

Circ-104916 is downregulated in gastric cancer and suppresses migration and invasion of gastric cancer cells

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Abstract: Circular RNAs are a large class of noncoding RNA that have shown huge capabilities as gene regulators. Recent evidence suggest that circular RNAs are associated with many diseases, especially cancer. However, little attention has been focused on the expression and function of circular RNA in gastric cancer (GC). In this study, we demonstrate that the expression of circ-104916 is downregulated in GC tissues and cell lines. A lower expression of circ-104916 appeared in deeper invasion depth, higher tumor stage and more frequent lymphatic metastasis patients. Overexpression of circ-104916 effectively inhibited the proliferation, migration and invasion abilities of GC cells in vitro. Western blot showed that circ-104916 overexpression upregulated E-cadherin and downregulated N-cadherin, Vimentin and Slug, indicating that circ-104916 was involved in the epithelial–mesenchymal transition process. Our results revealed that circ-104916 might be a novel potential tumor suppressor and biomarker of GC.

Keywords: circular RNA, noncoding RNA, circ-104916, gastric cancer, EMT

Introduction

Gastric cancer (GC) is the third leading cause of cancer death worldwide, which affects >400,000 patients/year in China.¹ More than 80% of the Chinese patients were diagnosed at advanced stages, on which surgery with curative intent required extended lymphadenectomy. Laparoscopic distal gastrectomy had been proved to be safe for advanced GC.² Even with advanced systematic therapy, the 5-year overall survival of patients with late-stage GC was not >20%.³ Therefore, it is of great significance to investigate the mechanism of GC progression and metastasis.

Circular RNA (circRNA) was first detected over 2 decades ago, which was considered a byproduct of splicing errors.⁴ With the development of high-throughput RNA sequencing and bioinformatics, it was clear that circular RNA was abundant and stable in eukaryotic cell.^{5,6} Moreover, studies had found out that circular RNA expression was dysregulated in pancreatic cancer,⁷ ovarian cancer,⁸ bladder cancer⁹ and hepatocellular cancer.¹⁰ CircRNA could act as microRNAs sponge to regulate gene expression. The most famous circular RNA was CDR1as, which contained >70 conventional binding sites and functioned as a super sponge for miR-7.¹¹ Recent studies showed that CDR1as was a risk factor of hepatic microvascular invasion in hepatocellular carcinoma (HCC)¹² and knockdown of CDR1as suppressed the HCC cell proliferation and invasion.¹³ However, the expression and function of circular RNA in GC are still unknown.

In our previous study, via microarray, we found that the expression of circ-104916 was reduced in GC.¹⁴ In this study, we found that circ-104916 was significantly

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downregulated in GC tissues by expanding the sample size. In vitro studies showed that circ-104916 could suppress the proliferation, migration and invasion capabilities of GC cells. This is the first study to investigate the role of circ-104916 in GC, which suggests that circ-104916 may be a potential tumor suppressor and biomarker of GC.

Materials and methods

Tissues samples

Human samples including paired cancer specimens and adjacent normal tissues were obtained from the patients who underwent surgical resection from November 2015 to May 2016 at Nanfang Hospital, Southern Medical University. All fresh samples were immediately frozen after resection and stored at -80°C until use. Tumors were staged according to the TNM staging classification of stomach carcinoma of American Joint Committee on Cancer (AJCC; 7th ed, 2010). None of the patients had received chemotherapy or radiotherapy before surgery. The study was approved by the Clinical Research Ethics Committee of Nanfang Hospital of Southern Medical University. Written informed consent was obtained from all participants.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the cells and tissues using TRIzol reagent (Takara, Tokyo, Japan) according to the manufacturer's protocol. After RNA extraction, reverse transcriptase (Takara) was used for synthesizing cDNA according to the manufacturer's instructions. The expression level of the circRNAs was evaluated by quantitative PCR (qPCR) using SYBR Green assay. Specific divergent primers were designed to amplify the circular transcripts. PCR was performed in 10 μL reaction volume, including 2 μL of cDNA, 5 μL of 2 \times Master Mix (Takara), 0.4 μL of forward primer (10 μM), 0.4 μL of reverse primer (10 μM) and 2.2 μL of double distilled water. The reaction was set at 95°C for 10 min for pre-denaturation and then at 95°C for 10 s and at 60°C for 60 s repeating 40 cycles. β -actin was used as a reference. We used the following primer sequences: circ-104916 – forward: 5'-GCTCGGTGACCTTGGTCTGG-3' and reverse: 5'-GCGTGTTGGGATGCCTCTGT-3' and β -actin – forward: 5'-TGGATCAGCAAGCAGGAGTA-3' and reverse: 5'-TCGGCCACATTGTGAAGT-3'. Both target and reference were amplified in triplicate wells. The relative level of each circRNA was calculated using the $2^{-\Delta\Delta C_t}$ method.

