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SP1-activated LINC01638 promotes proliferation and migration of gastric cancer cells by regulating epithelial-mesenchymal transition

X.-J. WU[1], Y.-H. YANG[2], K.-X. WANG[3]

[1]Department of Pathology, Weihaiwei People's Hospital, Weihai, China
[2]Department of Neurology, People's Hospital of Jinan, Jinan, China
[3]Department of Alimentary Tract, Yantai Yida Hospital, Yantai, China

Abstract. – OBJECTIVE: The aim of this study was to detect the expression of long intergenic non-protein coding ribonucleic acid 01638 (LINC01638) in gastric cancer (GC), and to explore its role and molecular mechanism in regulating the proliferation and metastasis of GC cells.

PATIENTS AND METHODS: The relative expression level of LINC01638 in 50 cases of GC tissues and paracancerous tissues and GC cells was determined using quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). After interference with the expression of LINC01638, the interference efficiency was detected via qRT-PCR, and the changes in GC cell proliferation ability, cell cycle distribution, and migration and invasion abilities were examined using colony formation assay, flow cytometry, and transwell assay, respectively. The possible transcription factors binding to the promoter region of LINC01638 were predicted using bioinformatics methods. After interference with specificity protein 1 (SP1), qRT-PCR was performed to detect the interference efficiency and the change in LINC01638 expression. Besides, the changes in the molecular markers for epithelial-mesenchymal transition (EMT) were detected using Western blotting assay after interfering with LINC01638 expression and using Western blotting after interfering with SP1.

RESULTS: The expression of LINC01638 was upregulated in 40 cases of GC tissues, and its expression level in GC cells was higher than that in normal gastric mucosal cells. After interfering with the expression of LINC01638, the colony formation assay results showed that the proliferation of GC cells was suppressed, it was found through flow cytometry that the cell cycle was arrested in G1/G0 phase, and transwell assay results manifested that the cell migration and invasion abilities declined. According to the bioinformatics and qRT-PCR results, the transcription factor SP1 contributed to the expression of LINC01638, and the expressions of molecular markers in the EMT signaling pathway were changed after interfering with LINC0168 and SP1.

CONCLUSIONS: In this study, it was verified through the *in vitro* experiments that LINC01638 promotes the migration and invasion of GC by regulating EMT.

Key Words:
LINC01638, Gastric cancer, Proliferation, Migration, EMT.

Introduction

Gastric cancer (GC) originates from gastric mucosal epithelial cells, and its morbidity rate ranks 5th worldwide and 2nd in China[1]. The development and progression of GC is a multi-step and multi-factorial pathological process involving the synergy of gene and environmental factors[2]. In recent years, as molecular biology, especially gene cloning technique has progressed rapidly, a study found that various molecules and biological events are involved in the development of GC and that the identification of potential biomarkers will help treat GC[3].

Long non-coding ribonucleic acids (lncRNAs) have always been regarded as "gene noises", and it has been denied that lncRNAs can exert biological functions in gene coding[4]. However, they affect the synthesis of deoxyribonucleic acids (DNAs) and proteins and the reverse transcription (RT) of RNAs to modulate chromosome remodeling, mRNA degradation, and other biological processes, thereby regulating gene expression[5,6].

LncRNAs are closely associated with the development and progression of GC. Long intergenic non-protein coding ribonucleic acid 00978

Corresponding Author: Kaixu Wang, BM; e-mail: 574244171@qq.com

(LINC00978) and CASCL5 induce cell cycle arrest, inhibit cell apoptosis, and promote cell proliferation to affect the progression of GC[7,8]. LncRNA DLX6-AS1 stimulates the invasion and metastasis of GC through the regulation of the MAP4K1 signaling pathway by FUS[9]. LINC01410 functions as endogenous competitive RNAs to inhibit miR-532-5p expression, thus persistently activating the downstream nuclear factor-κB (NF-κB) pathway and promoting the metastasis of GC[10]. Moreover, lncRNAs can influence cell autophagy, metabolism stress, and hypoxia to accelerate GC progression[11]. However, the expression and biological function of LINC01638 in GC have not yet been reported. Through the *in vitro* experiments, the present research group first found that LINC01638 was upregulated in GC tissues and cells and promoted the proliferation and metastasis of GC cells.

