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Circ_0032627 accelerates proliferation of gastric cancer cells through regulating miR-502-5p expression

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Abstract. – OBJECTIVE: To explore the biological functions of circ_0032627 in the progression of gastric cancer (GC).

MATERIALS AND METHODS: The expression level of circ_0032627 in GC cell lines and gastric mucosal cell lines was measured *via* quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). Methyl thiazolyl tetrazolium (MTT) assay, colony formation assay, and FACS were performed to examine the influences of circ_0032627 on the proliferation and apoptosis of GC cells. The relationship between circ_0032627 and micro ribonucleic acids (miRNAs) was predicted on-line using StarBase software, and whether circ_0032627 can act as the sponge of the selected miRNAs was verified *via* Dual-Luciferase reporter assay and qRT-PCR. Finally, MTT assay was conducted to detect the influences of the co-knockdown of circ_0032627 and the selected miRNAs on the proliferation of GC cells.

RESULTS: Compared with that in gastric mucosal cell lines, the expression level of circ_0032627 was upregulated in the selected four GC cell lines, and circ_0032627 knockdown substantially inhibited the proliferation of GC cells, but promoted their apoptosis. Circ_0032627 could act as a sponge of miR-502-5p, and miR-502-3p knockdown reversed the inhibitory effect of circ_0032627 on the proliferation of GC cells.

CONCLUSIONS: The expression level of circ_0032627 is raised in GC cells, and circ_0032627 affects the proliferation and apoptosis of GC cells by sponging miR-502-5p.

Key Words:
Gastric cancer, Circ_0032627, MiR-502-5p, Proliferation, Apoptosis.

Introduction

Gastric cancer (GC), a common malignancy, ranks 5th in the morbidity and mortality rates among the digestive system tumors worldwide, and serves as the 3rd leading cause of cancer related deaths in China[1,2]. The morbidity rate of GC is regionally specific. Over half of GC cases occur in East Asian countries, such as Japan and China[2]. Although chemotherapy is an efficacious treatment regimen, it still produces poor prognosis in most patients who are definitely diagnosed with tumor invasiveness and distant metastasis. As a long-term progressive disease, GC is associated with oncogene activation or inactivation of tumor suppressor genes[3]. Therefore, it is necessary to elucidate the molecular foundation in the progression of GC to confirm the potential diagnostic markers and therapeutic targets.

Circular ribonucleic acids (circRNAs) are a class of non-coding RNAs (ncRNAs) that play vital roles in the transcriptional and post-transcriptional gene expressions[4]. They are evolutionarily conservative in different species, and their expressions normally exhibit the specificity to tissues and development stages. With the rapid development of bioinformatics technology and high-throughput sequencing, large numbers of circRNAs have been discovered. They are crucial in multiple physiological and pathological processes, including cell proliferation, cycle, migration, invasion, metastasis, and carcinogenesis[5]. CircRNAs are involved in the development and progression of many malignant tumors, such as breast cancer[6], hepatocellular carcinoma[7], bladder cancer[8], and GC[9].

CircRNAs can act as the competitive endogenous RNAs (ceRNAs) to sponge miRNAs and then inhibit their functions. Such a novel mechanism regulating miRNA activity also provides a promising action mode for circRNAs. Since miRNAs regulate a series of biological

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processes, the sponging of circRNA will affect these biological behaviors as well[10]. MiRNAs are a major type of short ncRNAs (22 nt) that regulate the post-transcriptional gene expression through the direct base pairing with the targets in the messenger RNAs (mRNAs). CircRNAs can compete for the binding sites of miRNAs to influence the activity of miRNAs[11]. Huang et al[12] researched the functions of circRNAs in the resistance of GC to cisplatin, and they found that the upregulation of circAKT3 in the patients treated with cisplatin is significantly correlated with invasive features, and that circAKT3 acts as a sponge of miR-198 to promote the expression of PIK3R1, enabling PIK3R1 to promote deoxyribonucleic acid (DNA) damage repair *in vivo* and *in vitro* and to repress GC cell apoptosis. In this study, circ_0032627, a novel oncogene, was identified, and it served as a sponge of miR-502-5p to accelerate the proliferation of GC cells.

