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LINC01116 promotes the proliferation and inhibits the apoptosis of gastric cancer cells

J. CHEN[1], Z.-H. YUAN[2], X.-H. HOU[1], M.-H. SHI[1], R. JIANG[1]

[1]Department of Endoscopic Diagnosis and Treatment Center, Gansu Provincial Hospital, Lanzhou, Gansu, China
[2]Digestive Department, Gansu Provincial Hospital, Lanzhou, Gansu, China

Abstract. – OBJECTIVE: To detect the relative expression of long intergenic non-protein coding ribonucleic acid (LINC) 01116 in gastric cancer (GC) tissues and cells and analyze the correlations of LINC01116 expression with the clinicopathologic characteristics of patients and investigate the biological functions of LINC01116 *via in vitro* experiments.

PATIENTS AND METHODS: The quantitative Real Time Fluorescence-Polymerase Chain Reaction (qRT-PCR) was applied to detect the relative expression level of LINC01116 in 73 cases of tissues and cells in GC patients. The patients were divided into LINC01116 high expression group and LINC01116 low expression group, and the correlations of LINC01116 with patient's pathological characteristics were statistically analyzed. *In vitro* experiments [cell counting kit-8 (CCK-8) assay, colony formation assay, and flow cytometry] were adopted to investigate the influences of LINC01116 on the biological functions of GC cells.

RESULTS: According to the results of qRT-PCR, the expression of LINC01116 was upregulated in 54 out of 73 cases of tissues (fold change >1), and it was upregulated in GC cells compared with that in the normal gastric mucosal epithelial cells (GES-1). The statistical analysis manifested that the highly expressed LINC01116 was positively correlated with the tumor-node-metastasis (TNM) stage (p=0.008), lymph node metastasis (p=0.005), and depth of invasion (p=0.007) of the GC patients. The patients with high expression of LINC01116 in the GC tissues had a shorter survival time than those with low expression (p=0.017). After interference in the expression of LINC01116, it was shown in CCK-8 assay and colony formation assay that the proliferative capacity of the cells was decreased. The results of flow cytometry indicated that the cell cycle was arrested at the G1/G0 phase, and the apoptosis rate was increased.

CONCLUSIONS: LINC01116 is highly expressed in GC tissues and cells, and highly expressed LINC01116 indicates poor prognosis of the patients, promotes the proliferation, and inhibits the apoptosis of GC cells.

Key Words:
Gastric cancer (GC), LINC01116, Prognosis, Proliferation, Apoptosis.

Introduction

The incidence and development of gastric cancer (GC) is a complex process involving multiple factors and genes[1], whose etiology and pathogenesis have not been completely clarified yet. Radical resection is the only approach that is likely to cure GC at present, but due to the atypical symptoms of the disease in the early stage, most of the patients have been in the intermediate and advanced stage when diagnosed; consequently, they missed the best timing for operation. Besides, even though it can be treated by operation, the patients are highly vulnerable to relapse and metastasis after the operation. Therefore, it is extremely urgent to seek for new therapeutic methods for tumors.

Long non-coding ribonucleic acids (lncRNAs) are a category of RNA molecules with a transcript length of over 200 nt, which do not encode proteins but regulate the gene expressions at the epigenetic, transcriptional, and post-transcriptional levels in the form of RNAs[2]. LncRNAs were initially regarded as the "noise" of genomic transcription, without biological functions. Currently, it has been proven that lncRNAs can participate in such regulation processes as X chromosome silencing, genomic imprinting, and chromatin modification. Some reports[3,4] have revealed that lncRNAs are also implicated in the biological process of tumor cells, including proliferation, migration, and apoptosis.

Long intergenic non-protein coding RNA (LINC) 01666 is located in chromosome 2q31.1, with a total length of 1058 bp. Firstly, Fang et al[5] found that the expression of LINC01666 is upregulated in the epithelial ovarian cancer, and the highly expressed LINC01116 can promote the proliferation and invasion and inhibit the apoptosis of ovary cells. Subsequently, some scholars discovered that LINC01116 is able to control the expression of ESR1 through competitive ad-

Corresponding Author: Rui Jiang, MM; e-mail: jiangr6636@163.com

sorption on micro RNA (miR)-145 in the case of breast cancer, thus accelerating the incidence and development of the disease[6]. However, there is no report about the expression of LINC01116 and its biological functions in GC, so this research group took the lead in studying the expression of LINC01116 in GC tissues and cells. It was revealed that the expression was upregulated, further statistical analysis indicated that the highly expressed LINC01116 suggested poor prognosis of the GC patients, and *in vitro* experiment results manifested that LINC01116 played a role similar to an oncogene in GC cells. The outcomes of this research will provide biological targets for predicting the prognosis of GC patients and the theoretical bases for reversing the formation of the malignant phenotype of GC.

