

# LncRNA SNHG12 regulated the proliferation of gastric carcinoma cell BGC-823 by targeting microRNA-199a/b-5p

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**Abstract.** – **OBJECTIVE:** We aimed at detecting the expression of lncRNA SNHG12 in gastric carcinoma and at exploring its effect on the proliferative ability and metastasis of gastric carcinoma. Also, its mechanism was analyzed.

**PATIENTS AND METHODS:** A total of 54 pairs of gastric carcinoma and paracancerous tissues were harvested from patients in our hospital. Clinical data were used for analyzing the relationship between lncRNA SNHG12 and general information of patients with gastric carcinoma. Knockdown and overexpression experiments were carried out to investigate the relationship between the expression of lncRNA SNHG12 and the proliferation ability of BGC-823. RNA levels of possible target microRNAs were verified and further confirmed by RIP and CHIP experiments.

**RESULTS:** Higher lncRNA SNHG12 expression was observed in 6 gastric carcinoma tissues than the paracancerous tissues. Clinical data analysis demonstrated that highly expressed lncRNA SNHG12 was related to tumor size and TNM staging. Survival analysis showed that highly expressed lncRNA SNHG12 in patients with gastric carcinoma was negatively correlated to the overall survival time. Additionally, the expression of lncRNA SNHG12 was positively linked with the proliferative ability of BGC-823. RIP experiment confirmed the binding abilities of lncRNA SNHG12, microRNA-199a/b-5p and Argo2. The distinct interaction between lncRNA SNHG12 and microRNA-199a/b-5p was verified by CHIP experiment.

**CONCLUSIONS:** This study indicates that lncRNA SNHG12 may play an important role in tumorigenesis and may serve as a molecular target for the malignant gastric carcinoma.

*Key Words:*

Gastric carcinoma, lncRNA SNHG12, MicroRNA-199a/b-5p.

## Introduction

As a common epithelial carcinoma, gastric carcinoma is the fifth most common carcinoma worldwide and the third most common malignancy. The incidence of gastric carcinoma has always been on the rise in recent years. About one million new cases of gastric carcinoma occur each year, of which 50% of cases occur in East Asia, especially in China, seriously endangering people's health and lives. The annual incidence of gastric carcinoma in China is about 400,000, accounting for 42% of the total number of cases worldwide, and the overall mortality rate has been increasing year by year<sup>1,2</sup>. Therefore, finding reliable biomarkers and targets for gastric carcinoma is crucial. Previous studies have shown that gastric carcinoma is closely related to genetic mutations<sup>3</sup>, bacterial and viral infections<sup>4</sup> and diet<sup>5</sup>. Recently, lncRNA has drawn more and more attention because of its regulatory role in the transcriptional and post-transcriptional levels, which has become a hot topic in carcinoma research. lncRNAs are a kind of RNA molecules that encode less than 200 nucleotides and short peptides<sup>6,7</sup>. Studies<sup>8-10</sup> have confirmed that lncRNA is involved in a number of important signal transduction regulatory processes, such as chromatin modification, transcription activation, transcriptional interference, post-transcriptional regulation, and regulatory protein functions. With the advent of a new generation of gene sequencing technologies, a large number of lncRNAs have been discovered and drawn great attention. Although these lncRNAs have no function of protein coding, they can participate in various life activities through epigenetic regulation and other mechanisms<sup>11-13</sup>. Many scholars<sup>14,15</sup> have confirmed that the differentially expressed lncRNA has a close linkage with var-

ious diseases and dysfunctions, including tumors. In this study, highly expressed lncRNA SNHG12 was observed in gastric carcinoma tissues by quantitative PCR. The upregulation of lncRNA SNHG12 was correlated with tumor size, stage, etc. At the cellular level, lncRNA SNHG12 promoted the proliferation and metastasis of gastric carcinoma cells. In addition, we discussed the possible mechanism of lncRNA SNHG12 in promoting the proliferation of gastric carcinoma cells.

## Materials and Methods

### Tissue Samples

A total of 54 pairs of gastric carcinoma and paracancerous tissues were harvested from patients treated in our hospital from June 2015 to March 2017. Before acquisition, all experiments were approved by the Medical Ethics Committee and informed consent was signed. 54 cases were diagnosed as gastric carcinoma based on pathological results. All patients were untreated, without any other history of malignancy, and did not receive any anti-carcinoma treatment. The average age of patients was  $66.3 \pm 1.2$  years.

### Cell Culture

Gastric carcinoma cell lines (SGC-7901, BGC-823) and normal gastric epithelial cell line (GES-1) were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA). The gastric carcinoma cell lines (SGC-7901, BGC-823) and normal gastric epithelial cell line (GES-1) were provided by the American Type Culture Collection (ATCC, Manassas, VA, USA). Cell passage was performed when cell density was up to 80%, with an inoculation density of 1:2.

