

LncRNA SNHG7 promotes development of breast cancer by regulating microRNA-186

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Abstract. – **OBJECTIVE:** To elucidate the biological functions of long non-coding RNA (lncRNA) SNHG7 in breast cancer (BC), and its underlying mechanism in the occurrence and progression of BC.

PATIENTS AND METHODS: The expression of SNHG7 in 72 pairs of BC tissues and paracancerous tissues was detected by quantitative Real-time polymerase chain reaction (qRT-PCR). Correlation between SNHG7 expressions and pathological indicators of BC patients was analyzed. Similarly, SNHG7 expression in BC cell lines was determined by qRT-PCR as well. After constructing the small interfering RNA of SNHG7, cell proliferation, migration and invasion were determined by cell counting kit-8 (CCK-8), 5-Ethynyl-2'-deoxyuridine (EdU) incorporation assay. MicroRNA-186 expression in BC tissues and cells was assessed. Dual-luciferase reporter gene assay was conducted to verify the binding condition between SNHG7 and microRNA-186.

RESULTS: SNHG7 expression was higher in BC tissues than that of paracancerous tissues. High expression of SNHG7 was positively correlated to tumor stage, lymph node metastasis and distant metastasis, whereas not correlated to age, sex and tumor location of BC. Kaplan-Meier survival analysis revealed that higher expression of SNHG7 is correlated to the worse prognosis of BC patients. SNHG7 was highly expressed in BC cells as well. Knockdown of SNHG7 inhibited proliferative, invasive and migratory abilities of BC cells. QRT-PCR data showed that microRNA-186 is lowly expressed in BC tissues compared with that of paracancerous tissues. MicroRNA-186 was lowly expressed in BC cells as well. Both mRNA and protein levels of microRNA-186 were negatively correlated to SNHG7 in BC tissues. Finally, the dual-luciferase reporter gene assay demonstrated that SNHG7 could be directly targeted by microRNA-186.

CONCLUSION: SNHG7 is highly expressed in BC, which is correlated to tumor stage, lymph node metastasis and distant metastasis of BC patients. SNHG7 could promote malignant progression of BC by regulating microRNA-186.

Key Words:

SNHG7, MicroRNA-186, BC, Malignant progression.

Introduction

Breast cancer (BC) is a common malignant tumor in women and the leading cause of tumor death in women¹⁻³. GLOBOCAN reported that the incidence and mortality of BC in China account for 12.2% and 9.6% of the world, respectively⁴⁻⁶. With the improvement of living standards and lifestyle changes, the incidence rate of BC rises year by year and tends to occur early. In China, the incidence and mortality of BC are 21.6/100,000 and 5.7/100,100, respectively^{4,6}. Early diagnosis of BC helps to achieve the satisfactory therapeutic effect. However, the onset of BC is insidious, and pathological change in early stage is often atypical hyperplasia. Some BC patients are already in the advanced stage at the time of diagnosis. The different degrees of invasion and metastasis of BC severely restrict the therapeutic efficiency, posing great pain and economic burden to affected patients and their families^{1,7-9}. Therefore, it is particularly important to explore the pathogenesis of BC and search for specific biomarkers^{10,11}. Currently, mammography and detection of serological tumor markers such as CA153, CA27, CA29, CA549, CEA, BRCA1, BRCA2 and HER-2, are the main methods for early screening and clinical monitor-

ing of BC. However, mammography is not very popular in developing countries due to its high expense. Detection of serological tumor markers lacks of sufficient diagnostic sensitivity and specificity¹²⁻¹⁴. Recent studies found stable expressions of circulating nucleic acids (cfCNA), such as cell-free DNA, messenger RNA (mRNA) and long non-coding RNA (lncRNA) in blood, urine and cerebrospinal fluid. They are closely related to the occurrence and development of diseases, especially in tumors, and exert advantages of non-invasive diagnosis¹⁵⁻¹⁷. As non-invasive hallmarks for BC, lncRNAs have shown high clinical value and prospects¹⁸. Besides, non-coding RNAs are extensively reported in the pathological progression of BC¹⁵. Whether these non-coding RNAs could be served as non-invasive hallmarks deserves further explorations. LncRNA is a new type of RNA containing about 200-200,000 nucleotides without the function of encoding protein. LncRNA regulates gene expressions at epigenetic, transcriptional and post-transcriptional levels, and participates in various physiological processes by interacting with proteins and nucleic acids^{19,20}. A great number of studies²⁰⁻²² have shown that lncRNA is differentially expressed in a variety of cancer cells and exerts an important role in the initiation, development, invasion and metastasis of cancer. The processes of development and metastasis of BC involve multiple genes and factors, including microRNAs and lncRNAs¹⁵⁻¹⁸. Among them, lncRNA HOTAIR specifically promotes the metastasis of BC and is regarded as a milestone in researches on lncRNA regulation of BC^{23,24}. Regulatory effects of lncRNAs on BC vary a lot. Clarifying the molecular mechanisms of BC development and metastasis helps to establish reasonable prevention and treatment programs, improving long-term survival of BC patients¹⁸. LncRNA SNHG7 is overexpressed in a variety of tumors, and could promote tumor invasion and metastasis. Its expression closely correlates with tumor stage, pathological grade and prognosis of some tumors²⁵⁻²⁷. However, its role in BC has not been reported yet. Changes in microRNA expressions affect tumorigenesis, and are also closely related to tumor invasion and metastasis²¹. At present, more than 40 microRNAs have been found to be involved in the regulation of tumor metastasis. MicroRNA can affect the connection of tumor cells by regulating expressions of specific genes, which in turn affects the metastasis and infiltration of tumor cells^{28,29}. Functionally, microRNAs could be served as oncogenes or tumor-suppres-