Patients and Methods

Tissue Specimens

A total of 73 cases of surgically resected GC specimens were collected from the Department of General Surgery of Gansu Provincial Hospital from January 2014 to December 2017. The surgical specimens were immediately frozen in liquid nitrogen within 30 min after isolation and then transferred into a refrigerator at -80°C for long-term preservation. The inclusion criteria were: (1) patients with gastric adenocarcinoma confirmed by pathology, (2) those who provided tumor specimens containing normal paracarcinoma tissues (more than 2 cm away from the tumor margin), and (3) those who or whose families approved the acquisition of tumor specimens and signed the informed consent. Exclusion criteria: (1) patients who underwent radiotherapy or chemotherapy before the operation or (2) those complicated with other malignant tumors or gastric metastases. This research was approved by the Ethics Committee of the Gansu Provincial Hospital.

Cell Culture

The GC cell line MGC803, AGS, BSG823, SGC7901 and normal gastric mucosal epithelial cell line GES-l (Shanghai Cell Bank of Chinese Academy of Sciences, Shanghai, China) were seeded into a Roswell Park Memorial Institute-1640 (RPMI-1640) medium or Dulbecco's Modified Eagle's Medium (DMEM; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA), 100 μg/mL penicillin and 100 μg/mL streptomycin, followed by culture in a cell incubator with 5% CO_2 and saturated humidity at 37°C.

Reverse Transcription and Real Time Fluorescence quantitative-Polymerase Chain Reaction (qRT-PCR)

The total RNAs in the carcinoma tissues, paracarcinoma tissues, and human GC cells were extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and then reversely transcribed into complementary deoxyribonucleic acid (cDNA) *via* reverse transcription kit (Invitrogen, Carlsbad, CA, USA). With the cDNA obtained as the template, the qRT-PCR was performed in an ABI 7500 qRT-PCR instrument (Applied Biosystems, Foster City, CA, USA). The relative expression level of LINC01116 was detected through the $2^{-\Delta\Delta Ct}$ method, and the primer and interference sequences included: GAPDH forward, 5'-GGTCATCCCAGAGCTGAACG-3', reverse, 5'-TTGCTGTTG AAGTCGCAGGA-3'. Linc01116 forward: 5'-AAGTGGTACTGTCCCAAGTG CC, reverse: 5'-GCCCTCTCTTTTTTGGATTTCTCC-3 si-linc01116 #1 5'UAUUCCUUCA GCCAGUGGUC3', si-linc01116 #2 5'-GCAGUCUGUUCUGAACAUA-3', si-linc01116 #3 5'-GUCUGCAGCUGAGCCAGUAT-3'. The sequences and primers were designed and synthesized by Invitrogen (Carlsbad, CA, USA).

Cell Counting Kit-8 (CCK-8) Assay

Two groups of cells separately transfected with small interfering (si)-linc01116 and si-negative control (NC) were inoculated into a 96-well plate (3,000 cells/well), with 5 duplicated wells for each group. Later, the cells were cultured in the cell incubator. The Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecural Technologies, Kumamoto, Japan) was conducted at 0, 24, 48, 72, and 96 h, as follows: 10 μL of CCK-8 reagent was added into each well, and after incubation in the cell incubator for 2 h, the absorbance at the wavelength of 450 nm (A_{450}) was measured using a microplate reader.

Colony Formation Assay

The colony formation assay is performed as follows: the cells were inoculated into a 6-well plate (1,000 cells/well), with triplicate wells set for each group, and then cultured in the cell incubator for 10-14 d. The medium was replaced, and the

cell state was observed every 2-3 d. Next, the culture was terminated when the cloned cells grew into a suitable size, and the cells were washed with Phosphate-Buffered Saline (PBS) twice and fixed with cold methanol at 4°C for 15 min, followed by washing in PBS twice and staining with crystal violet solution (1 mL/well) for 20 min.