### siRNA Transfection and pcDNA Transfection

The cells were seeded in a 6-well plate and when cell confluence was about 60%, they were transfected with lipofectamine 2000 (pcDNA using Entranster-R4000). 10  $\mu$ L of lipofectamine 2000 and 10  $\mu$ L of siRNA-SNHG12 at the dose of 20 nM were added in 500  $\mu$ L of serum-free suspension (pcDNA was prepared with 4  $\mu$ g of Entranster-R4000 and 2.68  $\mu$ g of pcDNA-SNHG12). 1.5 mL of 1640 medium (Gibco, Rockville, MD, USA) was added to culture dishes, and the control group was added with the same amount of

lipofectamine 2000 and siRNA-NC (pcDNA was prepared with Entranster-R4000 and pcDNA-NC) as the experimental group. Culture medium was replaced 6 h after transfection. Si-SNHG12: forward 5'-GCAGUGUGCUACUGAACUUTT-3', reverse 5'-AAGUUCAGUAGCACACUGCTT-3'. si-NC: forward 5'-UUCUCCGAACGUGUCACGUTT-3', reverse 5'-ACGUGACACGUUCGAGAATT-3'.

### qRT-PCR

We used TRIzol method to extract total RNA according to the instruction. 50  $\mu$ L of reaction system were prepared according to the manual of qRT-PCR. The reverse transcription reaction was performed under the standard method: reverse transcription reaction at 50°C for 30 min and 92°C for 3 min. The obtained cDNA was subjected to PCR amplification based on the standard method: denaturation at 92°C for 10 s, annealing at 55°C for 20 s, extension at 68°C for 20 s and amplification for 40 cycles. Using  $\beta$ -actin gene as internal control, the expression level of lncRNA SNHG12 was obtained by  $2^{-\Delta\Delta C_t}$  method.  $\beta$ -actin, forward 5'-CTC-CATCCTGGCCTCGCT-GT-3', reverse 5'-GCT-GTCACCTTCACCGT-TCC-3'; SNHG12, forward 5'-TCTGGTGATCGAGGACTTCC-3', reverse 5'-ACCTCCTCAGTATCACACACT-3'; microRNA-199a-5p, stem-loop 5'-CTCAACTGGTGTCGT-GGAGTCGGCAATTTCAGTTGAGGAACAGG-3', forward 5'-ACACTCCAGCTGGGCCCAGT-3'; reverse 5'-TGGTGTCGTGGAGTCG-3'; U6, forward 5'-GCTTCGGCAGCACATATACTAAAAT-3', reverse 5'-CGCTTCACGAATTTGCGTGCAT-3'.

### Cell Counting Kit-8 (CCK-8) Assay

The transfection point was 0 h, and cells in control group and the treated group were inoculated into 96-well plates with six replicates per group.  $5 \times 10^3$  cells were seeded in each well of the 96-well plates. After 6 h, the activity of adherent cells was measured (0 h). Afterwards, 20  $\mu$ L of CCK-8 solution were supplemented to each well at 24, 48, 72 and 96 h, respectively, and placed at 37°C and 5% CO<sub>2</sub> incubator for 2-3 h. The absorbance (OD value) was recorded with a microplate reader at a wavelength of 450 nm. Only CCK-8 solution and culture media (no cells) were added in the blank control.

### Colony Formation Assay

The transfection point was 0 h, and liquid was changed after 6 h. 3 h later,  $3 \times 10^3$  cells were inoculated into medium dish and cultured at 37°C and

5% CO<sub>2</sub>. We changed the medium every 2 days until 14 days after treatment. With medium discarded, cells were washed with phosphate-buffered saline (PBS) twice, and fixed with 5% paraformaldehyde for 30 min; after that, the waste solution discarded. 1 mL of 0.1% crystal violet solution was given to each well. 30 min later, the crystal violet solution was discarded and washed with PBS until the eluate was clear. Photos were taken for counting.

### **Transwell Assay**

The BGC-823 and SGC-7901 cells were cultured in 6-well plates ( $2 \times 10^5$  cells per well). After 24-48 h post-transfection, 1 mL of the cell suspension was prepared in each well. After counting, the cell density was adjusted to  $3 \times 10^4$ . 300  $\mu$ L of the cell suspension were given to the transwell chamber, with 700  $\mu$ L of culture medium containing 20% fetal bovine serum (FBS) supplemented in the lower chamber, and incubated for 12-48 h. The cells were fixed by methanol, stained by crystal violet and then dried, and an inverted microscope was utilized to take pictures.

### **Luciferase Reporter Vector Construction**

After cells were grown to 60-70% confluence, they were then co-transfected with pMicroRNA-Reporter-SNHG12-WT or pMicroRNA-Reporter-SNHG12-MUT and microRNA-199a/b-5p mimic. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was utilized as instructed. 48 h later, treated cells were used for luciferase assay by dual luciferase reporter assay (Promega, Madison, WI, USA).