Materials and Methods

Materials

GC cell lines HGC-27, BGC-823, MGC-803, and SGC-7901 and the control GES1 human gastric mucosal cell line were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA); Dulbecco's Modified Eagle's Medium (DMEM) with high glucose, and fetal bovine serum (FBS) were purchased from HyClone (South Logan, UT, USA); the reverse transcription (RT) kit and SYBR Green quantitative Polymerase Chain Reaction (qPCR) kit were purchased from TaKaRa (Otsu, Shiga, Japan). MiRNA RT and qPCR kits were purchased from BioTNT (Shanghai, China); TRIzol RNA extraction reagent and Lipofectamine 2000 transfection reagent were purchased from Invitrogen (Carlsbad, CA, USA); methyl thiazolyl tetrazolium (MTT) assay kit, cell apoptosis assay kit and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Dual-Luciferase reporter assay kit and empty pmirGLO vector plasmid were purchased from Promega (Madison, WI, USA); microplate reader and real-time qPCR instrument were purchased from Bio-Rad (Hercules, CA, USA); and flow cytometer was purchased from BD (Franklin Lakes, NJ, USA). Small interfering RNAs (siRNAs, si-1#, si-2# and si-3#) specifically targeting and knocking down circ_0032627, siRNA negative control (si-NC), miR-502-5p mimics, and its NC (miR-NC) were designed and chemically synthesized by Suzhou GenePharma Co., Ltd. (Suzhou, China).

Cell Culture and Transfection

The cells used in the present study were cultured in DMEM supplemented with 10% heat inactivated FBS, 100 U/mL penicillin and 100 μg/mL streptomycin in a humid incubator with 5% CO_2 at 37°C. Then, the cells growing well were harvested, divided into different experimental groups, and transfected based on Lipofectamine 2000 transfection procedures.

Total RNA Extraction and qRT-PCR

The total RNAs were isolated from cells using TRIzol reagent, among which circRNAs were synthesized into complementary DNAs (cDNAs) using the RT kit (TaKaRa, Otsu, Shiga, Japan), while miRNAs were reversely transcribed using the RT kit (BioTNT). SYBR Green qPCR kit and miRNA qPCR kit (BioTNT, Shanghai, China) were employed for the relative quantification of circRNAs and miRNAs, respectively. All primer sequences (synthesized by Beijing Liuhe BGI Co., Ltd., Beijing, China) used in this study are as follows: circ_0032627 F: 5'-TACAGTCAAAACCCCAGCGT-3', R: 5'-GTTGTTAATGCTTTCTCCCACC-3'. GAPDH F: 5'-CAATGACCCCTTCATTGACC-3', R: 5'-TTGATTTTGGAGGGATCTCG-3'. miR-502-5p F: 5'-CAATGACCCCTTCATTGACC-3'. R: 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAG-3'. U6 F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3'. Finally, the relative expression levels of the target genes were calculated using $2^{-\Delta\Delta Cq}$.

Analysis of Cell Proliferation Via MTT Assay

Cell viability was evaluated *via* MTT assay. At 24 h after transfection, the cells were seeded into a 96-well plate at 2×10^3 cells/well and continued to be cultured for 24, 48, 72, and 96 h. At different time points, 0.5 mg/mL MTT was added into cell wells, followed by incubation at 37°C for 4 h. With the supernatant removed, the crystals were dissolved in 100 μL of DMSO. Finally, the absorbance at the wavelength of 570 nm was measured using a microplate reader, with sextuplicate wells set in each group.

Colony Formation Assay

In colony formation assay, the cells were inoculated into a 6-well plate at 24 after transfection and let grow until visible colonies were formed. After 10-12 d, the colonies were fixed using methanol and stained with hematoxylin. Finally, they were visually observed and manually counted under a microscope.

Detection of Cell Apoptosis Via Flow Cytometry

Cell apoptosis was detected using flow cytometry and Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis assay kit according to the manufacturer's scheme as follows. The cells were first digested using trypsin and harvested. Then, they were washed with PBS twice and re-suspended in the binding buffer, followed by incubation with Annexin V-FITC and PI in the dark at room temperature for 15 min. Finally, the stained cells were detected using BD FACSCalibur flow cytometer (Franklin Lakes, NJ, USA).

