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LncRNA GAS5 affects epithelial-mesenchymal transition and invasion of breast cancer cells by regulating miR-216b

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Abstract. – OBJECTIVE: To study the mechanism of lncRNA GAS5 affecting epithelial-mesenchymal transition and invasion of breast cancer cells by regulating miR-216b.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to detect the expressions of GAS5 and miR-216b in breast cancer and paracancerous tissues. The relationship between GAS5 and clinicopathological parameters of breast cancer patients was analyzed. The Dual-Luciferase reporter gene was used to detect the interaction between GAS5 and miR-216b and the transwell invasion assay was used to detect the invasive ability of lung cancer cells after GAS5 inhibition. Apoptosis assay was used to detect the apoptosis of breast cancer cells after GAS5 inhibition. Western blotting and immunofluorescence staining were used to detect the inhibition of GAS5 epithelial-mesenchymal transition.

RESULTS: Compared with paracancerous tissues, in breast cancer tissues, the expression of GAS5 was increased and the expression of miR-216b was decreased. As the patients enter the later stages of breast cancer, the expression level of GAS5 in breast cancer patients was significantly elevated. The expression of GAS5 in the tissues with lymph node metastasis of breast cancer was markedly increased. The inhibition of GAS5 can promote the apoptosis of breast cancer cells; GAS5 can specifically bind to the 3' UTR of miR-216b. The expression of GAS5 inhibited the expression of E-cadherin in breast cancer cells and significantly upregulated N-cadherin, which has been confirmed by immunofluorescence staining experiments.

CONCLUSIONS: GAS5 plays an important role in the development of breast cancer. GAS5 can target on miR-216b to regulate the biological behavior and epithelial-mesenchymal transition of breast cancer cells.

Key Words:
Breast cancer, Epithelial-mesenchymal transition, GAS5, Invasive behavior, MiR-216b.

Introduction

Breast cancer is currently the most common malignant tumor in women. In 2014, there were more than 200,000 newly diagnosed cases, and the estimated annual death toll was about 40,000[1]. The main causes of death are distal metastases and the lack of treatments in late stages[2]. Studies have shown that Erb2/HER2 is amplified and overexpressed in 25%-30% of human breast cancer[3]. In breast cancer subtypes, the prognosis of HER2-positive breast cancer is relatively poor, and the overall survival time is relatively reduced[4,5]. Although chemotherapy combined with trastuzumab significantly improved the prognosis of breast cancer patients and paved the way for the targeted treatment of breast cancer treatment, the duration of the median response was limited[6,7]. Although many key studies have been conducted to determine the association of breast cancer with multiple lncRNAs, little is known about the biological role of long-chain non-coding RNA GAS5 (lncRNA) in the progression of breast cancer.

LncRNA is a class of transcripts longer than 200 nucleotides with extremely limited

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protein coding potential[8]. LncRNAs have multiple functions in a wide range of biological processes, such as proliferation, apoptosis, cell migration, and cell invasion[9,10]. Although lncRNA has been reported to regulate tumor metastasis by mediating TGF-β pre-metastatic action and regulating EMT[11], and several lncRNA transcripts are involved in the carcinogenesis of breast cancer[12], the biological effects of lncRNAs in the treatment of breast cancer have not been well studied.

The abnormal expression of the growth arrest specificity 5 (GAS5) gene has a special role in the breast cancer. In addition to a variety of other non-coding RNAs, GAS5 also encodes other RNAs, including small nucleolar RNA, microRNAs (miRNAs), etc.[13]. LncRNA GAS5 is downregulated in a variety of cancers[14]. In these cancers, clinicopathological features are inversely correlated with lncRNA GAS5 levels, while low levels of lncRNA GAS5 usually indicate poor prognosis[15]. At the cellular level, lncRNA GAS5 promotes growth arrest and/or apoptosis in a variety of cell types, including hormone-sensitive and insensitive breast cancer cells[16,17], which may be responsible for its inhibition of tumors. Therefore, in this study, the level of lncRNA GAS5 expression in breast cancer tissues was downregulated compared to that in adjacent normal tissues. The overexpression of GAS5 in breast cancer cell lines can directly affect the epithelial-mesenchymal transition of breast cancer cells, which may infect the biological behavior of breast cancer cells.

