Circ_0049447 acts as a tumor suppressor in gastric cancer through reducing proliferation, migration, invasion, and epithelial-mesenchymal transition

Kai-Wen Tang, Zhe-Xu Guo, Zhong-Hua Wu, Cen Zhou, Jie Sun, Xin Wang, Yong-Xi Song, Zhen-Ning Wang

Department of Surgical Oncology and General Surgery, Key Laboratory of Precision Diagnosis and Treatment of Gastrointestinal Tumors, Ministry of Education, The First Affiliated Hospital of China Medical University, Shenyang, Liaoning 110001, China.

Abstract

Background: Although increasing abnormal expression of circular RNAs (circRNAs) has been revealed in various cancers, there were a small number of studies about circRNAs in gastric cancer (GC). Here, we explored the expression and function of a novel circRNA, circ_0049447, in GC.

Methods: A total of 80 GC tissues and non-tumorous tissues were collected from the First Affiliated Hospital of China Medical University. And all cells were cultured with 10% fetal bovine serum and incubated at 37°C and 5% CO_2. The expression of circ_0049447 was quantified by real-time polymerase chain reaction. The biological function of circ_0049447 on proliferation, migration, invasion, and epithelial-mesenchymal transition (EMT) was evaluated by cell counting kit-8 (CCK-8), colony formation assay, transwell migration and invasion assay, and Western blotting. Luciferase report assay was used to verify the direct binding between circ_0049447 and predicted microRNA (miRNA). Furthermore, a xenograft mouse model was used to validate the function of circ_0049447 *in vivo*.

Results: We demonstrated that circ_0049447 was downregulated in GC ($P < 0.001$). The area under the receiver operating characteristic curve reached 0.838, while sensitivity was 82.3% and specificity was 77.2%. CCK-8 and colony formation assay showed that overexpression of circ_0049447 could inhibit the proliferation ($P < 0.05$). Transwell migration and invasion assay showed upregulated circ_0049447 could impede migration in GC cells ($P < 0.05$). In addition, overexpression of circ_0049447 could impede GC cell EMT. Upregulation of miR-324-5p in GC specimens and direct binding between miR-324-5p with circ_0049447 proven by luciferase reporter assay indicated that circ_0049447 may inhibit GC by sponging certain miRNA.

Conclusion: Circ_0049447 acts as a tumor suppressor in GC through reducing proliferation, migration, invasion, and EMT, and it is a promising biomarker for diagnosis.

Keywords: Gastric cancer; CircRNAs; Biomarker

Introduction

Gastric cancer (GC) is one of the most common malignant neoplasms. Although its morbidity has declined over time, GC is still the fifth most commonly diagnosed cancer and ranks third as a cause of cancer-associated deaths.[1,2] There were over 1,000,000 new cases of GC and an estimated 783,000 deaths caused by GC worldwide in 2018.[2] Since there are no obvious symptoms for early stage GC, patients are often diagnosed at an advanced stage when many are already metastatic, rendering them inoperable or necessitating palliative chemotherapy.[3] Therefore, there is an urgent need to find novel targets that can promote GC diagnosis and therapy.

Access this article online

Quick Response Code:

Website: www.cmj.org

DOI: 10.1097/CM9.0000000000001494

Circular RNAs (circRNAs) are characterized as covalently closed loops of RNA without 5′ caps or 3′ tails.[4] As a result of this structure, circRNAs are more stable and not easily degraded by endonucleases.[5] With the development of high-throughput sequencing, many circRNAs have been recognized. Accumulating recent evidences have indicated that circRNAs are abnormally expressed in lung cancer, breast cancer, and other cancers.[6,7] CircRNAs have been reported to regulate gene expression, to sponge microRNAs (miRNAs) and proteins, and to act as scaffolds for circRNA-protein complexes.[8] It has been shown that circRNAs can regulate proliferation, invasion, metastasis,

Correspondence to: Prof. Zhen-Ning Wang, Department of Surgical Oncology and General Surgery, Key Laboratory of Precision Diagnosis and Treatment of Gastrointestinal Tumors, Ministry of Education, The First Affiliated Hospital of China Medical University, 155 North Nanjing Street, Heping District, Shenyang, Liaoning 110001, China
E-Mail: josieon826@sina.cn

Chinese Medical Journal 2021;134(11)

Received: 01-09-2020 **Edited by:** Li-Shao Guo

and stemness in cancer cells via sponging miRNAs. For example, upregulated circFGFR1 acts as a sponge for miR-381-3p and enhances the expression of CXCR4 to promote lung cancer progression and drug resistance.[9] In addition, Du *et al*[10] found that circSKA3 acts as a binding partner to Tks5 and integrin β1 in the formation of invadopodia to promote tumor invasiveness. Although abnormally increased expression of circRNAs has been observed in various cancers, there are a small number of researches on circRNAs in GC has emerged, suggesting that circRNAs are promising therapeutic targets for GC. However, research on circRNAs in GC is still in the initial stage. Therefore, the function and mechanism of circRNAs in GC still needs further exploration.

In this study, we explored the expression and function of a novel circRNA, circ_0049447, in GC. Circ_0049447 is located at chr19:11446123–11448136 and has a spliced length of 533 nt. We show that circ_0049447 is down-regulated in GC and that overexpression of circ_0049447 could reduce proliferation, migration, invasion, and epithelial-mesenchymal transition (EMT) in GC cells.

Methods

Ethical approval

The present study was approved by the Ethics Committee of the First Affiliated Hospital of China Medical University (No. AF-SOP-07-1.0-01). Patients signed an informed written consent form.

Specimens and clinicopathological information

GC tissue specimens were obtained from 80 patients undergoing radical gastrectomy in the First Affiliated Hospital of China Medical University from 2016 to 2017, including 61 male patients and 19 female patients. All GC specimens were selected from the central mucosa of the tumor, and normal adjacent tissues were selected from the location >5 cm from the tumor edge. To ensure specimen integrity and prevent degradation, all fresh specimens were frozen in liquid nitrogen tanks before storage at −80°C.

The clinicopathological information of all specimens was collected. The diagnosis of GC patients was confirmed by post-operative pathology. All patients in this study did not receive radiotherapy or chemotherapy before the operation. Clinicopathological information was used to analyze the significance and prognostic value of circ_0049447.

RNA preparation and real-time polymerase chain reaction (PCR)

Total RNA was extracted from specimens and cultured cells using the TRIzol reagent (Invitrogen, Waltham, MA, USA), then a nanophotometer (Implen, München, Germany) was used to measure RNA concentration and purity by ultraviolet (UV) spectrophotometry (A260/A280 > 1.8). The first strand of complementary DNA (cDNA) was synthesized using the PrimeScript™ RT reagent Kit (Takara, Japan) and real-time PCR was operated by using TB Green® *Premix Ex Taq™* II (Takara) as described.[11] The specificity of the PCR amplification product was validated by the melting curve for each experiment. Additionally, the product from the circRNA primers was validated by sequencing. Relative expression of circ_0049447 was normalized to a house-keeping gene (*ACTB*) and calculated using the $2^{-\Delta\Delta Ct}$ method.[12] Primers used are as follow: *ACTB* forward: 5′-GAGGCGTACAGGGATAGCAC-3′ and reverse: 5′-GTCACCAACTGGGACGACAT-3′; circ_0049447 forward: 5′-GTATGACATCGCCAATCAGG-3′ and reverse: 5′-AGAGGAGACGGGCGTCCAAG-3′.

Cell culture

Human GC cell lines AGS (American type culture collection [ATCC]), HGC-27 (Institute of Biochemistry, Cell Biology [IBCB], Chinese Academy of Sciences [CAS]), and MGC-803 (IBCB, CAS) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen) with 10% fetal bovine serum (FBS) and incubated at 37°C and 5% CO_2.

