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LINC00355 inhibits apoptosis and promotes proliferation of gastric cancer cells by regulating Wnt/β-catenin signaling pathway

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Abstract. – OBJECTIVE: To study the expression and biological functions of long intergenic non-protein coding ribonucleic acid 00355 (LINC00355) in gastric cancer (GC), and to explore its potential mechanism of action.

PATIENTS AND METHODS: The relative expression level of LINC00355 in 48 cases of GC tissues, the corresponding paracancerous tissues, and GC cells was determined using quantitative reverse transcription-polymerase chain reaction (qRT-PCR), and the interference efficiency of small interfering (si)-LINC00355 was detected *via* qRT-PCR. After knock-down of LINC00355, methyl thiazolyl tetrazolium (MTT) and 5-ethynyl-2'-deoxyuridine (EdU) assays were performed to detect the changes in the proliferation ability of GC cells, and the changes in the GC cell cycle distribution and apoptosis rate were examined by flow cytometry. Besides, Western blotting was conducted to verify the changes in the downstream signaling pathway of LINC00355.

RESULTS: Among 48 cases of GC tissues, there were 42 (87.5%) cases of LINC00355 expression up-regulation, and 6 (12.5%) cases of LINC00355 expression down-regulation. The qRT-PCR results revealed that the expression of LINC00355 was raised in 4 kinds of GC cells. After interference with LINC00355 expression, the MTT assay results indicated that the cell proliferation ability was inhibited, consistent with the EdU assay results. After LINC00355 was knocked down in GC cells, GC cells in experiment group had a higher apoptosis rate than those in si-NC group and arrested in the gap 0 (G0)/G1 phase. Moreover, it was found through Western blotting that the expressions of the molecular markers in the downstream wingless-INT (Wnt)/β-catenin signaling pathway were downregulated after interference with the expression of LINC00355.

CONCLUSIONS: LINC0035 exhibits an up-regulated expression in GC and regulates the Wnt/β-catenin signaling pathway to promote proliferation and inhibit apoptosis.

Key Words:
Gastric cancer, LINC00355, Proliferation, Apoptosis, Wnt/β-catenin signaling pathway.

Introduction

Gastric cancer (GC), one of malignancies with a high morbidity rate, is the second leading cause of cancer-related deaths[1,2]. Radical resection of early GC significantly raises the 5-year survival rate and overall survival rate of patients, while the postoperative 5-year survival rate of advanced GC patients remains lower than 30%[3]. Most patients in China have suffered from advanced GC due to the low diagnostic rate, so targeted therapy becomes the hotspot in the research field of GC[4]. However, there have not yet been targeted drugs with stable and reliable efficacy available for the long-term treatment of advanced GC now.

A variety of non-coding ribonucleic acids (ncRNAs), including long ncRNAs (lncRNAs), have important influences on the cellular physiological and pathological processes[5]. Liu et al[6] found that the dysregulation of lncRNA expression is closely associated with the development and progression of GC. LncRNAs are involved in multiple tumor signaling pathways, such as the Notch, mTOR, NF-κB and Wnt signaling pathways[7,8], and modulate cellular proliferation, resistance to drugs, apoptosis, invasion and tumorigenesis, cell cycle and metastasis. Additionally, the abnormal expression of lncRNAs has important clinical implications for the diagnosis of GC, and that they are associated with clinicopathological factors for GC, such as metastasis,

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infiltration, TNM stage, prognosis and differentiation degree, the most of which are related to GC metastasis and infiltration (61.70% and 53.19%, respectively)[9-11]. These GC-associated lncRNAs can serve as biomarkers for GC metastasis, thereby helping clinically diagnose and treat GC.

Long intergenic non-protein coding RNA 00355 (LINC00355), located on chromosome 13q21.31 and measuring 1,878 bp in full length, exhibits expression dysregulation in bladder cancer, prostate cancer, and colorectal cancer[12-14], but its expression in GC has not yet been reported. The present research group first found through *in vitro* experiments that the expression of LINC00355 was upregulated in GC tissues and cells and that highly expressed LINC0035 promoted the proliferation and repressed the apoptosis of GC cells.