### **RIP Assay**

The RIP assay was carried out as instructed by the Magna RIP<sup>TM</sup> RNA Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). Cells with 80-90% confluency were harvested and lysed by RIP lysis buffer. Afterwards, the cell extract was cultured in RIP buffer, which contained magnetic beads bound to the human anti-Go2 antibody or IgG. Cells were maintained with proteinase K to prepare the samples, followed by precipitation of the RNA. The obtained RNA was utilized for mRNA level detection of lncRNA SNHG12 and microRNA-199a/b-5p.

### **ChIP**

Cells were treated in phosphate-buffered saline (PBS) supplemented with 0.1% trypsin and 0.1% collagenase, and then cell lysis and streptavidin

bead pellet were treated by the Bioruptor instrument (Diagenode, Denville, NJ, USA). Cells transfected with SNHG12-WT and SNHG12-MUT were utilized as controls. We performed qRT-PCR for identifying and quantifying the precipitated RNA. The expression level was accessed with  $2^{-\Delta CT}$ , where  $\Delta CT = CT \text{ microRNA-199a/b-5p} - CT \text{ SNHG12}$ . LncRNA-SNHG12 ChIP probe sets were: 1. 5'-GAGGAAAAACCCGGCGAGTG/iSp18//3Bio/-3'; 2. 5'-ACATTCACCACCATCTC-GAG/iSp18//3Bio/-3'; 3. 5'-CAGTCCGAAGCGA-GAGAAGG/iSp18//3Bio/-3'.

### **Statistical Analysis**

We used statistical product and service solutions (SPSS 22.0, IBM, Armonk, NY, USA) statistical software for data analysis, GraphPad Prism 6.0 (La Jolla, CA, USA) for image editing. Comparison of measurement data was done using *t*-test, expressed as mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ). Classification data was analyzed using  $\chi^2$ -test. We considered  $p < 0.05$  as statistically significant. \* $p < 0.05$ , \*\* $p < 0.01$ .

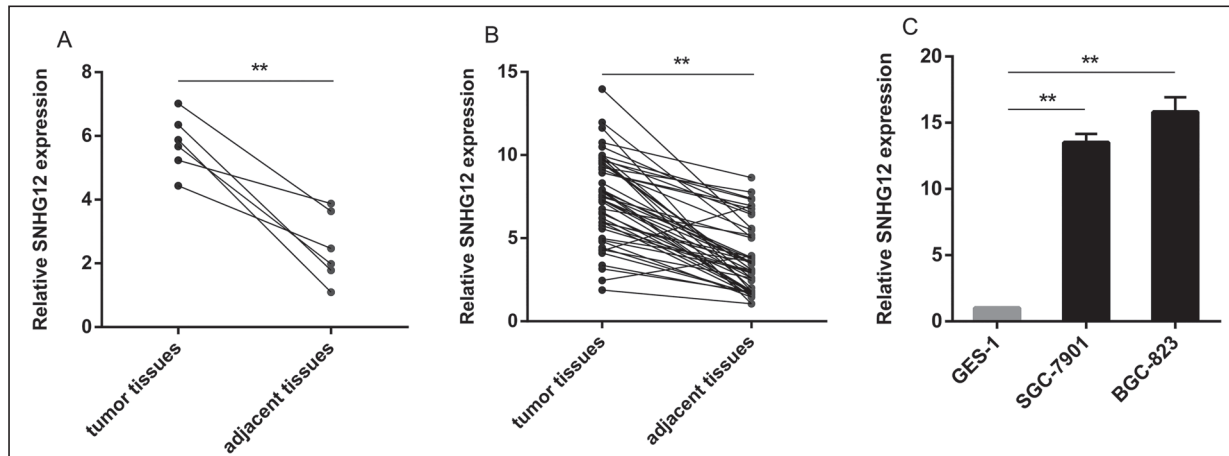
## **Results**

### **Expression of lncRNA SNHG 12 in Gastric Carcinoma Tissues and Cells**

The expression level of lncRNA SNHG12 in gastric carcinoma tissues and paracancerous tissues was accessed by qRT-PCR. The data revealed higher expression of lncRNA SNHG12 in 6 gastric carcinoma tissues than that in paracancerous ones (Figure 1A). Our subsequent validation in 48 gastric and paracancerous tissues showed the same result (Figure 1B). At the cellular level, we performed quantitative PCR to access the mRNA level of lncRNA SNHG12 in gastric carcinoma cell lines and normal gastric epithelial cell lines. The results illustrated a higher expression level of lncRNA SNHG12 in gastric carcinoma cell lines SGC-7901 and BGC-823 in comparison with normal gastric epithelial cells (Figure 1C). We concluded that lncRNA SNHG12 is upregulated in gastric carcinoma tissues and cell lines, which is likely to become a molecular marker of gastric carcinoma.