sor genes in multiple tumors^{29,30}. In this study, we aim to elucidate whether lncRNA SNHG7 could regulate invasion and migration of BC through mediating microRNA-186, so as to provide experimental basis for clinical treatment of BC.

Patients and Methods

Patients and BC Samples

72 BC patients diagnosed in our hospital were enrolled for collecting BC tissues and paraffin-embedded tissues. Samples were preserved at -80°C. Informed consent was signed from enrolled patients and their families. This study has been approved by the Ethics Committee of China Xiang'an Union Hospital.

Cell Lines and Reagents

BC cell lines (MCF-7, MDA-MB-231 and SKBR3) and normal mammary epithelial cell line (Hs578T) were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). MCF-7 and MDA-MB-231 cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Rockville, MD, USA). SKBR3 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) (HyClone, South Logan, UT, USA) containing 10% FBS. All cells were maintained in a 5% CO₂ incubator at 37°C. Cell passage was performed until 80-90% of confluence using 1× trypsin and EDTA (Ethylene Diamine Tetraacetic Acid).

Transfection

We authorized GenePharma (Shanghai, China) to construct negative control (si-NC) and si-SNHG7. Cells were seeded in 6-well plates and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions until 70% of density. Cells were harvested 48 h later for the following experiments.

Cell Counting Kit-8 (CCK-8)

Transfected BC cells for 48 h were seeded in the 96-well plate with 2000 cells per well. After cell culture for 6 h, 24 h, 48 h and 72 h, respectively, CCK-8 (Dojindo Laboratories, Kumamoto, Japan) reagent was added in each well. 2 hours later, the optical density (OD) value of each well was measured using a microplate reader.

5-Ethynyl-2'-Deoxyuridine (EdU) Proliferation Assay

Transfected BC cells for 24 h were incubated with 50 μmol EdU for 2 h, followed by staining with Aduro and DAPI. EdU assay was performed following the manufacturer's instructions (RiboBio, Nanjing, China). EdU-positive cells were observed and captured using a fluorescence microscope.

Transwell Assay

Transfected BC cells for 48 h were digested and resuspended in serum-free medium. Cell density was adjusted to $5.0 \times 10^5/\text{mL}$. Transwell chamber containing matrigel or not was placed in a 24-well plate. 200 μL of the cell suspension containing 1.0×10^5 cells was added to the upper chamber. 700 μL of medium containing 20% FBS was added to the lower chamber. 48 hours later, cells were fixed with 4% paraformaldehyde for 15 min after removal of the chamber. Cells were stained with 0.2% crystal violet for 20 min. The inner layer cells were carefully removed. 10 randomly selected fields of each sample were captured for calculating the amount of penetrating cells.

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

Total RNA was extracted from BC cell lines and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and RNA was reverse transcribed into cDNA using PrimeScript RT reagent (TaKaRa, Otsu, Shiga, Japan). qPCR was performed using SYBR[®] Premix Ex Taq[™] (TaKaRa, Otsu, Shiga, Japan), and Step One Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Primers used in this study was as follows: SNHG7, forward: 5'-TAGGGGACTGGGCTG-3', reverse: 5'-GGGTCTTAGGTTCCGACCA-3'; MicroRNA-186, forward: 5'-GCCCCAATGAAATTTTGTG-3', reverse: 5'-GGCTTCCGAACTGCGTGTCAT-3'; SNHG7, forward: 5'-CTCCATCCTGGCCTC-3', reverse: 5'-GCTGTCACCTTCACCGT-3'. Relative expression levels of mRNAs were calculated using the $2^{-\Delta\Delta C_t}$ method and analyzed by ABI Step One software (Applied Biosystems, Foster City, CA, USA).

