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Upregulation of circHIPK3 promotes the progression of gastric cancer *via* Wnt/β-catenin pathway and indicates a poor prognosis

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Abstract. – OBJECTIVE: To elucidate the regulatory effect of circular RNA HIPK3 (circHIPK3) on the progression of gastric cancer (GC) by regulating the Wnt/β-catenin pathway. We aim to reveal whether the abnormal expression of circHIPK3 could predict the poor prognosis of GC.

PATIENTS AND METHODS: CircHIPK3 level in GC tissues and paracancerous tissues was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Its level in GC cell lines (BGC, MGC, SGC and MKN) and gastric mucosal cell line (GES) was examined as well. The correlation between the circHIPK3 level and overall survival of GC was analyzed by the Kaplan-Meier method. Regulatory effects of circHIPK3 on the proliferative ability of GC cells were evaluated by Cell Counting Kit-8 (CCK-8) and colony formation assay, respectively. Migratory changes influenced by circHIPK3 were explored through wound healing assay. The expression levels of relative genes in the Wnt/β-catenin pathway were detected in GC cells with circHIPK3 knockdown. Finally, the Wnt/β-catenin pathway inhibitor PNU-74654 was applied for detecting its influence on cellular behaviors of GC cells.

RESULTS: CircHIPK3 level was higher in GC tissues relative to paracancerous tissues. Identically, its level was higher in GC cells compared with that of GES cells. The expression level of circHIPK3 was negatively correlated to the overall survival of GC patients. Silence of circHIPK3 weakened proliferative and migratory abilities of GC cells. In addition, circHIPK3 knockdown markedly downregulated levels of WNT1, TCF4 and β-catenin. Application of PNU-74654 weakened the abilities of GC cells to proliferate and migrate.

CONCLUSIONS: CircHIPK3 promotes GC cells to proliferate and migrate by regulating the Wnt/β-catenin pathway. Upregulation of circHIPK3 indicates poor prognosis of GC patients.

Key Words:
Gastric cancer, CircHIPK3, Wnt/β-catenin, Proliferation, Migration.

Introduction

Globally, gastric cancer (GC) is a common malignancy and has the highest incidence in the digestive tract. It is the fourth most common tumor in the world, and its mortality ranks the second of tumor death[1]. The incidence of GC has declined in the United States, Australia, New Zealand and other places in recent years. However, its prevalence gradually increases in Asia and eastern South America[2]. Although therapeutic methods for GC, such as radiotherapy, surgery and chemotherapy have been progressed, the postoperative 5-year survival is still low[3]. Early diagnosis and treatment of GC is an effective way to reduce mortality. At present, gastroscopy combined with pathological examination is still the gold standard for the diagnosis of GC. Unfortunately, the popularization of gastroscopy is limited due to economic factors, especially in less-developed areas. Tumor markers help to assist the diagnosis of GC, but could not completely replace gastroscopy owing to their low sensitivity and specificity. It is urgent to develop novel hallmarks for precisely diagnosing GC as early as possible[4].

Cyclic RNA (circDNA) is a recently discovered special, non-coding RNA (ncRNA). It was first discovered by Sanger et al[5] in a viroid RNA study of advanced plants in 1976. CircRNA is stably, abundantly and conservatively expressed in eukaryotes[6]. The great abundances of circRNAs varying from 100 nt to 4 kb have been extensively concerned[7]. Owing to the closed circular struc-

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ture, circRNA is not affected by exonuclease and is more stable than linear RNA[8]. As the microRNA sponge, circRNAs participate in protein binding, gene expression regulation, protein synthesis as translation templates and other crucial biological processes[9]. Research has shown the involvement of circRNAs in the occurrence and development of esophageal cancer[10], colon cancer[11], breast cancer[12], bladder cancer[13] and gastric cancer[14,15].

