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Circ-ZNF609 promotes carcinogenesis of gastric cancer cells by inhibiting miRNA-145-5p expression

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Abstract. – OBJECTIVE: To elucidate the expression pattern and biological function of circular RNA ZNF609 (circ-ZNF609) in gastric cancer (GC).

PATIENTS AND METHODS: Circ-ZNF609 expression in GC tissues and adjacent normal tissues (ANT) was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The regulatory effect of circ-ZNF609 on growth and metastasis of GC cells was evaluated through the Cell Counting Kit-8 (CCK-8), colony formation and transwell invasion assay, respectively. GC cell apoptosis influenced by circ-ZNF609 was examined by flow cytometry. The binding between circ-ZNF609 and miRNA-145-5p was verified by the Dual-Luciferase reporter gene assay. Finally, a series of rescue experiments were conducted to explore the mechanism of the circ-ZNF609/miRNA-145-5p axis in regulating GC progression.

RESULTS: QRT-PCR data revealed a higher level of circ-ZNF609 in GC tissues relative to ANT. Identically, circ-ZNF609 was highly expressed in GC cell lines relative to controls. The knockdown of circ-ZNF609 in BGC823 and MGC803 cells suppressed proliferative and invasive abilities. MiRNA-145-5p was predicted to be the target gene of circ-ZNF609 by bioinformatics, and further verified by the Dual-Luciferase reporter gene assay. Rescue experiments showed that miRNA-145-5p knockdown partially reversed the regulatory effect of circ-ZNF609 on growth and metastasis of GC cells.

CONCLUSIONS: Circ-ZNF609 promotes proliferative and invasive abilities of gastric cancer cells by inhibiting miRNA-145-5p expression as a ceRNA, thus accelerating gastric cancer progression.

Key Words:
Circ-ZNF609, MiRNA-145-5p, Gastric cancer, Proliferation, Invasion.

Introduction

Gastric cancer (GC) is a common malignancy globally, the incidence and mortality of which ranks fourth and second in malignant tumors, respectively[1]. The number of GC-induced deaths accounts for 12% of all malignancy-induced deaths. Surgery is still an effective therapeutic approach for GC, but the 5-year survival after standard radical resection is still not optimistic. The specific etiology and pathogenesis of GC have not been fully elucidated[2]. Hence, in-depth studies on the molecular and biological mechanisms of GC contribute to effectively improve the prognosis of GC patients.

Genomics researches[3] have shown that the higher the degree of biological evolution, the smaller the proportion of coding ribonucleic acid molecules and the higher the proportion of non-coding RNAs (ncRNAs). Human ncRNAs account for 97%-98% of the entire genomes. These ncRNAs exert vital roles in the regulation of biological processes, including DNA structure, RNA expression, protein translation, cellular differentiation, organ development, etc. Studies[4] on ncRNAs have been well concerned in recent years.

Circular RNA (circRNA) is an ncRNA with special structure recently discovered, which is abundantly present in the cytoplasm of eukaryotic cells and is produced by cleavage of pre-mRNA[5]. The closed loop structure of circRNA is different from that of traditional linear RNA; it does not contain the 5' cap and 3'poly(A). Most of the circRNAs are composed of exon sequences and highly conservative. The microRNA response element (MRE) in the sequence of circRNA endues the function

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of miRNA sponge. CircRNA can relieve the inhibitory effect of miRNA on target mRNAs, thus regulating mRNA expressions[6]. Previous studies have identified the involvement of circRNA in tumor development. For example, circRNA-100290 sponges miR-29 family to regulate oral cancer development[7]. Circ-ABCB10 promotes proliferation and progression of breast cancer by binding to miR-1271[8]. CircRNA-103809 regulates proliferative and migratory potentials of colorectal cancer cells by activating miR -532-3p/FOXO4 axis[9].

Circ-ZNF609 (ID: hsa_circ_0000615 in circBase) locates on 64791491-64792365, exerting an important role in promoting proliferative and metastatic potentials[10-12]. Its biological function has not been elucidated in GC. This study aims to elucidate the potential function of circ-ZNF609 in GC and provides novel perspectives for the diagnosis and treatment of GC.