Determination of Luciferase Activity

According to the StarBase, circ_0032627 sequences contain the potential blinding site of miR-502-5p. Wide-type circ_0032627 (circ_0032627-WT) and mutant circ_0032627 (circ_0032627-MUT) were constructed based on the predicted miR-502-5p binding sequences. Then, the cells were transfected with circ_0032627-WT or circ_0032627-MUT and miR-NC or miR-502-5p mimics for 48 h. Finally, the activity of Luciferases was determined according to the instructions of the Dual-Luciferase reporter assay kit (Promega, Madison, WI, USA).

Statistical Analysis

GraphPad Prism 5 software (La Jolla, CA, USA) was employed for statistical analysis. All data were expressed as mean ± standard deviation ($\bar{x} \pm s$). $p<0.05$ suggested that the difference was statistically significant.

Results

The Expression of Circ_0032627 Was Upregulated in Human GC Cell Lines

The role of circ_0032627 in the progression of GC was first explored, and the expression of circ_0032627 in GC cell lines (HGC-27, BGC-823, MGC-803, and SGC-7901) and human gastric mucosal cell line GES1 was detected *via* qRT-PCR. According to the results (Figure 1), the expression of circ_0032627 in four GC cell lines was notably higher than that in human gastric mucosal cell line ($p<0.05$). It is conjectured that the upregulation of circ_0032627 expression in GC cells may be associated with the development and progression of GC, and it is likely to play a role as an oncogene.

Influence of Circ_0032627 Knockdown on the Proliferation of SGC-7901 GC Cells

After the upregulation of circ_0032627 expression in GC cells was corroborated, the influence of circ_0032627 knockdown on the proliferation of SGC-7901 GC cells was examined. Circ_0032627 was first knocked down through transient transfection of siRNAs, and then, the knockdown efficiency was determined *via* qRT-PCR. The results revealed that compared with si-NC, three target sequences (si-1#, si-2# and si-3#) exhibited different degrees of knockdown ($p<0.05$), and the knockdown of si-3# was the most evident (Figure 2A), so it was selected for subsequent circ_0032627 knockdown experiments to study the biological functions of circ_0032627. The impact of circ_0032627 knockdown was detected *via* MTT assay, and it was found that circ_0032627 knockdown remarkably suppressed the proliferation of SGC-7901 GC cells ($p<0.05$) (Figure 2B).

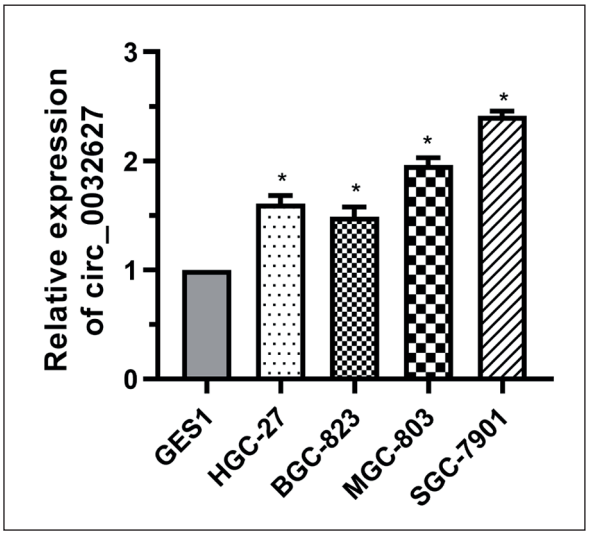

Figure 1. Circ_0032627 expression in GC cell lines (HGC-27, BGC-823, MGC-803, and SGC-7901) and human gastric mucosal cell line GES1 detected *via* qRT-PCR. (*$p<0.05$).