Patients and Methods

Tissue Acquisition and Cell Culture

From August 2018 to May 2019, 50 specimens of tissue samples were collected from the Affiliated Hexian Memorial Hospital of Southern Medical University, including breast cancer tissues and adjacent normal tissues. All samples were immediately stored in liquid nitrogen, and after surgery, stored at -80°C until RNA extraction. All diagnoses were supported by pathological and/or cytological evidence. Patient consent was obtained before thr use of the sample. The study protocol was approved by the Ethics Committee of the Affiliated Hexian Memorial Hospital of Southern Medical University.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from tissues or cells using TRIzol reagent, and cDNA was synthesized using reverse transcriptase (TaKaRa, Otsu, Shiga, Japan) and oligonucleotide (dT) according to the manufacturer's instructions. Real Time-PCR was performed using the SYBR Premix Ex Taq™ II kit (TaKaRa, Otsu, Shiga, Japan). The conditions of the Real Time-PCR were as follows: 94°C for 10 seconds, 94°C for 5 seconds, 52°C for 30 seconds, 72°C for 15 seconds, for 40 cycles. GAS5 primer sequences: 5'-GTGGAGGATCGGATTTTAGCAAACT-3' (forward) and 5'-CCTATGGGATCGGGCAGTG CAAAGT-3' (reverse). MiR-216b primer sequences: 5'-TTGAGCGGAGTCGGTAGGGCAAATCG-3' (forward) and 5'-GCCTATCTTTTAGGGCGAG CA-3' (reverse). U6 primer sequences: 5'-TTGAGCTATCGATGCTGAGCAAATCG-3' (forward) and 5'-GTACTCGAAACTGATCGAG CA-3' (reverse).

Western Blot Analysis

Total protein from cells was extracted using radioimmunoprecipitation assay (RIPA) buffer. Total protein concentration was measured by the BCA protein assay. Samples containing 30 μg of protein were loaded into each lane, separated on a 10% sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS PAGE) gel, and transferred to a nitrocellulose membrane for routine Western blot analysis. Anti-E-cadherin and anti-N-cadherin were used, and GAPDH was used as the internal reference. The membrane was washed and embedded with the corresponding HRP-labeled secondary antibody. Protein signals were detected using a SuperSignal West Pico chemiluminescent substrate, and the intensity of each band was quantified by ImageJ software. The experiment was repeated three times. Liposomes 2000 were purchased from Genechem Group, and Trizol was purchased from Ambion. The PCR kit was purchased from Kapa Biosystems. The luciferase activity detection kit was purchased from Promega Corporation. The luciferase reporter was synthesized by Promega Corporation. Transwell cells were purchased from Millipore, and Matrigel was purchased from Bio-Rad Laboratories.

Dual-Luciferase Assay

The wild type (Wt) and mutant (Mut) 3'-non-transcribed regions (3'-UTR) of miR-216b were designed by biotechnology companies, and cloned into plasmid, called miR-216b-WT and miR-216b-

Mut respectively. For the Luciferase assay, 1 x 10^5 cells were plated and cultured in 12 well plates to achieve approximately 70% confluence. The cells were co-transfected with GAS5 mimetic or NC, and the fluorescent expression activity of the miR-216b Wt/Mut reporter plasmid was examined. The Luciferase assay was performed 48 hours after transfection using the Dual-Luciferase reporter assay kit. The experiment was repeated three times.

Cell Cycle Experiment

Transfected cells were inoculated at 5 x 10^4 / well in 24 well plates. After 24 hours, the cells were further treated with si-GAS5 for 48 hours. Then, the cells were harvested, and the expression ratio of detected cell cycle was changed. Cell cycle data were obtained and analyzed by FACScan flow cytometry and CellQuest software.

Transwell Invasion Experiment

1x10^5 cells were suspended in 200 μL of serum-free Roswell Park Memorial Institute-1640 (RPMI-1640) medium, and then, inoculated into the upper chamber. In order to create a chemoattractant environment in the lower chamber, RPMI-1640 supplemented with 20% fetal bovine serum (FBS) was filled therein. After incubation for 24 hours in a cell culture incubator, the cells on the top surface of the insert were removed. The cells on the bottom surface were fixed with 4% polyoxymethylene, and the number of invading cells was counted after staining with 0.1% crystal violet. The experiment was repeated three times.