CircHIPK3 is a highly conserved and stable circRNA in many tissues, with 1099 bp in length[16]. Studies[17,18] have shown that circHIPK3 exerts a very important role in tumorigenesis. Abnormally expressed circHIPK3 can lead to abnormal proliferation, migration or invasion of tumor cells[19]. For example, circHIPK3 is upregulated in gallbladder carcinoma and promotes proliferative abilities by inhibiting miR-124 level[20]. CircHIPK3 is downregulated in osteosarcoma and inhibits osteosarcoma cell growth and metastasis[21]. Overexpression of circHIPK3 in colorectal cancer accelerates tumor proliferation and metastasis by targeting miR-7[18]. However, expression pattern and biological function of circHIPK3 in GC remains unknown. This study aims to investigate whether circHIPK3 is involved in the development of GC and its specific mechanisms.

Patients and Methods

Sample Collection

53 pairs of GC and paracancerous tissues were harvested from patients undergoing radical gastrectomy in our hospital. Tissues were immediately frozen in liquid nitrogen and stored at -80°C. None of the patients received preoperative radiation or chemotherapy. This study was approved by the Ethics Committee of Beijing Bo'ai Hospital. Signed informed consents were obtained from all participants before the study.

Cell Culture and Transfection

GC cell lines (BGC, MGC, SGC and MKN) and gastric mucosal cell line (GES) were provided by Cell Bank, Chinses Academy of Science (Shanghai, China). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL penicillin-streptomycin and maintained in a 5% CO_2 incubator at 37°C. Transfection vectors (si-NC, si-circHIPK3) and Wnt/β-catenin pathway inhibitor PNU-74654 were provided by RiboBio (Guangzhou, China). Cell transfection was conducted at 80-90% of confluence using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfection efficacy was verified at 48 h.

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

We extracted total RNA from cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and quantified using UV spectrophotometer. Extracted RNA (10 ng) was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using Prime Script RT Reagent (TaKaRa, Otsu, Shiga, Japan). The cDNA was amplified by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) at 50°C for 2 min, 95°C for 10 min and followed by 45 cycles of 95°C for 15 s and 60°C for 60 s. GAPDH was used as internal references. Primer sequences were as follows: CircHIPK3, F: 5'-CTACAGATCCGACCAGGAGTTC-3'; R: 5'-TGTGAACCAGCCACACTCTCAG-3'; WNT1, F: 5'-TGTTGACGGATTCCAAGAGT-3'; R: 5'-GAAGTAGACGAGGTCGTGAG-3'; TCF4, F: 5'-ACCACATGACTAGCAGGGATCT-3'; R: 5'-GGAGGAACTTTTCGGACTTTCT-3'; β-catenin, F: 5'-GGCAACCCTGAGGAAGAAGA-3'; R: 5'-AGCGTCAAACTGCGTGGAT-3'; GAPDH, F: 5'-GGAATCCACTGGCGTCTTCA-3'; R: 5'-GGTTCACGCCCATCACAAAC-3'.

Cell Proliferation Assays

Cells were seeded in the 96-well plate with 4.0×10^3 cells per well. Viability was determined at the appointed time points using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). The absorbance at 450 nm was recorded for plotting the proliferation curve.

Colony Formation Assay

The cells were seeded in a 6-well plate with 200 cells per well and incubated for 14 days. Subsequently, the cells were fixed in 100% methanol and dyed with 0.5% crystal violet for 20 min. Colonies were observed and calculated using the AID iSpot Reader (Autoimmun Diagnostika GmbH, Strassberg, Germany).

Wound Healing Assay

The cells were seeded in a 6-well plate with 4.0×10^6/well. Until 80% of confluence, an artificial wound was created in the confluent cell

monolayer using a 1 mL pipette tip. The images were taken at 0 and 24 h using an inverted microscope, respectively. The assay was terminated until wound closure in the control group.