Patients and Methods

Tissue Specimens

A total of 50 GC patients diagnosed and receiving surgical treatment in Weihaiwei People's Hospital from September 2016 to July 2018 were enrolled. After specimen resection, the cancer tissues and paracancerous tissues (the normal gastric mucosal tissues more than 5 cm away from the tumor tissues) were harvested, cut into small pieces, transported using a liquid nitrogen tank, and preserved in a refrigerator at -80°C for later use. The present study was approved by the patients themselves and the Ethics Committee of Weihaiwei People's Hospital.

Cell Culture

Human GC cell line (BGC823, MKN28, MKN45, AGS, SGC7901) and normal mucosal cell line (GES1; Cell Bank, Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China) were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) or Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). Then, the medium was placed in an incubator with 5% CO_2 and 95% humidity at 37°C, and the cells grew as a monolayer and passaged when the confluence reached 90%.

RNA Extraction and RT

Total RNAs were isolated from GC tissues and the control paracancerous normal tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, the concentration and purity of the RNAs were determined using a NanoDrop 2000 spectrophotometer. Subsequently, RT was conducted under the conditions set based on the instructions of the complementary deoxyribose nucleic acid (cDNA) synthesis kit (TaKaRa, Otsu, Shiga, Japan).

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

QRT-PCR was performed through the steps as specified in the kit (TaKaRa, Otsu, Shiga, Japan) under the following conditions: pre-denaturation at 95°C for 10 min, denaturation at 95°C for 15 s, and annealing at 60°C for 30 s for 40 cycles. Then, the dissolution curve was plotted, and the cycle threshold (Ct) of each sample was automatically analyzed using computer system. Finally, the relative expression level of lncRNAs was calculated using $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) F: 5'-AGCGAGCATCC CCCAAAGTT-3', R: 5'-GGGCACGAAGGCTCATCATT-3', Linc01638 F: 5'-CTGTCACCCATGA ACCTG CT-3', R: 5'-TACAGGGAGGTAGGCCAGTC-3', SP1 F: 5'-AGCACTACGCAGTCAG TCG-3', R: 5'-AACCGCAGCCTCGCTCAA-3'.

Cell Transfection

The cells were transfected using Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's specifications. The small interfering (si)-LINC01638 and si-negative control (si-NC) were purchased from Transheep (Shanghai, China).

Colony Formation Assay

Each group of cells was separately seeded into 6-well plates at 1×10^3 cells/well and cultured for about 15 d until the colonies were macroscopically visible. The resulting cells were washed with 40 g/L phosphate-buffered saline (PBS) for several times, fixed in 10 g/L paraformaldehyde for 30 min, stained with crystal violet dye for 10 min, dried in the air, and photographed, with triplicate wells set in each group.

Detection of Cell Cycle Using Flow Cytometry

The transfected cells and controls in logarithmic growth phase were harvested, washed using PBS and fixed in 75% ethanol at 4°C overnight. On the next day, the cells were incubated with RNases at 37°C for 30 min, and then stained with

propidium iodide (PI) for 30 min. The resulting products were collected, and the cell cycle was analyzed using a flow cytometer (FACSCalibur; BD Biosciences, Detroit, MI, USA). The data were presented as the percentage of cells in G0/G1, S and G2/M phases.

Transwell Assay

The cells treated through the designated experiment were first prepared into suspension at 1×10^6 cells/mL using serum-free medium, and 100 μL of the suspension was inoculated into the upper compartment of transwell with Matrigel paved onto the upper compartment, rather than the lower compartment (BD, Franklin Lakes, NJ, USA). Then, the lower compartment was added with 600 μL of medium containing 10% serum. After cell penetrating for 24 h, the transwell was taken out, and the cells in the upper compartment was wiped off, while those in the lower compartment were stained with 0.1% crystal violet, photographed under an upright microscope (Olympus, Tokyo, Japan), and counted in 5 fields randomly selected.