Cell culture

Human GC cell lines (AGS, MKN-28, NCI-N87 and MKN-45) and human gastric mucosa epithelial cells (GES-1) were propagated in Roswell Park Memorial Institute (RPMI) 1640 (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Capricorn, Ebsdorfergrund, Germany).

Circular RNA plasmid construction and transfection

We constructed the circular RNA plasmid used in this study. Human circ-104916 cDNA was synthesized by GENEWIZ (Suzhou, China) and cloned into pcDNA3.1. We used a 651-bp DNA fragment corresponding to exon 1, exons 3–6 and exon 8 of the *NEK6* gene and added 1 kb upstream and 200 bp downstream to the nonlinear splice sites. In addition, an 800-bp DNA stretch was added upstream of the splice acceptor site and inserted downstream in the reverse orientation.¹¹ The GC cells were seeded at six-well plates and then transfected at 48 h with pcDNA-hsa-circ-104916 (4 μg) or control plasmid (4 μg) using Lipofectamine 3000 Transfection Reagent according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA).

Cell proliferation assay

Cell proliferation was assayed using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. The transfected cells were plated in 96-well plates (1,500 cells/well). Cell proliferation was detected every 24 h according to the manufacturer's protocol. Briefly, 10 μL of CCK 8 solution was added to each well and incubated for 2 h at 37°C . The solution was then measured spectrophotometrically at 450 nm.

In vitro cell migration and invasion assays

A total of 1×10^5 BGC823 and MGC803 cells transfected for 48 h with 4 μg pcDNA-hsa-circ-104916 or pcDNA3.1 were plated in the upper chamber of transwell assay inserts (Corning Incorporated, Corning, NY, USA), which contained 200 μL of serum-free RPMI 1640 with a membrane (8 mm pores) to test migration. The lower chambers were filled with RPMI 1640 containing 10% FBS. The cells on the filter surface were fixed with methanol, stained with crystal violet and photographed with a digital microscope after 24 h. The cell numbers were calculated in five random fields for each chamber.

The transfected cells (1×10^6) were plated in the top chamber (Corning Incorporated) that was precoated with Matrigel (BD Biosciences, San Jose, CA, USA) in 200 μL serum-free RPMI 1640 to test cell invasion. There was

750 μ L of 10% FBS-RPMI 1640 in the bottom chambers. The invasion function was determined after 48 h.

Western blot assay

The cellular proteins were extracted 48 h after transfection. The cells in each well were lysed in 50 μ L of lysis buffer containing radioimmunoprecipitation assay (RIPA; 49.5 μ L) and protease inhibitor cocktail (0.5 μ L). Then, 30 μ g of protein was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis and transferred onto a polyvinylidene difluoride (PVDF) membrane. The primary antibodies used were anti-E-cadherin (1:1,000; Abcam, Cambridge, UK), anti-N-cadherin (1:5,000; Abcam), anti-Vimentin (1:1,000; Abcam), anti-Slug (1:1,000; Abcam), anti-Snail (1:1,000; Abcam) and anti-GAPDH (1:10,000; Proteintech group, Inc., Chicago, IL, USA). The membranes were washed and then incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000; Abcam) for 2 h at room temperature. The blots were visualized by enhanced chemiluminescence detection.

Statistical analysis

All statistical analyses were performed using SPSS 23.0 software (IBM Corporation, Armonk, NY, USA). Data were expressed as mean \pm standard error of the mean (SEM) from at least three separate experiments. Differences between groups were analyzed using Student's *t*-test analysis. Correlation between the clinicopathological data and circ-104916

was analyzed using chi-square test. *P*-value <0.05 was considered as statistically significant.