Western Blot

Cells were lysed for extracting total protein using radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China), quantified by bicinchoninic acid (BCA) method (Pierce, Waltham, MA, USA) and loaded for electrophoresis. After transferring on a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), it was blocked in 5% skim milk for 2 hours, incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Bands were exposed by electrochemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) and analyzed by Image J Software (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. The student *t*-test was applied for analyzing the intergroup differences. Categorical data were analyzed by Chi-square test. Kaplan-Meier was introduced for survival analysis. $p<0.05$ was considered statistically significant.

Results

Upregulation of CircHIPK3 in GC

QRT-PCR revealed a higher level of circHIPK3 in GC tissues relative to paracancerous tissues (Figure 1A). Compared with GES cells, circHIPK3 was highly expressed in GC cell lines as well (BGC, MGC, SGC, MKN; Figure

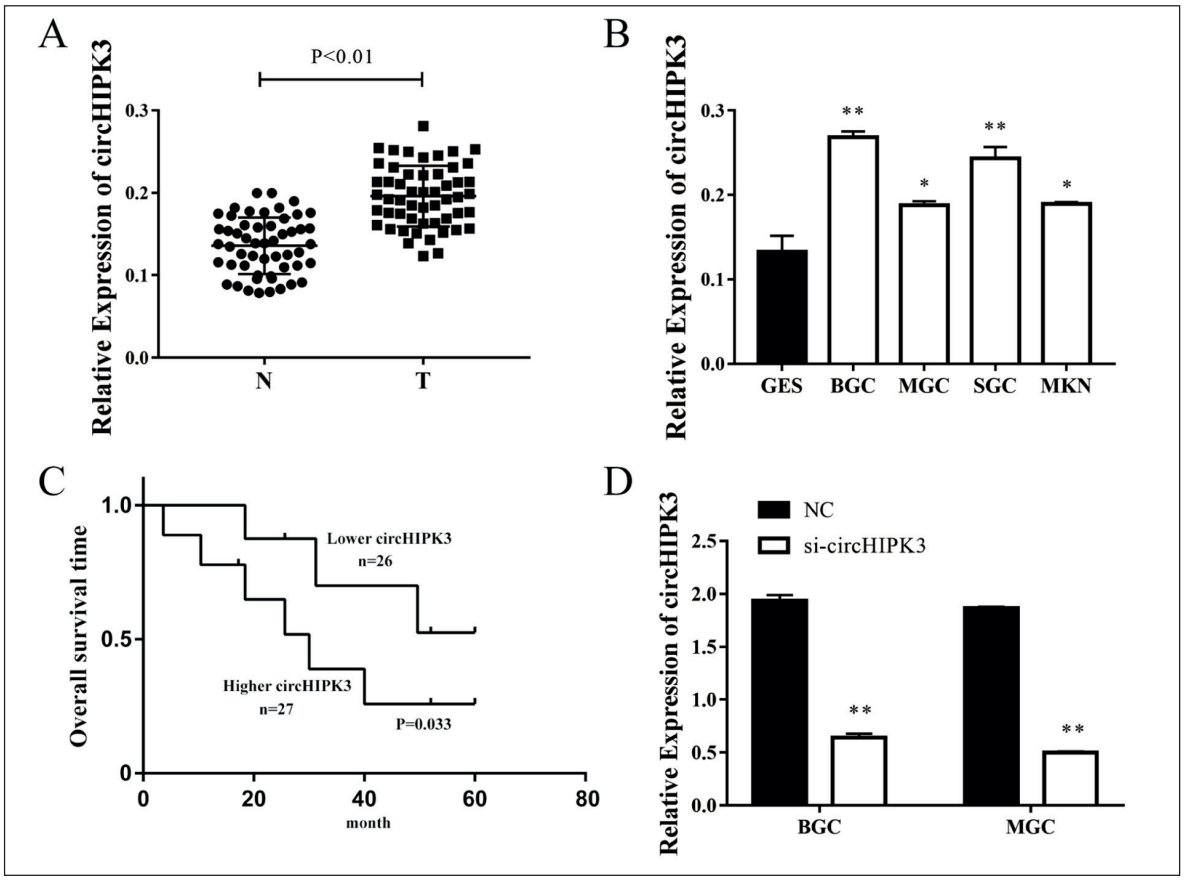

Figure 1. Upregulation of circHIPK3 in GC. **A,** QRT-PCR revealed higher level of circHIPK3 in GC tissues relative to paracancerous tissues. **B,** QRT-PCR revealed higher level of circHIPK3 in GC cell lines as