Western Blotting

After being treated through the designated experiment, the cells were collected and added with RIPA lysis buffer to extract total proteins therein. After protein quantification, 20 **μg** of proteins were taken from each sample, denatured at high temperature, and separated *via* sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, the separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), and the PVDF membrane was blocked using 5% skim powder, incubated with the primary antibody (1:2,000) overnight. On the next day, the membrane was washed using Tris-Buffered Saline with Tween-20 (TBST), incubated with the secondary antibody for 1 h, and washed again with TBST. Finally, chemiluminescent imaging was conducted.

Statistical Analysis

GraphPad Prism 7.0 software (La Jolla, CA, USA) was used to analyze data. Following the analysis of variance, One-way analysis of variance or non-unpaired *t*-test were performed. Data were expressed as mean ± standard deviation ($\bar{x} \pm s$). $p<0.05$ suggested statistically significant differences.

Results

The Expression of LINC01638 was Upregulated in GC

First, the expression level of LINC01638 in 50 cases of GC tissues was measured using qRT-PCR, and the results showed that compared with that in paracancerous tissues, the expression of LINC01638 was upregulated in 40 cases of GC tissues (Figure 1A). Then, the expression level of LINC01638 in GC cells was determined using qRT-PCR, and it was found that its expression was upregulated (Figure 1B). In this study, to investigate the biological function of LINC01638, LINC01638 siRNA sequences were designed, synthesized, and transiently transfected into GC cells, and 48 h later, the interference efficiency was detected using qRT-PCR. The results revealed the downregulation of LINC01638 expression (Figure 1C and 1D).

Biological Function of LINC01638 in GC

According to the colony formation assay results, the proliferation ability of GC cells was weakened after interference with LINC01638 expression (Figure 2A and 2B). Through the flow cytometry, it was found that the cells in si-LINC01638 group were distributed in G1/G0 phase in comparison with those in the si-NC group (Figure 2C and 2D). Besides, the transwell assay results manifested that after interfering with LINC01638 expression, the migration and invasion abilities of cells declined (Figure 2E and 2F).

Specificity Protein 1 (SP1) Promoted LINC01638 Transcription

The transcription factors, to which LINC01638 promoter region binds, were predicted *via* bioinformatics (http://jaspar.genereg.net/), and the efficiency of interference with SP1 was determined *via* qRT-PCR (Figure 3A) and Western blotting (Figure 3B). Moreover, the expression level of LINC01638 was measured after interfering with SP1 expression (Figure 3C).

SP1/LINC01638 Modulated Epithelial-Mesenchymal Transition (EMT)

After knockdown of LINC01638 expression in GC cells, the changes in the expressions of EMT molecular markers (E-cadherin, N-cadherin and vimentin) were evaluated using Western blotting, and it was discovered that after interfering with LINC01638 expression, the expression of E-cadherin was upregulated, while the expressions of