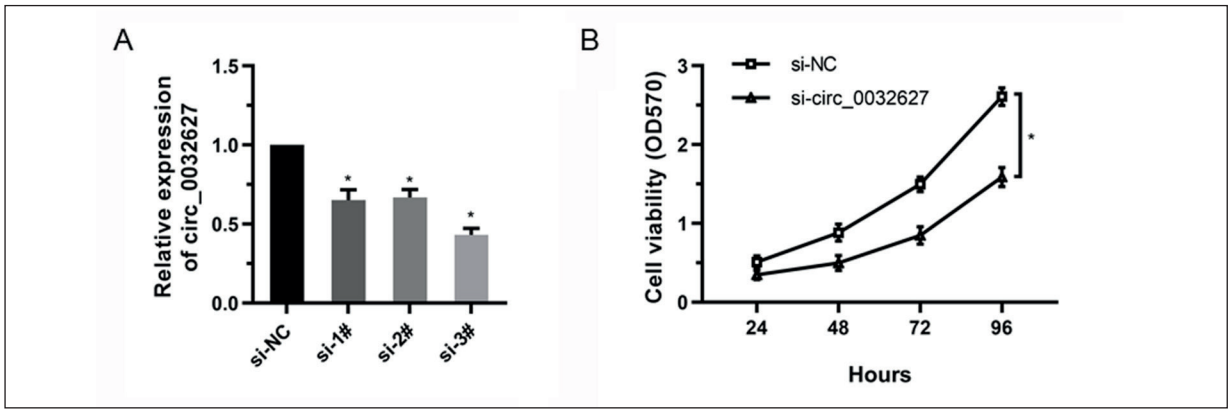

Figure 2. Influence of circ_0032627 knockdown on the proliferation of SGC-7901 GC cells. **A,** Knockdown efficiency determined *via* qRT-PCR. **B,** The impact of circ_0032627 knockdown on SGC-7901 GC cell proliferation detected *via* MTT assay. (*p<0.05).

Influence of Circ_0032627 Knockdown on the Colony Formation of SGC-7901 GC Cells

The influence of circ_0032627 knockdown on the colony formation of SGC-7901 GC cells was further verified *via* the colony formation assay, and according to the results (Figure 3), compared with that in the control group, the colony formation capacity was remarkably weakened in the knockdown group (p<0.05).

Impact of Circ_0032627 Knockdown on the Apoptosis Rate of SGC-7901 GC Cells

Annexin V-FITC/PI double staining was performed. Specifically, at 24 h after transfection, the impact of circ_0032627 knockdown on the apoptosis rate of SGC-7901 GC cells was evaluated *via* flow cytometry. As shown in Figure 4, the apoptosis rate in the knockdown group was evidently higher than that in the control group, suggesting that inhibiting circ_0032627 promotes the apoptosis of SGC-7901 GC cells.

Circ_0032627 Acted as a Sponge of MiR-502-5p

To detect whether circ_0032627 can function as a sponge of a certain miRNA as circRNAs do, the miRNAs containing the binding site of circ_0032627 were predicted using StarBase (Figure 5A). According to literature investiga-