well (BGC, MGC, SGC, MKN) relative to GES cell line. ***C,*** The Kaplan-Meier curve indicated that GC patients with high level of circHIPK3 had shorter overall survival than those with low level. **D,** Transfection of si-circHIPK3 sufficiently downregulated circHIPK3 level in BGC and MGC cells.

1B). Survival analysis was conducted based on the collected follow-up data. GC patients with high level of circHIPK3 tended to experience shorter overall survival than those with low level (Figure 1C). To elucidate the potential function of circHIPK3, we constructed si-circHIPK3 and tested its transfection efficacy. Transfection of si-circHIPK3 sufficiently downregulated circHIPK3 level in BGC and MGC cells (Figure 1D). It is demonstrated that circHIPK3 could be utilized as a hallmark for predicting the poor prognosis of GC.

Knockdown of CircHIPK3 Suppressed Proliferative and Migratory Capacities of GC

Viability curve illustrated that proliferative ability of BGC and MGC cells transfected with si-circHIPK3 was weakened (Figure 2A). Similarly, knockdown of circHIPK3 showed fewer colonies in GC cells relative to control, indicating the inhibited proliferative ability (Figure 2B). Wound healing assay was performed here to evaluate the influence of circHIPK3 on the migratory ability of GC. Wound closure was much more

