Long Non-Coding RNA SNHG19 Promotes Breast Cancer Progression by Regulating miR-299-5p

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Research

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Abstract

Background: Accumulating evidence has suggested that long noncoding RNA (lncRNA) played crucial roles in the development of human malignances including breast cancer. SNHG19 is a newly identified lncRNA which exerted oncogenic function in non-small cell lung cancer, but whether SNHG19 was involved the development of other cancer, such as breast cancer still unclear.

Methods: qRT-PCR was performed to examine the expression of SNHG19 and miR-299-5p in breast cancer tissues and cell lines. Cell proliferation was measure using CCK-8 and colony formation assay. Cell migration and invasion ability was detected by wound healing assay and transwell invasion assay. Bioinformatics analysis, dual luciferase reporter assay, RIP assay and Pull down assay were used to verify the direct binding between SNHG19 and miR-299-5p. The xenotransplantation mouse model was established to explore the effect of SNHG19 on breast cancer tumor growth *in vivo*.

Results: We found that SNHG19 expression level was up-regulated in breast cancer tissues and cell lines, while miR-299-5p expression was down-regulated in breast cancer tissues and it was negatively correlated with SNHG19 expression. Silence of SNHG19 inhibited breast cancer cells proliferation, migration and invasion *in vitro*. Moreover, SNHG19 knockdown suppressed tumor growth of breast cancer cells *in vivo*. Mechanistically, SNHG19 acted as a ceRNA (competitive endogenous RNA) to sponge miR-299-5p. Finally, the rescue assays further confirmed that miR-299-5p inhibitor reversed the inhibitory effects of SNHG19 knockdown on breast cancer cell proliferation, migration and invasion.

Conclusions: In conclusion, our findings proved that SNHG19 promoted breast cancer progression via sponging miR-299-5p and might function as promising prognostic indicator and therapeutic target for breast cancer.

Background

Breast cancer, one of the most common and aggressive gynecological malignancy, was considered to be the most leading causes of neoplastic cancer associated death in humans [1, 2]. Although the noticeable development of multiple therapeutic strategies, such as surgical resection, chemotherapy and radiation therapy *et al* has been made, the prognosis for breast cancer patients is still extremely dismal [3]. This dismal situation can be attributed to the high heterogeneity of breast cancer cells as well as the increasing resistance to chemotherapeutic drugs [4]. Therefore, it is urgent needed to identify the underlying molecular mechanism of breast cancer progression and define novel diagnostic biomarkers and potential therapeutic targets.

LncRNA (long noncoding RNA) was defined as a heterogeneous group of transcripts that are longer than 200 nucleotides with little protein-coding ability [5]. Accumulating evidence indicated that lncRNA played crucial roles in various biological processes [6, 7]. A series of evidences further displayed that lncRNA showed anomalous expression in breast cancer and took part in initiation and progression of breast cancer [8].

SNHG19 was a novel lncRNA and was firstly reported that it was dysregulated in Alzheimer's disease and played pivotal regulatory neuronal functions [9]. Then, Li *et al* found that SNHG19 expression was up-regulated in triple-negative breast cancer and was associated with low survival possibility in triple-negative breast cancers patients using bioinformatics analysis [10]. Moreover, it was reported that SNHG19 facilitated the progression of non-small cell lung cancer through regulating the miR-137/E2F7 axis [11]. However, to date, the clinical significance, biological function and molecular mechanism of SNHG19 in tumors including breast cancer still unknown.

In our research, we evaluated SNHG19 expression in breast cancer tissues and in paired adjacent normal brain tissues. Moreover, we investigated the biological roles of SNHG19 in breast cancer cell proliferation, migration, and invasion both *in vitro* and *in vivo*. Finally, we examined the interaction between SNHG19 and miR-299-5p to elucidate the molecular mechanism of SNHG19 in breast cancer in detail. To the best of our knowledge, this the first study showed that oncogenic function of SNHG19 in the development of breast cancer. These findings offered new insights into the tumorgenesis and progression of breast cancer and may promote the development of novel anticancer therapies strategies.

Methods

Clinical samples

A total of 30 pairs of breast cancer tissues and matched adjacent normal tissues were sourced from patients undergoing resection surgery at Department of Neurosurgery, Western Central Hospital of Hainan Province, from Feb 2015 to Dec 2018. All patients did not receive chemotherapy or radiation before collecting specimens. All these participants signed informed consents prior to the samples collection. The samples from resection surgery were rapidly frozen and stored in liquid nitrogen until required. This study was approved by the Ethic and Research Committees of Western Central Hospital of Hainan Province.