### **Relationship Between the Expression Level of lncRNA SNHG12 and the Clinical Features of Gastric Carcinoma**

Clinical data analysis revealed that the expression level of lncRNA SNHG12 in gastric



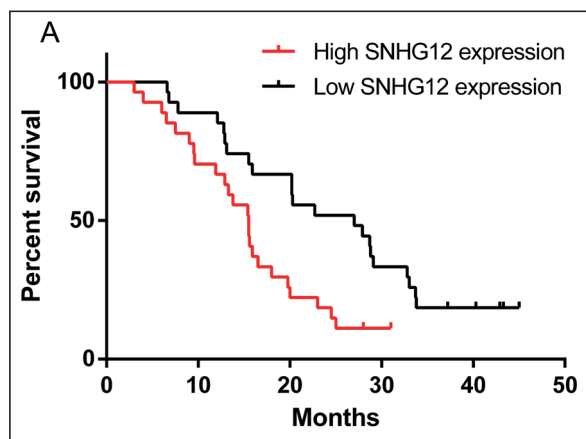
**Figure 1.** **A**, Gastric carcinoma patients (n=6) presented increased expression of lncRNA-SNHG12 in gastric carcinoma tissues relative to adjacent tissues. **B**, In 54 gastric carcinoma patients, the expression of lncRNA-SNHG12 in gastric carcinoma tissues was higher relative to adjacent tissues. **C**, The expression of lncRNA-SNHG12 in gastric carcinoma cell lines BGC-823 and SGC-7901 was increased relative to normal gastric epithelial cells GES-1. \* $p < 0.05$ , \*\* $p < 0.01$ .

carcinoma tissues was not significantly related with the age, gender, location of tumor and regional metastasis. However, lncRNA SNHG12 had significant correlation with the size and clinical stage of tumor. lncRNA SNHG12 expression in patients with high TNM stage was higher than that in patients with lower TNM stage (Table I). In addition, our analysis of survival time suggested a negative correlation be-

tween the expression level of lncRNA SNHG12 in patients with gastric carcinoma and their survival and relapse-free survival (Figure 2A). In general, clinical data showed that lncRNA SNHG12 in gastric carcinoma tissues is highly linked with the poor prognosis of patients with gastric carcinoma, which may be the molecular diagnostic biomarker and prognostic target of gastric carcinoma.

**Table I.** Clinicopathologic characteristics of patients.

| Characteristics       | N  | SNHG12              |                    | p-value |
|-----------------------|----|---------------------|--------------------|---------|
|                       |    | High NO. cases (27) | Low NO. cases (27) |         |
| Gender                |    |                     |                    | 0.586   |
| Male                  | 28 | 15                  | 13                 |         |
| Female                | 26 | 12                  | 14                 |         |
| Age                   |    |                     |                    | 0.4131  |
| ≤ 65                  | 25 | 14                  | 11                 |         |
| > 65                  | 29 | 13                  | 16                 |         |
| Stage                 |    |                     |                    | 0.008*  |
| I                     | 10 | 3                   | 7                  |         |
| II                    | 15 | 4                   | 11                 |         |
| III                   | 29 | 20                  | 9                  |         |
| Lymph node metastasis |    |                     |                    | 0.005*  |
| Negative              | 19 | 4                   | 15                 |         |
| Positive              | 35 | 23                  | 12                 |         |
| Tumor size            |    |                     |                    | 0.014*  |
| ≤ 5 cm                | 20 | 5                   | 14                 |         |
| > 5 cm                | 34 | 22                  | 13                 |         |
| HP infection          |    |                     |                    | 0.397   |
| Negative              | 22 | 11                  | 13                 |         |
| Positive              | 32 | 16                  | 14                 |         |



**Figure 2.** The overall survival of gastric carcinoma patients with higher expression of lncRNA SNHG12 was significantly lower than that of patients with relatively lower expression. \* $p < 0.05$ , \*\* $p < 0.01$ .

### ***Proliferative Ability of Gastric Carcinoma Cell Lines Was Inhibited After Reducing lncRNA SNHG12 Expression***

We transfected gastric carcinoma cell BGC-823 with small interfering RNA to knock down the expression of lncRNA SNHG12, and qRT-PCR data suggested that siRNA effectively reduced the expression of lncRNA SNHG12 in gastric carcinoma cell lines (Figure 3A). CCK-8 assay result demonstrated that, after lncRNA SNHG12 expression was reduced, the absorbance of cells was significantly reduced at the wavelength of 450 nm (Figure 3B). Colony formation assay indicated that the ability of cells to form a clone mass was decreased after reducing the expression of lncRNA SNHG12 (Figure 3C). Overexpression experiments presented the reverse results (Figure 3D-F).