Immunofluorescence Experiment

The slides of the cells were immersed in phosphate-buffered saline (PBS) three times for 3 min each time; the slides were fixed with 4% paraformaldehyde for 15 min, and the slides were immersed in PBS 3 times for 3 min each time; 0.5% polyethylene glycol octylphenyl ether (Triton X-100) was permeabilized at room temperature for 20 min; the slide was immersed in PBS 3 times for 3 min each time; the PBS was blotted dry, and normal goat serum was added to the slide, blocked at room temperature for 30 min; the blocking solution was absorbed by blotting paper without washing; enough amount of diluted primary antibody was added to each slide and put into the wet box, incubated at 4°C overnight; fluorescent secondary antibody was added and washed with PBS for 3 times, 3 min each time. The absorbent paper was used to absorb the excess liquid on the slide, and the diluted fluorescent secondary antibody was added dropwise, and the mixture was incubated at 20-37°C for 1 h in a wet box, and immersed in PBS for 3 times for 3 min each time. Since the adding of the secondary antibody, all subsequent steps were carried out in the dark. Re-stain of the nucleus: DAPI was added and incubated in the dark for 5 min, the nucleus of the specimen was stained. The liquid on the slide was blotted with absorbent paper and sealed with a sealing liquid containing anti-fluorescence quencher. The acquired image was then observed under a fluorescence microscope.

Statistical Analysis

Spearman's Rank was used for correlation analysis. Statistical analysis was performed using the Student's *t*-test. Data were expressed as mean ± standard deviation, and $p < 0.05$ was considered statistically significant.

Results

The Expression of GAS5 in Breast Cancer Tissues and Cell Lines

The results of quantitative Real Time-Polymerase Chain Reaction showed that the expression of GAS5 mRNA in breast cancer tissues was higher than that in normal tissues adjacent to the cancer tissue [(1.85±0.19) *vs.* (0.72±0.12), $p<0.05$], and the difference was statistically significant (Figure 1A). The expression level of miR-216b was different in the matched breast cancer and adjacent tissues, and the expression in the normal tissues adjacent to the cancer was relatively high [(2.18±0.21) *vs.* (0.52±0.28), $p<0.05$], with statistically significant difference (Figure 1B). The expressions of GAS5 and miR-216b were opposite in breast cancer tissues.

Relationship Between GAS5 and Clinical Pathological Parameters of Breast Cancer Patients

In this experiment, breast cancer tissues and adjacent normal tissues were compared for clinical pathological parameters. There was no difference in the expression level of GAS5 between breast cancer patients of different ages, and the difference was not statistically significant ($p>0.05$; Table I). In the later stage of breast cancer, the more evident expression level of GAS5 in breast cancer tumor tissues

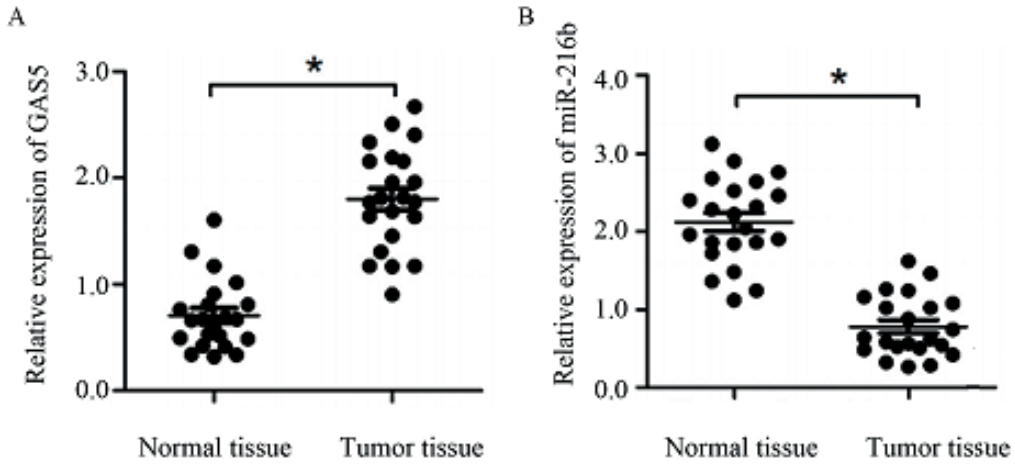

Figure 1. Expressions of GAS5 and miR-216b in breast cancer tissues. **A,** Expressions of GAS5 in breast cancer tissues. **B,** Expressions of miR-216b in breast cancer tissues, *$p<0.05$.