Cell culture

The normal breast epithelial cell line (MCF-10A) and human breast cancer cell lines (MCF-7, SKBR-3, MDA-MB-468 and MDA-MB-231) were bought from ATCC (American Type Culture Collection) and incubated in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS, 50 U/ml penicillin and 0.1 mg/ml streptomycin (Biowest). All the cell cultures were maintained at 37˚C under a humidified incubator with 5% CO2.

Constructs, synthesized oligos and transfection

shRNA (short hairpin RNA) targeting SNHG19 (sh-SNHG19), miR-299-5p mimics, inhibitors and their corresponding negative control were bought from by GeneChem (Shanghai, China). All the DNAs were inserted into pcDNA3.1. Finally, Lipofectamine 3000 (Thermo Fisher Scientific) were utilized to transfer the oligonucleotides and constructs into the MDA-MB-468 and MDA-MB-231 cells according to the manufacturer's protocol.

RNA extraction and quantitative real-time polymerase chain reaction

Total RNA from breast cancer tissues and cells was extracted using Trizol reagent (Invitrogen; Thermo Fisher Scientific, Ind.) according to the manufacturer's protocol. 2 μg RNA was reverse transcribed into cDNA using the PrimeScript RT Reagent kit (Invitrogen; Thermo Fisher Scientific, Ind.). qRT-PCR was undertaken using SYBR Green Master mix (Invitrogen; Thermo Fisher Scientific, Ind.) on ABI PRISM 7500 PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocol. GAPDH or U6 was used as controls and normalized the expression of mRNA and miRNA respectively. Primer sequences are provided in Table 1.

Table 1
Sequence of primers

Gene	Forward primer	Reverse primer
SNHG19	AACATGAGGGAATGAATGAG	TAGACCAAAACAGAAGGAAC
MiR-299-5p	ACACTCCAGCTGGGTGGTTTACCGTCCCAC	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGATGTATGT
GAPDH	CAAGTCATCCATGACAACTTTG	GTCCCCACCCTGTTGCTGTAG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

Luciferase reporter assay

Mut (mutant-type) or wt (wild-type) fragments of SNHG19 containing miR-299-5p targeting site was synthesized and cloned into a dual-luciferase reporter vector (pmirGLO, GenePharma, Shanghai, China). Similarly, luciferase vectors and miR-299-5p mimics or miR-299-5p NC together with Renilla plasmid were cotransfected into MDA-MB-468 and MDA-MB-231 cells by Lipofectamine 3000. 48 h after transfection, dual-luciferase assay (Promega, Madison, WI) was adopted to examine the renilla and firefly luciferases activity following the manufacturer’s protocol, and normalized to that of renilla luciferase activity.

RNA-binding protein immunoprecipitation assay

Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA, USA) was used for RIP (RNA-binding protein immunoprecipitation) assay. Cells were harvested and lysed, and lysis buffer containing magnetic beads was incubated with human anti-Ago2 antibody (Abcam, Cambridge, MA, USA) to conjugate the antibody to the magnetic beads. Then, proteinase K was added to digest the protein and the immunoprecipitated RNAs were isolated using Trizol reagent and measured

RNA pull-down assay

MDA-MB-468 and MDA-MB-231 cells were transfected a biotin-labeled miR-299-5p mimic or NC. 24 h later, the cells were collected and incubated with M-280 streptavidin magnetic beads (Invitrogen; Thermo Fisher Scientific, Ind.) at 4°C for 4 h with rotation. Then, the beads were washed with lysis buffer containing proteinase K (Invitrogen; Thermo Fisher Scientific, Ind.) and 10% SDS, and the supernatants were collected. RNA was isolated and coprecipitated RNA was detected by qRT-PCR assays.