Results

Downregulation of circNEK6 in human GC

In our previous study, the microarray results suggested that the expression of circRNAs in gastric carcinoma tissues was abnormal compared with adjacent normal tissues (Figure S1). The expression of circ-104916 was reduced in GC tissues. To confirm hsa-circ-104916 expression in GC, qRT-PCR was performed in 70 pairs of human GC specimens and their adjacent noncancerous tissue samples. More than 50% reduction in circ-104916 expression was observed in 65.7% (46/70) of GC tissues (Figure 1A). The expression of circ-104916 was significantly lower in GC tissues than in the corresponding adjacent noncancerous tissues (Figure 1B). These results suggested that circ-104916 was downregulated in GC tissues, which was consistent with our microarray result.

Correlation with the clinicopathological data and expression of circ-104916

To assess the correlation of circ-104916 expression with clinicopathological data, the level of circ-104916 in tumor tissue was categorized as low group or high group in relation to the media value of relative circ-104916 expression (0.36-fold, tumors/no cancers). Clinicopathological factors were analyzed in the high or low groups. The expression of circ-104916 was significantly associated with invasion depth,

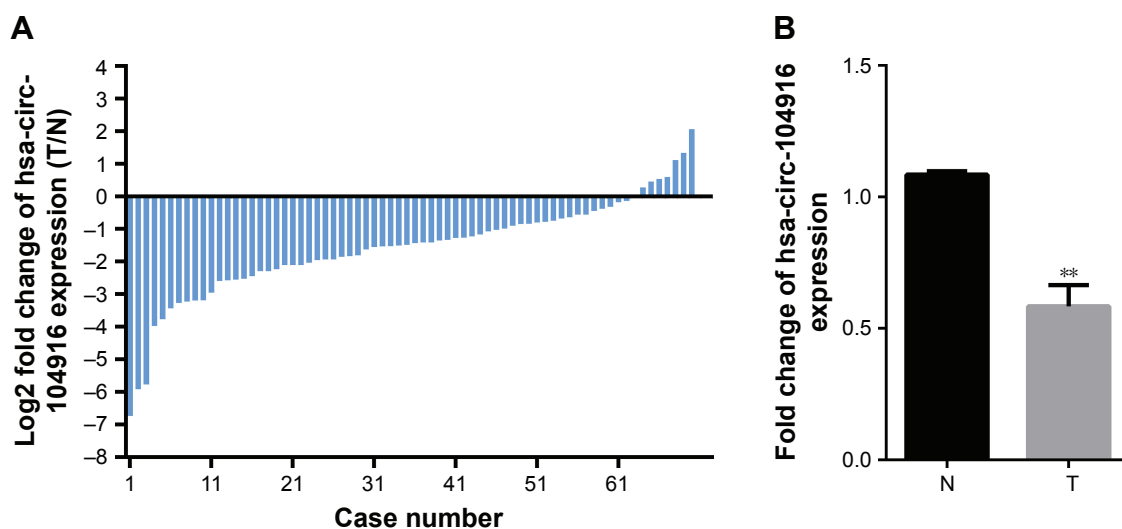


Figure 1 Circ-104916 expression in GC tissues.

Notes: (A) The expression of circ-104916 was validated by qPCR in seventy patients. Log₂ fold changes in circ-104916 expressions (T/N) of each case are plotted. (B) The expression level of circ-104916 was significantly downregulated in tumor tissues. ***P* <0.01 .

Abbreviation: qPCR, quantitative polymerase chain reaction.

tumor stage, lymphatic metastasis and nerve invasion (Table 1). However, there was no significant correlation between circ-104916 expression and other clinicopathological factors, such as age, histological grade and vascular invasion. Moreover, a lower expression of circ-104916 appeared in deeper invasion depth, higher tumor stage and more frequently lymphatic metastasis patients (Figure 2A–C). Taking together, our results showed that circ-104916 expression was associated with clinicopathological features and might be a potential biomarker of GC.