Plasmids and transfection

To construct overexpression vectors, full-length circ_0049447 cDNA was cloned into the pcDNA3.1 plasmid (circ_0049447 group). A pcDNA3.1 negative control plasmid with no functional cDNA was also constructed (pcDNA3.1 group). Transfections were performed using the Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's protocol. The cells were passaged at a 1:3 dilution with 400 μg/mL of G418 to screen the cells stably overexpressing circ_0049447. Transfection efficiency was confirmed by real-time PCR.

Cell counting kit-8 (CCK-8) proliferation assay

To check cellular proliferation capacity, 3×10^3 transfected cells were placed in a 96-well plate with 100 μL of cell suspension. Absorbance values were measured at 0, 24, 48, 72, or 96 h.

Colony formation assay

Transfected cells (1×10^3) were placed in a six-well plate and incubated, with culture media changed every 2 days. After 1 week, colonies were stained with crystal violet for 10 min. After washed with phosphate buffer saline, colonies were counted by the naked eye.

Migration and invasion assay

Cell migration and invasion activity were determined by using transwell (Corning, Corning, NY, USA) and matrigel (BD Biosciences, San Jose, CA, USA). Transfected GC cells (approximately 5×10^4) in 200 μL FBS-free medium were placed into the top chamber. For the invasion assay, 50 μL diluted matrigel was first added to the top chamber and incubated for 4 h at 37°C with 5% CO_2 before plating the cells. After 48 h of culture, the number of cells adhering to the bottom side was counted by using a microscope.

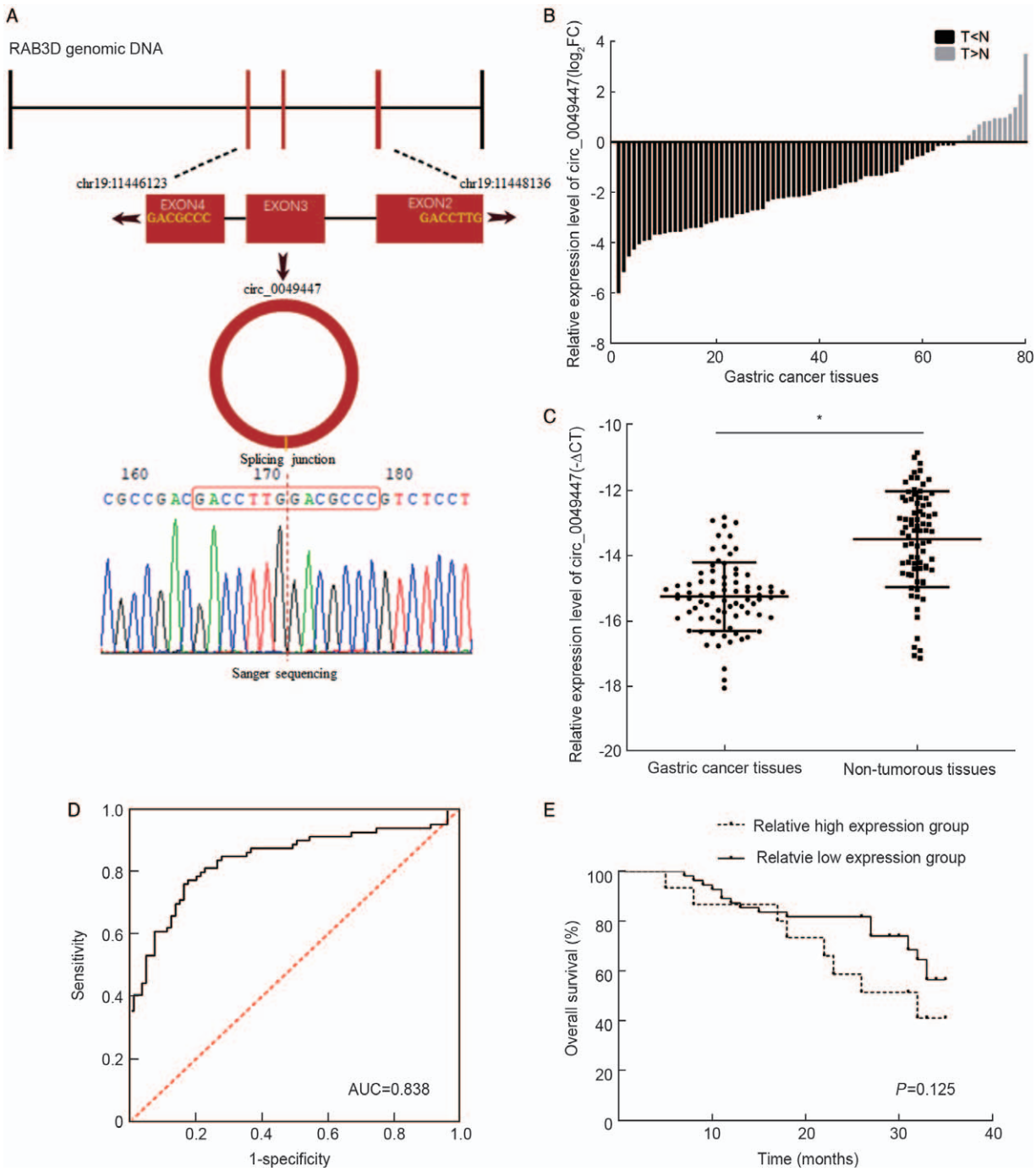

Figure 1: Expression of circ_0049447 in GC and its clinical value. (A) Simulated diagram and reverse splicing sequence of circ_0049447. (B) The percentage of low expression of circ_0049447 in tissue accounts for 83.75% (67/80). (C) Relative expression level of circ_0049447 in 80 GC tissues and in paired adjacent normal tissues ($n = 80$, $P < 0.001$). (D) The ROC curve of circ_0049447. The area under the curve (AUC) was up to 0.838, while sensitivity was 82.3% and specificity was 77.2%. (E) Kaplan-Meier survival analysis for OS of 80 patients with GC in the relatively low and high expression groups showed no significant difference between the two groups. $^{*}P < 0.001$. GC: Gastric cancer; OS: Overall survival; ROC: Receiver operating characteristic.

Western blotting

Cellular protein was collected by total protein extraction kit (KeyGen Biotech, Nanjing, China). Western blotting was performed using precast gels (KeyGen Biotech) according to the manufacturer's protocol. The proteins were incubated with primary antibodies: E-cadherin (SAB, #48355), N-cadherin (CST, 13116S), twist (Santa, sc-81417), vimentin (SAB, #41532), β-catenin (Santa, sc-7963), and β-actin (SAB, #21800).

Luciferase reporter assay

The MGC-803 cells were seeded in a 24-well plate overnight and then co-transfected with pmirGLO-circ_0049447 wide-type (circ_0049447 WT) or mutant (MUT) and miR-324-

5p mimics or negative control. Forty-eight hours later, the supernatant was obtained after lysis of cells. Luciferase activities were measured with the dual-luciferase reporter assay system (Promega, Madison, WI, USA).

Xenograft mouse model

Six-week-old male BALB/C nude mice were randomly divided into two groups ($n = 5$/group) and 2×10^6 GC cells were subcutaneously injected into the right axillary area. The volume of the tumor was measured once 2 days (volume = $0.5 \times$ length $\times$ width^2) and weight of tumor was recorded when the mice were sacrificed on day 14. Tumors were isolated and were further processed for immunohistochemistry.

Immunohistochemistry staining

After the *in vivo* tumor is prepared into the slide, immunohistochemistry staining was performed using immunohistochemical kit (ZSGB-BIO, Beijing, China) according to the manufacturer's protocol. And primary antibodies against β-catenin (Abcam, ab32572, Cambridge, England) and wnt1 (Abcam, ab15251) were used.