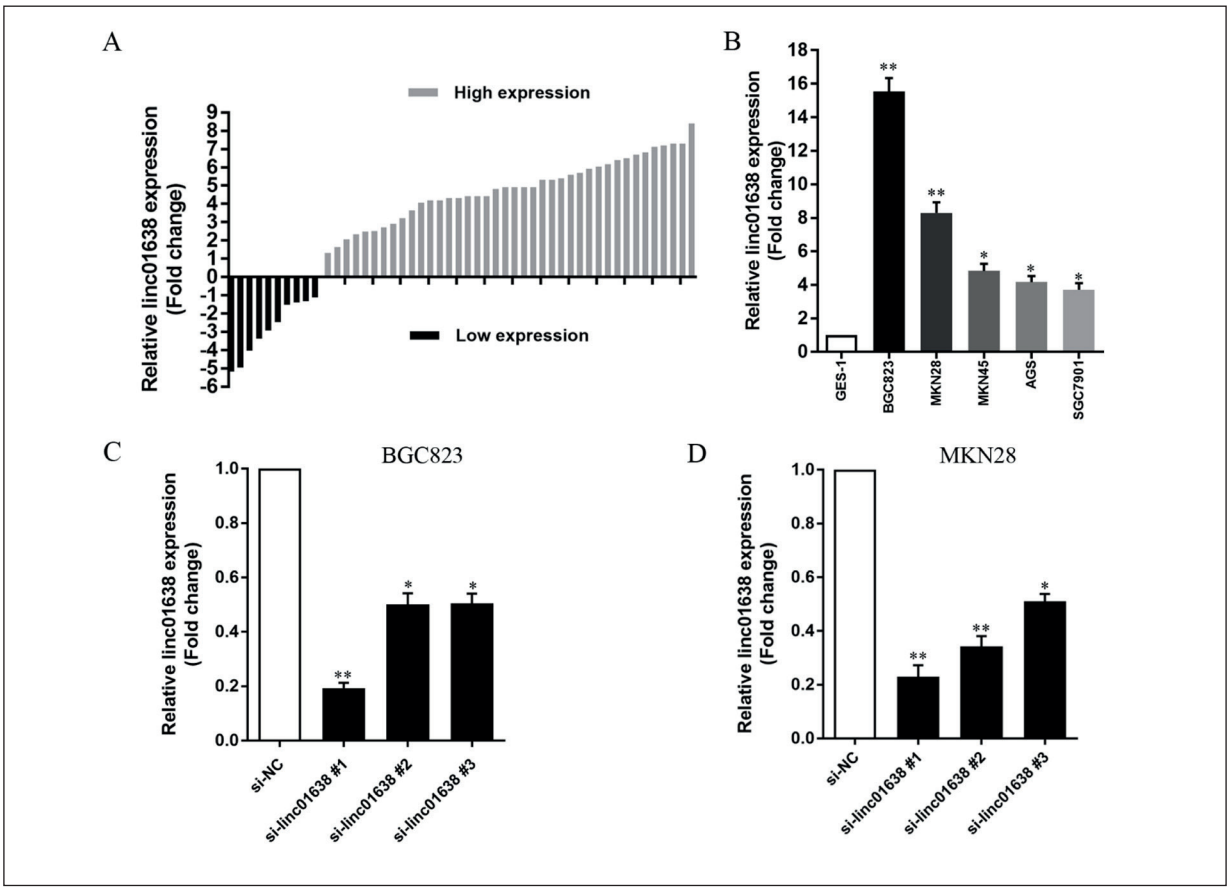

Figure 1. The expression of LINC01638 is upregulated in GC. **A,** According to the qRT-PCR results, 40 out of 50 cases of GC tissues exhibit the up-regulation of LINC01638 expression, with GAPDH as an internal reference. **B,** Based on the qRT-PCR results of RNAs extracted from GC cells, the expression level of LINC01638 is higher than that in normal gastric mucosal cells. **C,** and **D,** Interference efficiency determined *via* qRT-PCR.

N-cadherin and vimentin were downregulated (Figure 4A). Besides, the Western blotting results indicated the changes in the expressions of the EMT molecular markers after interfering with SP1 expression (Figure 4B).

Discussion

GC remains a common malignancy worldwide, especially in China[12]. Although there are many measures to treat GC, its mortality rate is still stubbornly high. Therefore, novel biomarkers and treatment methods are urgently needed to improve the therapeutic effect[13].

As non-coding genes extensively present in human bodies, lncRNAs regulate gene expression at the transcriptional, post-transcriptional, and epigenetic levels and in other biological processes, thereby participating in the development and progression of GC[14]. LINC01638, located on chromosome 22q12.1, has been reported to be upregulated and play a role as an oncogene in multiple tumors[15-17], such as colorectal cancer, prostate cancer, and hepatocellular carcinoma. Liu et al[15] found that LINC01638 promotes the development and progression of HER2-positive breast cancer, whereas LINC01638 was discovered by Luo et al[16] to stop the SPOP-mediated c-Myc degradation and activate the MTDH-Twist1 signaling pathway, thereby accelerating the progression of triple-negative breast cancer[18]. The present research group found through *in vitro* experiments that the expression of LINC01638 was raised in GC tissues and cells.

