LINC01088 regulates the miR-95/LATS2 pathway through the ceRNA mechanism to inhibit the growth, invasion and migration of gastric cancer cells

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Abstract

Background: In gastric cancer, a malignant condition with a dismal prognosis, long non-coding RNAs (LncRNAs) play a significant regulatory role. They often compete with microRNAs through the ceRNA mechanism to affect the expression of target mRNA. However, the specific clinical value and mechanism of action of LncRNA in gastric cancer are still unclear.

Methods: This study detected the expression and clinical value of LINC01088 in gastric cancer tissues. Furthermore, the biological functions of LINC01088 and the regulation mechanism of the miR-95/LATS2 pathway were explored.

Results: LINC01088 and LATS2 mRNA expression decreased, and miR-95 increased in gastric cancer tissues. LINC01088 has an excellent positive correlation with LATS2 mRNA, which may be a ceRNA pair; LINC01088 has binding sites with miR-95. Gene interference tests on gastric cancer cell lines revealed that LINC01088 could prevent gastric cancer cells from proliferating, invading, and migrating. The function of LINC01088 is achieved by regulating the miR-95/LATS2 pathway through the ceRNA mechanism.

Conclusion: The results of this study show that LINC01088 expression is significantly reduced in gastric cancer tissues and cell lines. LINC01088 inhibits gastric cancer cells’ proliferation, invasion, and migration by regulating the miR-95/LATS2 pathway via the ceRNA mechanism.

Keywords

gastric cancer, LINC01088, invasion, migration, proliferation, microRNA-95, large tumor suppressor kinase 2

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Introduction

When analyzing the structure of the human genome, researchers found that only some mRNAs are translated into protein and that a large number of RNAs are not translated into proteins, that is, nonfunctional sequences. On the other hand, non-coding RNAs have been discovered to regulate coding RNAs at the transcription level and so play a role in the incidence and progression of many diseases in recent years.1–3 Among non-coding RNAs, the function of long non-coding RNAs (lncRNAs) has been a research hotspot

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in recent years. RNAs having a length of more than 200 nucleotides are referred to as lncRNAs. They can be independently transcribed and are molecularly similar to mRNAs. They do not encode functional proteins themselves but can regulate downstream mRNAs. Studies have found that lncRNAs play important biological functions in preventing gastric cancer (GC). Recent findings indicate that lncRNAs can act as key signal transduction mediators in GC signal transduction pathways through interactions with proteins, RNA, and lipids. Studies have shown that lncRNAs can regulate mRNAs by competing with and sharing microRNA binding sites as competitive endogenous RNAs (ceRNAs) or through natural microRNA sponge adsorption and that these lncRNAs can act as ceRNAs or through natural microRNA sponge adsorption. In addition to tumor tissue, lncRNAs can also be directly obtained from healthy tissue and body fluids; the collection is simple and well tolerated by patients. Furthermore, the GC patients in the tissues and peripheral blood can regulate (up or down) the expression of some lncRNAs. As a result, lncRNAs might be employed as possible tumor indicators for GC diagnosis or therapeutic agents.

LINC01088 is a newly discovered tumor-associated lncRNA located in the 4q21.21 region. LINC01088 has been considerably downregulated in ovarian cancer tissue compared to non-cancerous tissue in studies. Zhang et al. showed that LINC01088 expression was reduced in ovarian cancer cells and that LINC01088 affected the posttranscriptional regulation of p21-activated protein kinase 4 (PAK4) by competitively inhibiting the expression of miR-24-1-5p, thereby inhibiting cell proliferation. However, studies of LINC01088 in GC have not been reported. Therefore, in this study, we first determined that LINC01088 was a downregulated lncRNA in GC, which was validated by comparing bioinformatics data. The biological roles of LINC01088 and the molecular processes behind its modulation of the incidence and development of GC were then investigated in vitro. This research has significant scientific relevance as well as therapeutic application possibilities for enhancing the survival of GC patients.