showed a more evident expression of GAS5 in patients with lymph node metastasis of breast cancer, with statistically significant differences ($p<0.05$; Table I).

Dual-Luciferase Assay Detection of the Relationship Between GAS5 and MiR-216b

In this experiment, the possibility of the direct interaction between GAS5 and miR-216b was predicted by bioinformatics prediction. Figure 2A showed that there were similar pairing sequences between the two. Furthermore, Dual-Luciferase reporter gene results showed (Figure 2B) that si-GAS5 can significantly inhibit the Luciferase activity of miR-216b to some extent, and it was concluded that si-GAS5 can specifically bind to the 3'UTR of miR-216b, thereby regulating its expression activity and level.

GAS5 Involvement in the Apoptosis of Breast Cancer Cells

The results of apoptosis experiments showed (Figure 3) that the cell cycle at G0/G1 phase of the si-GAS5 experimental group was significantly higher than that of the control group [(43.2±5.59)% *vs.* (18.3±3.28)%, $p<0.05$]. The difference between the two groups was statistically significant. The cell cycle also reflected that the S phase and G2/M phase of si-GAS5 were lower than those of the NC group [S phase (18.2±2.51)% *vs.* (25.6±3.65)%, $p<0.05$; G2/M phase (19.4±2.91)% *vs.* (28.1±3.95)%, $p<0.05$]. The difference between the two groups was statistically significant.

Table I. Relationship between expression of GAS5 and clinicopathological features in tissues of patients with breast cancer.

Clinicopathological data	No.	High expression of GAS5	Low expression of GAS5	*p*-value
Years				0.672
≤60	26	16	10	
>60	24	18	6	
Pathological staging				0.022
I	10	2	8	
II	12	4	8	
III	21	5	16	
IV	7	1	6	
Lymph node metastasis				0.003
No	29	8	21	
Yes	21	5	16	