Cell proliferation assay

Cell proliferation was examined by Cell Counting Kit-8 (CCK-8) and 5-ethynyl-20-deoxyuridine assays (EdU) assays. For CCK8 assay, cells were seeded in 96-well plates at the concentration of 3000 cells/well. Then a 10 μL of Cell Counting Kit-8 (CCK-8, Dojindo, Japan) was added after 24, 48, 72, and 96 h of incubation respectively. After 2 h, the plates was washed using PBS (phosphate-buffered saline) and the absorbance was measured at 450 nm through microplate reader (ELx800; BioTek Instruments, Inc, Winooski, VT, USA). EdU kit (RiboBio, Guangzhou, China) was used to perform Edu assay in accordance with the manufacturer's protocol. Briefly, cells were placed in 96-well plates and further incubated with 50μM EdU for 4 h. The cells were washed three times using PBS, fixed in 4% paraformaldehyde and incubated with 2 mg/ml glycine, followed by Apollo reaction cocktail for 30 min. The cell nucleus was stained with Hoechst 33342 for half an hour. Finally, the cells were observed under a fluorescence microscope, and the proliferation rate was calculated.

Wound-Healing Assay

A sterile pipette tip (p200) was used to create a linear wound when cells grown to 100% confluence. Then, cells were washed with PBS and cultured with DMEM without serum at 37°C for 24 h. Images were obtained at 0 h and 24 h after scratching using an inverted microscope (magnification x200, Nikon, Japan).

Transwell Invasion Assay

Transwell invasion assays were performed to determine the cell invasion potential using transwell plates (Corning, NY) that were coated with 50 μL of Matrigel (BD Biosciences, San Jose, CA, USA). Briefly, 1×10^5 cells were suspended in 300 μl serum-free medium and added to the upper chamber, while 800 μl complete medium was placed to the lower chamber. After 24 h incubation, cells on the upper surface of the membrane were scraped off. Cells on the lower side of chamber were fixed with methanol and stained with 1% crystal violet. The invaded cells were counted in at least five fields under a light microscope (magnification, x200, Olympus Corp)..

Tumor xenograft experiment

MDA-MB-231 cells (2×10^6) stably transfected with lv-sh-SNHG19 or lv-sh-NC were subcutaneously injected subcutaneously into left flank of 6-week-old female nude mice (n = 5 mice per group). The tumor sizes were measured every week. After 4 weeks, the mice were euthanized, the tumor tissues were excised and weighted, and qRT-PCR was performed to determine SNHG19 and miR-299-5p expression. Animal experiments were strictly obeyed the instruction of the Institutional Animal Care and Use Committee of the Western Central Hospital of Hainan Province.

Statistical analysis

All results are presented as mean ± SD from at least three independent experiments. One-way ANOVA or two-tailed Student's t-test or was performed for comparisons

between groups. Pearson's coefficient correlation was used to conduct expression correlation assays. A value of $P < 0.05$ was considered to be statistically significant.

Results

SNHG19 expression levels are up-regulated and miR-299-5p expression levels are down-regulated in breast cancer.

qRT-qPCR analysis was performed to determine the relative expression levels of SNHG19 and miR-299-5p in 30 pairs of breast cancer and adjacent normal tissues. The results revealed that SNHG19 expression levels were significantly up-regulated in breast cancer tissues compared with the corresponding normal tissues (Fig. 1A), while miR-299-5p expression levels were down-regulated in breast cancer (Fig. 1B). Additionally, the expression of miR-299-5p was remarkably negatively associated with SNHG19 expression in breast cancer tissues (Fig. 1C).

Negative association between SNHG19 and miR-299-5p expression levels.

Consistent to that in breast cancer tissues, SNHG19 was up-regulated in breast cancer cell line, while miR-299-5p was down-regulated in breast cancer cell line (Fig. 2A). MDA-MB-468 and MDA-MB-231 cells were selected for the subsequent studies because the conspicuously elevated expression of SNHG19 was noticed. The shRNAs against SNHG19 were transfected into MDA-MB-468 and MDA-MB-231 cells. SNHG19 expression was strikingly down-regulated in MDA-MB-468 and MDA-MB-231 cells compared with the matched controls (Fig. 2B). Moreover, silencing SNHG19 significantly up-regulated the expression levels of miR-299-5p (Fig. 2C). To further determine the association between SNHG19 and miR-299-5p, MDA-MB-468 and MDA-MB-231 cells were transfected with a miR-299-5p mimic or corresponding control. The up-regulated expression levels of miR-299-5p in the two cell lines were verified using qRT-qPCR (Fig. 2D). Notably, the data revealed that the overexpression of miR-299-5p markedly down-regulated SNHG19 expression levels (Fig. 2E). Contrarily, miR-299-5p inhibitor markedly up-regulated SNHG19 expression levels (Fig. 2F and 2G). Taken together, these results suggested a negative association between SNHG19 and miR-299-5p expression levels.

miR-299-5p is a direct target of SNHG19.