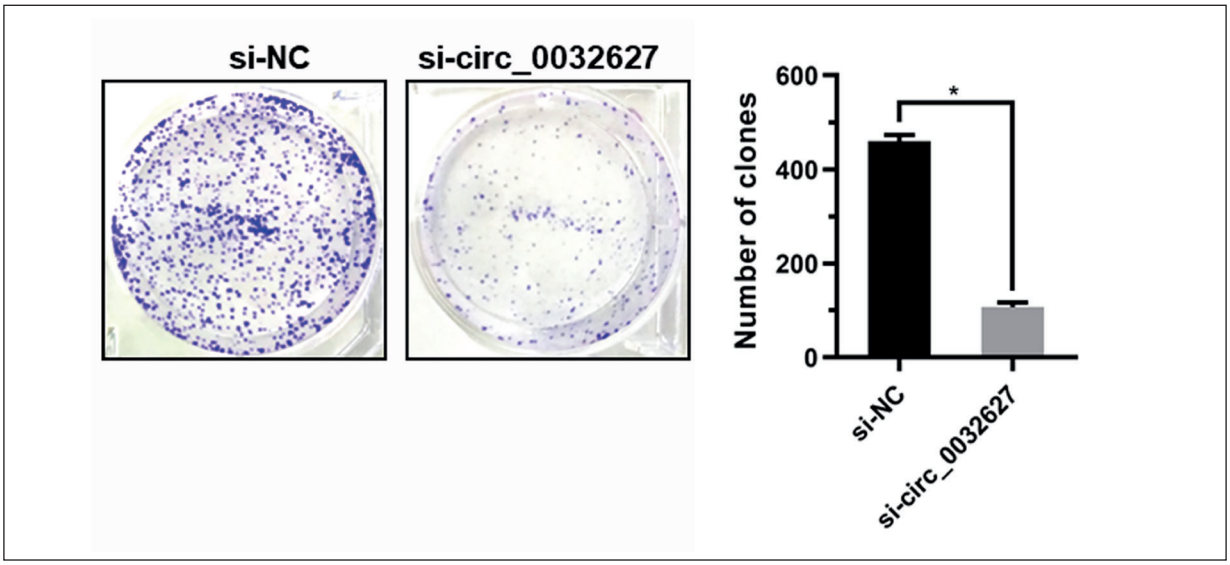

Figure 3. Influence of circ_0032627 on the colony formation of SGC-7901 GC cells (magnification: 10×). (*p<0.05).

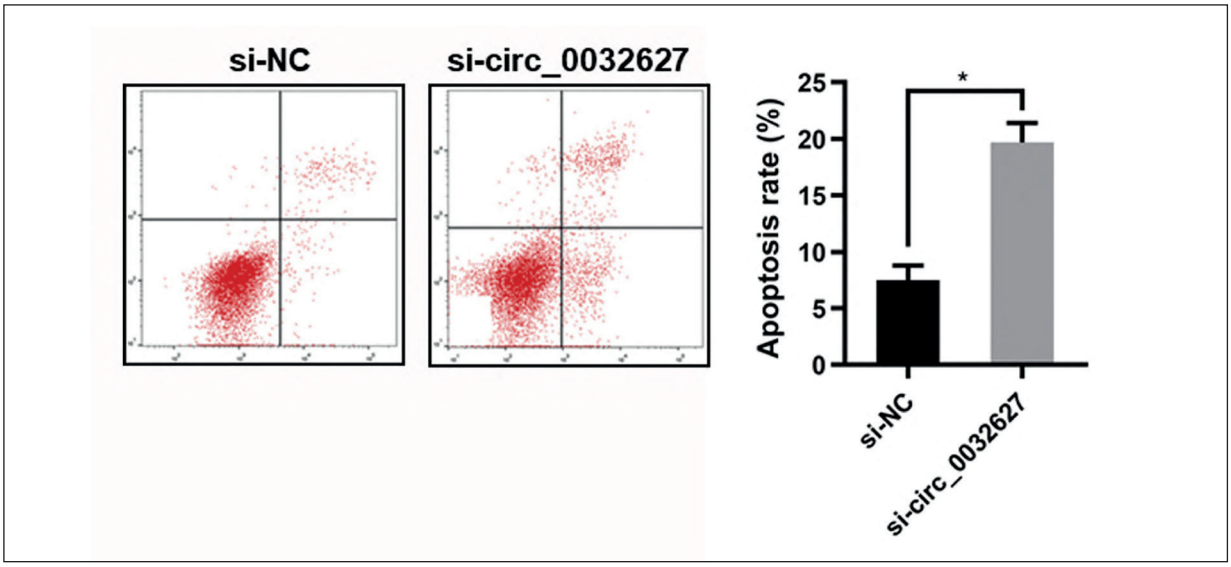

Figure 4. Impact of circ_0032627 knockdown on the apoptosis rate of SGC-7901 GC cells. (*p<0.05).

tion[13-14], among these miRNAs, miR-502-5p has downregulated expression in most tumor tissues, and it is speculated that circ_0032627 has an inhibitory effect on miR-502-5p expression. To verify this speculation, the circ_0032627 sequences containing the predicted miR-502-5p site or mutant site were cloned into empty vector plasmids, which were named as circ_0032627-WT and circ_0032627-MUT, respectively. Then, the activity of Luciferases was determined using Luciferase reporter system in each group, and the results showed that the transfection with miR-502-