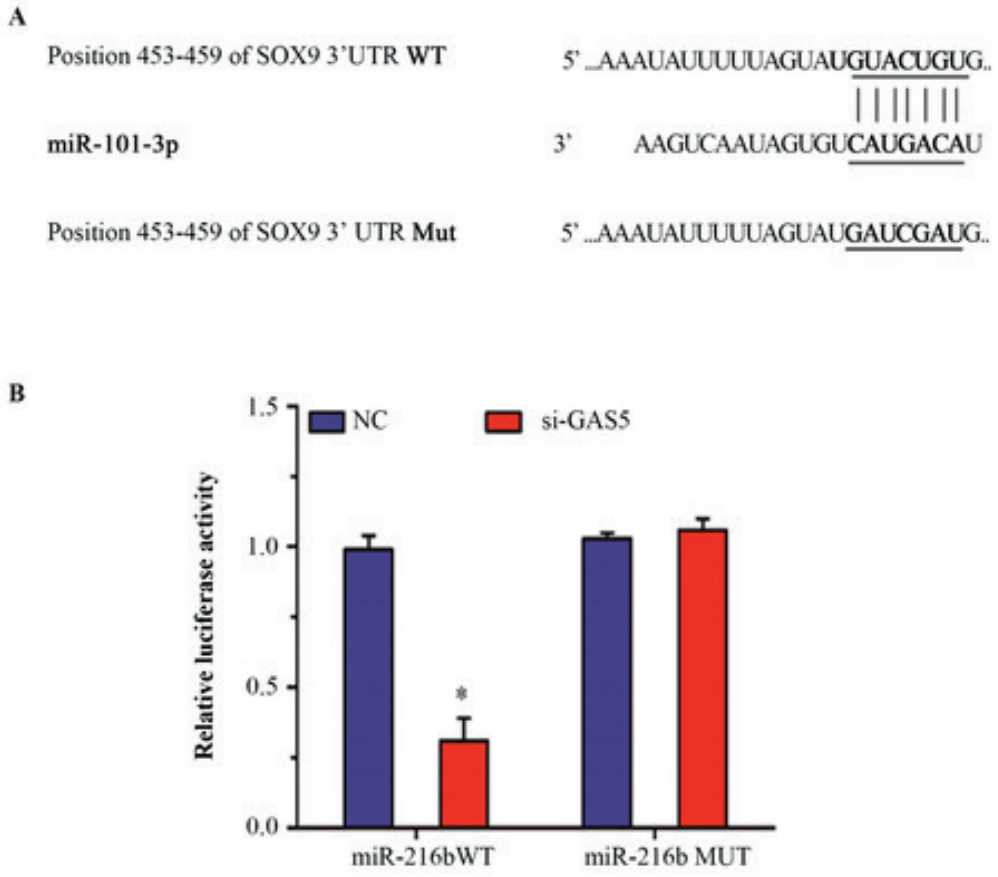

Figure 2. Dual-Luciferase assay to detect the correlation between GAS5 and miR-216b. **A,** Pairing sequences between GAS5 and miR-216b. **B,** Result of Dual-Luciferase assay, *p<0.05.

Effect of GAS5 on Invasion Behavior of Breast Cancer Cells

The results of transwell invasion showed (Figure 4) that the number of cells in the breast cancer cell (MCF-7) si-GAS5 group going through Matrigel was 72.54±8.12, which was significantly less than 156.52±13.24 in the NC group, the difference was statistically significant (p<0.05). The number of cells in the breast cancer cell (T47D) si-GAS5 group going through Matrigel was 102.92±12.62, which was significantly less than that in the NC group (195.92±12.75), and the difference was statistically significant (p<0.05). The inhibition of GAS5 can effectively inhibit the invasion ability of breast cancer cells.

LncRNA GAS5 Regulates Epithelial-Mesenchymal Transition in Breast Cancer Cells

In SiHa cells, the cells have a strong epithelial marker E-cadherin expression, but almost no strong expression of the mesenchymal marker N-cadherin. After transfection of breast cancer cells with si-GAS5, it was found that the expression of E-cadherin was partially decreased, and N-cadherin was also significantly increased (Figure 5). These trends were confirmed by immunofluorescence staining.

Discussion

In the current study, it has been reported that lncRNA GAS5 can promote the epithelial-mesenchymal transition process and invasion-metastasis cascade of breast cancer cells by competitive binding to miR-200c[18]. In addition, Hayes et al[19] have shown that lncRNA GAS5 has multiple effects on hepatocarcinoma cell invasion and apoptosis. This study also detected that lncRNA GAS can interfere with the invasiveness of breast cancer cells, accompanied by the emergence