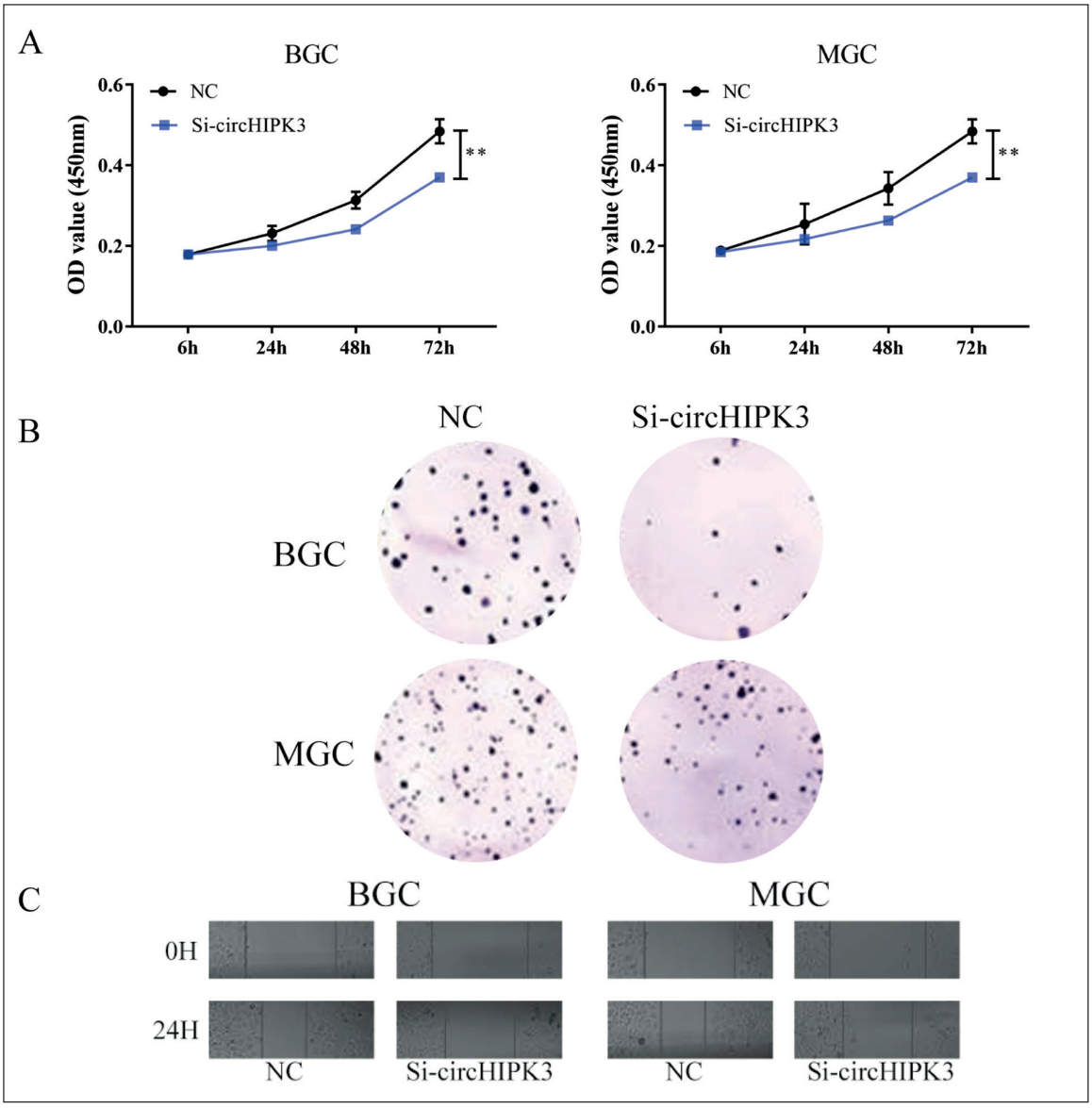

Figure 2. Knockdown of circHIPK3 suppressed proliferative and migratory capacities of GC. **A,** The CCK-8 assay showed that knockdown of circHIPK3 inhibited proliferative ability of BGC and MGC cells. **B,** Colony formation assay showed that knockdown of circHIPK3 showed fewer colonies in BGC and MGC cells. **C,** Wound healing assay showed that knockdown of circHIPK3 inhibited migratory ability of BGC and MGC cells.

pronounced in the control group compared with those transfected with si-circHIPK3, suggesting the inhibited migratory ability (Figure 2C).

Knockdown of CircHIPK3 Inhibited the Wnt/β-Catenin Pathway

The Wnt/β-catenin pathway is a classical proliferation-relative pathway. QRT-PCR data revealed the downregulated levels of WNT1, TCF4 and β-catenin in BGC and MGC cells transfected with si-circHIPK3 (Figure 3A, 3B). Identically, the protein levels of them were markedly downregulated by circHIPK3 knockdown in GC cells (Figure 3C).

Inhibition of the Wnt/β-catenin Pathway Suppressed Proliferative and Migratory Capacities of GC

To further clarify the influence of the Wnt/β-catenin pathway on proliferative and migratory capacities of GC, we applied its inhibitor PNU-74654 in GC cells. Application of PNU-74654 markedly inhibited the relative levels of WNT1, TCF4 and β-catenin in GC cells (Figure 4A). As the CCK-8 results indicated, PNU-74654 treatment greatly inhibited the viability in BGC and MGC cells (Figure 4B). Similar results were obtained in the colony formation assay, demonstrating the inhibited proliferative ability by PNU-74654 application (Figure 4C). Finally, PNU-74654 treatment was found to be capable of inhibiting the migratory rate of GC cells (Figure 4D). The above results clarified that circHIPK3 regulated GC progression *via* activating the Wnt/β-catenin pathway.