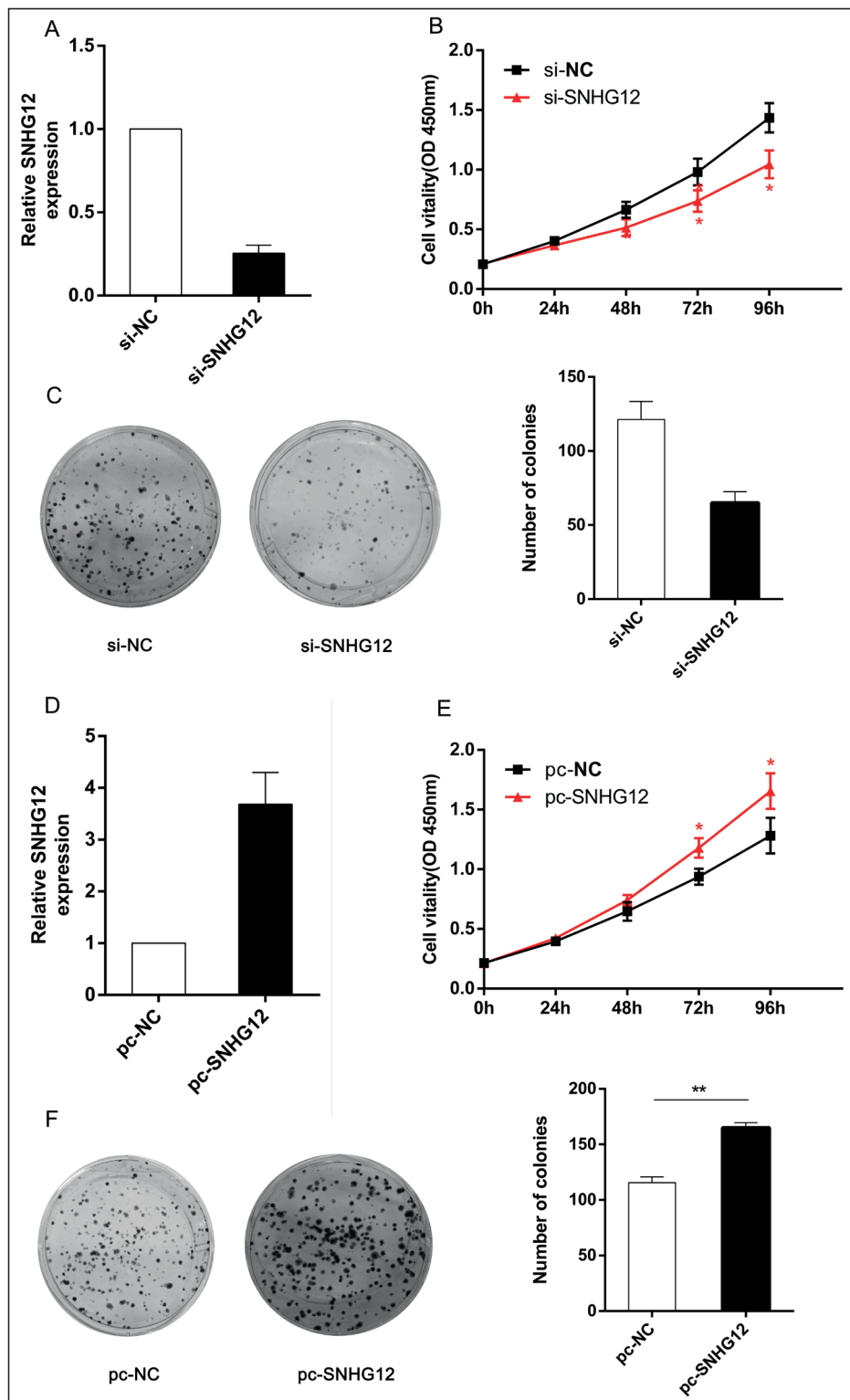
### ***SNHG12 Targeted to Inhibit microRNA-199a/b-5p***

In order to explore the mechanism of lncRNA SNHG12 regulating the proliferative ability of gastric carcinoma cell lines, we performed subcellular structure localization of lncRNA SNHG12. Nucleoplasmic separation experiment illustrated that lncRNA SNHG12 was mainly located in the cytosol of BGC-823 cells (Figure 4A). Subsequently, we predicted microRNA-199a/b-5p as the possible target of lncRNA SNHG12 using microRNAcode (<http://www.microRNAcode.org/>) (Figure 4B). MicroRNA-199a/b-5p was the most conserved among primates and mammals, and had a base sequence