Dual-Luciferase Reporter Gene Assay

SNHG7 sequence was cloned into the luciferase reporter plasmid pmirGLO and the mutant vector pmirGLO-SNHG7-mut (microRNA-186) was constructed. MCF-7 and SKBR3 cells were co-transfected with pmirGLO-SNHG7 and pmirGLO-SN-

HG7-mut (microRNA-186) or pmirGLO. Luciferase activity was determined followed by the manufacturer's instructions (Promega, Madison, WI, USA).

Statistical Analysis

GraphPad Prism 6 V6.01 software (La Jolla, CA, USA) was used for data analyses. Data were expressed as mean $\pm$ standard deviation. Differences between the two groups were analyzed using the *t*-test. One-way ANOVA was conducted to analyze differences among multiple groups, followed by post-hoc test. Kaplan-Meier method was used to calculate the survival time of BC patients, and the difference between different curves was compared by log-rank test. $P < 0.05$ was considered to be statistically significant.

Results

SNHG7 Was Highly Expressed in BC

To determine the role of SNHG7 in BC, we collected 72 pairs of BC tissues and paracancerous tissues. The expression of SNHG7 in BC tissues and paracancerous tissues was detected by qRT-PCR. The results showed that SNHG7 expression was higher in BC tissues than that of paracancerous tissues (Figure 1A, 1B). We speculated that SNHG7 may serve as an oncogene in BC. Meanwhile, we also detected SNHG7 expression in BC cell lines. Among them, SNHG7 was highly expressed in MCF-7 and SKBR3 cell lines (Figure 1C). Therefore, MCF-7 and SKBR3 cell lines were selected for subsequent experiments.

SNHG7 Expression was Correlated With Clinical Stage, Lymph Node Metastasis, Distant Metastasis And Overall Survival in BC Patients

Enrolled BC patients were assigned into two groups based on their expression level of SNHG7. The correlation between SNHG7 expression with age, sex, tumor location, tumor stage, lymph node metastasis and distant metastasis was analyzed. It is indicated that high expression of SNHG7 is positively correlated to tumor stage, lymph node metastasis and distant metastasis, whereas not correlated to age, sex and tumor location of BC (Table I). Additionally, follow-up data were collected for survival analyses. Kaplan-Meier curves revealed that higher expression of SNHG7 is correlated to worse prognosis of BC patients (Figure 1D). We considered that SNHG7 may serve as a predictable hallmark for BC.

