIPK3 WT, circHIPK3 MUT, Nr4A2 WT, and Nr4A2 MUT, respectively. HT-29 and Caco-2 cells were co-transfected with the constructed plasmid and the miR-524-5p mimic or control mimic. A dual-luciferase reporter gene detection kit (KeyGENBioTECH, China) was used to measure luciferase activity.

Cck-8 Assay

HT-29 and Caco-2 cells were seeded into 96-well plates (4×10^3 cells). Absorbance measurements were performed using a CCK-8 kit (Dojindo, Kumamoto, Japan) at 24, 48, and 72 h after transfection. The optical density (OD) was measured at a wavelength of 450 nm using a microplate reader.

Cell Migration And Invasion Assays

Transfected cells were seeded into the upper chamber of a transwell plate at 4×10^5 cells/well, and 500 μL of medium containing 20% FBS was added to the lower chamber. The plates were then incubated at 37°C for 12 hours. After fixation and staining, the cells were photographed and quantitated using a microscope. For the invasion experiment, Matrigel (BD Biosciences, San Diego, CA, USA) was added to the upper chamber and solidified at 37°C. The transfected cells (5×10^5 cells/well) from each group were then seeded into the upper chamber. The remaining steps are the same as those described above.

Tunel Assay

Transfected HT-29 or Caco-2 cells were incubated with TUNEL working solution (Solarbio, Beijing, China) for 30 min at 37°C. After washing and fixing the cells, the nuclei were stained with 1X Hoechst. Apoptotic cells were analyzed by fluorescence microscopy.

Qrt-pcr

RNA samples from colon cancer tissues and cells were extracted using TRIzol reagent (Invitrogen, Grand Island, NY, USA), and reverse transcription was performed using the instructions provided by the reverse transcription kit (TaKaRa, Dalian, China). The primers list were as follows: 5’-
GGTCCGGCCAGTCATGTATC-3' (circHIPK3 forward), 5’-ACACAACTGCTTGCTCTACT-3' (circHIPK3 reverse); HIPK3 5’-TCACAAGTCTTTGCTACCC-3' (HIPK3 forward), 5’-CACAAGGCTCGGATAGTTTC-3' (HIPK3 reverse); 5’-GTGGGCTCTGTGCAGGAGGTATTTCAGCACAGAGCCACGAGG-3’ (miR-524-5p RT), 5’-CGCTACAAAGGGAAGACTT-3’ (miR-524-5p forward), 5’-GCAGGGTCCAGGTTATCC-3’ (miR-524-5p reverse); 5’-TCATCTCCCTCAGACTGGGG-3’ (Nr4A2 forward), 5’-TGTACCAAATGCCCCTGTCC-3’ (Nr4A2 reverse); 5’-GCTTCGGCAGCACATATACT-3’ (U6 forward), 5’-GCAGGGTCCAGGTTATCC-3’ (U6 reverse); 5’-GACCTGACCTGCCGTCTAG-3’ (GAPDH forward), 5’-AGGAGTGGGTGTCGCTGT-3’ (GAPDH reverse). GAPDH and U6 were used as internal controls.

**Rnase R Treatment**

Total RNA was treated with 3 U/mg RNase R (Epicentre Technologies, Madison, WI, USA) for 20 min at 37°C. Mock was used as a control. HIPK3 and circHIPK3 expression were measured using qRT-PCR.

**Western Blotting**

RIPA lysis buffer (Beyotime, Shanghai, China) was added to cell or tissue samples to lyse the protein. The protein was then run on a 10% SDS-PAGE gel and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA), blocked in 5% nonfat dry milk for 2 h, and incubated with diluted antibodies overnight at 4°C. The following primary antibodies were used: E2F1, Nr4A2, E-cadherin, N-cadherin, MMP-9, MMP-2, Caspase-3, CL Caspase-3, and GAPDH (Abcam, Cambridge, UK). Protein bands were visualized by chemiluminescence.

**In vivo tumor model**

BALB/c nude mice (4–5 weeks of age; Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were reared for 1 week in a specific pathogen-free environment. Nude mice were injected subcutaneously with 1×10^7 HT-29 cells to establish a human colon cancer xenograft model. When the tumor volume was about 0.5 cm^3, si-circHIPK3 and si-NC were injected into the local tumor mass every 3 days. Tumor size was measured every 7 days. Animal studies followed the Guide for the Care and Use of Laboratory Animal Resources (1996), National Research Council and were approved by the China Medical University Animal Ethics Committee (IACUC Issue No.16071). All procedures accepted supervision and inspection by the committee and the animal laboratory department.