Detection of Apoptosis via Flow Cytometry

The harvested cells were washed with pre-cooled PBS twice. According to the instructions of the Annexin V-fluorescein isothiocyanate (FITC) apoptosis kit (BD, Franklin Lakes, NJ, USA), the cells were resuspended in 1×binding buffer to 1×10^6 cells/mL first. Then, 100 μL of suspension was added into 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI), and incubated in the dark at room temperature for 15 min. Next, 400 μL of 1× binding buffer was added and mixed, and the mixture was transferred into a flow cytometry tube, followed by detection *via* a flow cytometer.

Cell Cycle Analysis

The transfected cells and control cells in the logarithmic growth phase were harvested through standard digestion with trypsin, then washed with PBS and fixed in 75% ethanol at 4°C overnight. The next day, the cells were incubated with RNase at 37°C for 30 min. After that, the cells were stained with PI for 30 min. The cultures were collected to analyze the cell cycle using the flow cytometer. The data were presented as the percentage of cells at the G0/G1, S, and G2/M phases of the cell cycle.

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 21.0 (IBM Corp., Armonk, NY, USA) software was employed for statistical analysis. The measurement data were presented as ($\bar{x}\pm s$). The paired *t*-test was used for comparison of LINC01116 expression level between the tumor group and non-tumor group, and the One-way analysis of variance or Mann-Whitney test was adopted for comparison among multiple groups. χ^2-test or Fisher's exact test was utilized for comparison of the enumeration data, and the survival curves were plotted using the Kaplan-Meier method, while the prognostic factors were subjected to univariate or multivariate Cox model analysis. $p<0.05$ suggested that the difference was statistically significant.

Results

Upregulated LINC01116 Expression in GC Tissues

First, the relative expression level of LINC01116 in the tissues of 73 GC patients was detected by means of qRT-PCR. Compared with that in paracarcinoma tissues, the expression of LINC01116 was upregulated in 54 cases of GC tissues (Figure 1A). Next, qRT-PCR was conducted again to measure the relative expression level of LINC01116 in GC cells, and it was shown that LINC01116 expression was upregulated in GC cells in comparison with that in GES-1 (Figure 1B). With the mean fold change of LINC01116 expression as the cutoff point, 73 patients were divided into LINC01116 high expression group (n=32, fold change >3.9) and LINC01116 low expression group (n=41, fold change <3.9) (Figure 1C). The analysis by the Chi-square test revealed that the expression level of LINC01116 was positively correlated with the tumor-node-metastasis (TNM) stage (p=0.008), lymph node metastasis (p=0.005), and depth of invasion (p=0.007) of the GC patients, but had no correlations with the patients' age, gender, tumor location, and other variables (Table I).

Analysis on Correlation Between LINC01116 Expression and Patient's Survival

According to the Kaplan-Meier survival analysis, the patients with high expression of LINC01116 in GC tissues had a shorter survival time than those with low expression (Figure 1D). The Cox's proportional hazards regression model was adopted for univariate analysis of the survival data of the 73 patients. The results manifested that the highly expressed LINC01116 ($p<0.05$) and TNM stage ($p<0.01$) exhibited a statistical significance. Later, the multivariate Cox model analysis indicated that the TNM stage and linc01116 expression could serve as the independent prognostic factors for GC (Table II).

Impact of LINC01116 on GC Cell Proliferation

The impacts of LINC01116 on the biological functions of GC cells were investigated *via in vitro* experiments. Firstly, the LINC01116-specific interference sequences were designed and synthesized. 48 h later, the RNAs in the experimental group and control group were collected and reversely transcribed into cDNAs, and the inter-