Statistical analysis

Data are presented as mean ± standard deviation. All statistical analyses in this study were performed using SPSS 22.0 software (IBM Corp, Armonk, NY, USA) and GraphPad Prism 8. Student's *t* test, Mann-Whitney *U* test, and Kruskal-Wallis *H* test were used to evaluate the significance of variance. The Kaplan-Meier method with Wilcoxon test was used to evaluate survival.

Results

Expression of circ_0049447 in GC and its relationship with clinicopathological characteristics

To detect circ_0049447 expression in GC, we designed primers according to its reverse spliced sequence. To confirm the primers' specificity, we verified the sequence of the real-time PCR product by Sanger sequencing and found that the primers have a high specificity [Figure 1A]. Next, we examined the circ_0049447 expression in 80 pairs of GC specimens using real-time PCR. The results indicated that circ_0049447 was significantly downregulated in GC ($P < 0.001$) [Figure 1B]. The mean $-\Delta$CT value of circ_0049447 in GC tissues and non-tumorous tissues were -15.25 ± 1.05 and -13.50 ± 1.47, respectively [Figure 1C]. Since circ_0049447 was significantly downregulated in GC, we further explored the relationship between circ_0049447 expression and clinicopathological characteristics of GC patients. We found a significant correlation between circ_0049447 expression and pN stage ($P = 0.029$), but no significant correlations were observed between its expression and other characteristics such as age ($P = 0.136$), location ($P = 0.751$), tumor size ($P = 0.15$), histological grade ($P = 0.869$), or tumor-node-metastasis stage ($P = 0.275$) [Table 1]. To evaluate the diagnostic value of circ_0049447 in GC, we constructed a receiver operating characteristic (ROC) curve. The area under the ROC curve (AUC) reached 0.838, while sensitivity was 82.3% and specificity was 77.2% [Figure 1D]. The results suggest that circ_0049447 has good diagnostic efficiency and that it may be useful as a potential biomarker. We next used the Kaplan-Meier analysis to estimate the correlation between circ_0049447 expression and overall survival (OS) for GC patients, but we found no significant difference in OS ($P = 0.125$) between the two groups [Figure 1E]. Overall, we found that circ_0049447 is downregulated in GC and it is a promising biomarker for GC diagnosis.

Table 1: Association between the expression of circRNA circ_0049447 and the clinicopathological features in 80 patients with GC.

Characteristics	*n*	Expression level*	*P* values
Age			0.136
≤61 years	40	0.22 (0.12–0.44)	
>61 years	40	0.37 (0.11–0.96)	
Gender			0.655
Male	61	0.27 (0.10–0.72)	
Female	19	0.23 (0.14–0.89)	
Location			0.751
Upper	8	0.39 (0.23–0.72)	
Middle	33	0.31 (0.09–0.92)	
Lower	39	0.23 (0.12–0.63)	
Tumor size			0.150
≤4.3 cm	39	0.22 (0.10–0.61)	
>4.3 cm	41	0.33 (0.14–0.92)	
Macroscopic type†			0.776
Borrmann I + II	5	0.28 (0.17–1.51)	
Borrmann III + IV	68	0.27 (0.128–0.86)	
Histological grade			0.850
Good	20	0.24 (0.11–0.72)	
Poor	60	0.27 (0.12–0.74)	
pT stage			0.197
T_1	6	0.10 (0.08–0.29)	
T_2	9	0.27 (0.10–0.49)	
T_3	18	0.24 (0.08–0.81)	
T_4	47	0.33 (0.15–0.92)	
pN stage			0.029
N_0	24	0.39 (0.09–1.02)	
N_1	8	0.08 (0.06–0.20)	
N_2	22	0.26 (0.11–0.70)	
N_3	26	0.29 (0.15–0.70)	
pTNM stage			0.275
I	10	0.10 (0.08–0.70)	
II	20	0.38 (0.13–1.02)	
III	50	0.27 (0.13–0.65)	
Invasion into lymphatic vessels			0.696
Negative	19	0.21 (0.09–0.91)	
Positive	61	0.28 (0.13–0.72)	

*Median relative expression (25th–75th percentile). †Seven patients were early GC. circRNA: Circular RNA; GC: Gastric cancer; p: Pathological; TNM: Tumor-node-metastasis.

Overexpression of circ_0049447 inhibits GC cell proliferation

To explore the function of circ_0049447 in GC cells, we performed gain-of-function assays by transfecting

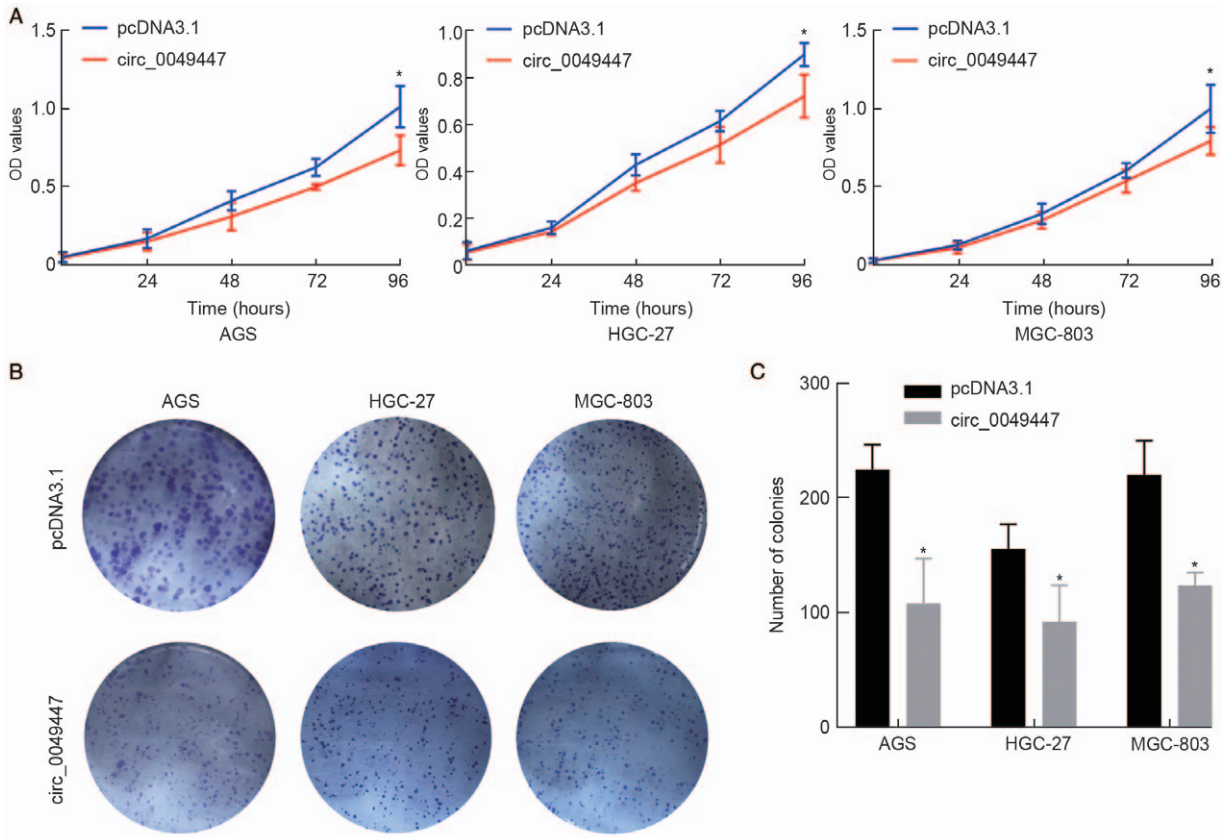

Figure 2: Upregulation of circ_0049447 inhibits proliferation in GC. CCK-8 assay (A) and colony formation assay (B, C) in AGS, HGC-27, and MGC-803 cell lines showed upregulating the expression of circ_0049447 can inhibit proliferation in GC. $^{*}P < 0.05$. CCK-8: Cell counting kit-8; GC: Gastric cancer.

pcDNA3.1-circ_0049447 (circ_0049447 group) or negative control (pcDNA3.1 group) into AGS, HGC-27, and MGC-803 human GC cell lines. The CCK-8 assay indicated that circ_0049447 overexpression significantly inhibited the proliferative capacity of AGS, HGC-27, and MGC-803 cells compared with negative controls ($P < 0.05$). The colony formation assay also showed decreased numbers of colonies in the circ_0049447 group compared with the pcDNA3.1 group ($P < 0.05$). Thus, we demonstrated that increased expression of circ_0049447 can decrease proliferation in GC cells [Figure 2].