Patients and Methods

Tissue Specimens

A total of 48 pairs of GC tissue specimens were collected from Yantaishan Hospital, and the patients were pathologically diagnosed with GC and preoperatively received no radiochemotherapy, targeted therapy or immunotherapy. Following surgical resection, GC tissues and the paracancerous tissues 5 cm beyond were preserved in liquid nitrogen at -180°C for subsequent experiments. This investigation was approved by the Ethics Committee of Yantaishan Hospital. Signed written informed consents were obtained from all participants before the study.

Cell Culture

Human GC cell lines BGC-823, MGC-803, AGS, and SGC-7901, and a normal gastric epithelial cell line GES-1 were purchased from the China Center for Type Culture Collection, Wuhan University (Wuhan, China). GC cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) or Roswell Park Memorial Institute-1640 medium (RPMI-1640) containing 10% fetal bovine serum (FBS; Abcam, Cambridge, MA, USA) and 1% penicillin/streptomycin, while normal gastric epithelial cells in the refined KSFM mixed at 1:1 (Abcam, Cambridge, MA, USA).

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The primer sequences were designed by Shanghai GenePharma Co., Ltd. (Shanghai, China): LINC00355: Sense: ACAGGACTCCATGGCAAACG, Antisense: ATGAAGAAAGCCTGGTGC, and glyceraldehyde -3-phosphate dehydrogenase (GAPDH): Sense: CCACAGTCCATGCCATCAC, Anti-sense: TCCACCACCCTGTTGCTGTA. Total RNAs were isolated from tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and synthesized into complementary deoxyribonucleic acid (cDNA) with reverse transcriptase [TaKaRa Biotechnology Co., Ltd., (Dalian, China)] and oligonucleotides according to the manufacturer's specifications. Then, qRT-PCR was performed using SYBR Premix ExTaq™ II kit [TaKaRa Biotechnology Co., Ltd., (Dalian, China)] under the following conditions: 94°C for 10 s, 94°C for 5 s, 52°C for 30 s, annealing at 52°C for 30 s, and 72°C for 15 s, for 40 cycles.

Cell Transfection

When the GC cells grew to about 50% confluence, they were co-transfected with Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA) diluted by Opti-MEM (Invitrogen, Carlsbad, CA, USA) and small interfering (si)-negative control (NC) or si-LINC00355, and 6 h later, the liquid in the culture plate was aspirated and discarded. Then, the cells continued to be cultured in the medium containing 10% fetal bovine serum for 48 h. Finally, the transfection efficiency was determined using qRT-PCR.

Methyl Thiazolyl Tetrazolium (MTT) Assay

The GC cells were first inoculated into a 96-well plate until adherence. Then, the cells transfected for 48 h through "experimental step 4" were incubated with MTT for 4 h and added with dimethyl methylene at 150 μL/well, and the 96-well plate was placed on a micro-oscillator to dissolve crystals. Finally, the optical density (OD) was measured at a wavelength of 560 nm using a microplate reader (Olympus, Tokyo, Japan).

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

The GC cells in the logarithmic phase were harvested, and with the density adjusted to 8×10^5 cells/mL, the cells were seeded into a 24-well plate at 400 μL/well and cultured in an incubator

for growing on a glass slide. After adherence, transfection was performed for 48 h. Subsequently, as specified in the EdU kit (Beyotime, Shanghai, China), the resulting cells were incubated with EdU solution for 2 h, fixed in 4% paraformaldehyde, and stained by Click-iT reactant, with 0.5% Triton X-100 (Solarbio, Beijing, China) as the penetration enhancer, followed by DNA counter-staining with 4',6-diamidino-2-phenylindole (DAPI) (Beyotime, Shanghai, China). Finally, images were captured under a fluorescence microscope in the dark. Cell proliferation rate = the number of red fluorescent cells/that of blue fluorescent cells ×100%.

Cell Apoptosis

The GC cells in the logarithmic phase were collected, inoculated to a 25-cm^2 culture flask, transfected and cultured in an incubator with 5% CO_2 at 37°C for 48 h. Then, the cell supernatant was harvested and washed using cooled phosphate-buffered saline (PBS) for 3 times. The resulting cells were digested using ethylenediaminetetraacetic acid (EDTA)-free trypsin, collected and detected using a flow cytometer according to the instructions of Annexin V-propidium iodide (PI) apoptosis kit (BestBio, Shanghai, China) within 1 h to analyze the cell apoptosis.