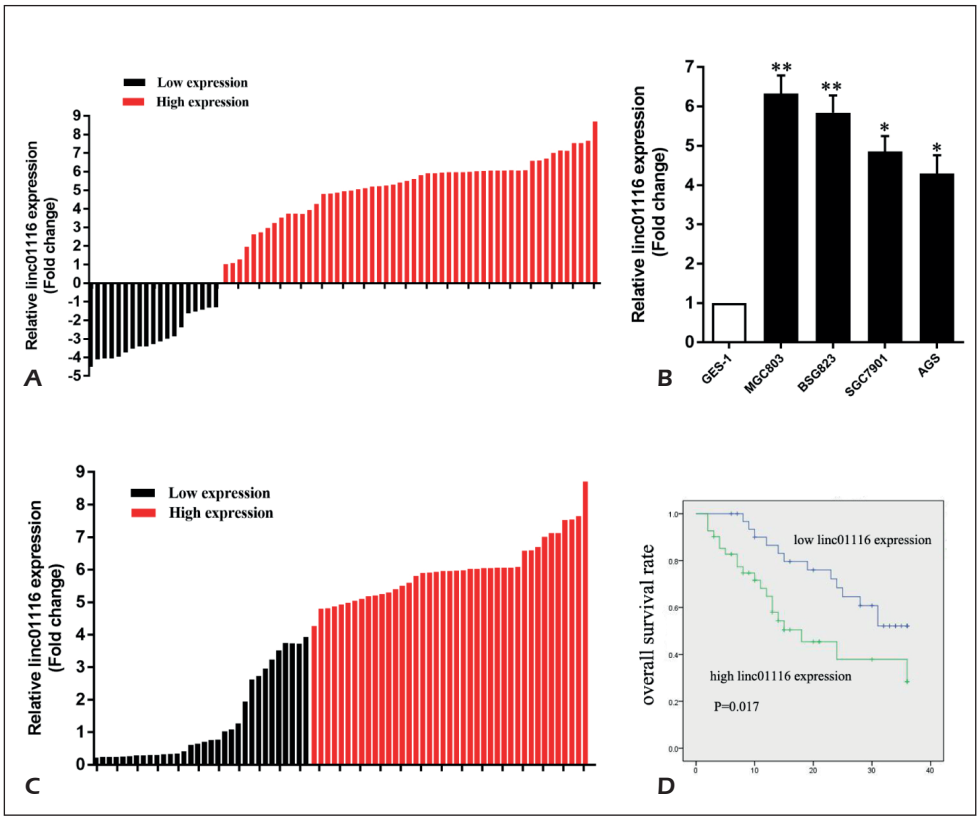

Figure 1. Upregulated LINC01116 expression in GC suggests poor prognosis. **A**, Relative expression level of LINC01116 in 73 cases of GC tissues detected via qRT-PCR, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference. **B**, Relative expression level of LINC01116 in GC cells detected via qRT-PCR, with GAPDH as the internal reference. **C**, 73 patients are divided into LINC01116 high expression group and LINC01116 low expression group with the mean fold change of LINC01116 expression in tissues as the cutoff point. **D**, Correlation between expression level of LINC01116 and survival time of GC patients investigated via Kaplan-Meier survival analysis. (**p<0.01, *p<0.05).

ference efficiency was determined *via* qRT-PCR (Figures 2A and 2B). Secondly, the CCK-8 assay was adopted to explore the impact of LINC01116 on cell proliferation, and it was shown that the proliferative capacity of the cells declined after the interference in LINC01116 expression (Figures 2C and 2D). The same results as those in CCK-8 assay were obtained in the colony formation assay (Figures 2E and 2F).

Impacts of LINC01116 on GC Cell Cycle and Apoptosis

The si-LINC01116 and si-NC were transiently transfected into GC cells, which were harvested 48 h later, and the distribution of the GC cell cycle was determined through flow cytometry. Compared with that in the control group, the cell cycle was arrested at the G1/G0 phase in the experimental group (Figures 3A and 3B). Then, the cells were treated using the same methods for 48 h, and the apoptosis rate was measured by means of flow cytometry. It was indicated that the apoptosis rate was raised in the si-LINC01116 group (Figures 3C and 3D).

Discussion

It was estimated[7] that there would be about 1,033,700 new cases and about 782,700 deaths of GC in 2018 according to the Global Cancer Statistics, and the incidence and the mortality rates of the disease rank the 5th and 3rd, respectively, among those of all the malignant tumors. China has a high incidence of GC, with approximately 410,000 new cases every year, accounting for nearly 40% around the world[8]. Due to the insidious onset and atypical clinical manifestations of GC in most cases, the majority of the GC patients have been in the intermediate and advanced stage when diagnosed, missing the best timing for radical resection, so the clinical treatment effect is far from satisfactory[9].