Patients and Methods

Sample Collection

GC tissues (n=40) and ATN (n=40, resected from >5 cm away from tumor edge) were surgically resected from patients who were pathologically diagnosed GC in our hospital. None of the GC patients received anti-tumor therapy. Signed informed consents were obtained from all participants before the study. This study was approved by the Ethics Committee of the Provincial Hospital Affiliated to Shandong University.

Cell Culture

Normal gastric epithelial cell line (GES1) and GC cell lines (MKN45, AGS, BGC823, MGC803 and SGC7901) were provided by Cell Bank, Chinese 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 and 100 μg/mL streptomycin and maintained in a 5% CO_2 incubator at 37°C.

Transfection

The cells in the logarithmical period were seeded into 6-well plates. Transfection of circ-ZNF609 siRNA, miRNA-145-5p inhibitor or negative control (GenePharma, Shanghai, China) was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) until 60-80% of confluence. The fresh medium was replaced 6 hours later.

RNA Extraction and RNase R Digestion

Total RNA was extracted from cells or tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) and quantified using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). 1 mg RNA was incubated in 3 units of RNase at 37°C for 10 min. RNA was extracted using phenol and chloroform and washed in ethanol.

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

Reverse transcription of complementary deoxyribose nucleic acid (cDNA) was conducted using PrimeSeript TM RT reagent kit (TaKaRa, Otsu, Shiga, Japan). Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed by TB Green TM Premix ExTaq TM II (TaKaRa, Otsu, Shiga, Japan) on an ABI7900 instrument (Applied Biosystems, Foster City, CA, USA). The relative level of the target gene was calculated using the $2^{-\Delta\Delta CT}$ method. Primer sequences were: Circ-ZNF609, F: 5'-CAGCGCTCAATCCTTTGGGA-3'; R: 5'-GACCTGCCACATTGGTCAGTA-3'; MiRNA-145-5p, F: 5'-CCTTGTCCTCACGGTCCAGT-3'; R: 5'-AACCATGACCTCAAGAACAGTATTT-3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), F: 5'-AGCCACATCGCTCAGACAC-3'; R: 5'-GCCCAATACGACCAAATCC-3'; U6, F: 5'-CTCGCTTCGGCAGCAGCACATATA-3', R: 5'-AAATATGGAACGCTTCACGA-3'.

Cell Counting Kit-8 (CCK-8)

The cells were seeded in the 96-well plate with 5×10^3 cells per well. Viability was determined at the appointed time points using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). Optical density (OD) at 450 nm was recorded for plotting the proliferation curve.

Colony Formation Assay

The cells were seeded in a 6-well plate with 300 cells per well and incubated for 10-14 days. Colonies were washed with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) twice, fixed in 4% paraformaldehyde and dyed with a Giemsa solution for 30 min. Colonies containing over 50 cells were counted. Colony formation rate (%) = (colony number / inoculated cell number) ×100%.

Transwell Invasion Assay

Transfected cells for 48 h were adjusted to 1×10^5/mL. 100 μL/well suspension was applied in the upper side of a Matrigel-coated transwell

chamber (Millipore, Billerica, MA, USA). In the bottom side, 600 μL of medium was applied. After 24 h incubation, invasive cells were fixed in 4% paraformaldehyde, dyed with crystal violet and counted using a microscope.

Flow Cytometry

The cells were seeded in a 6-well plate with 2×10^5 cells per well overnight, digested in Ethylene Diamine Tetraacetic Acid (EDTA)-free trypsin and centrifuged at 1000 rpm for 5 min. The precipitate was washed in pre-cold PBS twice, resuspended in 75% ethanol and fixed at -20°C overnight. At the other day, cells were washed, centrifuged, incubated with 10 mg/L PI and 500 μL of PBS containing 0.1% RNase A in the dark for 10 min. Before flow cytometry determination, cells were dyed with 5 μL of Annexin V-FITC (fluorescein isothiocyanate) and 10 μL of Propidium Iodide (PI) in the dark for 5-15 min. The apoptotic rate was finally determined.