Cell Cycle

The GC cells in the logarithmic phase were harvested at an adjusted density of 5×10^5 cells/mL and assigned to experiment group and control group. After culture for 48 h, 1×10^6 cells were taken, centrifuged at 300×g for 5 min, washed using PBS, mixed evenly with 5 mL of 75% ice ethanol, fixed, and let stand at -20°C overnight. Then, the resulting cells were centrifuged again at 300×g for 10 min to remove ethanol and rinsed using PBS. Subsequently, 1 mL of cells suspended in PBS were added with 5 μL of RNases (10 mg/mL), subjected to warm bath at 37°C for 1 h, stained with 5 μL of PI staining solution (10 mg/mL) at room temperature in the dark for 30 min, and detected using the flow cytometer (Partec AG, Arlesheim, Switzerland).

Western Blotting

The cells were collected from experiment group and control group and lysed to extract proteins. Then, the proteins were quantified, and sample solution was prepared for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred onto membranes, blocked and incubated with primary antibodies (diluted at 1:2,000). After the membranes were washed, the horseradish peroxidase (HRP)-labeled goat anti-rabbit and anti-rat IgG secondary antibodies (1:5,000) were added, followed by color development and fixation using enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific, Waltham, MA, USA). Finally, the protein bands were scanned and quantified using an automatic gel imaging analysis system.

Statistical Analysis

The above experiments were conducted in triplicate, and the results were averaged and presented as ($\bar{x} \pm s$). Data were processed by Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA), and inter-group comparisons were made using *t*-test. $p<0.05$ indicated statistically significant differences.

Results

LINC00355 Expression Was Upregulated in GC Cells

First, the relative expression level of LINC00355 in GC tissues were determined using qRT-PCR, and the results showed that compared with paracancerous tissues, GC tissues exhibited the up-regulation of the relative expression level of LINC00355 (Figure 1A), and the relative expression level of LINC00355 was up-regulated in 42 cases of GC tissues and down-regulated in 6 cases of GC tissues (Figure 1B). Then, the relative expression level of LINC00355 in GC cells was measured *via* qRT-PCR again, and it was found that its relative expression level was elevated in 4 kinds of GC cells compared with that in normal gastric mucosal cells (Figure 1C). To explore the biological functions of LINC00355, interfering sequences were designed and synthesized, and the interference efficiency was determined (Figure 1D).

Si-LINC00355 Inhibited GC Cell Proliferation

To investigate the influences of LINC00355 on GC cells, it was found through the MTT assay that the proliferation ability of GC cells was weakened after the expression of LINC00355 was lowered (Figure 2A and 2B). Moreover, ac-