A dual luciferase reporter assay was performed to further determine whether miR-299-5p was a direct target of SNHG19. The results of the dual luciferase reporter assay revealed that the miR-299-5p mimic markedly decreased the relative luciferase activity in the cells transfected with SNHG19-wt, but not in the cells transfected with SNHG19-mut or NC (Fig. 3A and 3B). In addition, the result of anti-AGO2 RIP assay suggested that SNHG19 was conspicuously enriched in Ago2-containing miRNPs (Fig. 3C). Moreover, the results showed that more SNHG19 were pulled down by the biotin-labeled miR-299-5p mimic compared with that in negative control group (Fig. 3D). Taken together, these data demonstrated that SNHG19 directly bind to miR-299-5p.

Effect of SNHG19 and miR-299-5p on breast cancer cell proliferation, migration, and invasion.

The biological effects of SNHG19 and miR-299-5p on breast cancer cell proliferation were further investigated. The results of the CCK-8 assay discovered that the knockdown of SNHG19 significantly inhibited the proliferation of MDA-MB-468 and MDA-MB-231 cells; however, this inhibitory effect was reversed when the cells were co-transfected with sh-SNHG19 and the miR-299-5p inhibitor (Fig. 4A). Similarly, the results of the colony formation assay further confirmed that the knockdown of SNHG19 significantly inhibited the proliferation of MDA-MB-468 and MDA-MB-231 cells, and that the inhibitory effect could be reversed following the co-transfection of the cells with sh-SNHG19 and the miR-299-5p inhibitor (Fig. 4B). It was further determined whether SNHG19 and the miR-299-5p affected breast cancer cell migration and invasion. The results of wound-healing assay and Transwell invasion assays demonstrated that silencing of SNHG19 could suppress invasive abilities of breast cancer cells. Similar to the trends observed in the proliferative capacity, the effects on cell migration and invasion were also reversed following the co-transfection with sh-SNHG19 and the miR-299-5p inhibitor (Fig. 5A and 5B).

SNHG19 knockdown inhibits breast cancer tumor growth in vivo.

To further verify the *in vitro* results, a subcutaneous xenograft tumor model was established by injecting stable sh-NC or sh-SNHG19 cells into nude mice. Consistent with the *in vitro* findings, the *in vivo* study demonstrated that the volume and weight

of the tumors in the sh-SNHG19 group were significantly reduced compared with the sh-NC group (Fig. 6A-C). Moreover, the expression levels of SNHG19 were down-regulated, while miR-299-5p expression levels were up-regulated, in the sh-SNHG19 group compared with the sh-NC group (Fig. 6D and 6E). These findings suggested that SNHG19 knockdown may inhibit breast cancer tumor growth *in vivo*.

Discussion

Tumor growth and invasion are important aggressive behavior of human cancers [12, 13]. A large number of studies have revealed that lncRNA can function as promoter or inhibitor of human cancer growth and invasion [3, 14]. LINC01133, down-regulated in breast cancer, restrained breast cancer invasion and metastasis via inhibiting SOX4 transcription through binding with EZH2 [3]. LncRNA DANCR exerted crucial regulatory role in apoptosis and autophagy of breast cancer cells through DANCR/miR-758-3p/PAX6 pathway [15]. LncRNA CBR3-AS1 regulated adriamycin resistance of breast cancer cells by sponging miR-25-3p and regulating MAPK signal pathway [16].

SNHG19 was firstly identified lncRNA in 2019 using gene array datasets and bioinformatics analysis, it was reported as age-associated expressions lncRNA, which was significantly positively associated with Braak stage of Alzheimer's disease [9]. Following, Li *et al*, performed bioinformatic analysis using public datasets and found that SNHG19 was one of the high expression lncRNAs in triple negative breast cancer and was correlated with poorer prognosis of triple negative breast cancer patients [10]. Moreover, SNHG19 expression was up-regulated in non-small cell lung cancer and promoted non-small cell lung cancer progression both *in vitro* and *in vivo* through miR-137/E2F7 axis [11]. Similarly to the previous findings, our results showed that SNHG19 was up-regulating in breast cancer tissues and the following functional studies further suggested that SNHG19 promoted breast cancer cells proliferation, migration and invasion. Finally, *in vivo* study confirmed that SNHG19 knockdown inhibited breast cancer growth *in vivo*.