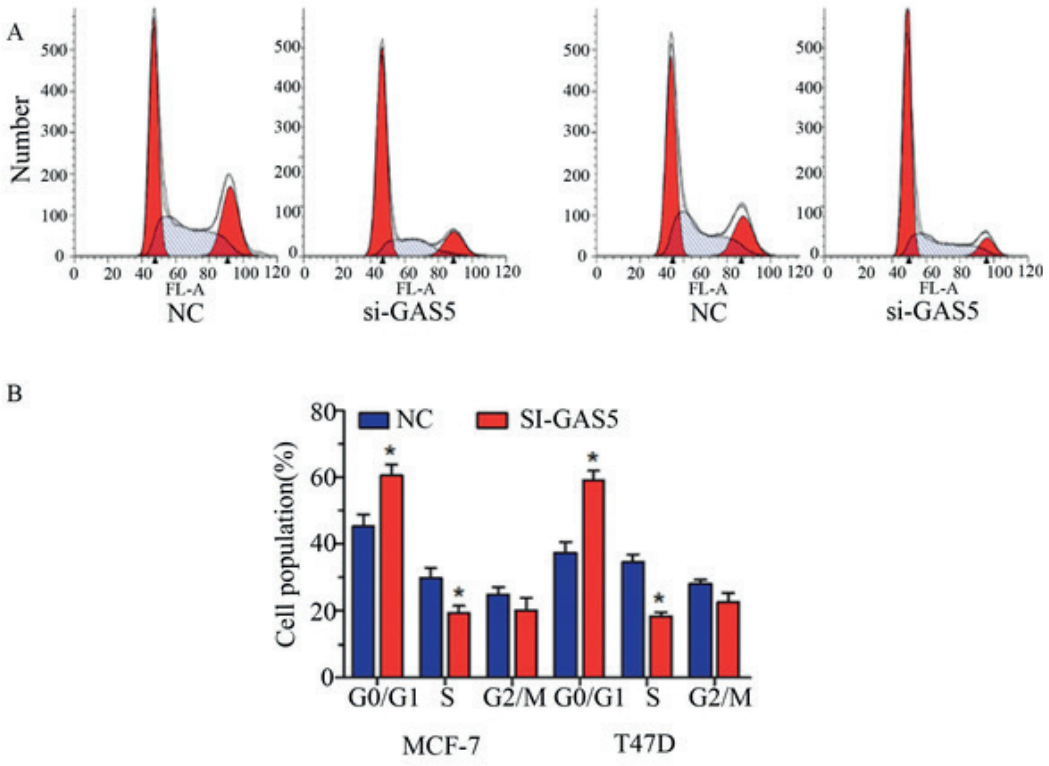

Figure 3. Effect of GAS5 on apoptosis behavior of breast cancer cells. **A,** Result of cell cycle experiment. **B,** Quantification analysis of cell apoptosis, *$p<0.05$.

of epithelial-mesenchymal transition. Xu et al[20] pointed out that lncRNA GAS5 can mediate TGF-β signaling induced epithelial-mesenchymal transition in hepatocellular carcinoma and further metastasis, suggesting that lncRNA GAS5 plays an important role in the biological behavior of tumor cells.

To further indicate the mechanism of action of lncRNA GAS5 in breast cancer cells, this study investigated the relationship between lncRNA GAS5 and miR-216b. Dual-Luciferase assay confirmed that lncRNA GAS5 and miR-216b were upstream and downstream. The molecular activity of miR-216b can be directly inhibited by transcriptional activation of lncRNA GAS5. Notably, this effect was dependent on competitive binding to miR-216b, further indicating that lncRNA GAS5 acts as an upstream acting element in breast cancer cells. These results demonstrate that lncRNA GAS5 plays a direct regulatory role by regulating the expression of miR-216b in breast cancer.

Many miRNAs have been shown[21] to be associated with biological invasion behavior in breast cancer. In particular, miR-216b has been shown[22] to mediate distant metastatic behavior of breast cancer cells. Notably, there may be further negative feedback mechanisms between lncRNA GAS5/miR-216b[23]. On the one hand, the negative feedback between lncRNA GAS5 and miR-200c may be more complex than we currently know. LncRNA GAS5 can act as a ceRNA to upregulate the expression of pathway proteins in breast cancer. On the other hand, lncRNA GAS5 can amplify its effect on epithelial-mesenchymal transition in breast cancer cells through the lncRNA GAS5/ZNF217/TGF-β signaling pathway. Therefore, this study boldly speculated that lncRNA GAS5/miR-216b is involved in the metastatic behavior of breast cancer cells, and subsequent studies further investigated the negative feedback mechanism between them.