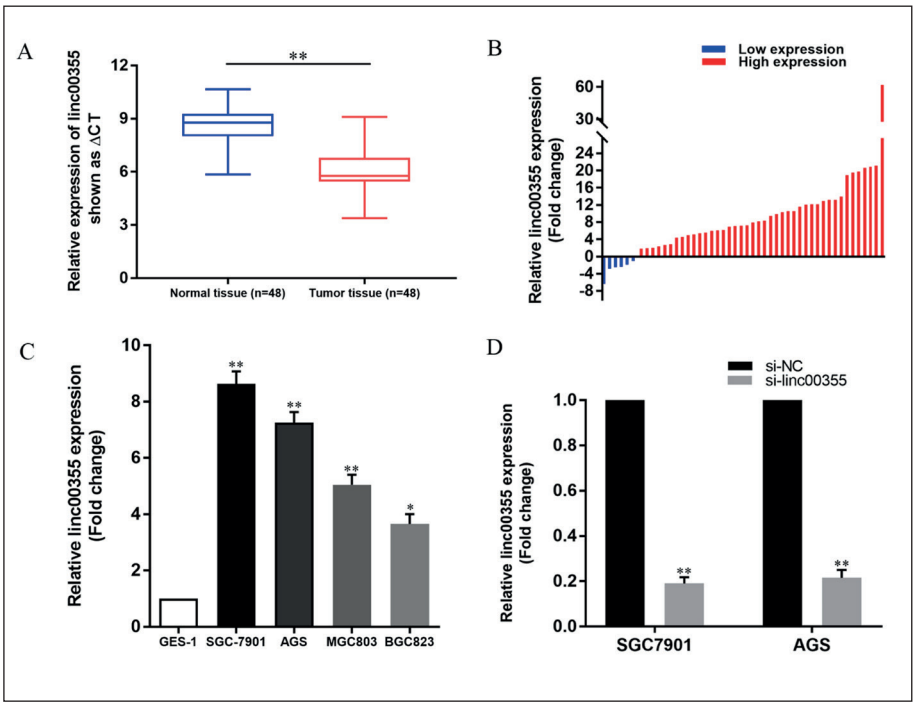

Figure 1. The relative expression of LINC00355 is upregulated in GC cells. **A,** Compared with that in paracancerous tissues, the relative expression of LINC00355 is elevated in GC tissues based on the qRT-PCR results. **B,** LINC00355 expression is up-regulated in 42 out of 48 GC tissue samples, and down-regulated in 6 out of 48 GC tissue samples. **C,** The results of qRT-PCR show that the expression of LINC00355 in GC cells is higher than that in normal gastric mucosal cells. **D,** Relative expression level of si-LINC00355 determined *via* qRT-PCR.

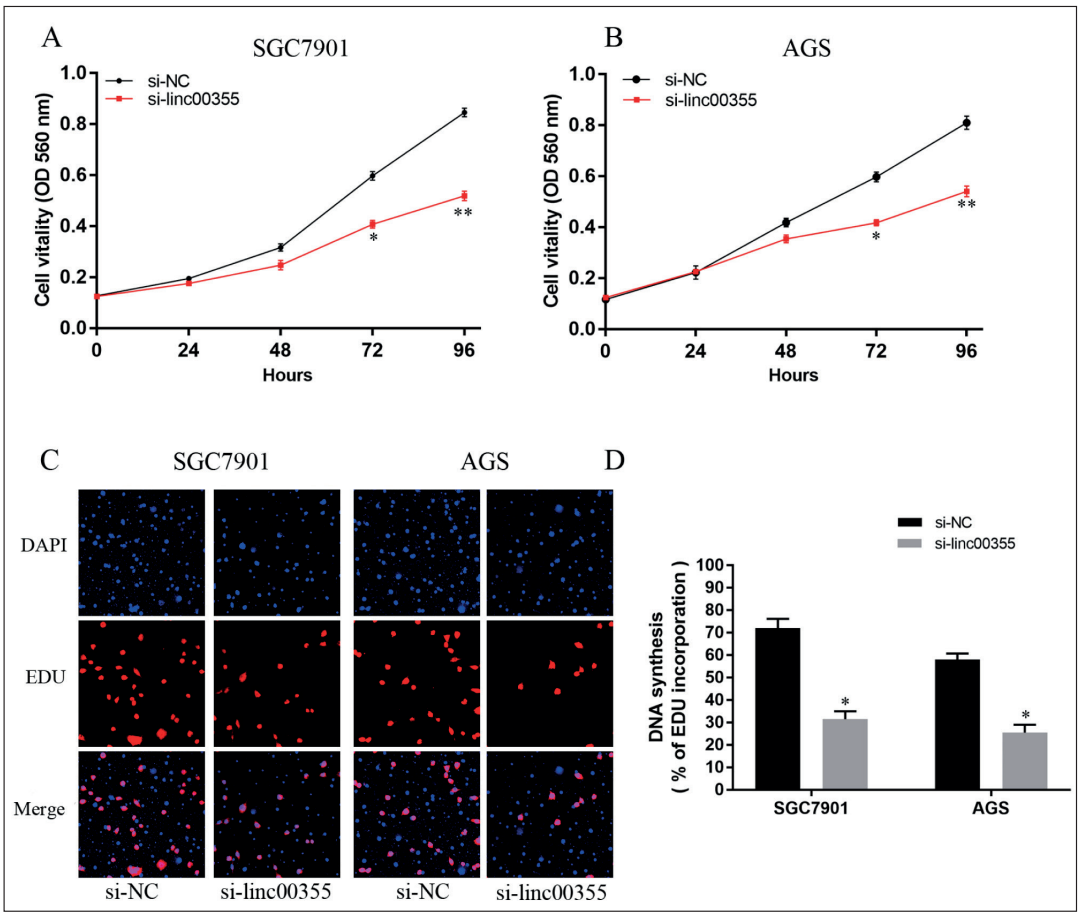

Figure 2. Si-LINC00355 represses the proliferation of GC cells. **A,** and **B,** According to the MTT assay results, the proliferation of GC cells is inhibited after interference with LINC00355 expression. **C,** and **D,** The results of EdU assay manifest that the proliferation rate of GC cells in si-LINC0035 group is lower than that in si-NC group (magnification: 40×).

cording to the EdU assay results, compared with that in si-NC group, the proliferation rate of GC cells declined in si-LINC00355 group (Figure 2C and 2D).