Overexpression of circ_0049447 impedes GC cell migration, invasion, and EMT

We next examined GC cell motility after upregulating circ_0049447 expression using transwell assays. Transwell migration assay showed fewer cells migrated from the transwell chambers in the circ_0049447 group compared with the pcDNA3.1 group in AGS, HGC-27, and MGC-803 cells, suggesting that increased expression of circ_0049447 could inhibit migration capacity in GC cells ($P < 0.05$) [Figure 3A and 3B]. Similarly, overexpression of circ_0049447 also significantly impeded invasion ability in GC cells ($P < 0.05$) [Figure 3C and 3D].

As EMT can lead to cancer cell migration and invasion,[13] we detected the expression of EMT-associated markers via Western blotting. After overexpression of circ_0049447, the mesenchymal markers N-cadherin, twist, vimentin, and β-catenin were downregulated, whereas the epithelial marker E-cadherin was upregulated [Figure 3E]. Taken together, these insinuate that overexpression of circ_0049447 can reduce GC migration, invasion, and EMT.

Prediction of miRNAs bound by circ_0049447

Current evidence indicates that circRNAs can act as competitive endogenous (ce) RNAs that bind with miRNAs to modulate the expression of their downstream gene targets.[14] This is especially seen in circRNAs derived from exons.[15] circ_0049447 is composed of RNA derived from exons of RAB3D. Given that circ_0049447 may affect GC progression by acting as a ceRNA to sponge miRNAs and regulate their downstream targets, it is important to identify the signaling axis in the ceRNA network. To explore whether certain miRNAs can bind to circ_0049447 to potentially mediate GC processes, we used Circinteractome, circBank, and RegRNA to predict potential miRNA-binding partners.[16-18] Six miRNAs were co-predicted by all three databases [Figure 4A and 4B]. According to these results, circ_0049447 may act as a sponge for certain miRNAs whose validated functions include potential roles in GC development[19-37] [Table 2]. To further explore the underlying mechanisms, we detected the expression of these predicted miRNAs in

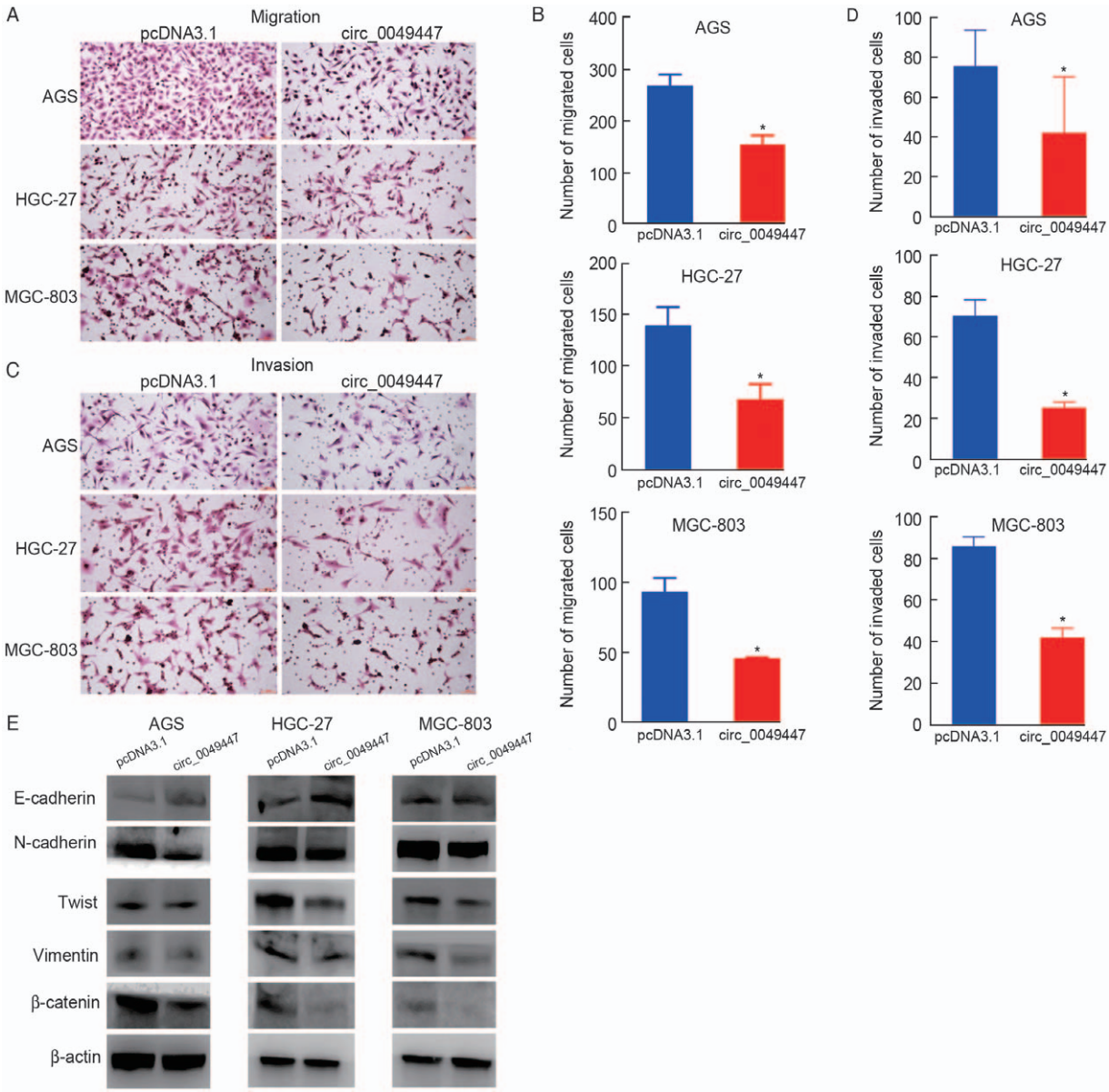

Figure 3: Overexpression of circ_0049447 impedes migration, invasion, and EMT in GC. (A, B) Transwell migration assay showed overexpressed circ_0049447 can inhibit migration in GC. (C, D) Transwell invasion assay showed upregulated circ_0049447 can impede invasion in GC. (E) Western blotting showed high expression of circ_0049447 downregulated the mesenchymal markers N-cadherin, twist, vimentin, and β-catenin whereas upregulated E-cadherin. $^{*}P < 0.05$. GC: Gastric cancer; EMT: Epithelial-mesenchymal transition.