Dual-Luciferase Reporter Gene Assay

Potential binding sites between miRNA-145-5p and circ-ZNF609 were predicted by bioinformatics. Based on the binding sequences, wild-type or mutant-type circ-ZNF609 3'UTR was inserted into the promoter region of pGL3. One day prior to transfection, cells were seeded in the 24-well plate with 5×10^5 cells per well. The cells were co-transfected with 0.12 μg Luciferase vector and 40 nM miRNA-145-5p mimics for 48 h, and finally subjected to determination of Luciferase intensity.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 24.0 software (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Continuous variables between the two groups were analyzed by the *t*-test. The comparison between groups was made using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference) and $p<0.05$ was considered statistically significant.

Results

High Expression of Circ-ZNF609 in GC

Circ-ZNF609 expression in GC tissues and adjacent normal tissues (ANT) was determined by qRT-PCR. It is shown that circ-ZNF609 was highly expressed in GC tissues (Figure 1A). Furthermore, pairing analysis indicated that circ-ZNF609 expression markedly increased in tumor tissues of 90% GC patients (Figure 1B). These results demonstrated the crucial role of abnormally expressed circ-ZNF609 in GC.

Identification of Circ-ZNF609

We examined circ-ZNF609 expression in GC cell lines by qRT-PCR. As shown in Figure 2A, circ-ZNF609 was highly expressed in GC cells relative to GES1 cells. Among them, the expression difference of circ-ZNF609 was pronounced in BGC823 and MGC803 cells, which were selected for further experiments. Constructed circ-ZNF609 plasmid was further transiently transfected into GC cells. Random primers and oligo-dT primers were used for reverse transcription. Since samples containing poly-A barely expressed circRNA, the relative expression of the linear template was higher compared to that of the circular template (Figure 2B). In addition, circ-ZNF609 was effectively resistant against RNase R digestion (Figure 2C). Thus, circ-ZNF609 was identified to be a circRNA indeed.

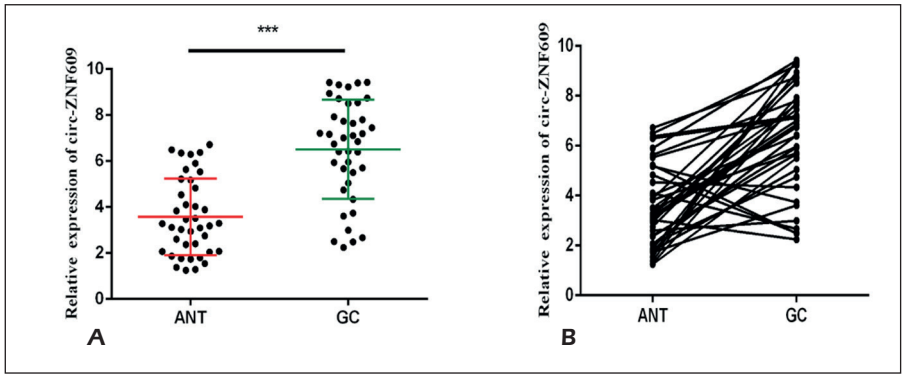

Figure 1. High expression of circ-ZNF609 in GC. **A**, Circ-ZNF609 expression was higher in GC tissues than that of adjacent normal tissues (ANT) determined by qRT-PCR. **B**, Pairing analysis indicated that circ-ZNF609 expression markedly increased in tumor tissues of 90% (36/40) GC patients.