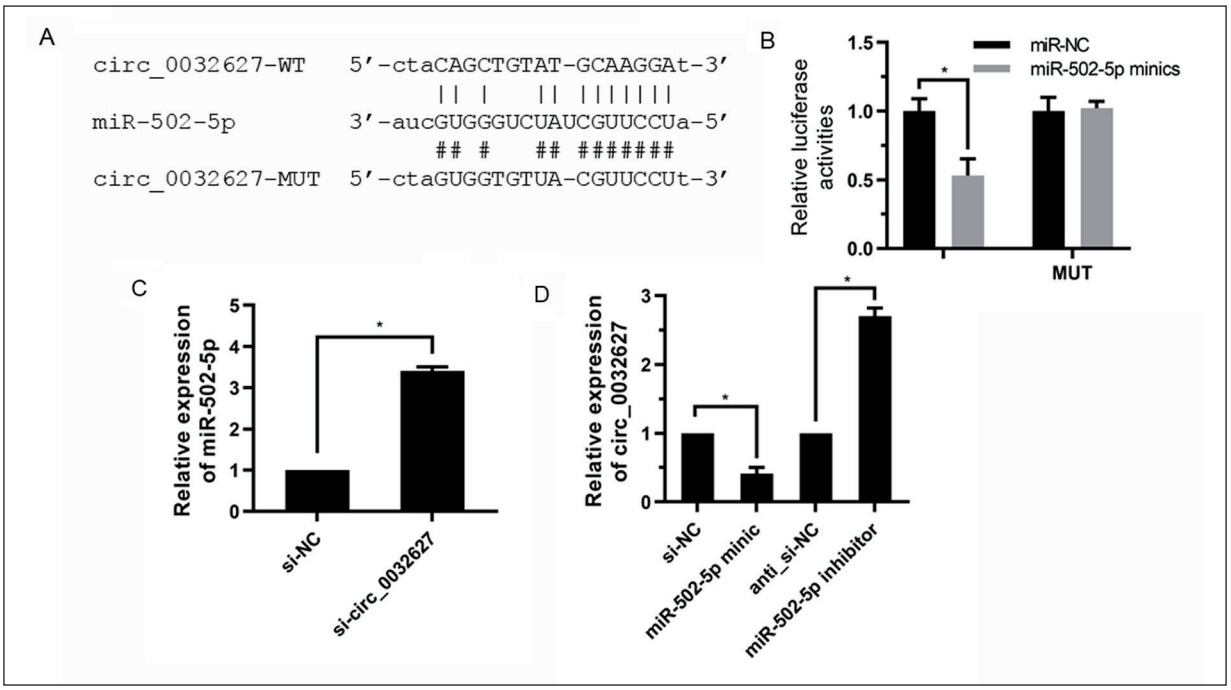

Figure 5. Circ_0032627 acts as a sponge of miR-502-5p. **A,** The binding sites between circ_0032627 and miR-502-5p predicted using StarBase. **B,** Interaction between circ_0032627 and miR-502-5p detected *via* Luciferase activity assay. **C-D,** Regulatory effect of circ_0032627 on miR-502-5p mRNA level examined *via* qRT-PCR. (*p<0.05).

5p markedly weakened the activity of Luciferases in circ_0032627-WT group (p<0.05), but had no evident influence on that in circ_0032627-MUT group (Figure 5B). Furthermore, the qRT-PCR verification results manifested that circ_0032627 knockdown remarkably increased the expression level of miR-502-5p (p<0.05) (Figure 5C). Likewise, the transfection with miR-502-5p knockdown or overexpression plasmids had evident influences on the expression level of circ_0032627 (p<0.05) (Figure 5D). The above results imply that circ_0032627 acts as a sponge of miR-502-5p to inhibit its expression.

MiR-502-5p Was Involved in Circ_0032627-Mediated Inhibition of SGC-7901 GC Cell Proliferation

It is speculated that circ_0032627 mediates the inhibition of SGC-7901 GC cell proliferation through miR-502-5p. Circ_0032627 and miR-502-5p were knocked down together in SGC-7901 GC cells, and the influence on SGC-7901 GC cell proliferation was evaluated *via* MTT assay. It was found that miR-502-3p knockdown reversed the inhibitory effect of circ_0032627 on the proliferation of SGC-7901 GC cells ($^*p$<0.05) (Figure 6).

Discussion

GC remains one of the most common malignant tumors all around the world now. Due to GC's manifestations and insufficient sensitivity to radiotherapy and chemotherapy in the advanced stage, the total survival rate of patients is below 30%, still far from satisfactory[15]. It is, therefore, vital to improve early diagnosis, develop effective prognostic markers, and find out new molecular therapeutic targets for gastric cancer.