MGC-803 cells overexpressed circ_0049447. miR-324-5p, the most downregulated after overexpressing circ_0049447, was chosen for further analysis [Figure 4C]. Next, we used luciferase reporter assays to check whether miR-324-5p directly targets circ_0049447. A significant reduction of luciferase activity was detected in MGC-803 cells co-transfected with miR-324-5p mimics and circ_0049447 WT, but not with the MUT vector [Figure 4D]. Then, we detected the expression of miR-324-5p in 30 pairs of GC tissues. The results indicated that the expression of miR-324-5p in GC tissues was upregulated [Figure 4E]. Then, we searched the target genes directly regulated by miR-324-5p that have been reported in tumors and detected the expression of them in MGC-803 cells. So far, only *FBXO11* and *PTPRD* are directly negatively regulated by miR-324-5p in lung cancer and thyroid cancer, respectively.[23,24] We found *PTPRD* was upregulated in MGC-803 cells overexpressed circ_0049447, while *FBXO11* had no differences [Figure 4F]. It has been shown that upregulated miR-324-5p can directly target *PTPRD* and promote migration and invasion in thyroid cancer.[24] And these results were consistent with our prediction that circ_0049447 inhibits GC by sponging certain miRNA and implied a mechanism by which circ_0049447 may regulate migration and invasion in GC [Figure 4G].

Upregulation of circ_0049447 impedes GC growth in vivo

To further explore whether circ_0049447 could influence the progression of GC *in vivo*, we used a subcutaneous xenograft model in nude mice. The volume of the tumor was measured

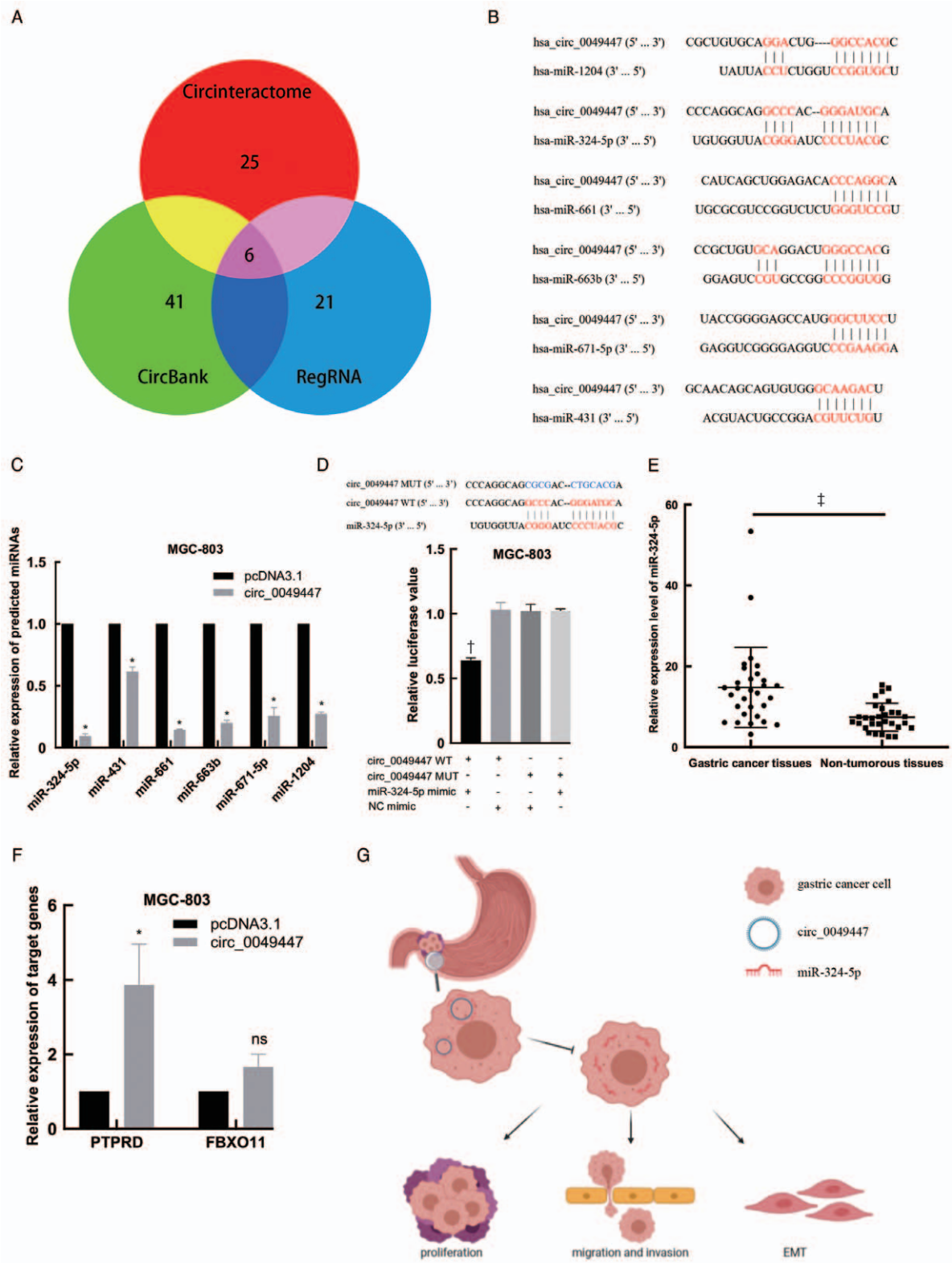

Figure 4: Prediction of miRNAs binding to circ_0049447 and mechanism simulated diagram of circ_0049447. (A, B) Predicting potential miRNAs by Circinteractome, circBank, and RegRNA and binding sites with circ_0049447. (C) Relative expression of predicted miRNAs in MGC-803 cell overexpressed circ_0049447, miR-324-5p is the most downregulated, $^{*}P < 0.05$. (D) Schematic illustration of circ_0049447 luciferase reporter vectors and luciferase activity of circ_0049447 WT or MUT after transfection with miR-324-5p mimics or negative control in MGC-803 cells, $^{\dagger}P < 0.01$. (E) Relative expression of miR-324-5p in 30 pairs of GC tissues, $^{\ddagger}P < 0.001$. (F) Relative expression of the validated target gene of miR-324-5p in MGC-803 cells overexpressed circ_0049447, $^{*}P < 0.05$; ns: No significant difference. (G) Mechanism simulated diagram of circ_0049447 in GC. GC: Gastric cancer; miRNAs: MicroRNAs.

Table 2: Prediction of miRNAs binding to circ_0049447 and their validated functions in cancer.

Potential miRNAs	Target genes	Function	Tumor types	Reference
miR-1204	*ZNF418*	Promote proliferation, inhibit apoptosis	Hepatocellular carcinoma	[19]
	NR3C2	Aggravate proliferation, suppress apoptosis	Glioblastoma	[20]
	PITX1	Promote proliferation	Non-small cell lung cancer	[21]
	VDR	Promote proliferation, invasion, and EMT	Breast cancer	[22]
miR-324-5p	*FBXO11*	Chemotherapy resistance	Non-small cell lung cancer	[23]
	PTPRD	Promote migration and invasion	Papillary thyroid carcinoma	[24]
miR-661	*RB1*	Promote proliferation and metastasis	Non-small cell lung cancer	[25]
	RUNX3	Promote proliferation and invasion		[26]
	INPP5J	Promote proliferation	Ovarian cancer	[27]
	Nectin-1, *StarD10*	Promote invasion	Breast cancer	[28]
miR-663b	*APC2*	Promote proliferation, migration, and invasion	Colorectal cancer	[29]
	TNK1	Promote proliferation and stemness		[30]
	ERF	Promote proliferation and EMT	Bladder cancer	[31]
	SMAD7	Promote proliferation and EMT	Nasopharyngeal carcinoma	[32]
	TP73	Promote proliferation, inhibit apoptosis	Osteosarcoma	[33]
miR-671-5p	*TRIM67*	Promote proliferation, migration, and invasion	Colon cancer	[34]
	APC	Promoted migration and invasion	Clear cell renal cell carcinoma	[35]
	CDR1	Promote proliferation, migration, and invasion	Glioblastoma	[36]
miR-431	*DAB2IP*	Promote migration and invasion	Pancreatic neuroendocrine cancer	[37]