Materials and methods

Clinical organization

This study is an experimental cell study. A total of 48 patients diagnosed with GC and treated surgically at the Fourth Hospital of Hebei Medical University, China, from January 2017 to June 2020 were selected as the study subjects. None of them received radiotherapy or chemotherapy, and no other tumors were combined. The patients were 27 males and 21 females, whose ages ranged from 40 to 75 years old, with a median age of 62. During surgery, tumor tissue and paracancerous tissue were collected and placed in liquid nitrogen and transferred as soon as possible to a −80°C freezer for storage. All patients signed informed consent, and the experiment was reviewed and approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University.

Bioinformatics analysis

Data collection and differential expression analysis for LINC01088: The data for this study came from The Cancer Genome Atlas (TCGA) database, which created a GC dataset. Gene expression data were obtained from the TCGA database through the R package “TCGAbiolinks,” which was specifically developed for comprehensive analysis with Genomic Data Commons (GDC) data. The R package DESeq2 was used to identify differentially expressed genes (DEGs) based on the original read count expression profile. After correction, genes with a p-value less than .05 and an expression change greater than 1-fold were identified as DEGs.

Cell lines and main reagents

The China Facility for Type Culture Collection (CCTCC) provided the human GC cell lines MKN28, SGC7901, BGC823, andGES-1, cultivated and passaged in our hospital’s scientific research center. MTT, RPMI 1640, trypsin, and TRizol were purchased from Invitrogen (USA); matrix metalloproteinase-2 (MMP-2), MMP-9, NM23, N-cadherin, E-cadherin, Twist, Zeb1, vimentin, large tumor suppressor kinase 2 (LATS2), and β-actin antibodies were purchased from Sigma (USA) (Table 1). PCR primers were designed and synthesized by Sangon Biotech Co., Ltd. (China).

Cell line culture

The cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂ incubator and digested and passaged with a 0.25% trypsin solution. Experiments were performed when the cells reached a confluency of 60%–80%.

RT-PCR and fluorescence quantitative PCR detection

Total RNA was isolated from tissues or cells using the TRizol one-step procedure, and 2 μg of RNA was used to reverse-transcribe template cDNA. Two microliters of the reverse transcription product was used for PCR to detect the mRNA expression level of target molecules. The
thermal cycling parameters of the PCR system (final volume, 20 μL) were according to the kit’s instructions. The primers were used to test the specificity in the experiment after BLAST alignment were designed by Primer 5.0. The primer sequences are provided in Table 2. The agarose gel electrophoresis (1.5%) evaluated the RT-PCR products. Fluorescence quantitative PCR results were calculated based on the $2^{-\Delta\Delta Ct}$ method. U6 was used as the reference gene for microRNA, and the internal reference gene for mRNA is β-actin.

**MTT method to detect cell viability**

A single-cell suspension was made for each batch of cells, the cell concentration was adjusted to $5 \times 10^4$ cells/ml, and the cells were plated in 96-well plates. For each group, 20 μL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (5 mg/mL) was added. After 4 h of culture, 150 μL of DMSO was added to replace the culture medium and shaken for 15 min. Absorbance (OD) was measured at 570 nm using a microplate reader. The experiment was repeated 3 times.

**Wound assay**

Cells were sown in 24-well plates with a concentration of $5 \times 10^5$ cells/mL. After adhering to the plates, the cells were treated based on the experimental design for each group. The cells were cultured for 48 h, after which the culture medium was aspirated. Serum-free culture medium was added after a 200 μL pipette tip scratched cells. After being cultured for 24 h, the cells were washed with PBS 3 times to remove dead and unadhered cells and wound healing was assessed under a microscope. The cell migration distance and rate were calculated. The experiment was repeated 3 times.

**Transwell cell invasion test**

Cell invasion assay was performed using a 24-well Transwell plate chamber. The pore size of the polycarbonate membrane in the chamber is 8 μm, and 100 μL of Matrigel was coated in the upper chamber. The prepared chamber was sterilized by ultraviolet (UV) irradiation for 2 h to polymerize the Matrigel fully. BGC823 cells in the logarithmic growth phase were digested to prepare suspensions; the cells ($1 \times 10^6$ cells/ml) were seeded into 6-well plates. When the cells reached a confluency of 60%–70%, each group of cells was treated per the experimental design. After 24 h, 200 μL of cells for each group were inoculated into the upper Transwell chamber, the chamber was placed on a 24-well plate, and the lower chamber was added to the culture medium. After 24-h incubation, the upper chamber Matrigel and cells were wiped off with a cotton swab, and the membrane was fixed with methanol for 10 min. The cells in the chamber were observed under an inverted phase contrast microscope coupled with a camera (×200). Five fields of view were randomly selected to count the number of penetrating cells.