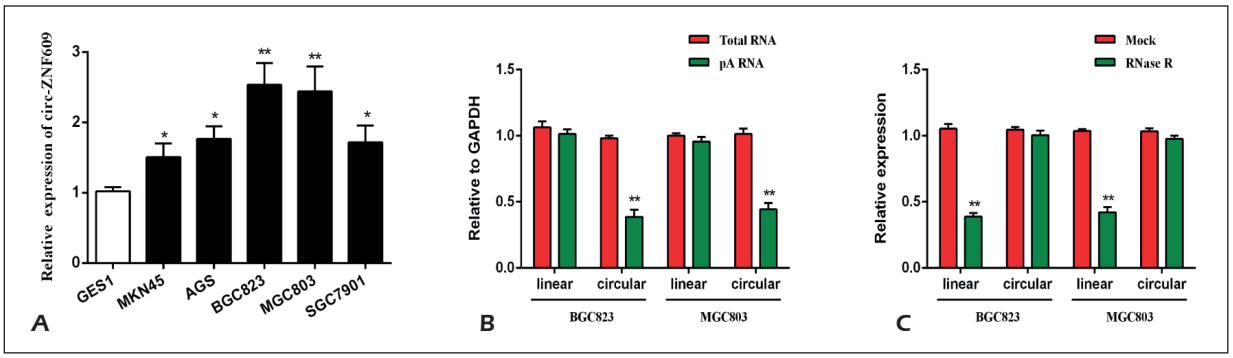

Figure 2. Identification of circ-ZNF609. **A**, Circ-ZNF609 was highly expressed in GC cells relative to GES1 cells. **B**, Relative expression of the linear template was higher compared to that of circular template. **C**, Circ-ZNF609 was effectively resistant against RNase R digestion.

Circ-ZNF609 Bound to MiRNA-145-5p

Since circ-ZNF609 was highly expressed in GC, we constructed circ-ZNF609 siRNA to knock down its expression. We determined linear transcript and circular transcript of circ-ZNF609 after transfection of circ-ZNF609 siRNA in GC cells. QRT-PCR data revealed a remarkable decrease in the circular expression of circ-ZNF609 relative to linear level (Figure 3A). A potential binding target of circ-ZNF609 was subsequently predicted by bioinformatics, and miRNA-145-5p was selected (Figure 3B). Luciferase intensity remarkably decreased in GC cells co-transfected with wild-type circ-ZNF609 3'UTR and miRNA-145-5p mimics. In comparison, co-transfection of mutant-type circ-ZNF609 3'UTR and miRNA-145-5p mimics did not show a significant change in Luciferase intensity (Figure 3C). Hence, we verified the binding between circ-ZNF609 and miRNA-145-5p. By determining miRNA-145-5p expression, it was lowly expressed in GC (Figure 3D). A negative correlation was identified be-