APC: Adenomatous polyposis coli; *APC2*: Adenomatous polyposis coli 2; *CDR1*: Cerebellar degeneration-related protein 1; *DAB2IP*: DAB2-interacting protein; EMT: Epithelial-mesenchymal transition; *ERF*: Ets2-repressor factor; *FBXO11*: F-box protein 11; *INPP5J*: Inositol polyphosphate-5-phosphatase J; miRNAs: MicroRNAs; *NR3C2*: Nuclear receptor subfamily 3 group C member 2; *PITX1*: Paired like homeodomain 1; *PTPRD*: Protein tyrosine phosphatase receptor delta; *RB1*: RB transcriptional corepressor 1; *RUNX3*: Runt-related transcription factor 3; *SMAD7*: SMAD family member 7; *TNK1*: Tyrosine kinase non-receptor 1; *TP73*: Tumor protein p73; *TRIM67*: Tripartite motif-containing 67; *VDR*: Vitamin D receptor; *ZNF418*: Zinc-finger protein 418.

once 2 days and the weight of the tumor was recorded when the mice were sacrificed. Compared with the negative control group, we discovered that mean tumor volume and weight in the circ_0049447 group were significantly smaller, indicating that circ_0049447 can impede GC proliferation *in vivo* [Figure 5A–D]. In addition, we used immunohistochemical analysis to detect the β-catenin and wnt1 in the tumor from the xenograft model. The results indicated that β-catenin and wnt1 were significantly inhibited in MGC-803 cells overexpressed circ_0049447, which may affect the growth of the tumor [Figure 5E].

Discussion

circRNAs were initially considered to be splicing errors when they were discovered in the 1970s.[38] With the progress of RNA-sequencing technologies and bioinformatics over the past few decades, an increasing number of circRNAs have been identified. circRNAs have received additional focus due to their stability, conserved properties, and abundance.[39] Recently, more biological functions have been attributed to circRNAs, including important roles in development and physiological conditions. For example, Shen *et al*[40] discovered that three circRNAs were differentially expressed in rat lung tissues at various points of embryonic development, and they suggested that these circRNAs participate in lung development. circRNAs have also been implicated in many diseases including cancer,[41] diabetes,[42] Alzheimer disease,[43] and others. For example, upregulation of circRNA_102231 aggravated lung cancer, and it was found to be associated with poor prognosis.[44] For the past few years, a small number of researches on circRNAs in GC have emerged, suggesting that circRNAs are promising therapeutic targets for GC. For example, it was suggested that circ_103809 promotes GC cell migration and invasion.[45] However, research on circRNAs in GC is still in the initial stage, which means that further exploration is needed to fully define the effects of circRNAs in GC.

There are currently no published reports regarding circ_0049447 in any diseases or cancers, including GC. Here, we show for the first time that circ_0049447, a novel circRNA, is downregulated in GC. Moreover, the AUC of circ_0049447 for GC tissues reached 0.838, while sensitivity was 82.3% and specificity was 77.2%. These results indicate that circ_0049447 may play a promising role in GC diagnosis as a potential biomarker conducive to GC screening. Although the expression of circ_0049447 in GC tissues was correlated with the pN stage, its expression level was much lower in the N_1 stage than that in N_2 and N_3 stage. Therefore, we further compared GC patients with and without N metastasis, and the results showed that there was no difference between the two groups. We thought that significantly low expression of circ_0049447 in patients with N_1 stage may be due to the low proportion of GC patients in this subgroup.

We validated that overexpression of circ_0049447 can decrease the proliferation of GC cell lines using CCK-8 and colony formation assays. We also found that migration and invasion capacities were impeded in GC cell lines after upregulation of circ_0049447. While exploring potential mechanisms for how circ_0049447 affects cell motility, we found that circ_0049447 could modify the EMT pathway

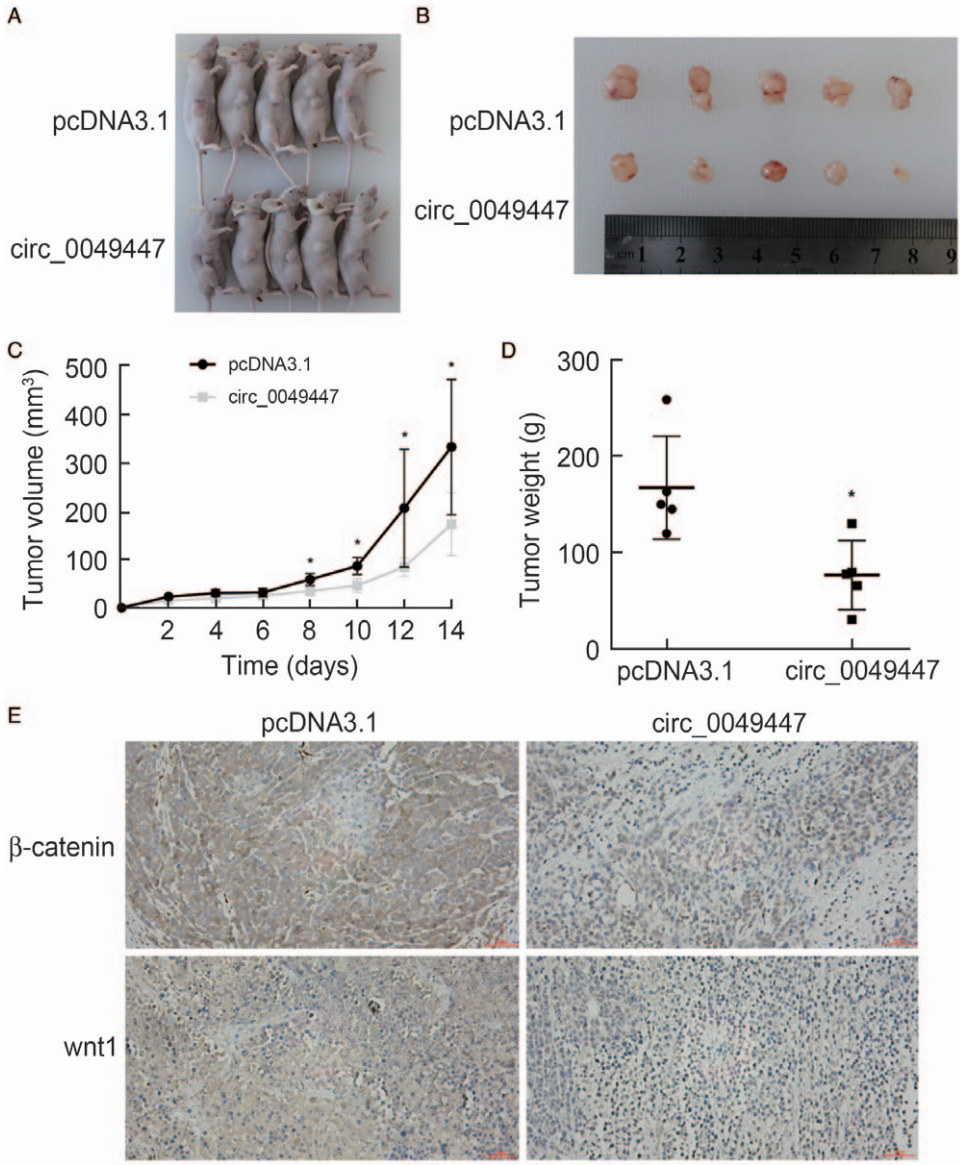

Figure 5: Upregulation of circ_0049447 impedes GC growth *in vivo*. (A) Representative images of *in vivo* tumors of nude mice. (B) Images of the tumor of each group at the endpoint of the experiment. (C) The tumor growth curves of *in vivo* tumor volumes. $^{*}P < 0.05$. (D) The mean weight of tumor in each group. $^{*}P < 0.05$. (E) Immunohistochemical staining of β-catenin and wnt1 *in vivo* tumor. GC: Gastric cancer.