### Table 1. Antibodies’ information.

| Gene       | Product number |
|------------|---------------|
| MMP-2      | SAB5701187    |
| MMP-9      | SAB5700152    |
| NM23       | SAB5402009    |
| N-cadherin | SAB5701190    |
| E-cadherin | SAB5700789    |
| Twist      | SAB571071     |
| Zeb1       | SAB5701068    |
| Vimentin   | SAB5701293    |
| LAT52      | SAB5701812    |
| β-actin antibodies | A1978 |

### Table 2. Sequences of each primer.

| Gene       | Forward primer (5’-3’) | Reverse primer (5’-3’) |
|------------|------------------------|------------------------|
| LINC01088  | CCTGGCTATCTGGAGTTTCC   | TCATATCGACCGGGAAGTTTA  |
| miR-95     | CTCTTCTCTGTTGCTATCTAG  | AGTATGGCATCCTCTGGCCTC  |
| MMP-2      | AGAAGGCTGTGGTCTCTGGA   | AAAGGACCGGTCTACTGGCT   |
| MMP-9      | GGCACATAGGGCCCTTTAA    | TCACATCGCTCTCCAGCTCA   |
| NM23       | TTGACGCTACCTCCTATGATC  | TTTGCATCTCATACAGATCTC  |
| N-Cadherin | ATGAAAGACCCATCAGCG     | CCTGCTACACCCTCACTA    |
| E-Cadherin | GCTCCTCTGGCTTCTCTCG    | CCCGCTCTCCTTCTACTAG   |
| Vimentin   | GCAGACGCTAACAAACGACA   | AATCCCATCACCACACAGC   |
| Twist      | GCTCAGCTACGCTTCTCG     | GTACCCGTTCTGCTGATC   |
| Zeb1       | TAGTTGCTCCCTGTGAGTTAC  | GCATCGATCGGCTCTCCAGCTG|
| β-actin    | CATCCATCCCATGAGTACCCC  | AGCTCGATGCAATCAGCTAG  |
| snRNA U6   | GCTTCCGACGCA-CATATATCTAAA | GGCTGAGAATC-GAATTCCA |

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Western-blot detection of the target protein

After protein quantification using the BCA method, 12% polyacrylamide gel electrophoresis (PAGE) and electro-transferred to poly (vinylidene fluoride) (PVDF) membranes were used to separate the protein extracted from each group of samples. After transfer, membranes were blocked with 5% skim milk powder prepared with TBS containing 0.1% Triton X-100 at RT for 1 h, and incubated with diluted target molecule primary antibodies or β-actin (internal reference) primary antibody at 4°C overnight; then, the membranes were washed three times with TBST before being treated for 1 hour at room temperature with the matching horseradish peroxidase-labeled secondary antibodies. Chemiluminescence color development was used to visualize the bands, which were scanned for integrated absorbance analysis.

Double luciferase reporter gene analysis

The bioinformatics software (StarBase v2.0, miRcode, and RNAhybrid) was used to predicate the potential binding sites of LINC01088, and miR-95–5p primers were designed based on the prediction results. To create the reporter vector LINC01088-Wt, the amplified segment was introduced into the luciferase reporter gene plasmid. The predicted fragment of miR-95–5p was modified to establish the mutant reporter vector LINC01088-Mut. The wild-type reporter vector, mutant reporter vector, miR-95–5p mimic, and NC-mimic were cotransfected into GC BGC823 cells using Lipofectamine™ 2000. The cells were harvested 48 h after transfection, and luciferase activity was measured for statistical purposes. The same method was used to verify the regulatory relationship with LATS2.

Statistical analysis

Research data are expressed as x ± s. The analysis of variance (ANOVA) and Dunnett’s t-test was used the SPSS 19.0 software. p < .05 was considered statistically significant.