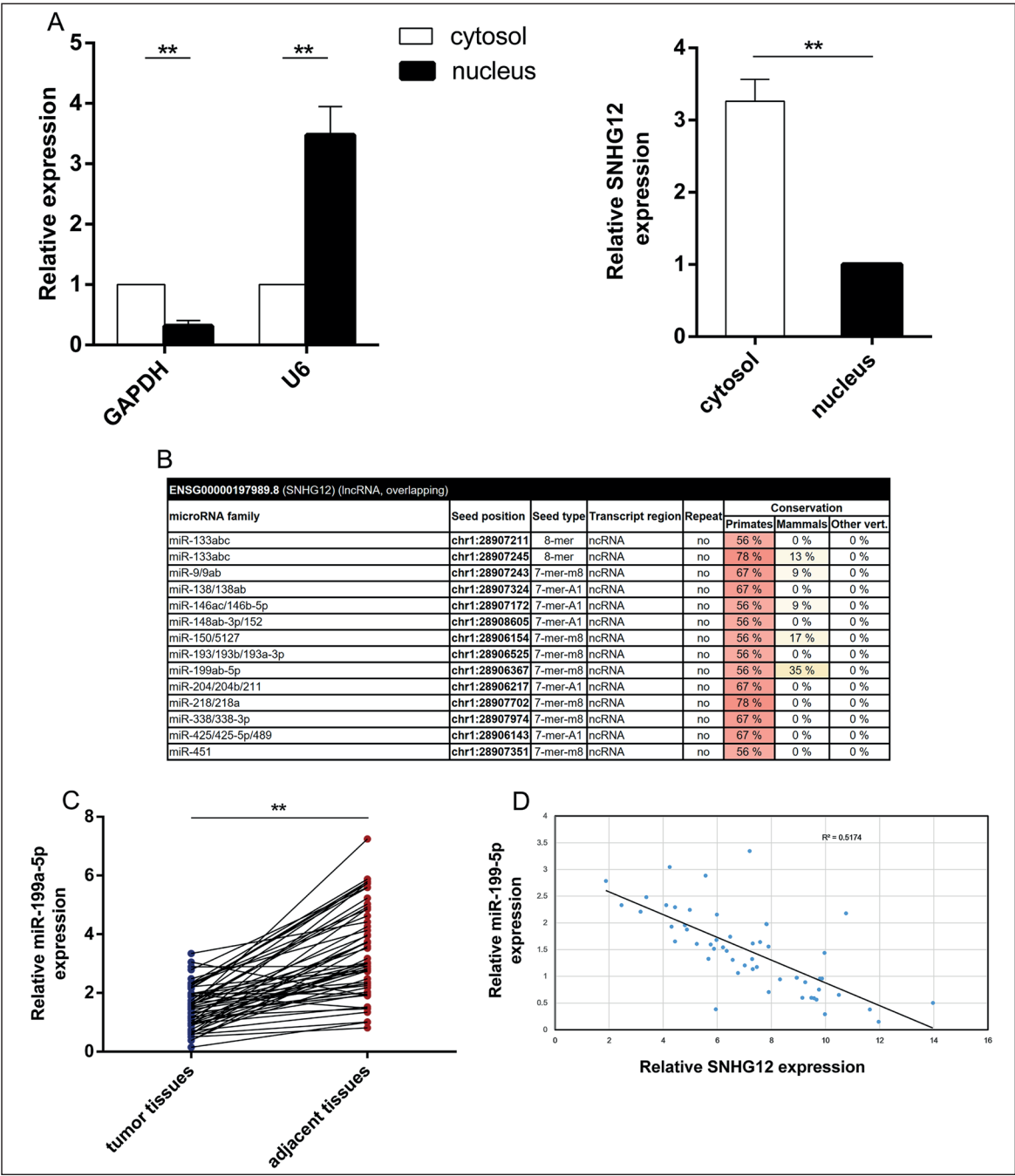
covalently bound to lncRNA SNHG12. Since microRNA-199a-5p and microRNA-199b-5p have homologous sequences, we performed a quantitative assay of microRNA-199a-5p in gastric carcinoma (Figure 4C); also, a correlate analysis was performed. Our data suggested that the expression level of lncRNA SNHG12 in gastric carcinoma tissue was negatively correlated with microRNA-199ab-5p (Figure 4D). In conclusion, lncRNA SNHG12 can target microRNA-199a/b-5p in the cytoplasm.