Recently, accumulating studies suggested that lncRNA exert its functions through bingding with microRNAs as ceRNA [17, 18]. For example, LINC00665 promoted the development of breast cancer via miR-379-5p/LIN28B axis [19]. LncRNA HAND2-AS1 retained triple negative breast cancer development directly targeting miR-106a-5p and reducing MSCs secretion [20]. LncRNA HOTTIP facilitated the maintenance of breast cancer cell stemness through miR-148a-3p/WNT1 axis [21]. In this study, an online database was used to find potential target miRNAs of SNHG19 and selected miR-299-5p. Previous studies have established the inhibitory function of miR-299-5p in human cancers, including head and neck squamous cell carcinoma [22], breast cancer [23], papillary thyroid cancer [24]. In SNHG19-knockdown breast cancer cells, the expression of miR-299-5p was significantly increased. Additionally, the expression of miR-299-5p was negatively associated with SNHG19 expression in breast cancer tissues. The results of luciferase reporter assay, RIP and RNA pull-down assay further confirmed that SNHG19 directly bound to miR-299-5p. Finally, the results of functional rescue assay indicated that SNHG19 knockdown inhibited breast cancer cell proliferation, migration and invasion through regulating miR-299-5p.

Conclusions

In conclusion, we demonstrated that SNHG19 expression is up-regulated in breast cancer. Moreover, SNHG19 promotes breast cancer proliferation, migration and invasion partly through regulating miR-299-5p. These results suggested that SNHG19 is an important oncogenic player in the development of breast cancer and may be a promising therapeutic target for breast cancer patients.

Declarations

Ethics approval and consent to participate: Written informed consent was obtained from all participants prior to the sample collection. The present study was approved by the Ethic and Research Committees of Western Central Hospital of Hainan Province. All methods are in accordance with relevant guidelines and regulations. All animal experiments were performed in strict accordance with the principles and procedures approved by The Animal Experimentation Ethics Committee of the

Western Central Hospital of Hainan Province and were conducted in accordance with the Western Central Hospital of Hainan Province guidelines for the care and use of experimental animals strictly and in compliance with ARRIVE guidelines.

Consent for publication: Not applicable

Availability of data and materials: The datasets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: ZJ conceived and designed the experiments. HL and ZJ performed the experiments. HL analyzed the data and wrote the paper. Authorship must be limited to those who have contributed substantially to the work reported. All authors read and approved the final manuscript.