Circ-104916 suppresses proliferation of GC cells in vitro

To detect the function of circ-104916 in the progression of GC, we performed gain of function assay. First, we analyzed circ-104916 expression in human GC cell lines. The expression of circ-104916 in AGS, MKN45, BGC823, MGC803 and 7901 was significantly lower than that in the normal gastric mucosa cell line GES-1 (Figure 3A), which was consistent with our clinical findings that human GC tissues had low levels of circ-104916. According to the endogenous expression of circ-104916 in five GC cell lines, we transfected pcDNA-hsa-circ-104916 or negative control in BGC823 and

MGC803 cell lines. After 48 h of treatment, the expression of circ-104916 was effectively increased (Figure 3B). Then, we assessed the effect of circ-104916 on the proliferation of GC cells through CCK-8 assay. The result showed that overexpression of circ-104916 could suppress the proliferation of BGC823 and MGC803 cell lines (Figure 3C). Our results indicated that circ-104916 was downregulated in GC cells and could inhibit the proliferation of GC cells.

Circ-104916 suppresses GC cell migration and invasion

Migration and invasion assays were used to determine the change of migration and invasion capability of BGC823 and MGC803 cells, which were both transfected with pcDNA-circ-104916. The migration assay indicated that overexpression of circ-104916 significantly suppressed the migration capability of BGC823 and MGC803 cells (Figure 4A). Meanwhile, the invasion assay showed that overexpression of circ-104916 could inhibit the invasion capability of BGC823 and MGC803 cells (Figure 4B). These results suggested that circ-104916 could inhibit the migration and invasion of GC cells.

Circ-104916 suppresses GC cell invasion and migration via the epithelial–mesenchymal transition (EMT) process

To further explore the underlying mechanism of decreased invasion and migration by circ-104916, EMT molecules were evaluated. The upregulation of E-cadherin was observed in circ-104916 overexpression of GC cells. Additionally, the downregulation of N-cadherin and Vimentin were also observed in circ-104916 overexpression of GC cells. The decreased expression of Slug was accompanied with circ-104916 overexpression (Figure 4C). In conclusion, our results suggested that circ-104916 might suppress GC cell migration and invasion via inhibiting the EMT process.

Discussion

In recent years, with the rapid development of RNA sequencing, researches have discovered that circRNA is abundant and especially expresses in a cell type or development stage.⁵ Circular RNAs can modulate cellular physiology as microRNA sponges. For instance, the circ-ITCH suppresses esophageal squamous cell carcinoma via the Wnt/ β -catenin pathway.¹⁵ Circ-001569 could promote proliferation and invasion of colorectal cancer via target mir-145.¹⁶ Previous studies also elaborated that circRNA could affect cell processes by forming RNA–protein complexes.^{17,18} For example,

Table 1 Correlation with the clinicopathological data and expression of circ-104916

Characteristics	Low	High	P-value
Total	35	35	
Age (years)			0.339
<60	15	20	
≥60	20	15	
Sex			0.045
Male	27	18	
Female	8	17	
Differential status			0.785
Undifferentiated/poorly	25	27	
Moderate/well	10	8	
Nerve invasion			0.019
Yes	29	19	
No	6	16	
Vascular invasion			0.338
Yes	19	14	
No	16	21	
T stages			0.001
T1	1	12	
T2–T4	34	23	
N stage			0.019
N0	6	16	
N1–N3	29	19	
TNM stages			0.015
I–II	9	20	
III–IV	26	15	