Results

The expression of LINC01088 and miR-95 in GC tissue and the bioinformatics analysis results

The qRT-PCR results revealed that LINC01088 expression was considerably lower in human GC tissue than in paracancerous tissue (p < .01) (Figure 1(a)). Human GC tissue
has considerably higher miR-95 expression than paracancerous tissue ($p < .01$) (Figure 1(b)). The functional analysis of LINC01088 indicated that LINC01088 might directly regulate miR-95 expression (Figure 1(c)). According to the bioinformatics findings, LINC01088 expression in human GC tissue was much lower than in paracrine tissue (Figure 1(d)). Correlation analysis indicated that LINC01088 was coexpressed with LATS2 in GC, presumably as a ceRNA (Figure 1(e)).

Expression of LINC01088 and miR-95 in cell lines

From Figure 2, we found that the LINC01088 expression in GES-1 cells was higher than that in GC cell lines; LINC01088 expression was the lowest in the GC cell line BGC823 ($p < .01$) (Figure 2(a)). MiR-95 expression was higher in GC cell lines than in GES-1 cells, according to the qRT-PCR data; miR-95 expression was highest in the GC cell line BGC823 ($p < .01$) (Figure 2(b)).

The regulatory relationship between LINC01088 and miR-95 in BGC823 cells

The qRT-PCR results showed that when LINC01088 was overexpressed in BGC823 cells, LINC01088 levels rose considerably ($p < .001$) (Figure 3(a)). The qRT-PCR results indicated that after LINC01088 overexpression in BGC823 cells, miR-95 significantly decreased in BGC823 cells ($p < .05$) (Figure 3(b)). The analysis of dual-luciferase reporter genes showed that LINC01088 directly regulated the expression of miR-95, a finding that was consistent with the bioinformatics results (Figures 3(c) and (d)).

LINC01088 inhibition on the viability, invasion, and migration of BGC823 cells

The MTT assay results indicated that after LINC01088 overexpression in BGC823 cells, cell viability significantly decreased ($p < .05$) (Figure 4(a)). After LINC01088 overexpression in BGC823 cells, the invasion and migration abilities of BGC823 cells significantly decreased ($p < .05$) (Figures 4(b) and (c)). After LINC01088 overexpression in BGC823 cells, invasion- and migration-related genes and proteins changed significantly ($p < .01$) (Figures 4(d) and (e)).

miR-95 overexpression of on LATS2 expression in MKN28 cells

The target gene binding site sequences predicted that a miR-95 binding site was present in the 3'UTR of LATS2 mRNA (Figure 5(a)). After transfection of miR-95 mimics into MKN28 cells, miR-95 expression rose considerably ($p < .01$) (Figure 5(b)). LATS2 mRNA and protein expression significantly decreased after miR-95 mimic transfection ($p < .01$) (Figure 5(c)). The analysis of dual-luciferase reporter genes indicated that miR-95–5p had a direct regulatory effect on LATS2 mRNA ($p < .05$) (Figure 5(d)).

miR-95 inhibition on LATS2 expression in BGC823 cells

After miR-95 inhibitor transfection, the expression of miR-95-5p in BGC823 cells fell considerably, according to qRT-PCR data ($p < .01$) (Figure 6(a)). After miR-95 inhibitor transfection, LATS2 mRNA and protein expression in BGC823 cells rose considerably ($p < .05$) (Figure 6(b)). The activity of BGC823 cells significantly decreased after miR-95 inhibitor transfection ($p < .05$) (Figure 6(c)). After miR-95 inhibitor transfection, BGC823 cells' invasion and migratory activities were reduced ($p < .05$) (Figures 6(d) and (e)). After BGC823 cells were transfected with miR-95 inhibitor, the mRNA and protein expression levels of genes involved in cell invasion and migration altered ($p < .01$) (Figures 6(f) and (g)).
LATS2 overexpression on the viability, invasion, and migration of BGC823 cells

The results indicated that LATS2 mRNA and the relevant protein expression in BGC823 cells significantly increased after LATS2 was overexpressed ($p < .01$) (Figure 7(a)). The activity of BGC823 cells significantly decreased after LATS2 was overexpressed ($p < .05$) (Figure 7(b)). Cell invasion and migration activity decreased after LATS2 overexpression in BGC823 cells ($p < .05$) (Figures 7(c) and (d)). After LATS2 overexpression in BGC823 cells, the levels of mRNA and protein expression of genes involved in cell invasion and migration altered ($p < .01$) (Figures 7(e) and (f)).