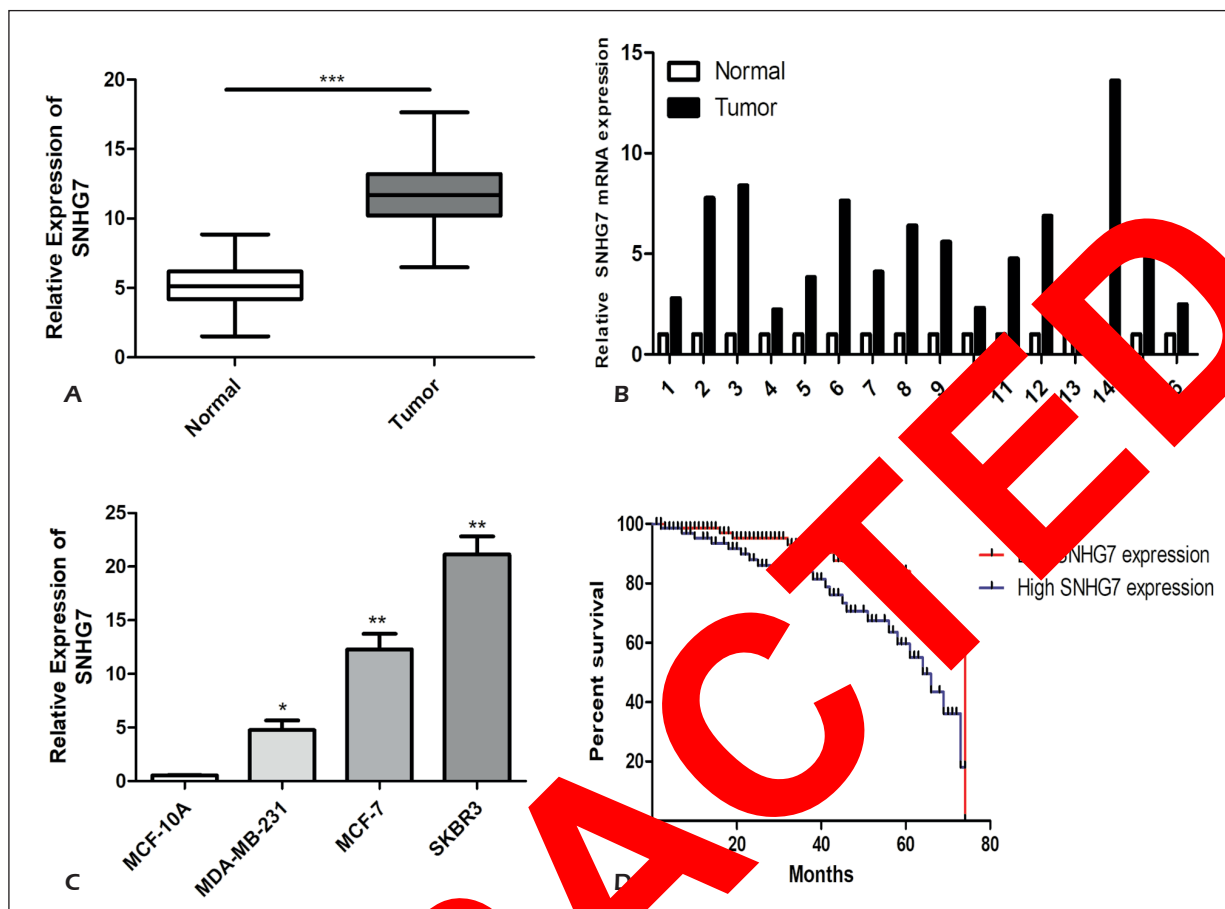


Figure 1. SNHG7 was highly expressed in BC tissues. **A**, Expression level of SNHG7 in BC tissues and paracancerous tissues. **B**, Expression level of SNHG7 in BC tissues. **C**, Expression level of SNHG7 in BC cell lines. **D**, Survival curves of SNHG7 expression in BC patients. Data were expressed as mean $\pm$ SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table I. Association of SNHG7 expression with clinicopathologic characteristics of breast cancer.

Parameter	Number of cases	SNHG7 expression		p-value
		Low (%)	High (%)	
Age (years)				0.355
< 60	30	19	11	
≥ 60	42	22	20	
Gender				0.180
Male	35	23	12	
Female	37	18	18	
Stage				0.014
T1-2	41	29	12	
T3-4	31	13	18	
Lymph node metastasis				0.028
No	43	29	14	
Yes	29	12	17	
Distance metastasis				0.041
No	58	38	20	
Yes	14	5	9	

Knockdown of SNHG7 Inhibited Proliferation of BC Cells

To investigate the biological function of SNHG7 in BC, we constructed a small interfering RNA for SNHG7. After transfection of si-SNHG7 in MCF-7 and SKBR3 cell lines, qRT-PCR assay was performed to verify the interference efficiency. Significant difference in SNHG7 expression was found between BC cells transfected with si-SNHG7 or si-NC ($p < 0.05$, Figure 2A, 2B). The cell growth curve was detected by CCK-8 assay after interference with SNHG7 in MCF-7 and SKBR3 cell lines. CCK-8 results showed that the proliferative rate of BC cells markedly decreases after SNHG7 knockdown ($p < 0.05$, Figure 2C, 2D). EdU assay obtained the similar results (Figure 2E), indicating that SNHG7 has a significant effect on proliferation of BC cells.

Knockdown of SNHG7 Inhibited Migration and Invasion of BC Cells

After transfecting si-SNHG7 in BC cells, transwell assay was conducted to detect migratory and invasive abilities. It is shown that cell migratory and invasive abilities markedly decrease after interference with SNHG7 in MCF-7 and SKBR3 cell lines ($p < 0.05$, Figure 3). We concluded that SNHG7 exerts a regulatory effect on migration and invasion of BC cells.

SNHG7 Modulated microRNA-186 Expression in BC

Subsequently, we further explored how SNHG7 promoted malignant progression of BC. Through bioinformatics analysis, we found that SNHG7 may interact with microRNA-186. qRT-PCR data showed that microRNA-186 is lowly expressed in BC tissues compared with that of paracancerous tissues (Figure 4A). Similarly, microRNA-186 was also lowly expressed in BC cell lines (Figure 4B). We then elucidated whether there was an interaction between microRNA-186 and SNHG7 in BC. MicroRNA-186 expression was remarkably upregulated in BC cells after SNHG7 knockdown (Figure 4C). To further verify their interaction, we detected expression levels of microRNA-186 and SNHG7 in 72 pairs of BC tissues by qRT-PCR. Negative correlation between microRNA-186 and SNHG7 was found in BC tissues at both mRNA and protein levels (Figure 4D).