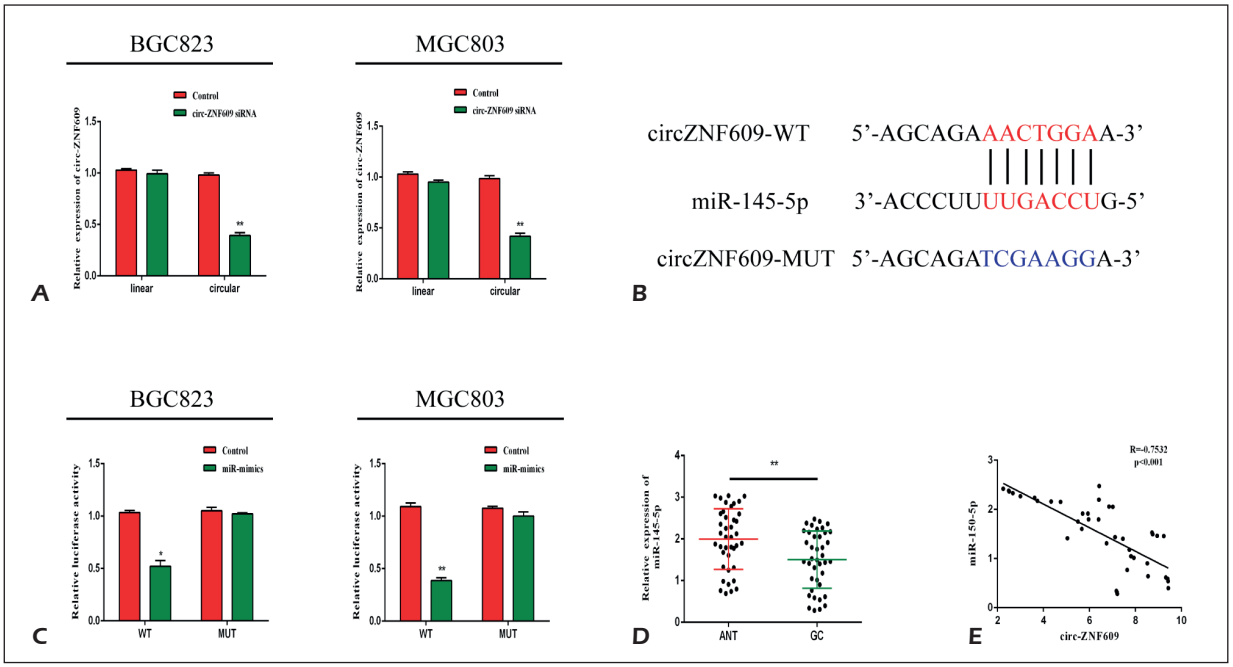

Figure 3. Circ-ZNF609 bound to miR-145-5p. **A**, Transfection of circ-ZNF609 siRNA in GC cells decreased circular expression of circ-ZNF609 relative to linear one. **B**, Potential binding sequences of circ-ZNF609 to miR-145-5p. **C**, Luciferase intensity remarkably decreased in GC cells co-transfected with wild-type circ-ZNF609 3'UTR and miR-145-5p mimics. In comparison, co-transfection of mutant-type circ-ZNF609 3'UTR and miR-145-5p mimics did not show a significant change in Luciferase intensity. **D**, MiR-145-5p expression was lower in GC tissues relative to ANT. **E**, A negative correlation between expressions of circ-ZNF609 and miR-145-5p in GC tissues.

tween the expressions of circ-ZNF609 and miRNA-145-5p in GC tissues (Figure 3E).

Knockdown of Circ-ZNF609 Inhibited Proliferative and Invasive Abilities but Induced Apoptosis of GC Cells, Which Was Reversed by MiRNA-145-5p Inhibitor

A series of rescue experiments were conducted to evaluate the potential function of circ-ZNF609 and miRNA-145-5p in GC. First, we examined miRNA-145-5p expression in GC cells transfected with circ-ZNF609 siRNA or circ-ZNF609 siRNA+miRNA-145-5p mimics. We found that miRNA-145-5p expression was upregulated after transfection of circ-ZNF609 siRNA, which was reversed by co-transfection of circ-ZNF609 siRNA and miRNA-145-5p mimics (Figure 4A). CCK-8 and colony formation assay indicated an inhibited proliferation in GC cells with the circ-ZNF609 knockdown, but was reversed by miRNA-145-5p overexpression (Figure 4B, C). Similar trends were obtained in determining cell invasion as well (Figure 4D). Knockdown of circ-ZNF609 greatly induced apoptosis of GC cells, but was further inhibited by miRNA-145-5p over-