Influences of si-LINC00355 on GC Cell Cycle Distribution and Apoptosis

Subsequently, the effects of the interference with LINC00355 expression on GC cell cycle distribution and apoptosis were explored. After the cells were treated as above, it was discovered through the flow cytometry that compared with those in si-NC group, the GC cells in si-LINC0035 group were arrested in the gap 0 (G0)/G1 phase (Figure 3A and 3B) and had an increased apoptosis rate (Figure 3C and 3D).

LINC00355 Regulated the Wnt/β-Catenin Signaling Pathway to Affect the Biological Behaviors of GC Cells

To explore the potential molecular mechanism by which LINC00355 influences the biological behaviors of GC cells, the changes in the expressions of crucial molecular markers in the downstream signaling pathway were detected *via* Western blotting. According to the results, after interference with LINC00355 expression, the expressions of the biomarkers in the pathway down-regulated (Figure 4).

Discussion

GC remains one of the gastrointestinal tract malignancies with the highest morbidity rate worldwide. In particular, the prognosis of advanced GC is still not satisfactory, posing a huge threat to human health[15]. Tumor recurrence, metastasis and chemotherapy resistance are the major causes of the failure in the treatment of patients, resulting in less favorable comprehensive efficacy[16].

LncRNAs are a class of RNA molecules of over 200 nt in length, without the function of directly encoding proteins, and their major functions are