Direct Target Effect of microRNA-186 on SNHG7

Since there was a negative interaction between SNHG7 and microRNA-186, we speculated that a

direct target may exist between them. We cloned the SNHG7 sequence into the luciferase reporter plasmid pmirGLO and constructed the mutant vector pmirGLO-SNHG7-mut (microRNA-186). MCF-7 and SKBR3 cells were co-transfected with pmirGLO-SNHG7 and pmirGLO-SNHG7-mut (microRNA-186) or pmirGLO. Dual-luciferase reporter gene assay revealed that overexpression of microRNA-186 markedly decreased the luciferase activity in cells transfected with wild-type SNHG7 vector, whereas no significant difference was found in those transfected with mutant-type or empty vector (Figure 5). The above results demonstrated that SNHG7 could be directly targeted by microRNA-186.

Discussion

BC is a malignant tumor with a high incidence and poor prognosis. As a major therapeutic method, chemotherapy exerts a very important role in the treatment of BC¹⁻³. However, tumor patients often experience chemotherapy tolerance or multi-drug resistance^{4,5}. In this case, the altered characteristics of tumor cells lead to less sensitive to chemotherapeutic drugs and, thereafter, affect the long-term prognosis⁷. Hence, it is of great significance and clinical value to explore the mechanism of chemotherapy tolerance in BC⁸. However, the mechanism of chemotherapy tolerance in malignant tumors is very complicated. There have been some research results on the mechanism of drug resistance in tumors, such as altered expressions of ATP-binding cassette transporters, elevated anti-apoptosis, phenotypic transformation, EMT, etc.^{6,8}. However, these findings still could not comprehensively explain the occurrence of drug resistance. It is considered that multiple molecular mechanisms are involved in the chemotherapeutic drug tolerance of malignant tumors, including microRNAs, lncRNAs and various signaling pathways¹⁶⁻¹⁸. Scholars¹⁹⁻²¹ suggested that lncRNAs may be involved in the regulation of tumor cell behaviors, including cell invasiveness and proliferation. The specific roles of lncRNAs in regulating malignant progression of tumors are rarely reported¹⁸. To explore the role of lncRNA in the malignant progression of BC, we analyzed differentially expressed lncRNAs in MCF-7 cells using bioinformatics. LncRNA SNHG7 was finally screened out. We showed that SNHG7 knockdown could promote the malignant progression of BC.