Table I. Correlation between linc01116 expression and clinicopathological characteristics of gastric cancer patients (n = 73).

Characteristics	Linc01116 Low No. Case (%)	Linc01116 High No. Case (%)	*p* Chi-squared test p-value
Age (years)			
>60	12	23	0.157
≤60	20	18	
Sex			
Male	15	27	0.152
Female	17	14	
Tumour size (cm)			
>4	18	16	0.163
≤4	14	25	
Differentiation			
Well	2	3	0.512
moderate	13	8	
Poor	11	25	
undiffer	6	5	
TNM staging			
I+II	19	11	0.008*
III+IV	13	30	
Lymph lode metastasis			
No	22	10	0.005*
Yes	10	27	
Tumor location			
Gastric antrum	15	15	0.756
Corpora ventriculi	10	19	
Preventriculus	7	7	
Invasion degree			
T1	11	6	0.007*
T2	11	8	
T3	5	14	
T4	5	13	

*Overall $p<0.05$.

LncRNAs refer to a kind of non-coding RNA molecules that do not encode proteins but have extensive regulatory effects on the gene expression, which can be implicated in the regulation of multiple vital activities at the epigenetic, transcriptional, and post-transcriptional levels[10]. Studies[11-13] over the past few years have discovered that lncRNAs are abnormally expressed in

Table II. Univariate and multivariate analysis of over-survival in gastric cancer patients (n=73).

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Sex	0.852	0.427-1.700	0.650			
Age	0.672	0.338-1.339	0.259			
Tumor size (cm)	1.435	0.718-2.869	0.306			
Differentiation	0.968	0.650-1.444	0.875			
Invasion degree	0.955	0.706-1.292	0.767			
Lymph lode metastasis	0.988	0.497-1.963	0.972			
Tumor location	0.828	0.525-1.303	0.414			
TNM staging	3.238	1.483-7.070	0.003*	2.946	1.331-6.519	0.008*
Linc01116 expression	2.387	1.138-4.802	0.021*	2.201	0.966-4.299	0.042*

HR, hazard ratio; 95% CI, 95% confidence interval, * Overall $p<0.05$.