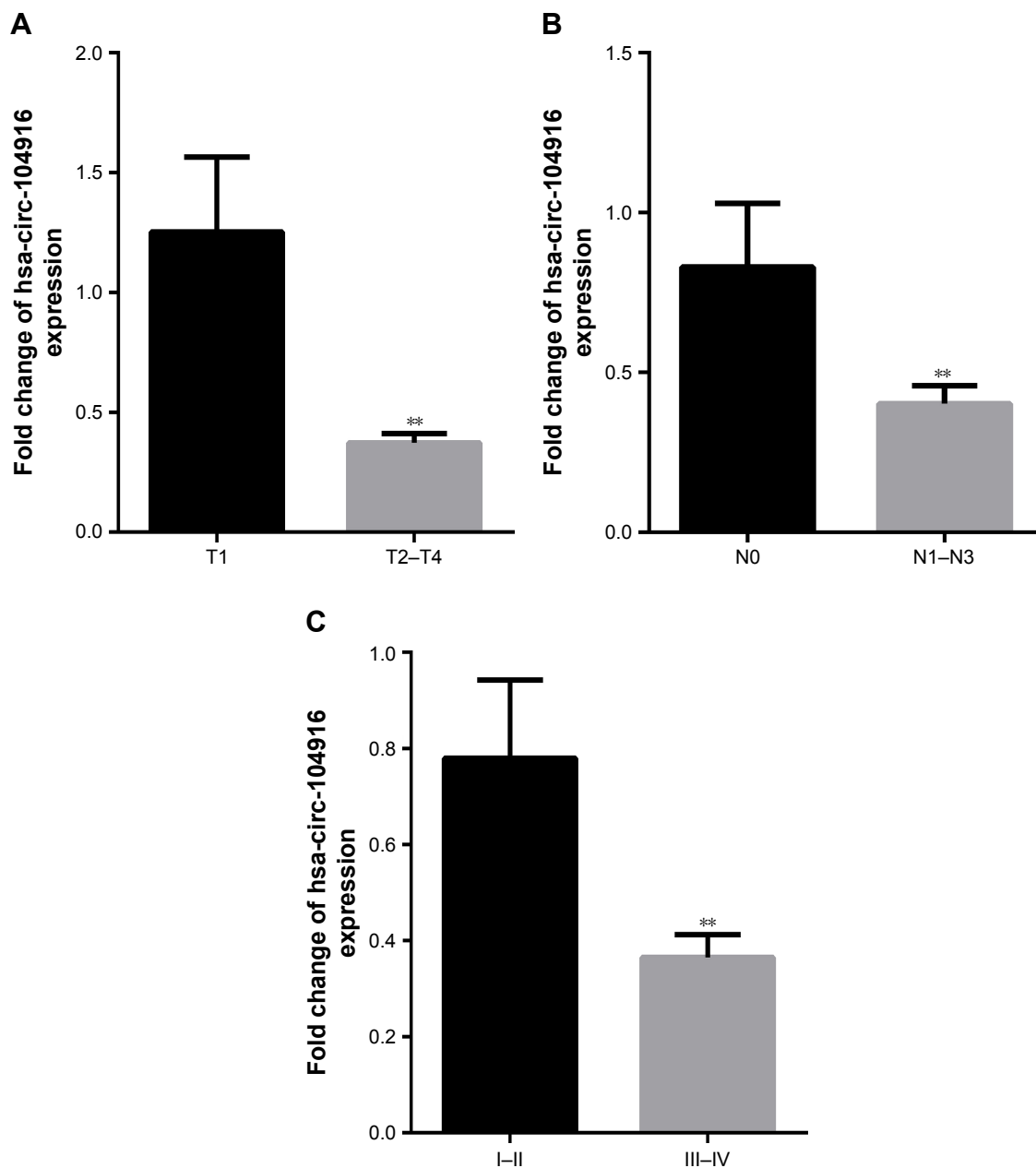


Figure 2 Clinicopathological factors with the expression level of circ-104916.

Notes: (A) The expression of circ-104916 was much lower in the deeper invasion depth patients. (B) Circ-104916 expression was downregulated in the patients with more frequent lymphatic metastasis. (C) The expression of circ-104916 was lower in higher tumor-stage patients. ** $P < 0.01$.

Circ-Foxo3 not only could block cell-cycle progression via forming ternary complexes with p21 and CDK2¹⁷ but also induces cell apoptosis via binding MDM2 and P53.¹⁸ Recent studies revealed that circular RNA could regulate transcription or splicing. Circular intronic RNA may have a *cis*-regulatory role on their parent coding genes.¹⁹ Thus, circRNA may be a core participant in physiology and pathology processes. To the best of our knowledge, there were few studies about circular RNA in GC. Only one study showed that circ-002059 in GC was significantly associated with

TNM stage and distal metastasis.²⁰ However, the effect and function of circRNA in GC remains unknown.

