

Long noncoding RNA Lnc01614 promotes the occurrence and development of gastric cancer by activating EMT pathway

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Abstract. – OBJECTIVE: To investigate the expression characteristics of long noncoding ribonucleic acid (lncRNA) lnc01614 in gastric cancer, so as to further investigate its roles in the occurrence and development of gastric cancer and its potential regulatory mechanism.

PATIENTS AND METHODS: Quantitative Real-time polymerase chain reaction (qRT-PCR) was used to detect the expression levels of lnc01614 in 79 pairs of gastric cancer tissues and normal adjacent tissues. The correlations of lnc01614 expression with pathological parameters of gastric cancer and prognoses of patients were also analyzed. QRT-PCR was employed to further verify the expression of lnc01614 in gastric cancer cells. Lnc01614 knockdown expression models were established using small interfering RNA (siRNA) in gastric cancer cell strains (SGC-7901 and AGS). The effect of lnc01614 on the biological function of gastric cancer cells was analyzed by Cell Counting Kit-8 (CCK-8) and transwell invasion and migration assay. Lastly, Western blotting was performed to explore its potential mechanism.

RESULTS: The results of qRT-PCR showed that the expression of lnc01614 in gastric cancer tissues was significantly higher than that in normal tissues. Compared with patients with low expression of lnc01614, patients with high expression of lnc01614 had higher tumor staging, greater lymph node metastasis and distant metastasis rates, and lower overall survival rate. In comparison with the negative control si-NC group, cell proliferation, invasion and migration abilities in lnc01614 knockdown expression group (si-lnc01614) were significantly decreased. Western blotting results indicated that si-lnc01614 group exhibited increased E-cadherin expression, and significantly reduced vimentin and snail expression.

CONCLUSIONS: Lnc01614 was upregulated in gastric cancer and significantly correlated with gastric cancer staging, lymph node metas-

tasis, distant metastasis and poor prognosis. Lnc01614 may promote the proliferation, invasion and migration capabilities of gastric cancer through the regulation of epithelial-mesenchymal transition (EMT) pathway.

Key Words:

Long noncoding RNA, lnc01614, Gastric cancer, Prognosis.

Introduction

Gastric cancer is one of the most common malignant tumors of human beings¹. In recent years, diagnosis and treatment techniques for gastric cancer have been continuously improved, but the overall five-year survival rate is still not optimistic. The efficacy of neoadjuvant chemoradiotherapy still needs long-term observation^{2,3}. Many studies have shown that the occurrence and development of gastric cancer are extremely complex. Advanced gastric cancer is more common, especially in China. Therefore, improving the early diagnosis of gastric cancer and exploring new mechanisms of the occurrence and development of gastric cancer are of great importance⁴⁻⁶.

Long noncoding RNA (lncRNA) is a noncoding RNA newly discovered in recent years, and its transcript length is more than 200 nucleotides⁷. lncRNA has various biological functions, which has been reported to be involved in the regulation of many diseases, especially in the tumors⁸⁻¹⁰. Currently, it has been proved that lncRNA is abnormally expressed in various tumors such as breast cancer, bladder cancer, melanoma, liver cancer and colorectal cancer, and plays key regulatory functions in tumorigenesis and progres-

sion¹¹⁻¹³. There are also more and more researches reporting lncRNA in the field of gastric cancer. For example, H19 is highly expressed in gastric cancer tissues and promotes the proliferation of gastric cancer cells. The level of H19 expression is correlated with the progression and poor prognosis of gastric cancer¹⁴. Another work¹⁵ showed that lncRNA GAPLINC is highly expressed in gastric cancer, which is closely related to the change in cluster of CD44. CD44 is a marker promoting tumor proliferation, migration, and angiogenesis. The inhibition of CD44 may cause reduced abilities of lncRNA GAPLINC in promoting cell migration and proliferation.

In this study, the expressions of lncRNA 01614 in 79 pairs of gastric cancer tissues and adjacent tissues were analyzed. The effect of lnc01614 on the biological function of gastric cancer cells was explored at the same time. According to the results, lnc01614 may serve as a new target in the treatment of gastric cancer.

Patients and Methods

Patients and Tumor Samples

A total of 79 pairs of tumor and adjacent tissue specimens were collected from gastric cancer patients receiving surgical resection. According to the American Joint Committee on Cancer (AJCC) Tumor Node Metastasis (TNM) Staging for Gastric Cancer (the 7th edition), all patients enrolled were diagnosed with gastric cancer by postoperative pathological analysis. All the patients did not receive anti-cancer treatment such as radiotherapy and chemotherapy before surgery. This study was approved by the Ethics Oversight Committee. Patients and their families have been fully informed that their specimens would be used for scientific research and signed the relevant informed consent.