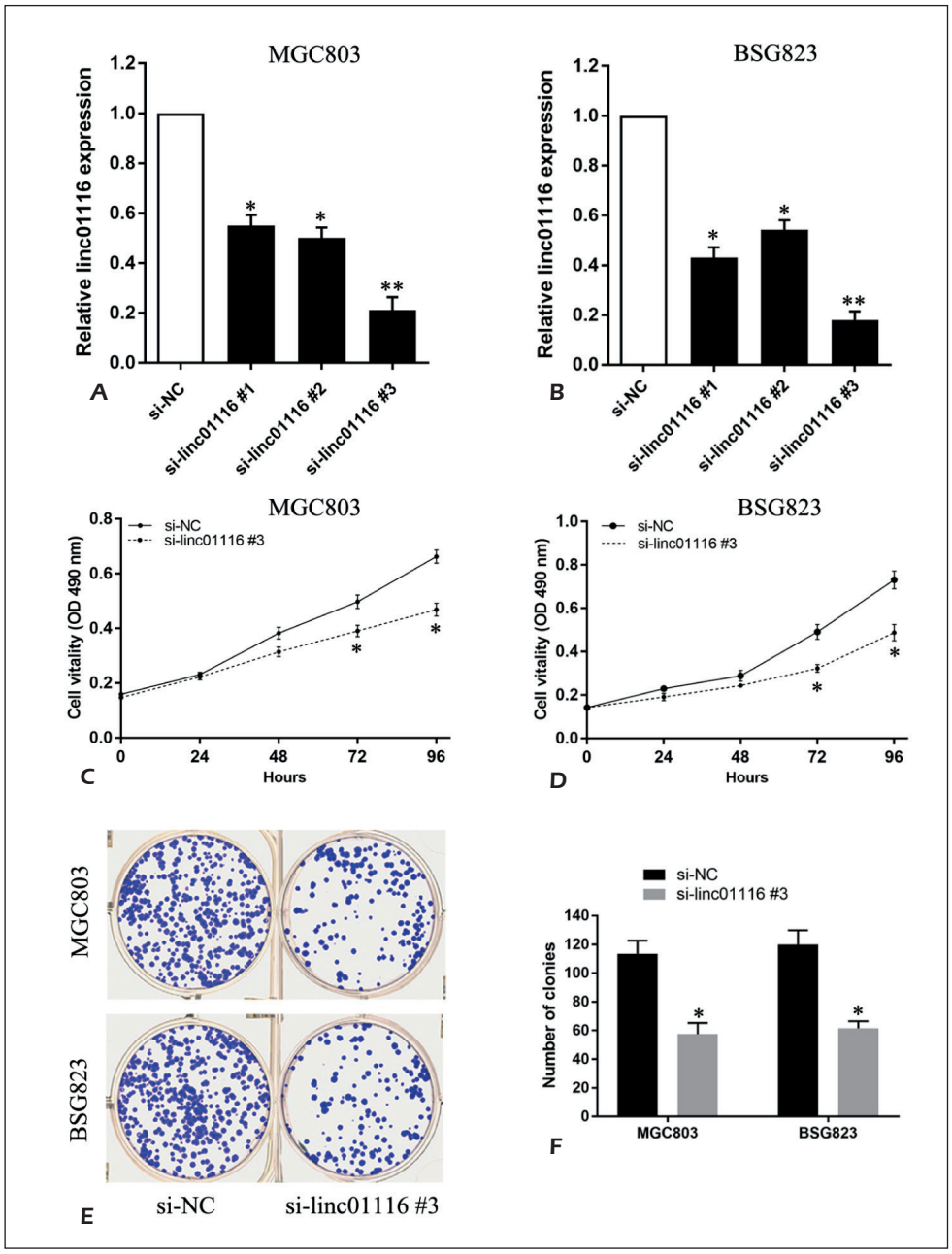

Figure 2. Impact of LINC01116 on GC cell proliferation. **A-B**, LINC01116 expression in MGC803 and BSG823 cells was determined via qRT-PCR. **C-D**, Impact of LINC01116 on GC cell proliferation detected via CCK-8 assay. It is shown that the proliferative capacity of the cells declines after the interference in LINC01116 expression. **E-F**, The results of colony formation assay indicate that the proliferative capacity of the cells is weakened in si-LINC01116 group compared with that in si-NC group (magnification: 40×). (**p<0.01, *p<0.05).

various tumor tissues and play crucial roles in promoting and maintaining the progression of tumors, becoming the hotspots of research on tumors. It is generally believed that lncRNAs have 4 modes of actions, namely signal transduction, molecule blocking, guiding role, and central platform, and they can participate in the epigenetic and transcriptional or post-transcriptional regulation of the genes through the interaction among the 4 modes[14,15].

According to literature, the expression of lncRNA Sox2ot is upregulated in the GC tissues, which can be regarded as a biomarker for poor prognosis of GC patients[16]. LncRNA SNHG5 is