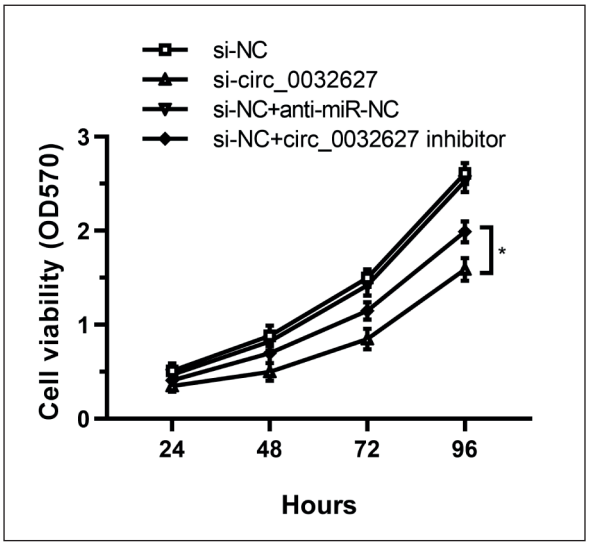

Figure 6. MiR-502-5p is involved in circ_0032627-mediated inhibition of SGC-7901 GC cell proliferation. ($^*p$<0.05).

CircRNAs, the ncRNA family members discovered recently, are characterized by covalently closed continuous loops and resistance to RNase R digestion[16] and stay highly stable in various cells. In recent years, circRNAs have received growing attention as the high-throughput sequencing and bioinformatics technology develop rapidly. They are involved in tumorigenesis by regulating a variety of biological processes, including cell proliferation, apoptosis, invasion, and differentiation[11]. Li et al[17] found that the expression of circPVT1 is upregulated in non-small cell lung cancer (NSCLC) tissue samples and cells, and that it inhibits miR-125b to activate E2F2 expression and promote NSCLC cell growth and migration. Wang et al[18] screened differentially expressed circRNAs in glioblastoma multiforme (GBM) using gene chip technology and discovered that circMMP9 was increased by EIF4A3 in GBM. Besides, circMMP9, as an oncogene, directly targets miR-124 to promote the proliferation, migration, and invasion of GBM cells. Ding et al[19] by investigating the role of circ_DONSON in the progression of GC found that its expression is upregulated and positively correlated with advanced TNM stage and poor prognosis. Silencing circ_DONSOC substantially inhibits the proliferation, migration, and invasion of GC cells, while promoting their apoptosis. The mechanism analysis revealed that circ_DONSOC recruits NURF complex to the SOX4 promoter and trigger its transcription, thereby accelerating the progression of GC.

The present study mainly explored the biological functions of circ_0032627 in the progression of GC, and it was found that the expression of circ_0032627 in GC cell lines was notably higher than that in human gastric mucosal cell lines. It is speculated that the increase in circ_0032627 expression in GC cells may be associated with the development and progression of GC, and circ_0032627 is likely to act as an oncogene. Circ_0032627 was knocked down through transient transfection of siRNAs, and then, the influence of circ_0032627 knockdown on the proliferation of SGC-7901 GC cells was examined.

According to data, knocking down circ_0032627 remarkably repressed the proliferation of GC cells, but promoted their apoptosis, suggesting that circ_0032627 functions as an oncogene in the progression of GC.

CircRNAs can play a role as ceRNAs and serve as miRNA sponges to regulate the progression of tumors[20]. Lu et al[21] reported a novel circRNA hsa_circ_0001368 never reported before, and they found that its expression is basically downregulated in GC tissues and cells and that such downregulation is related to the poor prognosis of GC patients. Studies have established that hsa_circ_0001368 suppresses tumors through the miR-6506-5p/FOXO3 axis in GC and can act as a potential target for the treatment of GC. The present study predicted and corroborated that the circ_0032627 and miR-502-5p are interrelated and verified that circ_0032627 acts as a sponge of miR-502-5p in GC cells and affects the biological functions of GC cells through miR-502-5p.

Conclusions

In summary, the results of the present study demonstrated that circ_0032627 is upregulated in GC cells, and it accelerates the development and progression of GC by sponging miR-502-5p, giving more options of the potential targets in treating GC and understanding the development and progression mechanisms of this disease as well.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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