In our study, via microarray, we first found that circ-104916 was significantly lower in the GC tissue than the adjacent normal tissues. Through database CircBase,²¹ we figured out that circ-104916 was generated from chromosomal region 9q33.3, which was back spliced by exon 1, exon 3, exon 4, exon 5, exon 6 and exon 8 of NEK6. Mature circ-104916 transcript is a circular RNA molecule of 651nt. Then, we validated the expression of circ-104916

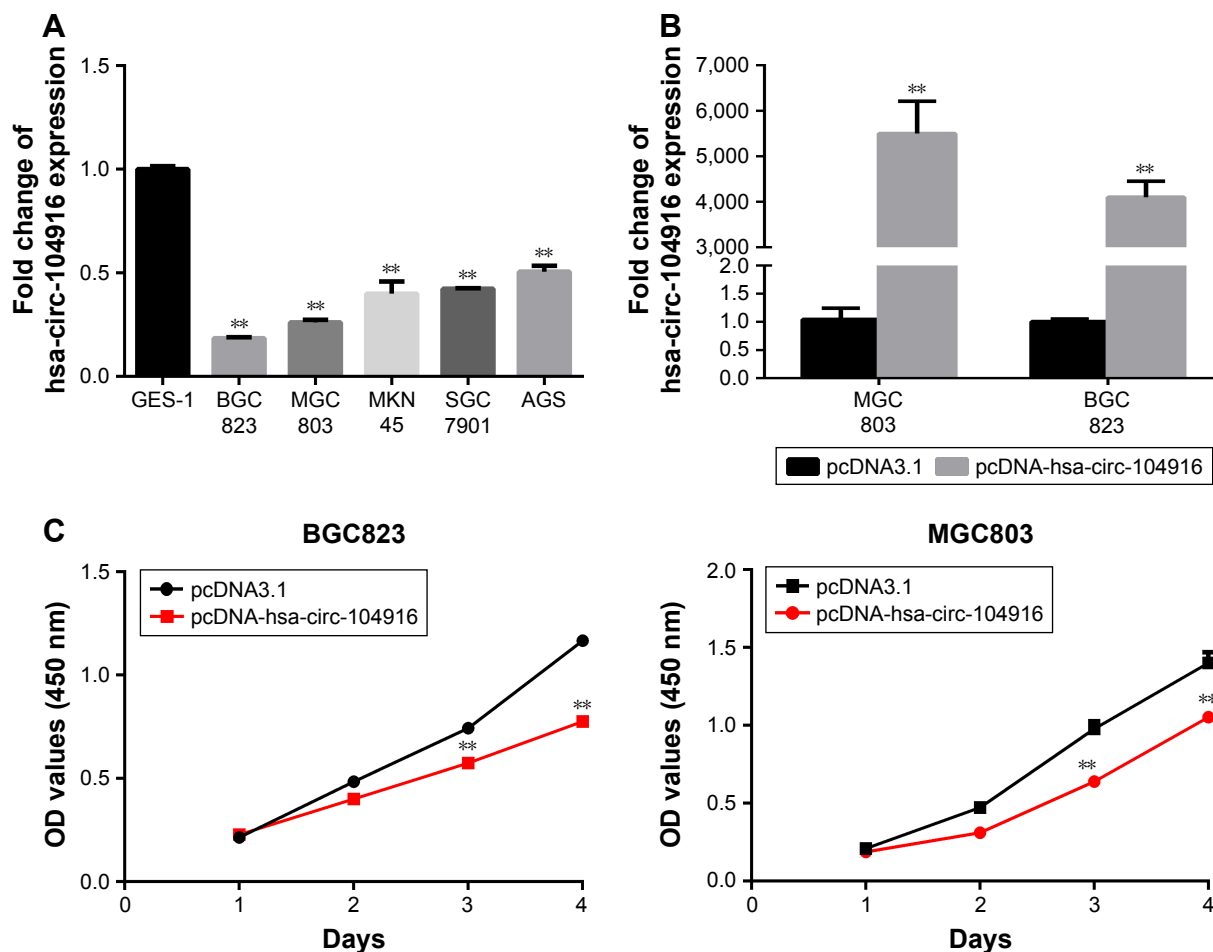


Figure 3 Circ-104916 overexpression suppressed the proliferation of GC cells.

Notes: (A) Circ-104916 was more downregulated in GC cell lines (BGC823, MGC803, MKN45, SGC7901, AGS) than in the normal gastric mucosa cell line (GES-1). (B) The effects of the pcDNA-hsa-circ-104916 plasmid on the expression of circ-104916 in GC cell lines. (C) Effect of circ-104916 on cell proliferation in vitro by CCK-8 assay. Error bars represent mean \pm SEM from three independent experiments. $**P < 0.01$.