Cell lines and Reagents

Four gastric cancer cell strains (BGC-823, SGC-7901, AGS and MKN28) and one normal gastric mucosa epithelial cell strain [gastric epithelial cell strain-1 (GES-1)] were purchased from Shanghai Model Cell Bank (Shanghai, China). Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies Corporation (Carlsbad, CA, USA). Cell culture was performed in a 5% CO₂ incubator at 37°C. DMEM containing 10% fetal bovine serum (FBS) was used as medium.

Transfection

Negative control si-NC and siRNA containing lnc01614 interference sequence (si-lnc01614) were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). Cells were plated in a 6-well plate and cultured until the cell density was up to 70%. SiRNA transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the instructions, and after 48 h, cells were collected for quantitative Real-time polymerase chain reaction (qRT-PCR) analyses and cell function assays.

Cell Proliferation Assay

After 48 h of transfection, cells were collected, plated into a 96-well plate (with 2,000 cells per well) and cultured. Cell counting kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) reagent was added at 1, 2, 3 and 4 d after culture, respectively, for 2 h of further incubation, and cells were placed in a microplate reader to measure the optical density (OD) value of each well at an absorption wavelength of 490 nm. Last, data were analyzed.

Transwell Cell Migration and Invasion Assay

Cells transfected for 48 h were selected, trypsinized and resuspended in serum-free medium. Cell density was adjusted and diluted with 2.0*10⁵/mL after cell counting. Transwell chambers with or without matrigel were placed in a 24-well plate. 200 µL cell suspension were added into the upper chamber, and 500 µL medium containing 10% FBS were added to the lower chamber. Then, chambers were cultured in an incubator at 37°C. After 48 h, chambers were taken out, fixed with 4% paraformaldehyde for 30 min, stained with crystal violet for 15 min, and washed with phosphate-buffered saline (PBS). After that, the inner surface of the basilar membrane of chambers was carefully cleaned to remove endothelial cells. Stained transmembrane cells on the external surface of the basilar membrane of chambers were observed under a microscope, and five visual fields were randomly selected for counting.

Quantitative Real-time PCR (qRT-PCR)

Total RNA was extracted from gastric cancer cell lines and tissues via TRIzol reagents (Invitrogen, Carlsbad, CA, USA), and RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA) using Primescript^{RT}

Reagents (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was performed using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) and StepOne Plus Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The following primers were used for qRT-PCR: lnc01614: forward: 5'-GACTGAGACACTTGGACGAC-3', reverse: 5'-TGCCTTGTTGACCTTCCTGT-3'; -actin: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-GCTGATCCACATCTGCTGGAA-3'. Data were analyzed using ABI StepOne software (Applied Bio-Systems, Foster City, CA, USA), and relative messenger RNA (mRNA) levels were calculated using the 2- $\Delta\Delta C_t$ method.

Western Blotting

Transfected cells were lysed with cell lysis buffer and centrifuged at 14000 g for 15 min at 4°C. The total protein concentration was calculated by bicinchoninic acid (BCA) protein detection kit (Pierce, Rockford, IL, USA). The extracted protein was separated using a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The Western blotting analysis was performed according to standard procedures. Primary antibodies were E-cadherin, vimentin, snail, and GAPDH. Secondary antibodies were anti-mouse and anti-rabbit. All antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Statistical Analysis

SPSS 20.0 (SPSS IBM, Armonk, NY, USA) was used for data treatment. Data were expressed as ($\bar{x} \pm s$). *t*-test was employed for continuous variables, and χ^2 -test or Fisher's exact test was applied to analyze categorical variables. Kaplan-Meier method was used to assess the prognosis and survival time of patients, and Log-rank test was adopted to compare the differences among curves. $p < 0.05$ suggested that the difference was statistically significant.

Results

Lnc01614 was Highly Expressed in Gastric Cancer Tissues and Cell Lines

In this study, qRT-PCR was employed to detect the expressions of lnc01614 in 79 pairs of gastric cancer tissues and adjacent tissues and gastric cancer cell lines. The results showed that the expression of lnc01614 in gastric cancer tissues was significantly higher than that in adjacent tissues, and the difference was statistically significant (Figure 1A). Lnc01614 expression level was significantly enhanced in gastric cancer cells compared with that in normal gastric mucosa epithelial cells (GES-1) (Figure 2A). Lnc01614 expression levels in SGC-7901 and AGS cells were the highest, so these two cell strains were chosen for subsequent experiments.