**Immunohistochemical Analysis**

Tumor tissue obtained from the mice was fixed, embedded, sectioned (thickness, 4 μm), and subjected to immunohistochemical experiments. Tumor sections were incubated with Ki67 (Abcam) primary antibody overnight at 4°C, followed by secondary antibody. The sections were then stained with diaminobenzidine
for color development. Immunopositive proteins were observed under a light microscope at 200x and 400x magnification.

Statistical analysis

All experimental data are presented as the mean ± standard deviation of at least three independent experiments. All statistical analyses were performed using SPSS version 20.0 software (IBM Corp., Armonk, NY, USA). Statistical differences between groups were calculated using a one-way analysis of variance (ANOVA) or the Student's t-test. A p-value < 0.05 was considered statistically significant.

Results

Circhipk3 Was Overexpressed In Colon Cancer Cells

QRT-PCR results showed that circHIPK3 was significantly higher in colon cancer cells (SW480, Caco-2, HT-29, HCT116, and RKO) than in normal colon cells (HCoEpiC) (Fig. 1A). In addition, it was confirmed that circHIPK3 was more resistant to RNase R than linear HIPK3 mRNA, suggesting that it can be defined as a circular RNA (Fig. 1B and C).

Circhipk3 Knockdown Inhibited Colon Cancer Cells Proliferation, Migration, And Invasion, And Induced Apoptosis

CircHIPK3 expression was significantly decreased in HT-29 and Caco-2 cells following transfection with si-circHIPK3, indicating that the silencing experiments were effective (Fig. 2A). In addition, after the transfection of si-circHIPK3, HT-29 and Caco-2 cell proliferation decreased significantly by 72 h (Fig. 2B and C). Migration and invasion experiments showed a reduction in the number of migrating and invasive HT-29 and Caco-2 cells after transfection with si-circHIPK3 (Fig. 2D and E), while TUNEL experiments showed a higher number of apoptotic HT-29 and Caco-2 cells following transfection (Fig. 2F). Western blot results showed that Nr4A2 protein expression was also significantly lower after si-circHIPK3 transfection (Fig. 2G). These results indicated that circHIPK3 silencing impacted the proliferation, migration, invasion, and apoptosis of colon cells, highlighting an important role for circHIPK3 in colon cancer.

Circhipk3 Targeted Mir-524-5p

StarBase was used to predict complementary sequences in WT-circHIPK3 and miR-524-5p (Fig. 3A). The constructed dual-luciferase reporter vector containing wild-type or mutant circHIPK3 was then transfected into HT-29 and Caco-2 cells with a miR-524-5p mimic or a control mimic. While circHIPK3 WT luciferase activity was significantly lower in the miR-524-5p mimic group, circHIPK3 MUT luciferase activity was similar between the miR-524-5p mimic and control mimic groups (Fig. 3B and C). QRT-PCR showed that
miR-524-5p expression was significantly lower in colon cancer cells (SW480, Caco-2, HT-29, HCT116, and RKO) than in normal colon cells (HCoEpic) (Fig. 3D). In addition, miR-524-5p expression was significantly higher after transfection of HT-29 and Caco-2 cells with si-circHIPK3 (Fig. 3E). Thus, circHIPK3 can reverse regulate miR-524-5p expression in colon cancer cells.

**Mir-524-5p Knockdown Reversed The Inhibitory Effect Of Circhipk3 Knockdown On Colon Cancer Cells**

CircHIPK3 knockdown was shown to enhance the expression of miR-524-5p in HT-29 and Caco-2 cells, and this was reversed by transfection of a miR-524-5p inhibitor (Fig. 4A). CCK-8 assay results showed that the reduction in si-circHIPK3 cell proliferation was reversed by knockdown of miR-524-5p in HT-29 and Caco-2 cells (Fig. 4B and C). The number of migrating and invasive cells were significantly reduced in HT-29 and Caco-2 cells transfected with si-circHIPK3, and this was reversed by the knockdown of miR-524-5p (Fig. 4D and E). In addition, the number of apoptotic cells was significantly higher in HT-29 and Caco-2 cells transfected with si-circHIPK3, and this was reversed by knockdown of miR-524-5p (Fig. 4F). Meanwhile, Nr4A2, E2F1, N-cadherin, MMP-2, MMP-9, and CL Caspase-3 expression were increased after the downregulation of miR-524-5p in HT-29 and Caco cells transfected with si-circHIPK3. E-cadherin expression was reduced after miR-524-5p downregulation in HT-29 and Caco cells transfected with si-circHIPK3 (Fig. S1). Thus, miR-524-5p knockdown reversed the inhibitory effects of si-circHIPK3 on colon cancer cell proliferation, migration, and invasion, and its stimulatory effect on apoptosis.