Discussion

In China and across the world, GC is a malignant tumor with a high morbidity and fatality rate. Although significant advances have been achieved in the complete diagnosis and treatment of GC, overall therapy effectiveness for GC remains poor. When most GC patients are diagnosed, they are already at an advanced stage of the disease, and surgery will not be able to cure them totally. The effects of traditional adjuvant treatment measures such as chemotherapy and radiotherapy are also limited. Some targeted medications and immunological drugs, such as programmed death 1 (PD-1) and programmed cell death ligand 1 (PD-L1), have recently been used in clinical trials to treat GC, with promising results, but the comprehensive treatment effect is still not satisfactory. Therefore, the screening and identification of novel molecules closely related to GC progression significantly improve clinical treatment effects.

There is increasing evidence that lncRNAs play key roles in human diseases and cell development. The dysregulation of these lncRNAs can lead to the occurrence and progression of tumors. In the bioinformatics analysis, we identified a GC-associated lncRNA, LINC01088, reduced expression in GC tissues and cell lines. Our analysis of clinical revealed that LINC01088 expression was lower in GC tissue and cell lines, which matched the bioinformatics findings. Overexpression of LINC01088 decreased cell...
proliferation, invasion, and metastasis. These findings suggest that LINC01088 can prevent the development of GC.

Studies have shown that the ceRNA mechanism may be an important means by which lncRNAs exert their functions. lncRNAs can affect the expression levels of free miRNAs through competitive binding to common miRNAs and further affect the mRNAs regulated by miRNAs. In studies of GC, the ceRNA mechanism has been confirmed as the role played by some lncRNAs. In this study, we used bioinformatics techniques to determine that LINC01088 might form a ceRNA pair with LATS2 mRNA and that LINC01088 might play a role by competing with endogenous miRNAs. MiR-95-5p was shown to be a competitive target of LINC01088 after further bioinformatics study and luciferase reporter gene discovery. Studies have found that miR-95 promotes osteosarcoma progression and is involved in GC processes. However, its specific relationship with GC is still unclear. In this study, by inhibiting or overexpressing LINC01088 in GC cell lines, we found that miR-95-5p expression exhibited opposite changes. Because LINC01088 showed anticarcinogenic effects in GC cells in part...
through miR-95-5p, our findings demonstrated the importance of the connection between LINC01088 and miR-95-5p in carcinogenesis.

Ordinarily, lncRNAs function through ceRNAs that inhibit miRNA targets. Using an online prediction database, we revealed that LATS2 is a potential target of miR-95-5p. We used luciferase reporter gene assays to establish that LATS2 is a direct target of miR-95-5p and that miR-95-5p targets and binds LATS2 mRNA at its 3′ UTR. Furthermore, a miR-95-5p mimic reduced LATS2 mRNA and protein expression, whereas a miR-95-5p inhibitor boosted LATS2 mRNA and protein expression. LATS2 (large tumor suppressor kinase 2) is an upstream regulator of the Hippo pathway, which plays an important role in the formation, growth, and differentiation of cells, tissues, and organs by regulating cell proliferation and apoptosis and the renewal of stem cells. According to studies, the LATS2 gene is located at chromosome 13q12.11 and has the tumor suppressor gene functions; its expression is downregulated in lung cancer, prostate cancer, and GC, and has an inhibitory influence on tumor development. LATS2 expression was discovered to be decreased in GC