Abbreviations: GC, gastric cancer; CCK-8, Cell Counting Kit-8; OD, optical density; SEM, standard error of the mean.

in 70 matched samples. The result was coincident with the microarray finding. Moreover, deeper invasion depth, higher tumor stage and more frequent lymphatic metastasis patients had a lower expression of circ-104916. These results indicated that circ-104916 might be a potential biomarker for GC.

In in vitro experiments, the results suggested that circ-104916 could suppress the proliferation, migration and invasion abilities of GC cells. Therefore, circ-104916 may be a potential tumor suppressor and play a core role in GC invasion and metastasis. EMT is a critical cellular progress in cancer metastasis.²² What is more, a previous study had reported that hundreds of circRNAs were regulated during human EMT.²³ The key events in EMT are dissolution of the epithelial cell-cell junction, downregulation of an epithelial gene expression signature and activation of genes that help to define the mesenchymal phenotype and increased cell protrusions and motility.²⁴ By Western blot, we found that

the epithelial molecule (E-cadherin) was upregulated and the mesenchymal molecule (N-cadherin, Vimentin) was downregulated after circ-104916 overexpression. Slug (also known as Snail2) is a member of the SNAIL family of zinc finger transcriptional repressors, which can repress the E-cadherin expression.²⁵ In our data, circ-104916 could decrease Slug expression. Taking together, the results suggested that circ-104916 might have epithelial-specific functions through decreasing the Slug expression.

Our study has several limitations. First, we did not perform loss of function because of the low efficiency of siRNA resulting from the high GC rate around the back-splice sites. Second, these conclusions were based on the responses of two cell lines and may not reflect the process in the organism. The conformance in mice needs to be accomplished in the future.

In summary, our data first found that circ-104916 was downregulated in GC tissues and the expression of