Discussion

Gastric cancer (GC) is a common malignancy that endangers human health, with 1,000,000 newly onsets per year. China is one of the areas with a high incidence of GC. The annual incidence

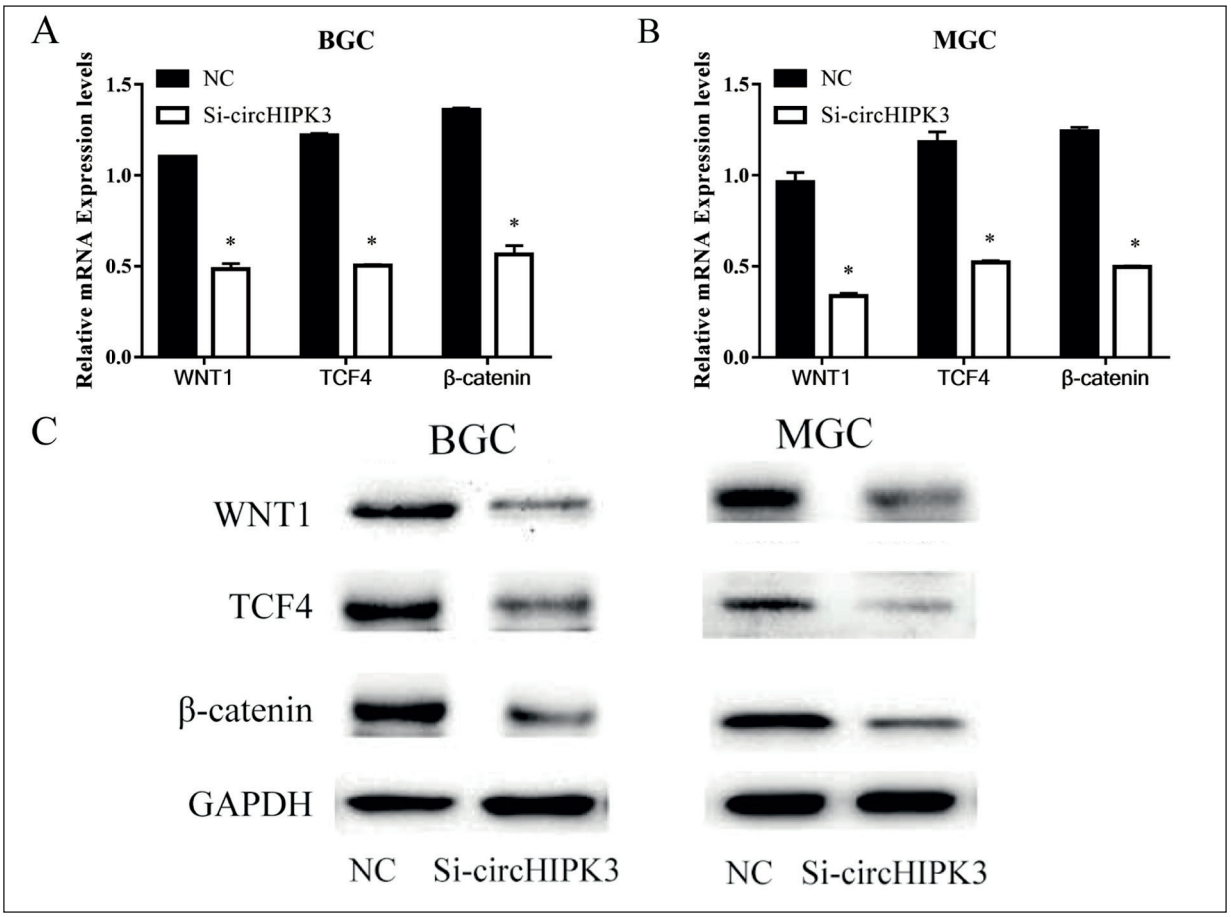

Figure 3. Knockdown of circHIPK3 inhibited the Wnt/β-catenin pathway. **A-B,** QRT-PCR data revealed the downregulated mRNA levels of WNT1, TCF4 and β-catenin in BGC and MGC cells transfected with si-circHIPK3. **C,** Western blot analysis revealed the downregulated protein levels of WNT1, TCF4 and β-catenin in BGC and MGC cells transfected with si-circHIPK3.