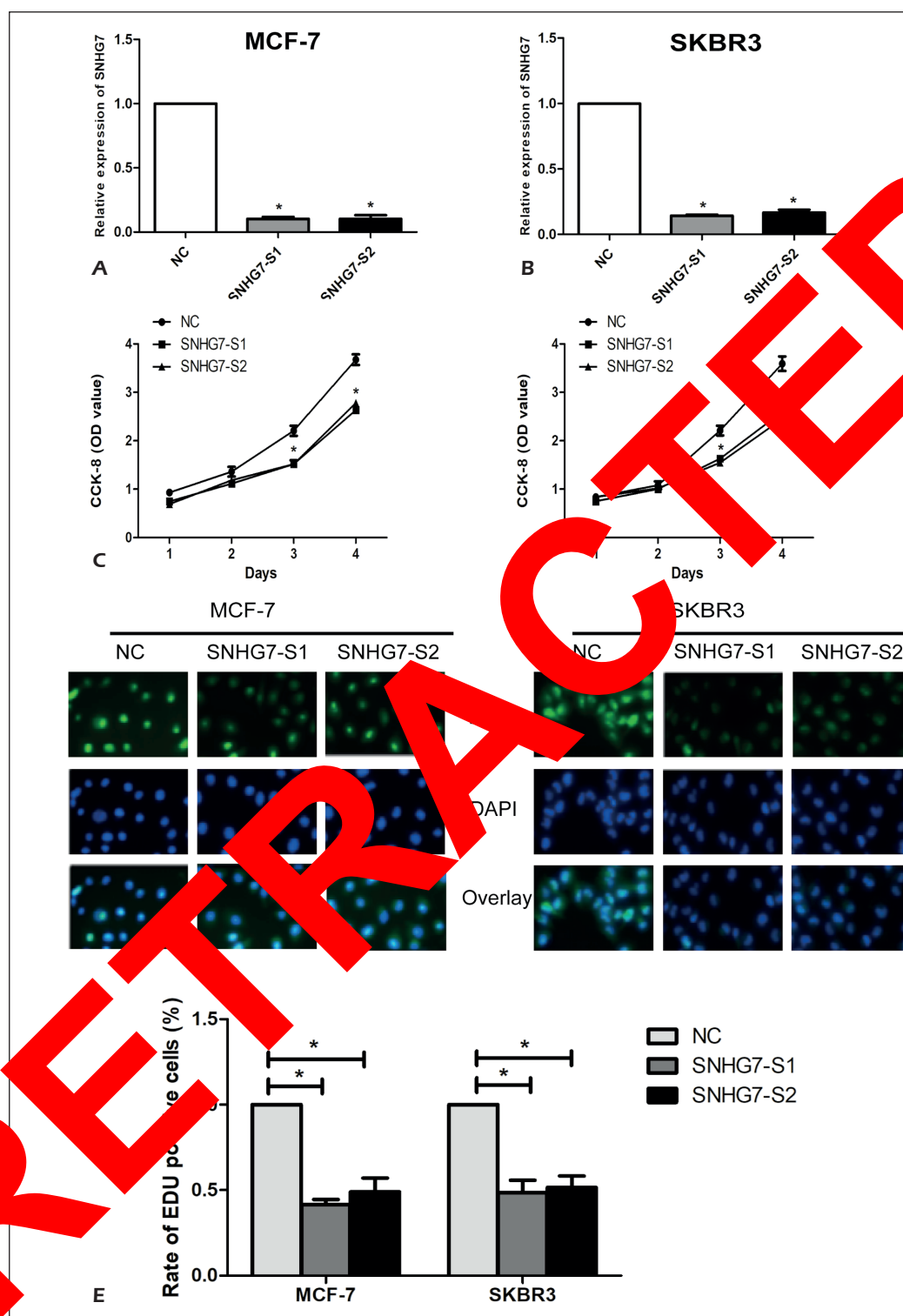


Figure 2. Knockdown of SNHG7 inhibited proliferation of BC cells. **A**, Transfection efficacy of si-SNHG7 in MCF-7 cells. **B**, Transfection efficacy of si-SNHG7 in SKBR3 cells. **C-D**, Proliferative rate of MCF-7 and SKBR3 cells detected by CCK-8 assay after transfection of si-SNHG7. **E**, Proliferative rate of MCF-7 and SKBR3 cells detected by EdU assay after transfection of si-SNHG7. Data were expressed as mean $\pm$ SD, * p <0.05, ** p <0.01, *** p <0.001.

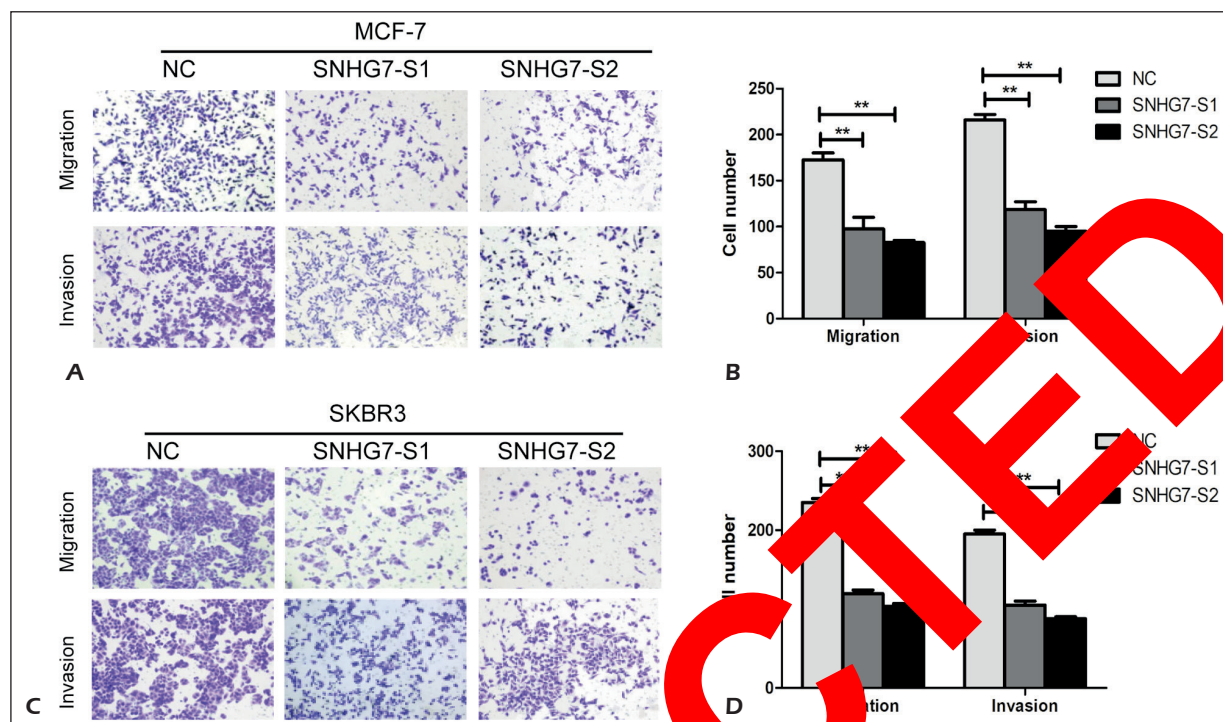


Figure 3. Knockdown of SNHG7 inhibited migration and invasion of MCF-7 cells after transfection of si-SNHG7. **C-D**, Invasive and migratory abilities of SKBR3 cells after transfection of si-SNHG7. Data were expressed as mean $\pm$ SD, * p <0.05, ** p <0.01, *** p <0.001.