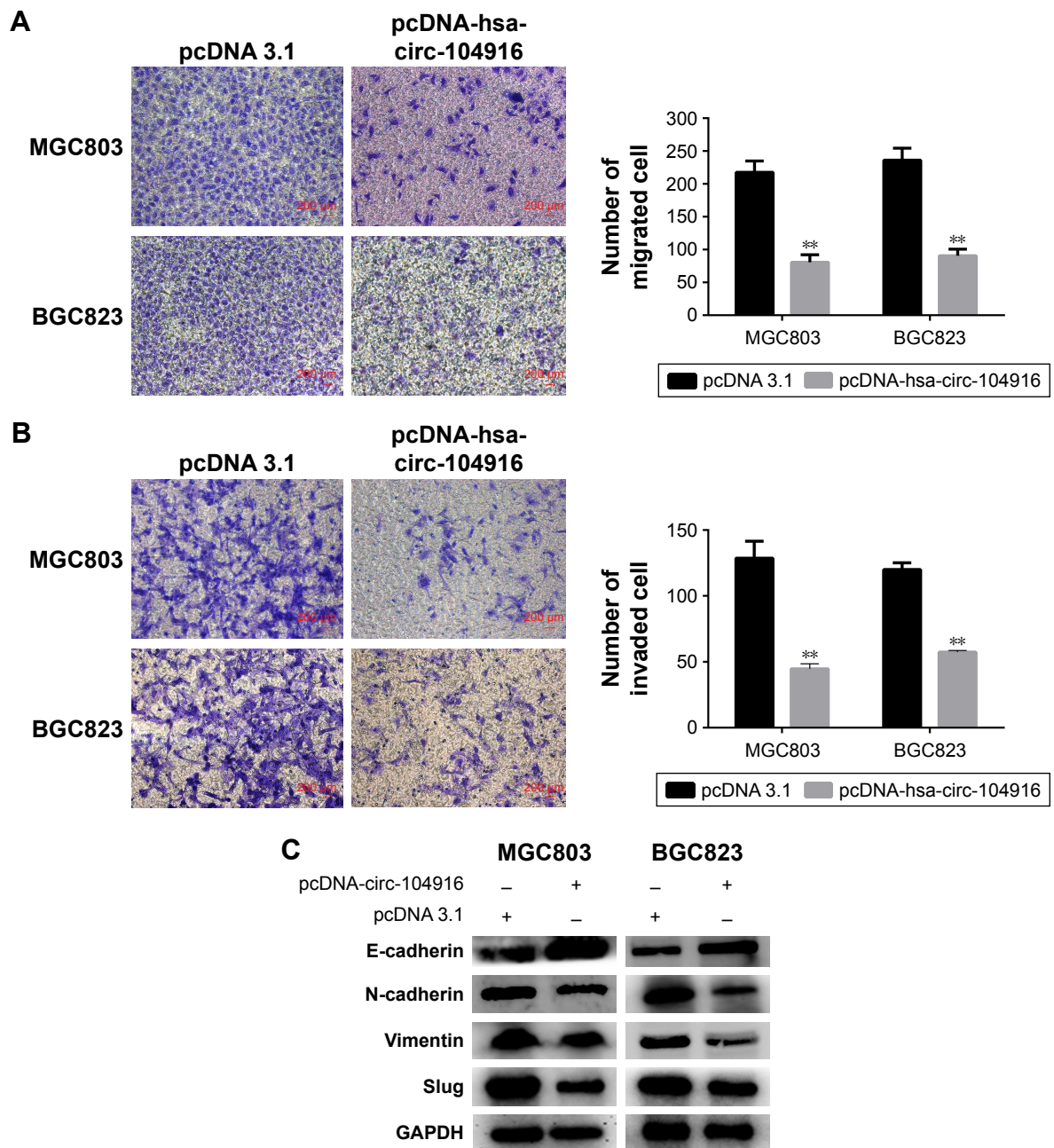


Figure 4 Circ-104916 inhibited the migration and invasion of GC cells.

Notes: (A) After transfecting pcDNA-hsa-circ-0067934, GC cells showed reduced migration in transwell migration assays. (B) Circ-104916 also suppressed the invasion ability of BGC823 and MGC803 cells. $**P < 0.01$. (C) After transfecting pcDNA-hsa-circ-104916, the protein level of key molecules of EMT was assessed by Western blot.

Abbreviations: GC, gastric cancer; EMT, epithelial–mesenchymal transition.

circ-104916 was related to invasion depth, tumor stage and lymphatic metastasis. The overexpression of circ-104916 suppressed the migration and invasion of GC cells through altering the EMT process. Our research suggested that circ-104916 might be a novel potential tumor suppressor and biomarker of GC.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

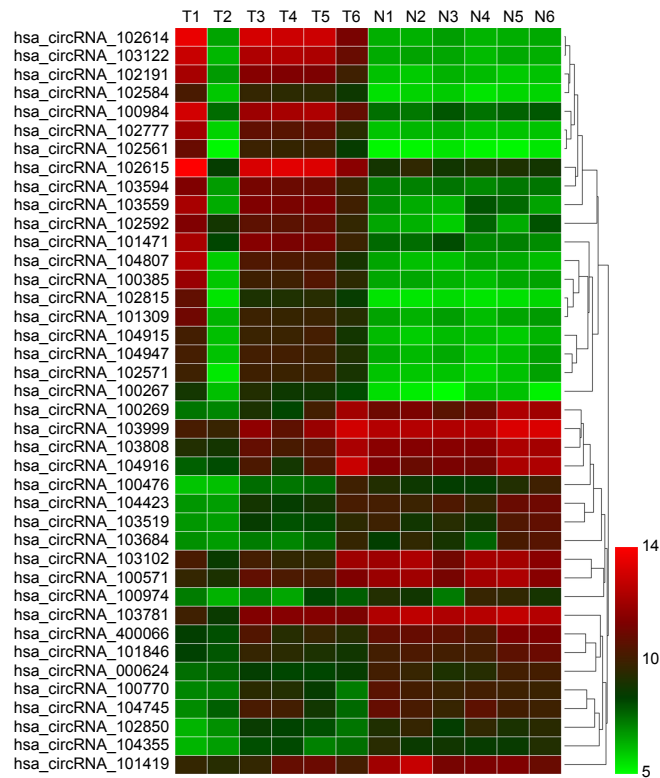


Figure S1 Hierarchical clustering analysis of the most up- and down-regulated circRNAs.

Notes: Each column represents a sample, and each row represents a circRNA. Red strip represents a high relative expression, and green strip represents a low relative expression. T represents GC tissues and N represents normal gastric mucosa.

Abbreviation: GC, gastric cancer.

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