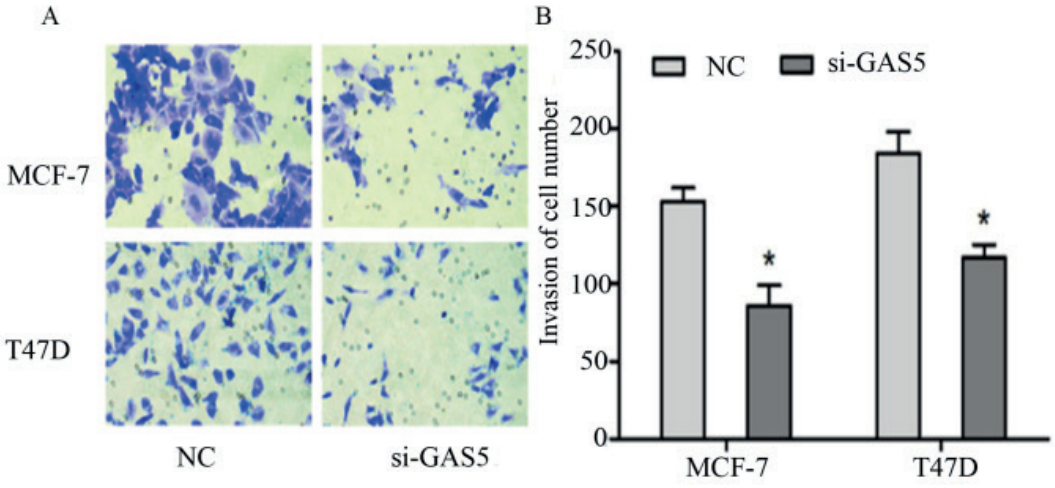

Figure 4. Effect of GAS5 on invasion behavior of breast cancer cells (magnification 400x). **A,** Results of transwell invasion assay. **B,** Quantification of results of transwell invasion, *p<0.05.

Conclusions

This study has shown that lncRNA GAS5 is a key regulator of breast cell behavior. The results of this study are important for us to understand the mechanisms of EMT and metastatic behavior in breast cancer. As a direct target of lncRNA GAS5, miR-216b mediates the role of lncRNA GAS5 in breast cancer cell invasion-metastasis cascades. LncRNA GAS5 may serve as a novel biomarker in breast cancer and a potential therapeutic molecular target for the treatment of breast cancer.

Conflict of Interests

The Authors declare that they have no conflict of interests.

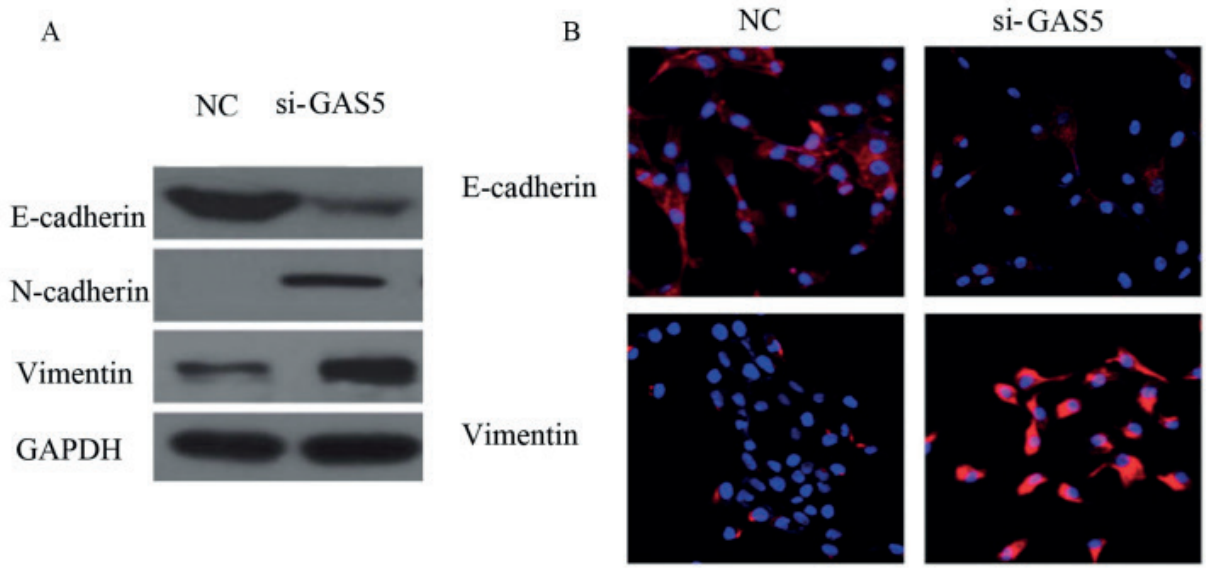

Figure 5. LncRNA GAS5 regulates epithelial-mesenchymal transition in breast cancer cells (magnification 400x). **A,** Result of Western blot. **B,** Immunofluorescence staining, *p<0.05.

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