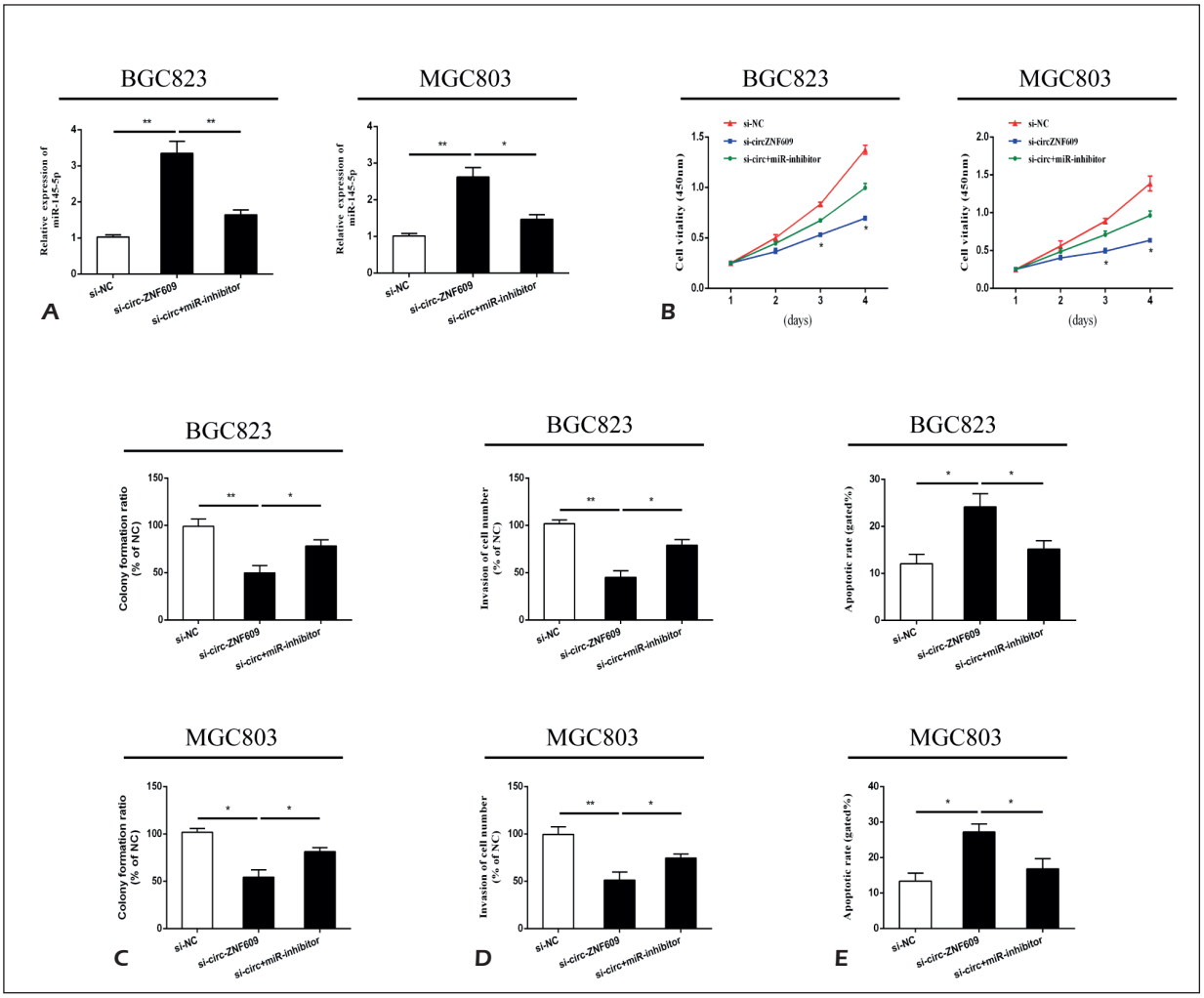

Figure 4. Knockdown of circ-ZNF609 inhibited proliferative and invasive abilities but induced apoptosis of GC cells, which was reversed by miR-145-5p inhibitor. GC cells were transfected with si-NC, circ-ZNF609 siRNA or circ-ZNF609 siRNA+ miR-145-5p mimics. **A**, MiR-145-5p expression was upregulated after transfection of circ-ZNF609 siRNA, which was reversed by co-transfection of circ-ZNF609 siRNA and miR-145-5p mimics. **B-C**, CCK-8 **B**, and colony formation assay **C**, indicated an inhibited proliferation in GC cells with circ-ZNF609 knockdown, but was reversed by miR-145-5p overexpression. **D**, Invasive ability was inhibited after transfection of circ-ZNF609 siRNA, which was reversed by co-transfection of circ-ZNF609 siRNA and miR-145-5p mimics. **E**, Apoptosis was induced after transfection of circ-ZNF609 siRNA, which was reversed by co-transfection of circ-ZNF609 siRNA and miR-145-5p mimics.

expression (Figure 4E). These results indicated that circ-ZNF609 may promote the proliferative and invasive abilities, but inhibited apoptosis of GC cells by binding to miRNA-145-5p.