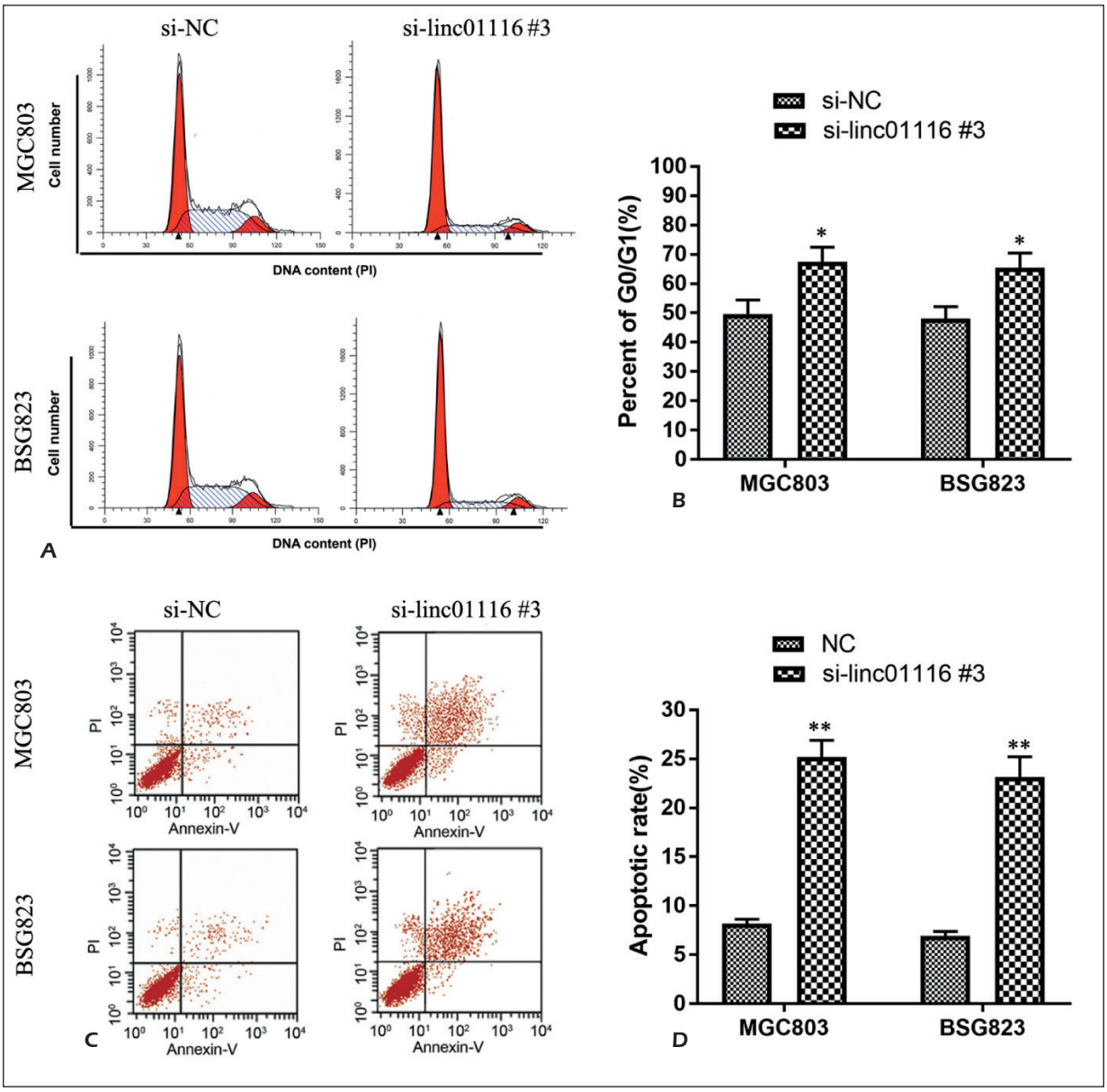

Figure 3. Impacts of LINC01116 on GC cell cycle and apoptosis. **A-B**, The results of flow cytometry manifest that the cell cycle is arrested at the G1/G0 phase after the interference in LINC01116 expression. **C-D**, The results of flow cytometry manifest that the apoptosis rate is increased after the interference in LINC01116 expression. (**p<0.01, *p<0.05).

capable of repressing the occurrence and development of GC through targeted regulation on MTA2 expression[17]. Yan et al[18] found that lncRNA HOTAIR can control the PI3K/AKT pathway to facilitate the cisplatin resistance of GC by adsorption on miR-126. It was found for the first time by this research group that the expression of LINC01116 was upregulated in GC tissues and cells and positively associated with the clinical stage and other factors of the patients. Besides, the highly expressed LINC01116 implied poor prognosis of the patients. Furthermore, the results of *in vitro* experiments manifested that the interference in LINC01116 expression could suppress the proliferation and apoptosis of GC cells. It was reported in the literature that LINC01116 can targetedly regulate the expression of IL-6R through miR-520a-3p in osteosarcoma, thus promoting cell proliferation and invasion[19]. In addition, in the case of glioma, LINC01116 is able to accelerate the occurrence and development of tumors *via* targeted regulation on VEGFA[20]. In subsequent

studies of this research, the molecular mechanisms of upregulated LINC01116 expression in promoting the proliferation and inhibiting the apoptosis of the GC cells will be deeply explored, thereby providing a theoretical basis for the targeted treatment of GC in the clinic.

Conclusions

In this study we demonstrated that LINC01116 is highly expressed in GC tissues and cells, and highly expressed LINC01116 indicates poor prognosis of the patients, promotes the proliferation, and inhibits the apoptosis of GC cells.

Conflict of Interests

The authors declared that they have no conflict of interests.

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