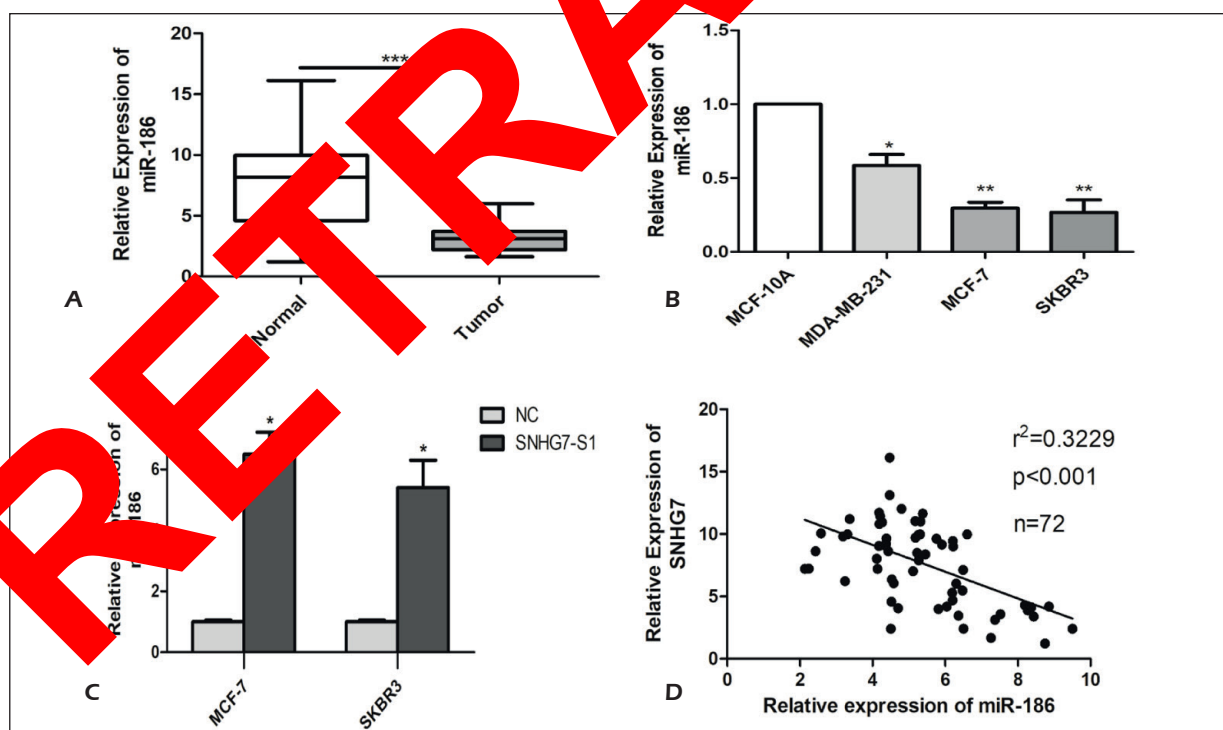


Figure 4. SNHG7 modulated microRNA-186 expression in BC. **A**, MicroRNA-186 expression in BC tissues and paracancerous tissues. **B**, MicroRNA-186 expression in BC cells. **C**, MicroRNA-186 expression in MCF-7 and SKBR3 cells after transfection with si-SNHG7. **D**, Negative correlation between SNHG7 and microRNA-186 in BC. Data were expressed as mean $\pm$ SD, * p <0.05, ** p <0.01, *** p <0.001.