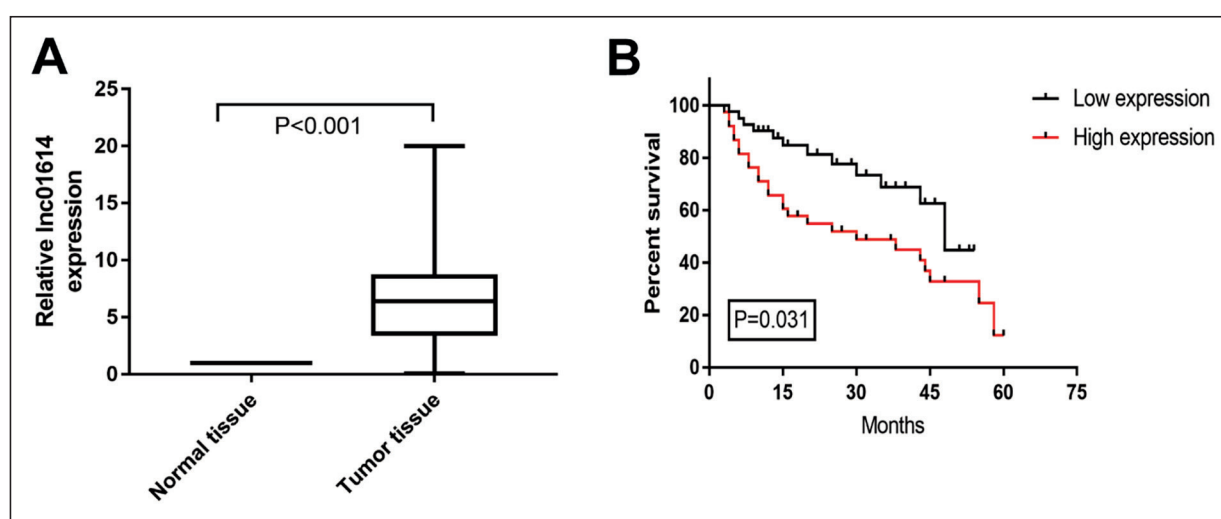


Figure 1. **A**, The expression of lnc01614 in gastric cancer tissue was significantly increased; **B**, Kaplan-Meier survival curves of patients with gastric cancer based on lnc01614 expression. Patients in the high expression group had a significantly more unfavorable prognosis than those in low expression group.