by inhibiting mesenchymal phenotypes and facilitating epithelial phenotypes. After transfection with the circ_0049447 overexpression plasmid, the mesenchymal markers N-cadherin, twist, vimentin, and β-catenin were downregulated, whereas E-cadherin was upregulated, suggesting that circ_0049447 regulates GC cell migration and invasion through modulating the EMT pathway. In addition, the subcutaneous xenograft model also indicated that circ_0049447 can impede GC growth *in vivo*. Therefore, circ_0049447 acts as a tumor suppressor gene in GC. Increasing evidence indicates that circRNAs can act as ceRNAs to mediate the expression of downstream genes.[46] It has been shown that circRNAs derived from exons are especially adept at acting like miRNA sponges, such as circ5615 which promotes colorectal cancer progression by effectively sponging miR-149-5p.[47] circ_0049447 is derived from three exons, which means that circ_0049447 may also exert its function through sponging miRNA. In the current study, we confirmed circ_0049447 could impede the proliferation, migration, and invasion in GC cells, and sponge miR-324-5p. Previous studies have reported miR-324-5p could promote migration, invasion and inhibit apoptosis to enhance chemotherapy resistance.[23,24] Based on our founding and previous studies, we hypothesized that circ_0049447 could influence some specific phenotypes like apoptosis to impede chemotherapy resistance in GC through sponging miR-324-5p. In the future, we will focus on these phenotypes to further explore the underlying mechanisms of circ_0049447 in GC. Considering the anti-tumor effects of circ_0049447, future research should test whether recover its expression *in vivo* may allow physicians to target oncogenic miRNAs *via* circRNA nanosponge to help treat tumors in the future.

Acknowledgement

The authors thank the Department of Surgical Oncology of the First Affiliated Hospital of China Medical University for providing human gastric tissue samples.

Funding

This work was supported by grants from the Natural Science Foundation of China (No. 81872031), the Major R&D Plan of Liaoning Province (No. 2019JH1/10300007), the Xingliao Talents Program in Liaoning Province (No. XLYC1807164), and the Scientific and technological innovation talents program of Shenyang (No. RC190202).

Conflicts of interest

None.

References

1. Arnold M, Park JY, Camargo MC, Lunet N, Forman D, Soerjomataram I. Is gastric cancer becoming a rare disease? A global assessment of predicted incidence trends to 2035. Gut 2020;69:823–829. doi: 10.1136/gutjnl-2019-320234.
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018;68:394–424. doi: 10.3322/caac.21492.
3. Venerito M, Vasapolli R, Rokkas T, Malfertheiner P. Gastric cancer: epidemiology, prevention, and therapy. Helicobacter 2018;23 (Suppl 1):e12518. doi: 10.1111/hel.12518.
4. Vicens Q, Westhof E. Biogenesis of circular RNAs. Cell 2014;159:13–14. doi: 10.1016/j.cell.2014.09.005.
5. Jeck WR, Sorrentino JA, Wang K, Slevin MK, Burd CE, Liu J, *et al*. Circular RNAs are abundant, conserved, and associated with ALU repeats. RNA 2013;19:141–157. doi: 10.1261/rna.035667.112.
6. Sun X, Wang M, Xu R, Zhang D, Liu A, Wang Y, *et al*. Prognostic model based on circular RNA circPDK1 for resected lung squamous cell carcinoma. Transl Lung Cancer Res 2019;8:907–919. doi: 10.21037/tlcr.2019.11.20.
7. Pan G, Mao A, Liu J, Lu J, Ding J, Liu W. Circular RNA hsa_circ_0061825 (circ-TFF1) contributes to breast cancer progression through targeting miR-326/TFF1 signalling. Cell Prolif 2020;53:e12720. doi: 10.1111/cpr.12720.
8. Chen LL. The expanding regulatory mechanisms and cellular functions of circular RNAs. Nat Rev Mol Cell Biol 2020;21:475–490. doi: 10.1038/s41580-020-0243-y.
9. Zhang PF, Pei X, Li KS, Jin LN, Wang F, Wu J, *et al*. Correction to: circular RNA circFGFR1 promotes progression and anti-PD-1 resistance by sponging miR-381-3p in non-small cell lung cancer cells. Mol Cancer 2020;19:21. doi: 10.1186/s12943-020-1131-y.
10. Du WW, Yang W, Li X, Fang L, Wu N, Li F, *et al*. The circular RNA circSKA3 binds integrin beta1 to induce invadopodium formation enhancing breast cancer invasion. Mol Ther 2020;28:1287–1298. doi: 10.1016/j.ymthe.2020.03.002.
11. Song YX, Sun JX, Zhao JH, Yang YC, Shi JX, Wu ZH, *et al*. Non-coding RNAs participate in the regulatory network of CLDN4 via ceRNA mediated miRNA evasion. Nat Commun 2017;8:289. doi: 10.1038/s41467-017-00304-1.
12. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001;25:402–408. doi: 10.1006/meth.2001.1262.
13. Bera A, Lewis SM. Regulation of epithelial-to-mesenchymal transition by alternative translation initiation mechanisms and its implications for cancer metastasis. Int J Mol Sci 2020;21:4075. doi: 10.3390/ijms21114075.
14. Wang Y, Han D, Zhou T, Zhang J, Liu C, Cao F, *et al*. Melatonin ameliorates aortic valve calcification via the regulation of circular RNA CircRIC3/miR-204-5p/DPP4 signaling in valvular interstitial cells. J Pineal Res 2020;69:e12666. doi: 10.1111/jpi.12666.
15. Han B, Chao J, Yao H. Circular RNA and its mechanisms in disease: from the bench to the clinic. Pharmacol Ther 2018;187:31–44. doi: 10.1016/j.pharmthera.2018.01.010.
16. Dudekula DB, Panda AC, Grammatikakis I, De S, Abdelmohsen K, Gorospe M. CircInteractome: a web tool for exploring circular RNAs and their interacting proteins and microRNAs. RNA Biol 2016;13:34–42. doi: 10.1080/15476286.2015.1128065.
17. Glazar P, Papavasileiou P, Rajewsky N. circBase: a database for circular RNAs. RNA 2014;20:1666–1670. doi: 10.1261/rna.043687.113.
18. Huang HY, Chien CH, Jen KH, Huang HD. RegRNA: an integrated web server for identifying regulatory RNA motifs and elements. Nucleic Acids Res 2006;34:W429–W434. doi: 10.1093/nar/gkl333.
19. Wang L, Sun L, Wang Y, Yao B, Liu R, Chen T, *et al*. miR-1204 promotes hepatocellular carcinoma progression through activating MAPK and c-Jun/AP1 signaling by targeting ZNF418. Int J Biol Sci 2019;15:1514–1522. doi: 10.7150/ijbs.33658.
20. Zhao X, Shen F, Ma J, Zhao S, Meng L, Wang X, *et al*. CREB1-induced miR-1204 promoted malignant phenotype of glioblastoma through targeting NR3C2. Cancer Cell Int 2020;20:111. doi: 10.1186/s12935-020-01176-0.
21. Jiang W, He Y, Shi Y, Guo Z, Yang S, Wei K, *et al*. MicroRNA-1204 promotes cell proliferation by regulating PITX1 in non-small-cell lung cancer. Cell Biol Int 2019;43:253–264. doi: 10.1002/cbin.11083.
22. Liu X, Bi L, Wang Q, Wen M, Li C, Ren Y, *et al*. miR-1204 targets VDR to promotes epithelial-mesenchymal transition and metastasis in breast cancer. Oncogene 2018;37:3426–3439. doi: 10.1038/s41388-018-0215-2.
23. Ba Z, Zhou Y, Yang Z, Xu J, Zhang X. miR-324-5p upregulation potentiates resistance to cisplatin by targeting FBXO11 signalling in non-small cell lung cancer cells. J Biochem 2019;166:517–527. doi: 10.1093/jb/mvz066.
24. Yang Y, Xia S, Zhang L, Wang W, Chen L, Zhan W. MiR-324-5p/PTPRD/CEBPD axis promotes papillary thyroid carcinoma progression via microenvironment alteration. Cancer Biol Ther 2020;21:522–532. doi: 10.1080/15384047.2020.1736465.
25. Liu F, Cai Y, Rong X, Chen J, Zheng D, Chen L, *et al*. MiR-661 promotes tumor invasion and metastasis by directly inhibiting RB1 in non small cell lung cancer. Mol Cancer 2017;16:122. doi: 10.1186/s12943-017-0698-4.
26. Wang Y, Li Y, Wu B, Shi C, Li C. MicroRNA-661 promotes non-small cell lung cancer progression by directly targeting RUNX3. Mol Med Rep 2017;16:2113–2120. doi: 10.3892/mmr.2017.6827.
27. Zhu T, Yuan J, Wang Y, Gong C, Xie Y, Li H. MiR-661 contributed to cell proliferation of human ovarian cancer cells by repressing INPP5J expression. Biomed Pharmacother 2015;75:123–128. doi: 10.1016/j.biopha.2015.07.023.
28. Vetter G, Saumet A, Moes M, Vallar L, Le Bechec A, Laurini C, *et al*. miR-661 expression in SNAI1-induced epithelial to mesenchymal transition contributes to breast cancer cell invasion by targeting Nectin-1 and StarD10 messengers. Oncogene 2010;29:4436–4448. doi: 10.1038/onc.2010.181.
29. Xiao F, Chen W, Yu C, Zhao G. MicroRNA-663b enhances migration and invasion by targeting adenomatous polyposis coli 2 in colorectal carcinoma cells. Oncol Lett 2020;19:3701–3710. doi: 10.3892/ol.2020.11482.
30. Hong S, Yan Z, Wang H, Ding L, Song Y, Bi M. miR-663b promotes colorectal cancer progression by activating Ras/Raf signaling through downregulation of TNK1. Hum Cell 2020;33:104–115. doi: 10.1007/s13577-019-00294-w.
31. Yin X, Zheng X, Liu M, Wang D, Sun H, Qiu Y, *et al*. Exosomal miR-663b targets Ets2-repressor factor to promote proliferation and the epithelial-mesenchymal transition of bladder cancer cells. Cell Biol Int 2020;44:958–965. doi: 10.1002/cbin.11292.
32. Wang M, Jia M, Yuan K. MicroRNA-663b promotes cell proliferation and epithelial mesenchymal transition by directly targeting SMAD7 in nasopharyngeal carcinoma. Exp Ther Med 2018;16:3129–3134. doi: 10.3892/etm.2018.6576.
33. Shu Y, Ye W, Gu YL, Sun P. Blockade of miR-663b inhibits cell proliferation and induces apoptosis in osteosarcoma via regulating TP73 expression. Bratisl Lek Listy 2018;119:41–46. doi: 10.4149/BLL_2018_009.
34. Jin W, Shi J, Liu M. Overexpression of miR-671-5p indicates a poor prognosis in colon cancer and accelerates proliferation, migration, and invasion of colon cancer cells. Onco Targets Ther 2019;12:6865–6873. doi: 10.2147/OTT.S219421.