Figure 5. The effect of overexpression of miR-95 on the expression of LATS2 in MKN28 cell line. A The predicted results of target gene binding site sequence analysis show that there is a miR-95 binding site in the 3′UTR region of LATS2 mRNA. B The expression of miR-95 was significantly increased after transfection of miR-95 mimics in MKN28 cells (p < .01). C The mRNA and protein expression of LATS2 was significantly reduced after miR-95 mimics transfection (p < .01). D Analysis of dual-luciferase reporter genes showed that miR-95-5p has a direct regulatory effect on LATS2 mRNA (p < .05).
Figure 6. The effect of inhibiting miR-95 on the expression of LAST2 in BGC823 cell line. A The results of qRT-PCR showed that the expression of miR-95-5p in BGC823 cells was significantly reduced after miR-95 inhibitor was transfected into BGC823 cells (p < .01). B The expression of LAST mRNA and protein in BGC823 cells was significantly increased after miR-95 inhibitor was transfected (p < .05). C MTT results showed that the activity of BGC823 cells was significantly reduced after miR-95 inhibitor was transfected (p < .05). D, E After BGC823 cells were transfected with miR-95 inhibitor, the cell invasion and migration activity was weakened (p < .05). F, G The mRNA and protein expression of genes related to cell invasion and migration changed after BGC823 cells were transfected with miR-95 inhibitor ("p < .05, ""p < .01).
Figure 7. Overexpression of LAST2 in the BGC823 cell line affects the viability, invasion and migration of BGC823 cells. A The results of qRT-PCR and Western blot showed that the expression of LAST2 mRNA and protein in BGC823 cells was significantly increased after LAST2 was overexpressed ($p < .01$). B After overexpression of LAST2 in BGC823 cells, the cell activity was significantly reduced ($p < .05$). C, D The cell invasion and migration activity was weakened after LAST2 was overexpressed in BGC823 cells ($p < .05$). E, F The mRNA and protein expressions of genes related to cell invasion and migration changed after overexpression of LAST2 in BGC823 cells ($^{*}p < .05$, $^{**}p < .01$).
cells, and LATS2 overexpression in cells prevented cell invasion and migration and altered the expression of associated genes and proteins. These findings suggest that the LATS2 gene, as a tumor suppressor gene, has a strong inhibitory effect on GC cell invasion; however, the specific mechanism of action still needs to be explored in depth.

Conclusion
This study found that LINC01088 is a kind of lncRNA with tumor suppressor function and has a target relationship with miR-95-5p. LINC01088 is downregulated in gastric cancer and has a tumor growth-inhibiting effect by regulating the miR-95-5p/LATS2 pathway, which may better understand the function of the lncRNA–miRNA–mRNA ceRNA network of LINC01088/miR-95-5p/LATS2 in the growth of gastric cancer. This work was the first to describe the role of LINC01088 in the development of gastric cancer and the molecular mechanism by which miR-95-5p regulates LATS2.

Limitations
1. Although the relationship between the three molecules we studied, most of them were at the transcriptional level, and the research on directly regulated proteins was relatively insufficient. Besides, the practical application value of this study still needed large-scale clinical validation.
2. The clinical sample size of this study is small, and statistical methods are not used to predict before the study, which needs to be further improved in future research.

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Informed consent
Before the research, written informed consent was obtained from subjects in our research.

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# Appendix

## Notation

| Abbreviations | Definition |
|---------------|------------|
| LncRNAs       | Long non-coding RNAs |
| ceRNA         | competing endogenous RNAs |
| MTT           | (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| qRT-PCR       | quantification real-time polymerase chain reaction |
| RT-PCR        | reverse transcription PC |
| LATS2         | large tumor suppressor kinase 2 |
| GC            | gastric cancer |
| TCGA          | The Cancer Genome Atlas |
| GDC           | Genomic Data Commons |
| DEGs          | differentially expressed genes |
| MMP-2         | matrix metalloproteinase-2 |
| EDTA          | ethylenediaminetetraacetic acid |
| DMSO          | dimethyl sulfoxide; |
| OD            | Absorbance |
| PBS           | phosphate-buffered saline; |
| UV            | ultraviolet; |
| PAGE          | polyacrylamide gel electrophoresis |
| PVDF          | vinylidene fluoride; |
| TBST          | TBS containing 0.1% Triton X-100 |
| PD-1          | programmed death 1 |
| PD-L1         | programmed cell death ligand 1 |
| miRNA         | microRNA |