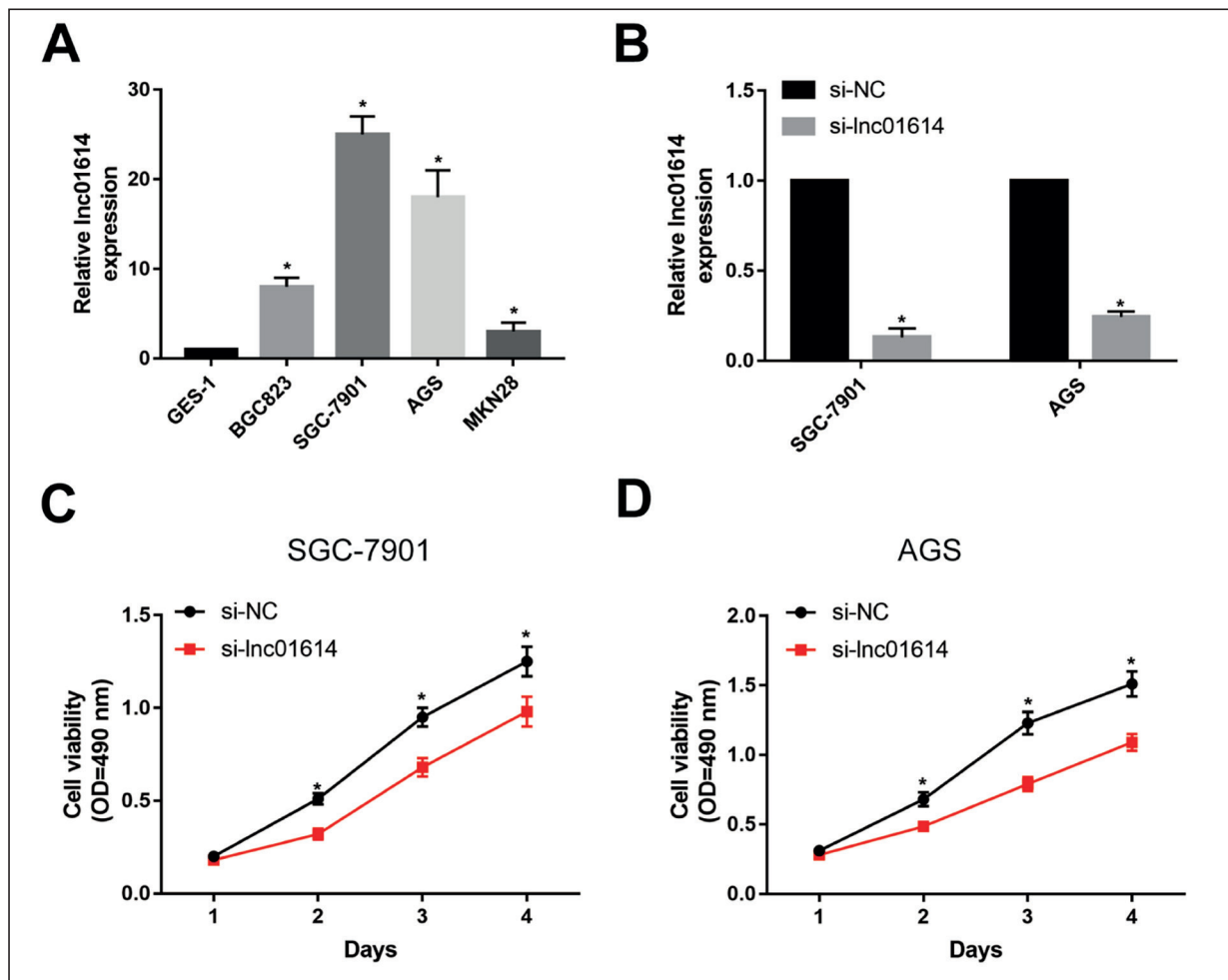


Figure 2. *A*, qRT-PCR analysis of lnc01614 expression in gastric cell lines and gastric mucosal epithelial cells GES-1; *B*, qRT-PCR were used to verify the efficiency of lnc01614 knockdown. *C*, *D*, Growth curve analysis showing the cell growth of SGC-7901 and AGS cells with lnc01614 knockdown.

lnc01614 Expression was Correlated with Clinical Stage, Lymph Node and Distance Metastasis and Overall Survival in Gastric Cancer Patients

According to the lnc01614 expressions in the gastric cancer tissues and adjacent tissues, lnc01614 expression was divided into high expression group and low expression group. The correlations of lnc01614 expression with age, gender, tumor depth, clinical stage, lymph node metastasis and distant metastasis of gastric cancer patients were analyzed by χ^2 -test. lnc01614 high expression was positively correlated with clinical stage, tumor depth, lymph node metastasis and distant metastasis, but had no relationship with age and gender (Table I). To investigate the relationship between lnc01614 expression and the prognosis of gastric cancer

patients, relevant follow-up data were collected. Kaplan-Meier survival curves revealed that the high expression of lnc01614 was significantly associated with poor prognosis of gastric cancer ($p = 0.031$; Figure 1B). These results suggested that lnc01614 may be used as a new biological indicator in the prediction of the prognosis of gastric cancer.

Knockdown of lnc01614 Inhibited Cell Proliferation

To explore the effect of lnc01614 on the proliferation of gastric cancer cells, lnc01614 interference expression models were first successfully constructed (Figure 2B). Then, CCK-8 was used to detect the proliferation of lnc01614 in control group (si-NC) and lnc01614 interference expression group (si-lnc01614). According to Figure 2C

Table I. Association of lncRNA01614 expression with clinicopathologic characteristics of gastric cancer.

Parameters	Number of cases	Lnc01614 expression		p-value
		Low (%)	High (%)	
Age (years)				0.941
< 50	35	18	17	
≥ 50	44	23	21	
Gender				0.523
Male	47	23	24	
Female	32	18	14	
Clinical stage				0.017
I-II	38	25	13	
III-IV	41	16	25	
Tumor depth				0.003
T1-T2	45	30	15	
T3-T4	34	11	23	
Lymph node metastasis				0.001
N0-N1	40	28	12	
N2-N3	39	13	26	
Distance metastasis				0.023
M0	66	38	28	
M1	13	3	10	

and Figure 2D, the si-Lnc01614 group had a clearly decreased cell proliferation rate in comparison with the si-NC group.