**Nr4a2 Acts As A Downstream Target Of Mir-524-5p**

Complementary WT-Nr4A2 and miR-524-5p sequences were predicted using StarBase (Fig. 5A). The constructed dual-luciferase reporter vector containing wild-type or mutant Nr4A2 was then transfected into HT-29 and Caco-2 cells using the miR-524-5p mimic or a control mimic. While Nr4A2 WT luciferase activity was significantly lower in the miR-524-5p mimic group, the Nr4A2 MUT luciferase activity was similar between the miR-524-5p mimic and control mimic groups (Fig. 5B and C). QRT-PCR and Western blot results indicated that Nr4A2 expression was significantly higher in colon cancer cells (SW480, Caco-2, HT-29, HCT116, and RKO) than in normal colon cells (HCoEpic) (Fig. 5D and E). In addition, Nr4A2 expression was significantly lower after transfection of HT-29 and Caco-2 cells with the miR-524-5p mimic (Fig. 5F and G). These data suggest that Nr4A2 is a downstream gene of miR-524-5p.

**Nr4a2 Overexpression Reversed The Inhibitory Effect Of Upregulated Mir-524-5p On Colon Cancer Cells**

Upregulated miR-524-5p inhibited Nr4A2 expression in HT-29 and Caco-2 cells, and this was reversed by transfection of Nr4A2 OE (Fig. 6A and B). CCK-8 assay results showed that the reduced proliferation of HT-29 and Caco-2 cells transfected with the miR-524-5p mimic could be reversed by upregulating Nr4A2
(Fig. 6C and D). HT-29 and Caco-2 cell migration and invasiveness following transfection with the miR-524-5p mimic were similarly reversed by overexpression of Nr4A2 (Fig. 6E and F). In addition, the number of apoptotic cells was significantly higher in HT-29 and Caco-2 cells transfected with the miR-524-5p mimic, and this was reversed by overexpression of Nr4A2 (Fig. 6G). These findings indicated that Nr4A2 overexpression reversed the inhibitory effects of the miR-524-5p mimic on colon cancer cell proliferation, migration, invasion, and its stimulatory role on apoptosis.

**CircHIPK3 promoted tumor growth in vivo**

HT-29 cells were subcutaneously injected into BALB/c nude mice to study the effects of circHIPK3 silencing on colon cancer growth. Transplanted tumors were significantly smaller in the si-circHIPK3 group than in the si-NC group (Fig. 7A and B) and the weight of the transplanted tumors was significantly lower (Fig. 7C). In addition, circHIPK3 and Nr4A2 expression was reduced in the si-circHIPK3 xenograft (Fig. 7D, F, and G). In contrast, miR-524-5p expression was significantly higher (Fig. 7E). Immunohistochemical staining confirmed that the expression of Ki67 was significantly inhibited in si-circHIPK3 xenografts (Fig. 7H). These findings indicated that silencing circHIPK3 suppresses colon cancer growth *in vivo*.

**Discussion**

Evidence indicates that circHIPK3 can serve as an important biomarker for CRC diagnosis and treatment[18]. Results of the current study demonstrate that circHIPK3 silencing inhibits colon cancer cell proliferation, migration, and invasion, promotes apoptosis, and suppresses tumor growth. Studies have shown that circHIPK3 has similar regulatory effects on the proliferation, migration, invasion, apoptosis, and angiogenesis of other malignant tumors such as osteosarcoma, cervical cancer, renal cancer, bladder cancer, gastric cancer, and glioma[19–24]. CircHIPK3 is also found to enhance its FMNL2-mediated migration, invasion, and proliferation-promoting effects by sponging miR-1207-5p in CRC cells[25]. Yin et al.[26] showed that knocking down circHIPK3 promoted temozolomide sensitivity in gliomas by regulating proliferation, metastasis, and apoptosis through miR-524-5p/KIF2A-mediated PI3K/AKT signaling. This finding demonstrated that circHIPK3/miR-524-5p has a regulatory effect on the biological behavior of colon cancer. The current study found that downregulating miR-524-5p reversed the inhibitory effects of si-circHIPK3 on the proliferation, migration, and invasion of colon cancer cells and its stimulatory effect on apoptosis.