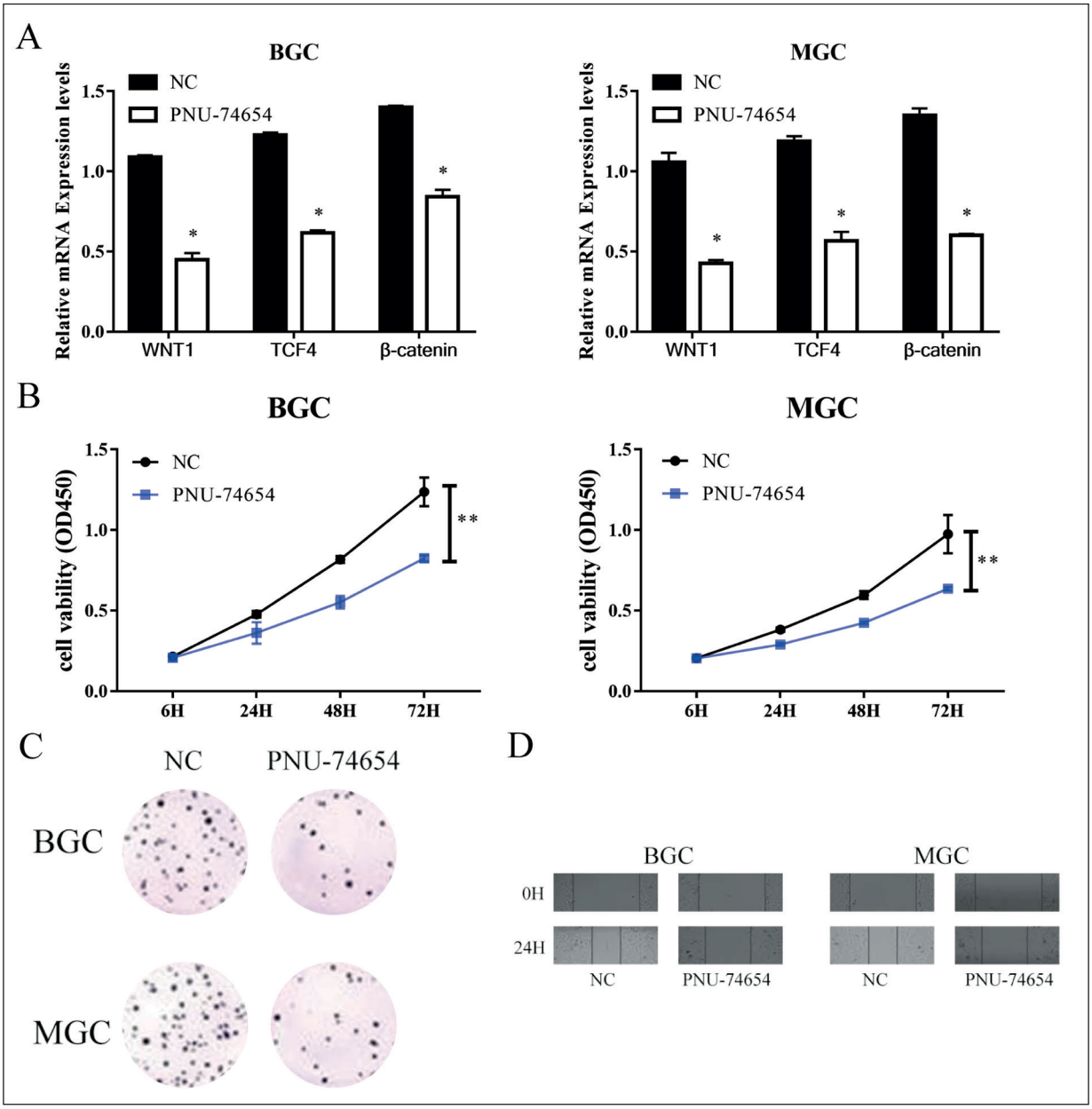

Figure 4. Inhibition of the Wnt/β-catenin pathway suppressed proliferative and migratory capacities of GC**. A,** Application of PNU-74654 markedly inhibited relative levels of WNT1, TCF4 and β-catenin in BGC and MGC cells. **B,** The CCK-8 results showed that PNU-74654 treatment greatly inhibited the viability in BGC and MGC cells. **C,** Colony formation assay showed that PNU-74654 treatment greatly inhibited the proliferation in BGC and MGC cells. **D,** Wound healing assay showed that PNU-74654 treatment greatly inhibited the migration in BGC and MGC cells.

and mortality of GC in China is over 400,000 and 300,000, respectively. GC is the second malignant tumor in China only after lung cancer, accounting for over 40% of GC-induced death globally[22-24]. The pathogenesis of GC is complex, involving multiple genes, factors and pathways. In particular, abnormal activation of pathways relative to growth and proliferation are of significance in the tumorigenesis of GC, including Notch, Ras, Jnk, mTOR and Wnt pathways[25]. The Wnt/β-catenin pathway has been well concerned in tumor researches.

Meanwhile, circRNAs are closely related to tumor proliferation, invasion and apoptosis[26,27]. For example, hsa_circ_0020397 has been found to promote proliferative and invasive abilities,

but inhibit apoptosis of colorectal cancer cells by targeting miR-138[27]. Multiple circRNAs related to the etiology of GC are downregulated. These abnormally expressed circRNAs finally lead to changes in biological genetic information, further resulting in the occurrence and development of GC. Their expression levels, importantly, are correlated to pathological indexes, such as tumor diameter, lymph node metastasis, distant metastasis and TNM stage[28]. Chen et al[29] pointed out the negative correlation between hsa_circ_0000190 level and tumor size of GC.