Knockdown of Lnc01614 Inhibited Cell Migration and Invasion

In this work, transwell migration and invasion assay were adopted to explore the impacts of lnc01614 on migration and invasion abilities of gastric cancer cells. Results of migration assay (Figure 3A and Figure 3B) showed that the number of cells penetrating the membrane in transwell chambers in lnc01614 knockdown group was markedly lower than that in si-NC group, indicating that the migration ability was inhibited. The result of invasion assay was consistent with that of migration assay (Figure 3C and Figure 3D).

Knockdown of Lnc01614 Inhibited the Expression of Epithelial-Mesenchymal Transition (EMT) Signaling Pathway

To analyze the potential mechanism of lnc01614 in the promotion of cell proliferation, invasion, and migration, Western blotting was performed to detect the expressions of key proteins (E-cadherin, vimentin and snail) in EMT pathway after knockdown of lnc01614. The results indicated that knockdown of lnc01614 remarkably increased the expression of E-cadherin but reduced vimentin and snail (Figure 4).

Discussion

More and more studies¹⁶ have shown that lncRNA plays an important role in the alteration of tumor biological characteristics. It can regulate the occurrence and development of tumors by affecting genomic transcriptional processes and epigenetic signals¹⁷. However, there are few reports of lncRNA in the field of gastric cancer research. Therefore, based on the previous theoretical basis, lnc01614 was found, and its expression in gastric cancer and biological significance was reported at the first time.

Firstly, the expressions of lnc01614 in 79 pairs of gastric cancer tissues and adjacent tissues were demonstrated in this study. The results showed that lnc01614 expression was significantly up-regulated and positively correlated with gastric cancer staging, lymph node metastasis, distant metastasis and poor prognosis. Therefore, it was thought that lnc01614 may have a tumor-promoting role in gastric cancer. To further investigate the effect of lnc01614 on the biological function of gastric cancer, we constructed lnc01614 knockdown expression models using siRNA. The results of CCK-8 and invasion and migration assay indicated that lnc01614 could promote the occurrence and development of gastric cancer.

Tumor metastasis is a complex and multi-step process that can be divided into two phases. Firstly, tumor cells to be transferred are de-ad-