The NR4A receptor subfamily is increasingly recognized as an important molecular link between chronic inflammation, altered immune cell responses, and cancer development[27, 28]. Studies indicate that the NR4A gene can act as an oncogene in lung, melanoma, and colorectal cancer (CRC), while exhibiting a tumor suppressor effect in acute myeloid leukemia (AML), breast cancer, and metastatic ovarian cancer[29–31]. In the current study, Nr4A2 overexpression reversed the inhibitory effects of miR-524-5p mimics on colon cancer cell proliferation, migration, and invasion, while promoting apoptosis. In summary, functional experiments showed that circHIPK3 inhibited the proliferation, migration, and
invasion of colon cancer cells through the miR-524-5p/Nr4A2 axis, and promoted apoptosis, thereby hindering the progression of colon cancer. These findings suggest that circHIPK3 could serve as a potential target for colon cancer therapy.

Declarations

Acknowledgments
None

Conflict of interest Statement

The author(s) declare no competing interest.

Author Contribution Statement

L. X. drafted the article, collected, analyzed, and interpreted the data. L.Z.T. and L.C.J. critically revised the article and contributed to data acquisition. Z.G.S. contributed to the provision and cultivation of cells. B.D.D. collected and analyzed the data. Z.Z. contributed to the concept and design of this study and is responsible for all aspects of the work.

Ethics Statement

The study was carried out in compliance with the ARRIVE guidelines. Animal studies were performed in compliance with the Guide for the Care and Use of Laboratory Animal Resources (1996), the National Research Council, and approved by the Animal Ethics Committee of the China Medical University (IACUC Issue No.16071). All procedures followed supervision and inspection by the Committee and the Laboratory Animal Department.

Funding Statement

This study was supported by grants from the National Natural Science Foundation of China (No. 81471809; No.81971639).

Data Availability Statement

The datasets are available from the corresponding author on reasonable request.

References


Figures
Figure 1

CircHIPK3 expression in colon cancer cells. A. Relative circHIPK3 expression in colon cancer cells. B and C. CircHIPK3 and HIPK3 mRNA levels in HT-29 and Caco-2 cells. *P<0.05 vs. the indicated group.

Figure 2
Effect of circHIPK3 silencing on colon cancer cell proliferation, migration, invasion, and apoptosis.  
A. Relative circHIPK3 expression in HT-29 and Caco-2 cells. The proliferation of  
B. HT-29 and  
C. Caco-2 cells after transfection with si-circHIPK3.  
D. Representative images of HT-29 and Caco-2 cell migration and an  
assessment of the number of migrated cells.  
E. Representative images of HT-29 and Caco-2 cell migration and an  
assessment of the number of invaded cells.  
F. The apoptotic capacity of HT-29 and Caco-2 cells transfected with si-circHIPK3.  
G. Expression of Nr4A2 in HT-29 and Caco-2 cells. Magnification ×200.  
*P <0.05 vs. NC group.

Figure 3

MiR-524-5p is a target of circHIPK3.  
A. StarBase predicted the binding site of miR-524-5p in circHIPK3.  
B and C. Relative luciferase activity in HT-29 and Caco-2 cells.  
D. Relative miR-524-5p expression in colon cancer cells.  
E. MiR-524-5p expression in HT-29 and Caco-2 cells after transfection with si-circHIPK3.  
*P <0.05 vs. the indicated group.
Figure 4

MiR-524-5p knockdown reverses the inhibitory effect of circHIPK3 knockdown on colon cancer cells.  
A. Relative miR-524-5p expression in HT-29 and Caco-2 cells.  
B and C. Proliferative capacity of HT-29 and Caco-2 cells.  
D and E. Representative images of cell migration and invasion.  
F. Representative images of apoptotic capacity. Magnification ×200. *P<0.05 vs. the indicated group.
**Figure 5**

**Nr4A2 acts as a downstream target of miR-524-5p.** A. StarBase predicted the binding site of miR-524-5p in Nr4A2. B and C. Relative luciferase activity of HT-29 and Caco-2 cells. D and E. Relative Nr4A2 expression in colon cancer cells. F and G. Nr4A2 expression in HT-29 and Caco-2 cells after transfection with the miR-524-5p mimic. *P* < 0.05 vs. the indicated group.
Overexpression of Nr4A2 reverses the inhibitory effect of upregulated miR-524-5p on colon cancer cells. A and B. Relative Nr4A2 expression in HT-29 and Caco-2 cells. C and D. Proliferative capacity of HT-29 and Caco-2 cells. E and F. Representative images of cell migration and invasion ability. G. Representative images of apoptotic capacity. Magnification ×200. *P<0.05 vs. the indicated group.
Figure 7

**Effect of miR-524-5p on tumor growth in vivo.**  
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigS1.tif