### ***lncRNA SNHG12 Had a Direct Interaction With the Sponge of microRNA-199a/b-5p***

Multiple researches have shown that lncRNA affects the post-transcriptional level of downstream mRNA by competitively binding target microRNA-199a/b-5p in the cytoplasm. To explore whether there is a direct effect between lncRNA SNHG12 and microRNA-199a/b-5p, a wild type SNHG12-luciferase reporter vector and a mutant lncRNA SNHG12 luciferase reporter vector were constructed. After transfection of microRNA-199a/b-5p mimic into gastric carcinoma cell lines, the luciferase activity of wild-type SNHG12-luciferase reporter vector was significantly decreased, while no significant changes were found in wild-type, indicating that there is an interaction between microRNA-199a/b-5p and lncRNA SNHG12 (Figure 5A). Previous studies showed that lncRNA can exert its ceRNA function by binding with Argo2 and target microRNA-199a/b-5p to form a silencing complex. Therefore, we speculated that lncRNA SNHG12 interacts with microRNA-199a/b-5p through a similar mechanism. To confirm this hypothesis, we performed RIP experiment and detected the binding abundances of lncRNA SNHG12, microRNA-199a/b-5p and Argo2 via qRT-PCR. The data illustrated that the expression abundance of lncRNA SNHG12 and microRNA-199a/b-5p in the Argo2 antigen-antibody complex were significantly increased relative to the control group (Figure 5B), suggesting that lncRNA SNHG12 and microRNA-199a/b-5p can be combined with Argo2. In addition, ChIP results further confirmed that wild-type SNHG12 could be co-immunoprecipitated with microRNA-199a/b-5p, while mutant lncRNA SNHG12 had no such effects (Figure 5C). To sum up, we considered that lncRNA SNHG12 can function as a sponge of microRNA-199a/b-5p to promote carcinoma development.



**Figure 3.** **A**, After transfection of si-SNHG12, the expression of lncRNA-SNHG12 in BGC-823 cell line was significantly reduced. **B**, CCK-8 assay showed that the proliferation of BGC-823 cell line was significantly inhibited after si-SNHG12 transfection. **C**, Colony formation assay showed that the ability of BGC-823 cells to form colony mass was significantly decreased after transfection with si-SNHG12. **D**, After transfection of pc-SNHG12, the expression of lncRNA SNHG12 in BGC-823 cell line was significantly increased. **E**, CCK-8 assay showed that mouse and human osteoblasts proliferated significantly after transfection of pc-SNHG12. **F**, Clone formation assay showed that the ability of BGC-823 cells to form clonal cell populations was significantly increased after transfection of si-SNHG12. \* $p < 0.05$ , \*\* $p < 0.01$ .