Tumor development is closely related to the infinite proliferation, invasion and metastasis of tumor cells. The Wnt/β-catenin pathway regulates the abnormal expressions of multiple effector molecules involved in differentiation, invasion and metastasis of GC[30]. Akaboshi et al[31] have shown that Wnt/β-catenin promotes the proliferative rate of GC cell line MKN45 and inhibits cell apoptosis by regulating HMGA1 expression. β-catenin can upregulate the expressions of SNAIL and ZEB1, thereafter participating in the occurrence of endometriosis due to EMT of uterine epithelial cells[32]. Methylation of secreted Frizzled Protein-related Protein 2 (sFRP2) effectively inhibits apoptosis of renal small cell carcinoma cells by activating Wnt/β-catenin[33]. It is suggested that targeted inhibition of Wnt/β-catenin may be one of the most effective approaches to anti-tumor therapy[34].

In this paper, circHIPK3 was upregulated in GC tissues and cell lines. By analyzing the follow-up data, we found that circHIPK3 level was negatively correlated to the overall survival of GC. After downregulation of circHIPK3, the proliferative and migratory capacities of GC were attenuated. Interestingly, we found the inhibited Wnt/β-catenin pathway after the circHIPK3 knockdown. To further elucidate the potential mechanism of circHIPK3 in regulating GC progression, the Wnt/β-catenin pathway inhibitor PNU-74654 was applied. Proliferative and migratory capacities of GC were markedly inhibited due to the application of PNU-74654. Hence, we believed that circHIPK3 regulated GC progression *via* activating the Wnt/β-catenin pathway.

Conclusions

CircHIPK3 promotes GC cells to proliferate and migrate by regulating the Wnt/β-catenin pathway. Upregulation of circHIPK3 indicates poor prognosis of GC patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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