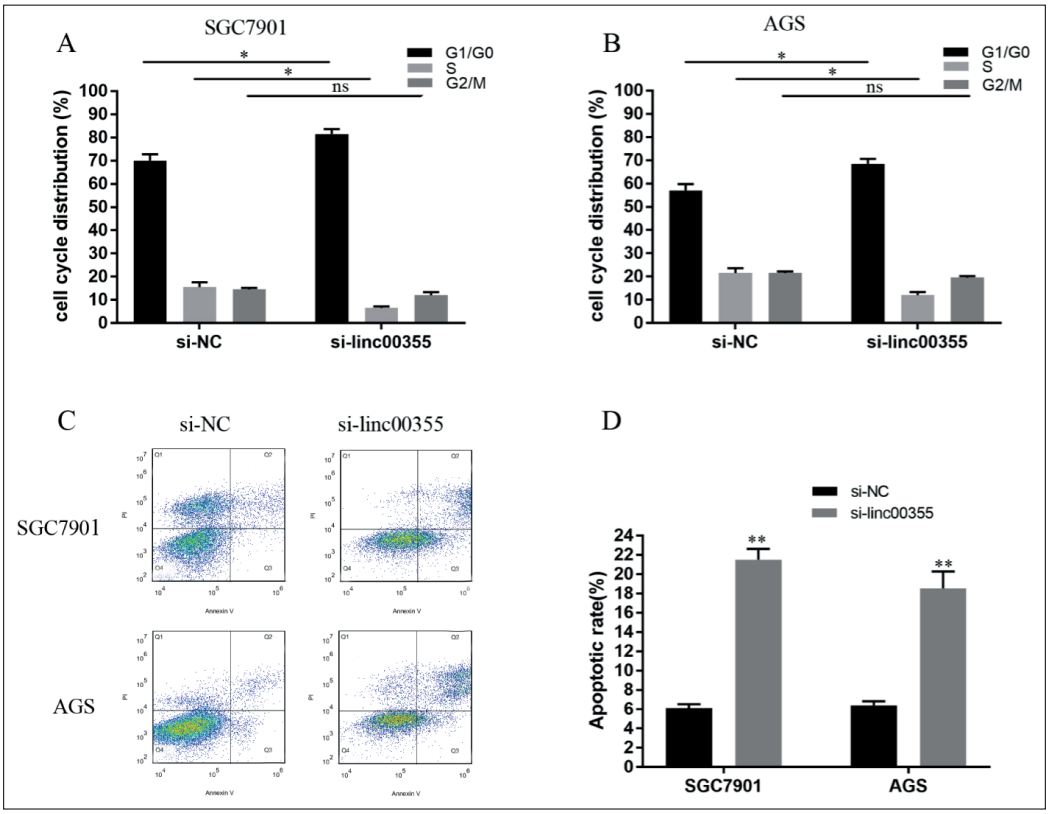

Figure 3. Influences of si-LINC00355 on GC cell cycle distribution and apoptosis. **A,** and **B,** After interference with LINC00355 expression, GC cells are arrested in the G0/G1 phase as indicated by the flow cytometry results. **C,** and **D,** After the transient transfection of si-NC and si-LINC0035 into GC cells for 48 h, it is found through the flow cytometry that the apoptosis rate of cells in si-LINC0035 group is higher than that in si-NC group.

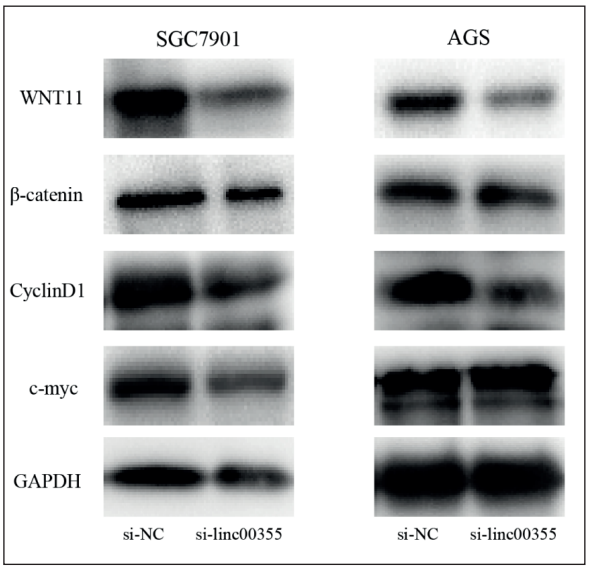

Figure 4. LINC00355 regulates the Wnt/β-catenin signaling pathway to affect the biological behaviors of GC cells. After interference with LINC00355 expression, the Western blotting results of the proteins extracted from experiment group and control group reveal the changes in the expressions of the molecular markers in the Wnt/β-catenin signaling pathway.

to regulate cell proliferation, differentiation and apoptosis through participating in RNA fragment shearing, chromosome modification and protein folding[17]. In recent years, lncRNAs have been found to play pivotal roles in the development and prognosis of tumors. They promote or inhibit the proliferation and metastasis of GC cells[18,19]. Therefore, delving well into the mechanisms of action of lncRNAs in GC is of great clinical significance for the development of novel treatment methods for GC.

According to literature reports, lncRNA LOC554202 modulates the expressions of P21 and E-cadherin to accelerate the proliferation and metastasis of GC cells. Gong et al[20] found that lncRNA UCA1 absorbs miR-203 to regulate the expression of ZEB2, thereby promoting the metastasis of GC cells, whereas Liu et al[21] reported that LINC00662 promotes the proliferation of GC cells by modulating the Hippo-YAP1 pathway. Moreover, it was discovered in the present study that the expression of LINC00335 was upregulated in GC and it promoted the proliferation of GC cells and inhibited their apoptosis.

The Wnt/β-catenin signaling pathway is a highly conservative signal transduction pathway closely associated with multiple diseases of humans and aberrantly expressed in various tumors, such as GC and lung cancer. Activating this pathway can spur the development and progression of tumors, while inhibiting this pathway can reduce the proliferation and metastasis of tumor cells and promote apoptosis[22]. In the current of Wnt signals, complexes are formed through a series of processes and can release β-catenin, and the β-catenin in cell nuclei can also bind to TCF/LEF proteins to activate c-Myc, cyclin D1 and other target genes[23]. Additionally, as important regulators, lncRNAs, such as lncRNA AFAP1-AS1, can be involved in the regulation of the Wnt/β-catenin signaling pathway[24]. In the present study, it was found through *in vitro* experiments that LINC00355 regulated the Wnt/β-catenin signaling pathway to promote the development and progression of GC as well.

Conclusions

In summary, the Wnt/β-catenin signaling pathway is involved in the progression of GC, and LINC0035 is able to stimulate the Wnt/β-catenin signaling to promote the proliferation of GC cells and repress their apoptosis. These findings provide a novel biomarker for the diagnosis and treatment of GC patients and treatment strategies.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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