35. Chi XG, Meng XX, Ding DL, Xuan XH, Chen YZ, Cai Q, *et al*. HMGA1-mediated miR-671-5p targets APC to promote metastasis of clear cell renal cell carcinoma through Wnt signaling. Neoplasma 2020;67:46–53. doi: 10.4149/neo_2019_190217N135.
36. Li X, Diao H. Circular RNA circ_0001946 acts as a competing endogenous RNA to inhibit glioblastoma progression by modulating miR-671-5p and CDR1. J Cell Physiol 2019;234:13807–13819. doi: 10.1002/jcp.28061.
37. Zhang T, Choi S, Zhang T, Chen Z, Chi Y, Huang S, *et al*. miR-431 Promotes metastasis of pancreatic neuroendocrine tumors by targeting DAB2 interacting protein, a Ras GTPase activating protein tumor suppressor. Am J Pathol 2020;190:689–701. doi: 10.1016/j.ajpath.2019.11.007.
38. Sanger HL, Klotz G, Riesner D, Gross HJ, Kleinschmidt AK. Viroids are single-stranded covalently closed circular RNA molecules existing as highly base-paired rod-like structures. Proc Natl Acad Sci U S A 1976;73:3852–3856. doi: 10.1073/pnas.73.11.3852.
39. Suzuki H, Tsukahara T. A view of pre-mRNA splicing from RNase R resistant RNAs. Int J Mol Sci 2014;15:9331–9342. doi: 10.3390/ijms15069331.
40. Shen YQ, Pan JJ, Sun ZY, Chen XQ, Zhou XG, Zhou XY, *et al*. Differential expression of circRNAs during rat lung development. Int J Mol Med 2019;44:1399–1413. doi: 10.3892/ijmm.2019.4299.
41. Meng S, Zhou H, Feng Z, Xu Z, Tang Y, Li P, *et al*. CircRNA: functions and properties of a novel potential biomarker for cancer. Mol Cancer 2017;16:94. doi: 10.1186/s12943-017-0663-2.
42. Wu H, Wu S, Zhu Y, Ye M, Shen J, Liu Y, *et al*. Hsa_circRNA_0054633 is highly expressed in gestational diabetes mellitus and closely related to glycosylation index. Clin Epigenetics 2019;11:22. doi: 10.1186/s13148-019-0610-8.
43. Akhter R. Circular RNA and Alzheimer's disease. Adv Exp Med Biol 2018;1087:239–243. doi: 10.1007/978-981-13-1426-1_19.
44. Zong L, Sun Q, Zhang H, Chen Z, Deng Y, Li D, *et al*. Increased expression of circRNA_102231 in lung cancer and its clinical significance. Biomed Pharmacother 2018;102:639–644. doi: 10.1016/j.biopha.2018.03.084.
45. Huang SS, Guo WX, Ren MS. Circular RNA hsa_circ_103809 promotes cell migration and invasion of gastric cancer cells by binding to microRNA-101-3p. Eur Rev Med Pharmacol Sci 2020;24:6064–6071. doi: 10.26355/eurrev_202006_21501.
46. Guo W, Mu K, Zhang B, Sun C, Zhao L, Dong ZY, *et al*. The circular RNA FAM169A functions as a competitive endogenous RNA and regulates intervertebral disc degeneration by targeting miR-583 and BTRC. Cell Death Dis 2020;11:315. doi: 10.1038/s41419-020-2543-8.
47. Ma Z, Han C, Xia W, Wang S, Li X, Fang P, *et al*. circ5615 functions as a ceRNA to promote colorectal cancer progression by upregulating TNKS. Cell Death Dis 2020;11:356. doi: 10.1038/s41419-020-2514-0.

How to cite this article: Tang KW, Guo ZX, Wu ZH, Zhou C, Sun J, Wang X, Song YX, Wang ZN. Circ_0049447 acts as a tumor suppressor in gastric cancer through reducing proliferation, migration, invasion, and epithelial-mesenchymal transition. Chin Med J 2021;134:1345–1355. doi: 10.1097/CM9.0000000000001494