Discussion

Detective rate of early-stage GC is insufficient, while the prognosis of advanced GC is poor. The mortality rate of GC is second only to liver cancer, pancreatic cancer and lung cancer, which greatly threatens human health[13]. Several aspects may be explained for the poor prognosis of GC. First of all, the early diagnostic rate of GC is relatively low. Secondly, surgical resection and chemotherapy are still the major therapeutic approaches of GC. However, local invasion, lymph node metastasis and distant metastasis of middle-stage and advanced GC extremely restrict the therapeutic efficacy. Thirdly, a comprehensive mechanism of GC has not been fully elucidated. Targeted molecular drugs of GC are lacking[14]. It is urgent to elucidate the molecular mechanism of GC, especially in its invasion and metastasis, so as to search for novel therapeutic targets.

In the human genome, only 2% are protein-encoding sequences. The majority of non-coding RNAs initially considered with few biological functions. CircRNA is a brand-new RNA transcript, and its function as ceRNA in tumors has been recently explored. Sry (Sex-determining region Y) is the earliest discovered circRNA containing 16 binding sites to miR-138, which serves as a molecular sponge for miR-138[15]. CircRNA CDR1as (cerebellar degeneration-related protein 1 transcript) contains over 70 binding sites to miR-7, which is also known as circRNA 7 (circular RNA sponge for miR-7)[16]. Functionally, CDR1as inhibits miR-7 activity by sponging it, thus regulating the downstream genes[17]. It is suggested that CDR1as serves as a regulator of neuronal activity and may be a potential target for the treatment of neurological diseases or other cancers.

Previous studies have shown the crucial functions of circRNAs in the development of GC. For example, circ-LARP4 suppresses GC cells to proliferate and invade by sponging miR-424-5p to regulate LATS1 expression[18]. Circ-0000745 is utilized as a diagnostic hallmark for GC[19]. Growth and migration of GC cells could be influenced by circ-0000096 level[20]. In this paper, circ-ZNF609 was highly expressed in GC tissues and cell lines. Moreover, circ-ZNF609 could elevate the proliferative and invasive capacities of GC cells. To further elucidate the molecular mechanism of circ-ZNF609 in regulating the progression of GC, we predicted its sponging miRNA through bioinformatics, and miRNA-145-5p was screened out.

MiRNAs are a class of endogenous, non-coding RNAs regulating target gene expressions at the post-transcriptional level by binding to 3'UTR of target mRNAs[21]. Multiple cellular processes involve miRNAs participation, including cell metabolism, growth, differentiation, proliferation, senescence and apoptosis. As oncogenes or tumor-suppressor genes, miRNAs are closely related to tumor development by mediating tumor-related pathways[22,23]. MiRNA-145-5p has been proved to be a tumor-suppressor gene in most types of tumors. It is reported that miRNA-145-5p is downregulated in non-small cell lung cancer, esophageal squamous cell carcinoma and bladder cancer[24-26]. Overexpression of miRNA-145-5p is able to inhibit invasiveness and lymph node metastasis of tumors. Jiang et al[27] pointed out that miRNA-145-5p is lowly expressed in GC, and N-cadherin is the direct target of miRNA-145-5p. The specific regulatory mechanism of miRNA-145-5p in GC, however, remains unclear.

We suggested that circ-ZNF609 regulated GC progression by sponging miRNA-145-5p. Subsequently, we found that circ-ZNF609 could competitively bind to miRNA-145-5p. Thus, the degradation of downstream target genes by microRNA-145-5p was eliminated, and the expressions of target genes were indirectly upregulated. Our results suggested that circ-ZNF609 promoted GC cells to proliferate and invade, while miRNA-145-5p presented the opposite functions on cellular potentials of GC cells. Through rescue experiments, we confirmed that circ-ZNF609 promoted growth and metastasis of GC cells by inhibiting miRNA-145-5p expression.

Conclusions

Circ-ZNF609 promotes proliferative and invasive abilities of gastric cancer cells by inhibiting miRNA-145-5p expression as a ceRNA, thus accelerating gastric cancer progression. Circ-ZNF609/miRNA-145-5p exerts a promising value in diagnosis and treatment of GC.

Conflict of Interest

The authors declare that they have no conflict of interest.

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