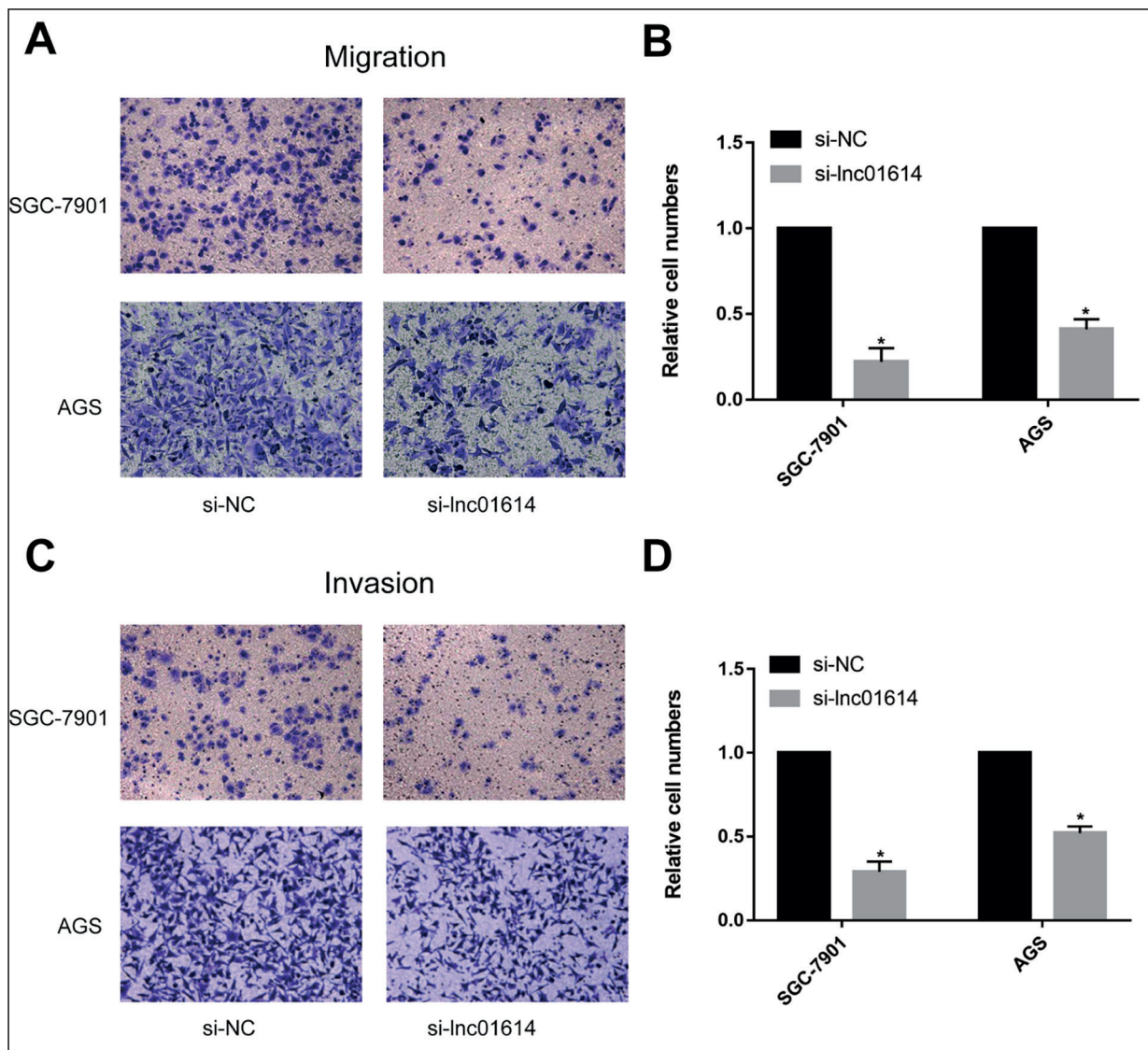


Figure 3. *A, B*, SGC-7901 and AGS cells transfected with si-lnc01614 displayed significantly lower migration capacity. *C, D*, SGC-7901 and AGS cells transfected with si-lnc01614 displayed significantly lower invasion capacity.

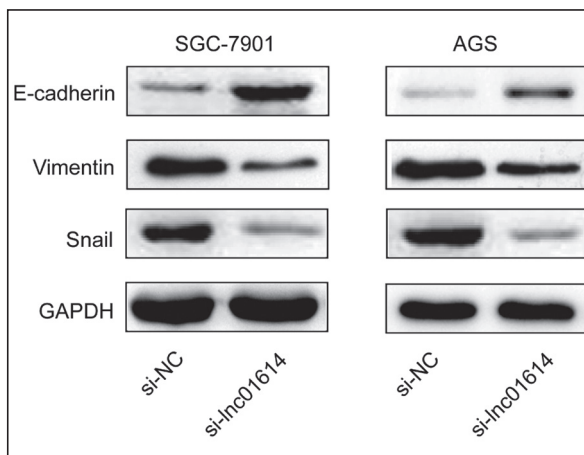


Figure 4. Knockdown of lnc01614 expression significantly decreased the expression of Vimentin and Snail, while increased the expression of E-cadherin.

hered from primary tumor. Secondly, metastases are planted in the distance, and more tumor cells are cloned. EMT, that is, epithelial-mesenchymal transition, plays an important role in tumor metastasis^{18,19}. Tumor cells obtain more capacitated stromal cell phenotypes through decomposition of cell-cell adhesions, thereby enhancing the metastases of cells to more distant places. During the process of EMT, epithelial cells lose their polarities, and intercellular connections become weaker. E-cadherin and zonula occludens-1 (ZO-1) are decreased. On the contrary, expression levels of some markers representing interstitial properties (such as snail, vimentin, and twist) are increased²⁰⁻²².

To explore whether lnc01614 could promote the occurrence and development of gastric cancer by regulating EMT, we detected expressions of E-cadherin, vimentin and snail in key EMT pathway after knockdown of lnc01614. The results revealed that E-cadherin expression was increased, while vimentin and snail expressions were significantly reduced after knockdown of lnc01614, suggesting that lnc01614 could promote the occurrence and development of gastric cancer through activation of EMT pathway.

Conclusions

The expression of lnc01614 in gastric cancer was distinctly increased and correlated with gastric cancer staging, lymph node metastasis, distant metastasis and poor prognosis. lnc01614 promoted the proliferation, invasion and migration capabilities of gastric cancer through regulation of EMT pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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