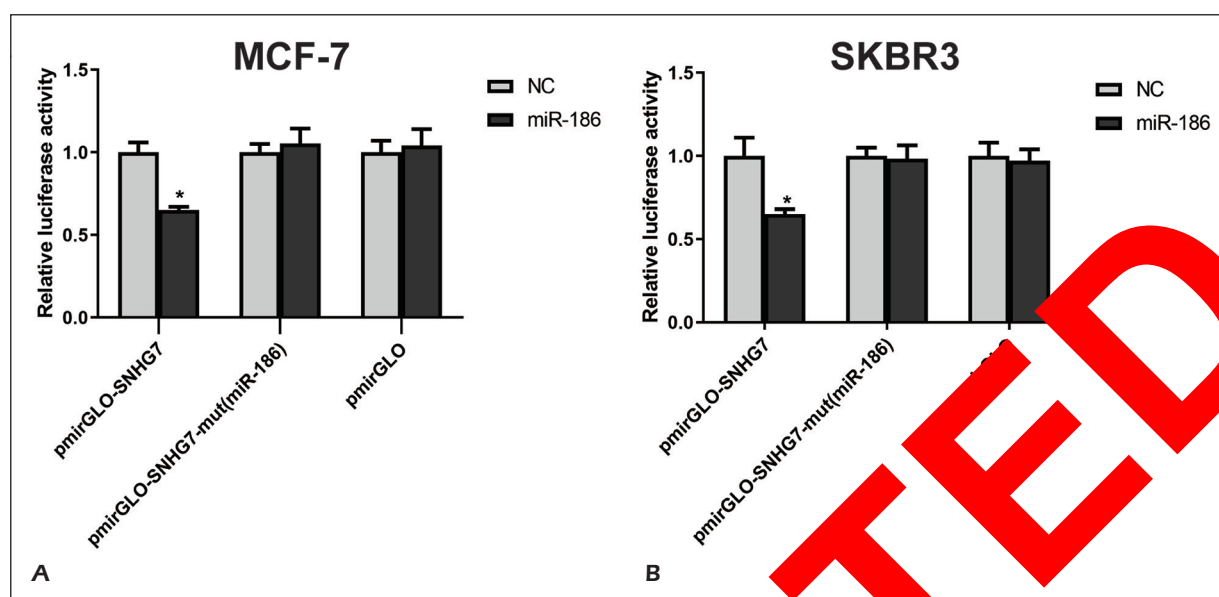


Figure 5. Direct target effect of microRNA-186 on SNHG7. **A**, Luciferase activity in MCF-7 cells. **B**, Luciferase activity in SKBR3 cells. Data were expressed as mean $\pm$ SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In addition to about 21,000 mRNAs that could encode proteins, there are 10,000-32,000 ncRNAs, 11,000 pseudogenes and 9,000 miRNAs in the human transcript. According to the length of non-coding RNAs, they are divided into short non-coding RNAs with less than 200 nt (miRNA and transfer RNA) and lncRNAs that are over 200 nt in length. LncRNAs are involved in various biological processes, including chromatin organization, transcriptional regulation, mRNA function regulation, mRNA stability, and protein function regulation^{14,15,18,19}. LncRNAs play a key role in cell development, abnormal expression of lncRNAs could be crucial in tumorigenesis¹⁸. Previous researches²⁵⁻²⁷ have shown that lncRNA SNHG7 is highly expressed in osteosarcoma, bladder cancer, and cervical cancer, which is served as an oncogene. In this study, we first examined the expression level of SNHG7 in BC tissue. Highly expressed SNHG7 was positively correlated to tumor size, lymph node metastasis, distant metastasis and poor prognosis of BC. We considered that SNHG7 may promote the malignant progression of BC. Subsequent CCK-8 and transwell assay indicated that SNHG7 markedly increases proliferative and invasive abilities of BC cells, whereas it did not affect the proliferative ability. Competitive endogenous RNA (ceRNA) regulatory mechanisms proposed that there is a complex interaction among mRNA, pseudogene, lncRNA and circRNA. CeRNA contains some common

binding sites of miRNA, and could competitively bind with common miRNAs with promotive or inhibitory effect on tumor development through MREs, thus releasing or reducing the inhibitory effect of miRNAs on other target genes^{31,32}. LncRNAs are served as oncogenes or tumor-suppressor genes. Previous studies^{18,20} have predicted the interaction of microRNA-186 with lncRNA SNHG7 by AGO-CLIP. Our experiments showed that microRNA-186 is lowly expressed in BC tissues. MicroRNA-186 could inhibit angiogenesis and cell invasion in BC cells. In this study, we found that SNHG7 sequence contains a microRNA-186 binding site through bioinformatics method. QRT-PCR and dual-luciferase reporter gene assay demonstrated that SNHG7 could directly bind to downstream of microRNA-186. Besides, SNHG7 vector lacking the mutated microRNA-186 binding site failed to enrich microRNA-186, further validating the binding site of SNHG7 to microRNA-186. In order to investigate the regulatory effects of SNHG7 and microRNA-186 on BC cells, we first transfected si-SNHG7 in BC cells. Knockdown of SNHG7 up-regulated microRNA-186 expression in BC cells. We speculated that the transcriptional activity of the SNHG7 locus may be regulated by microRNA-186, which still requires for further experiments. Our study indicated that lncRNA SNHG7 inhibits the expression of microRNA-186, thereby promoting migration and metastasis of BC cells.

Conclusions

SNHG7 is highly expressed in BC, which is correlated to tumor stage, lymph node metastasis and distant metastasis of BC patients. SNHG7 could promote malignant progression of BC by regulating microRNA-186.

Conflict of Interests:

The authors declared no conflict of interest.

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