Transcription factor SP1 belongs to SP1/Kruppel transcription factor family, which has more than 65% highly conserved regions and can bind to DNA[19]. SP1 is upregulated in gastric cancer and other multiple tumors, and its expression level is

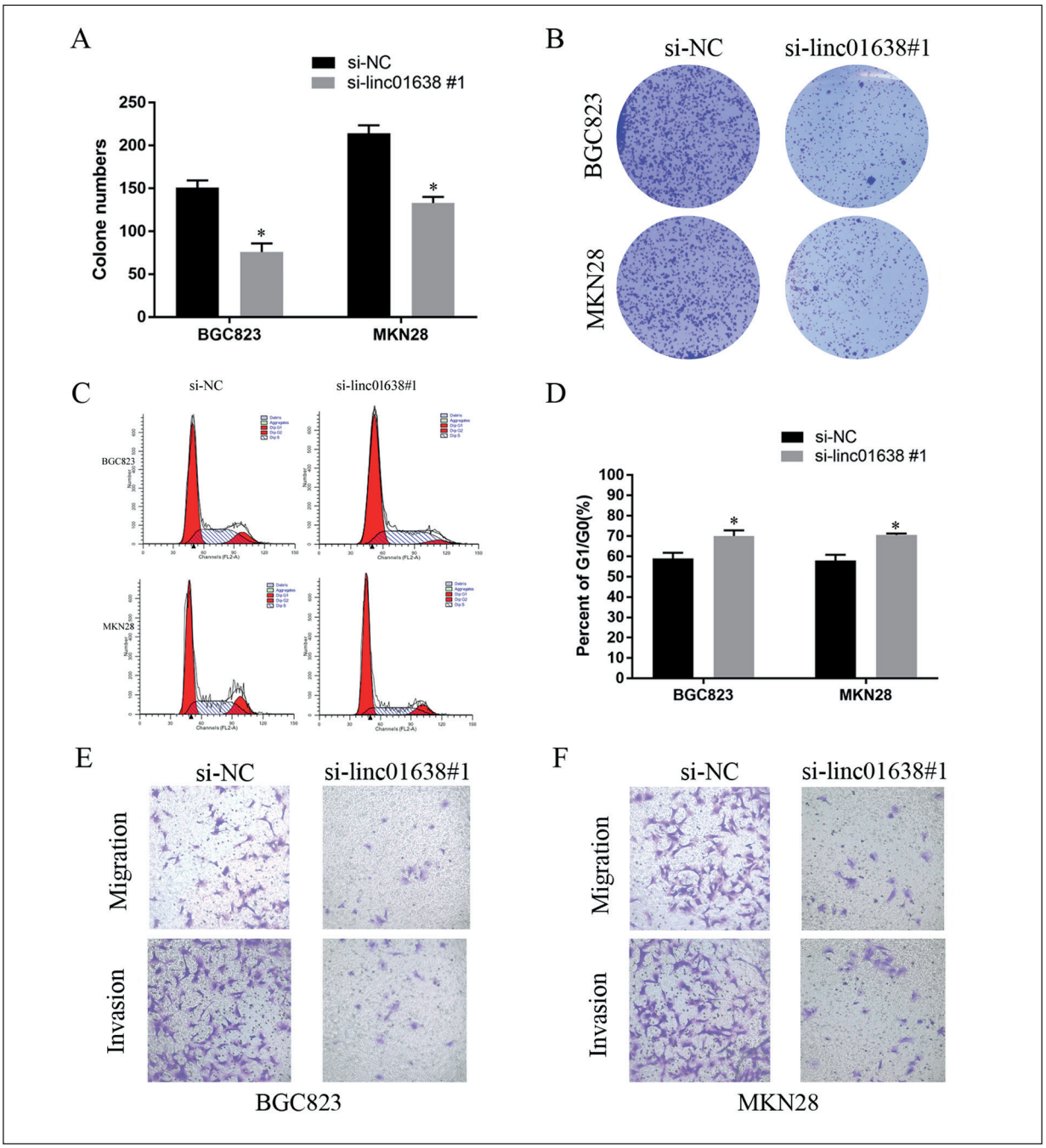

Figure 2. Biological function of LINC01638 in GC. **A,** and **B,** Based on the colony formation assay results, the proliferation ability of cells declines after interference with LINC01638 expression in GC cells (magnification: 40×). **C,** and **D,** Compared with that in si-NC group, the cell cycle in si-LINC-10638 group is arrested in G1/G0 phase according to the flow cytometry results. **E,** and **F,** The transwell results manifest that after the interference with LINC01638 expression, the migration and invasion abilities of cells are weakened (magnification: 40×).