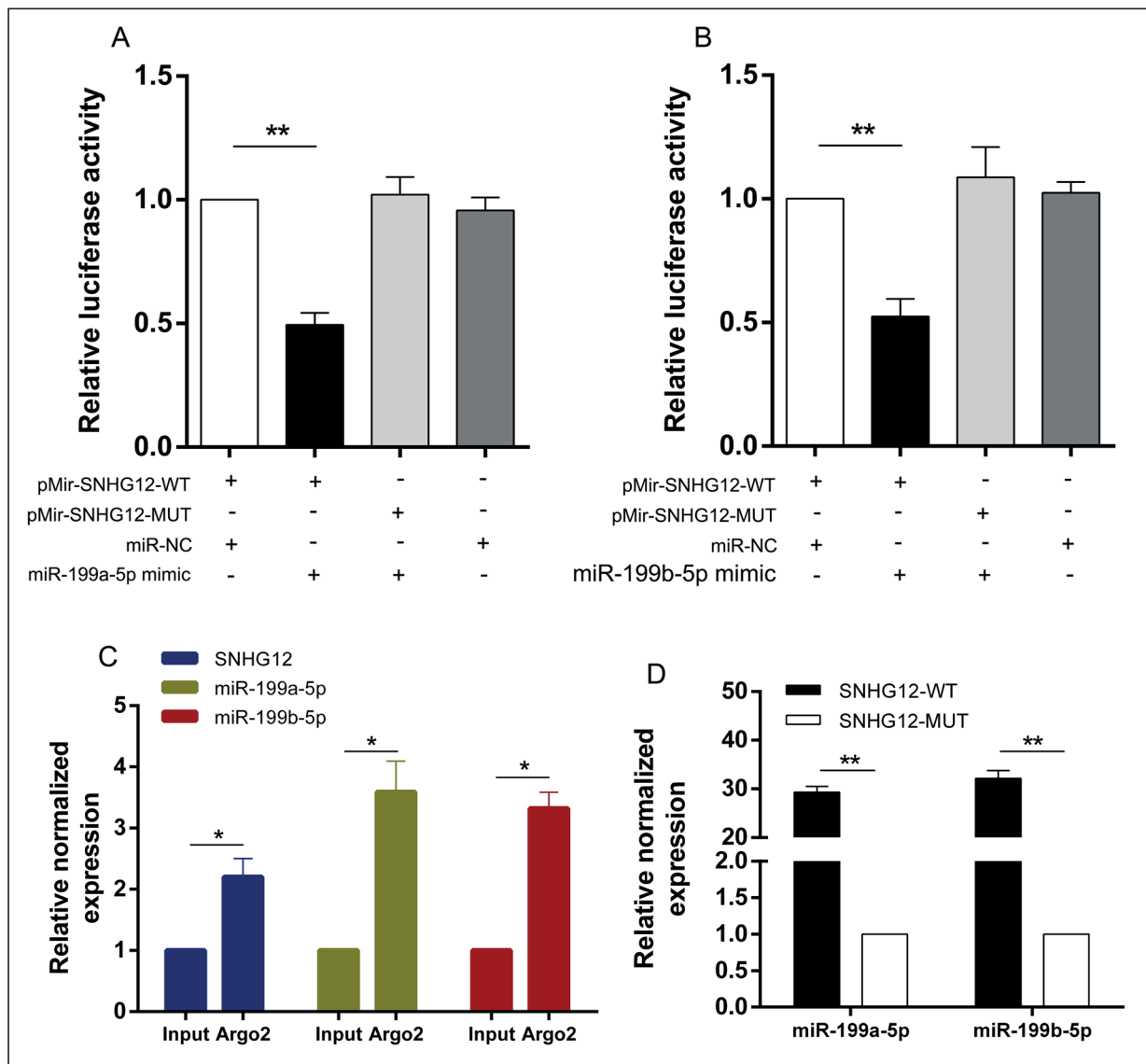


**Figure 4.** **A**, The results of nuclear isolation experiments showed that lncRNA SNHG12 mainly distributed in the cytoplasm. **B**, We predicted the possible target microRNA NAs of lncRNA SNHG12 using the microRNA code database. **C**, Results of qPCR showed that the expression of microRNA-199a-5p in gastric carcinoma tissues was lower than that in adjacent normal tissues. **D**, Correlation bivariate correlation analysis showed 54 pairs of gastric carcinoma lncRNA SNHG12 expression level was negatively related to the microRNA-199a-5p expression level. \* $p < 0.05$ , \*\* $p < 0.01$ .

### Discussion

An increasing number of works<sup>16,17</sup> indicated that lncRNAs have been identified as de-regulated in various physiological and patho-

logical processes as well as in malignancies. Meanwhile, numerous studies have demonstrated the function of lncRNAs in tumor development and prognosis<sup>18-20</sup>. Although the current research on the pathogenesis of lncRNA is not thorough



**Figure 5.** **A**, The wild-type and mutant lncRNA SNHG12 sequences were cloned into pMicroRNA-Reporter vector and co-transfected into BGC-823 cells with microRNA-199a/b-5p mimics or microRNA-NC to determine luciferase activity. **B**, The cRNA immunoprecipitation with anti-Ago2 antibody was used to assess the binding of endogenous Ago2 to RNA. The levels of lncRNA SNHG12 and microRNA-199a/b-5p were detected by qPCR. **C**, ChIP assay followed by RT-qPCR to detect the expression of microRNA-199a/b-5p.

enough, it is widely expressed in tumor cells as an ultra-conservative element in the evolution of species<sup>21</sup>. Such super conserved elements are greatly involved in the development of normal individuals, whereas their abnormal expression leads to the development of tumors<sup>22,23</sup>. Currently, studies were highly focused on the underlying function of lncRNA SNHG12 in the promotion of carcinoma, laying primary evidence for the dysregulation of lncRNA SNHG12 in gastric carcinoma. We demonstrated a higher expression

level of lncRNA SNHG12 in gastric carcinoma tissues in comparison with normal ones. In addition, based on a survival research carried out for 46-month for follow-up, our data illustrated that higher survival was observed in high lncRNA SNHG12 expression group ( $p < 0.05$ ), indicating that lncRNA SNHG12 may be a biomarker for predicting gastric carcinoma. Afterwards, the *in vitro* functional significance of lncRNA SNHG12 in gastric carcinoma cell lines was evaluated by RNAi and overexpression techniques. The data

showed that lncRNA SNHG12 enhances the proliferative activity of gastric carcinoma cells *in vitro*. We also observed that lncRNA SNHG12 is mainly distributed in the cytoplasm. First, we performed bioinformatics analysis, and results revealed that lncRNA SNHG12 may interact with microRNA-199a/b-5p. In addition, a negative correlation between lncRNA SNHG12 and microRNA-199a-5p was confirmed, and subsequently we demonstrated that microRNA-199a/b-5p binds to lncRNA SNHG12 by performing dual luciferase assays. Furthermore, an endogenous interaction between lncRNA SNHG12 and microRNA-199a/b-5p was observed through immunoprecipitation with Ago2 antibody in gastric carcinoma cells. ChIP analysis implied an interaction between lncRNA SNHG12 and microRNA-199a/b-5p. Finally, lncRNA SNHG12 regulated the expression of microRNA-199a/b-5p-related microRNA NAs, whereas microRNA-199a/b-5p has been shown in previous studies to promote its carcinoma-promoting effects through post-transcriptional regulation. Overall, these results indicated that lncRNA SNHG12 regulates microRNA-199a/b-5p of the target gene as an endogenous sponge or ceRNA.

## Conclusions

Current research indicates that lncRNA SNHG12 may play an important role in tumorigenesis and may serve as a molecular target for the malignant gastric carcinoma. In addition, we found that lncRNA SNHG12 acts as a sponge for microRNA-199a/b-5p to act on its downstream genes.

## Conflict of Interest

The Authors declare that they have no conflict of interests.

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