positively correlated with tumor grade and prognosis[20,21]. SP1 can promote the proliferation of gastric cancer cells by regulating lncRNA UCA1[22]. In this study, the upregulated expression of linc01638 was modulated by the transcription factor SP1 and promoted the proliferation, invasion, and metastasis of GC cells. However, limitation also existed in the present study. The follow-up period of the patients included was too short to analyze the relationship between the survival rate and the expression of

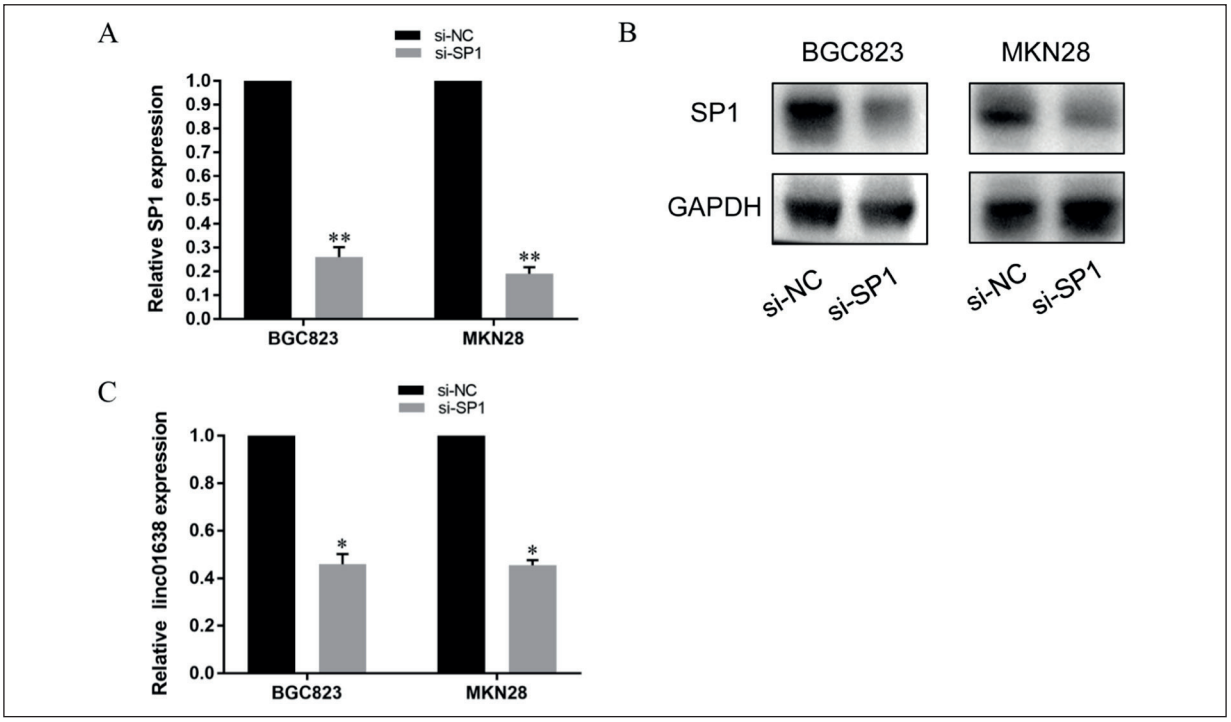

Figure 3. SP1 promotes the transcription of LINC01638. **A,** Interference efficiency of si-SP1 measured *via* qRT-PCR. **B,** Interference efficiency of si-SP1 determined using Western blotting. **C,** Changes in LINC01638 expression determined *via* qRT-PCR after interfering with SP1 expression.

linc01638. In our future research, we plan to conduct the analysis based on data of 5 years follow-up and also expand the clinical sample. Given this, we can only report the biological function and potential mechanism of linc01638 in gastric cancer.

EMT is not only able to be involved in the generation of tissues and organs in normal embryo development but can also accelerate the migration and invasion of tumor cells by altering the mesenchymal phenotypes of tumor cells[23].

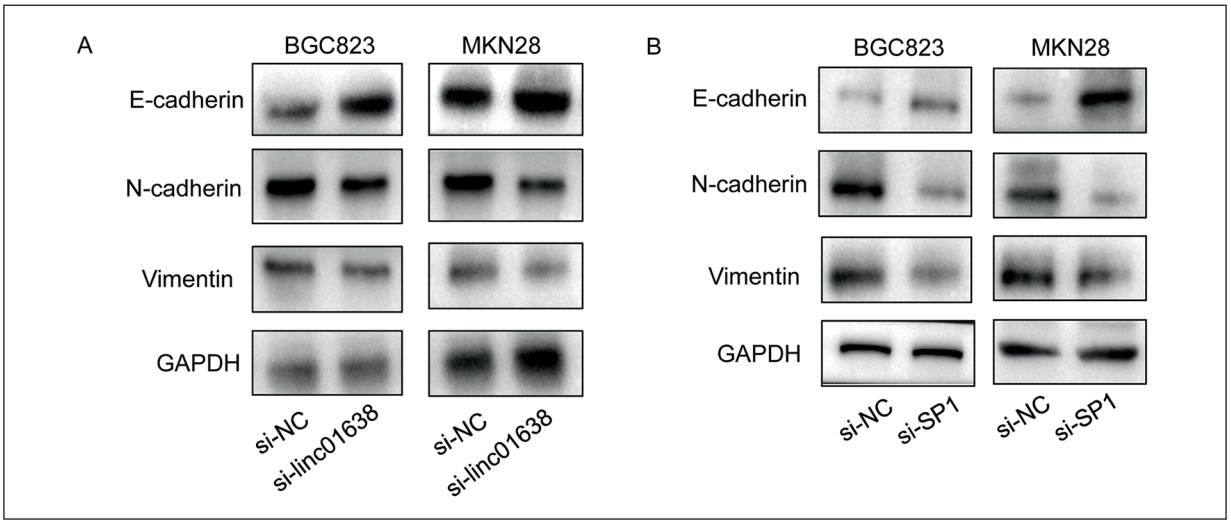

Figure 4. SP1/LINC01638 regulates EMT. **A,** Changes in the expressions of EMT molecular markers after interfering with LINC01638 expression evaluated *via* Western blotting. **B,** Changes in the expressions of EMT molecular markers after interference with SP1 expression evaluated *via* Western blotting.

E-cadherin, N-cadherin, and vimentin are considered as the regulators for tumor metastasis, and the dysregulation of their expressions is one marker for EMT. Some lncRNAs target E-cadherin or vimentin to regulate the EMT process. According to the findings of Xu et al[24], HOTAIR increases the expression level of Snail to promote the expressions of mesenchymal markers, such as vimentin and N-cadherin, and inhibit those of epithelial markers, like E-cadherin and ZO-1, thereby accelerating EMT. Other lncRNAs, such as HULC, LINC00152, and MALAT2, which are upregulated in hepatocellular carcinoma, promote the EMT process in GC as well[25,26].

Conclusions

In this study, it was verified that *in vitro* LINC01638 promotes the migration and invasion of gastric cancer by regulating EMT.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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