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Figures

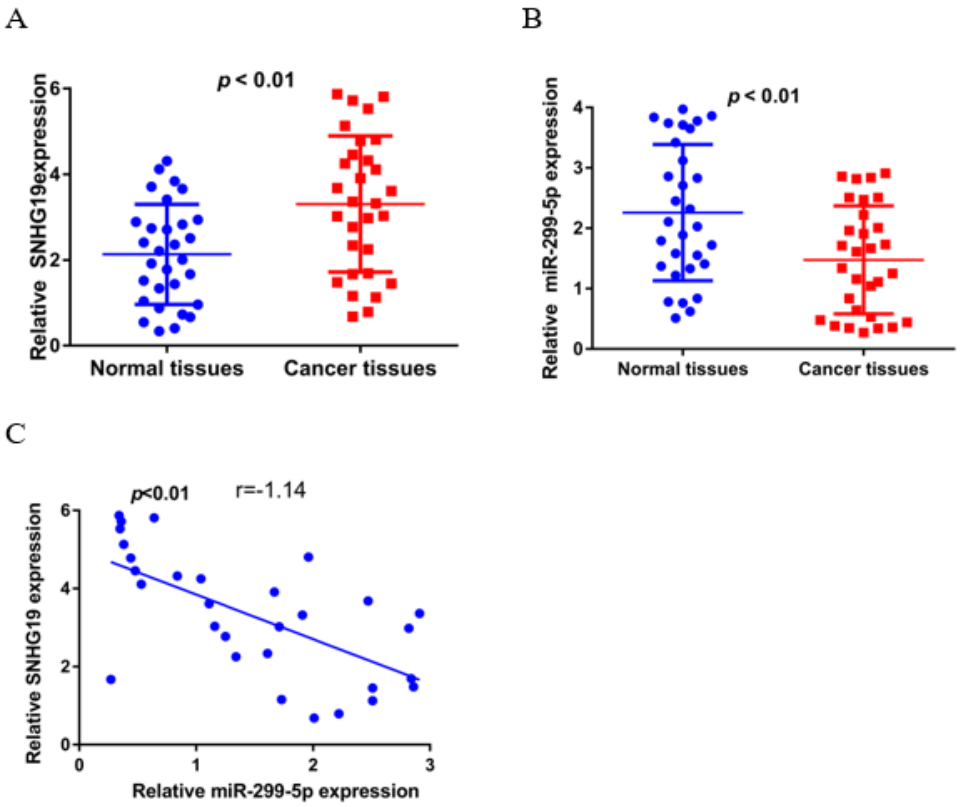

Figure 1

The expression of SNHG19 and miR-299-5p in breast cancer tissues. (A) The expression of SNHG19 in 30 pairs of breast cancer tissues and their adjacent normal tissues was detected by qRT-PCR. (B) The expression of miR-299-5p in 30 pairs of breast cancer tissues and their adjacent normal tissues was examined by qRT-PCR. (C) The correlation between SNHG19 and miR-299-5p was evaluated.

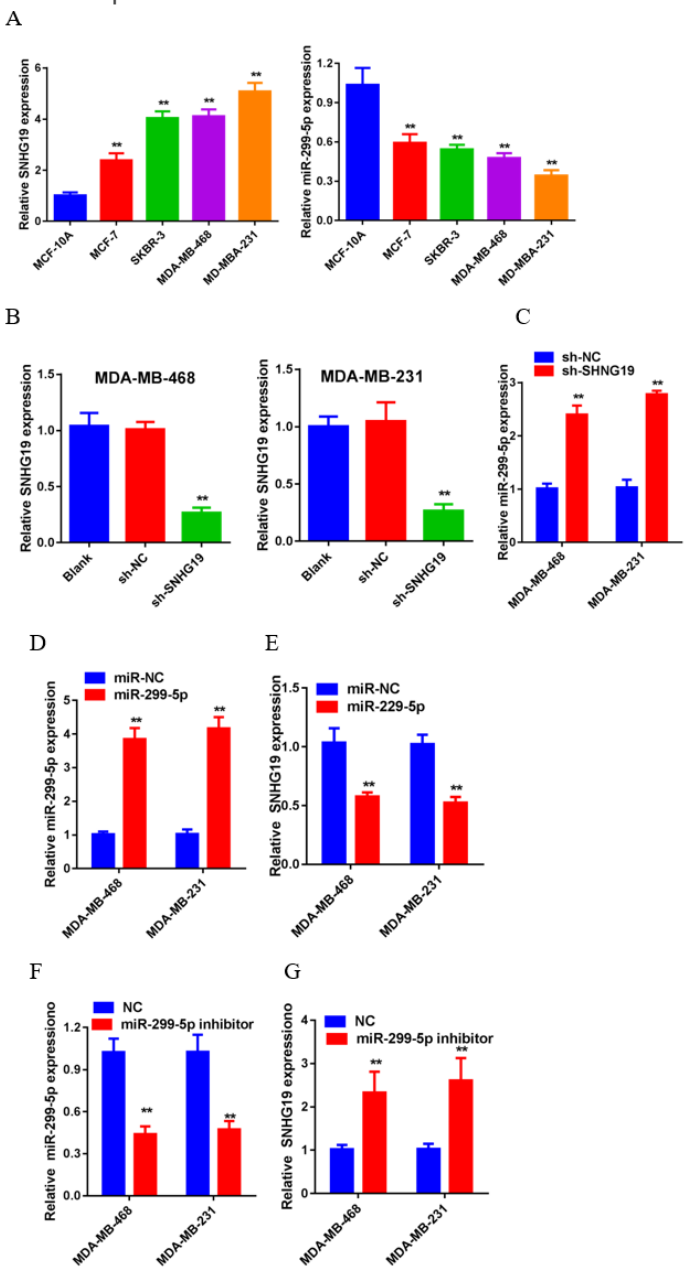

Figure 2

The reciprocal repression effect of SNHG19 and miR-299-5p. (A) The expression of SNHG19 was evaluated in four breast cancer cell lines. (B) The expression of SNHG19 in MDA-MB-468 and MDA-MB-231 cells lines transfected with sh-NC or sh-SNHG19 was determined by qRT-PCR. (C) Expression of miR-299-5p in MDA-MB-468 and MDA-MB-231 cells after the knockdown of SNHG19. (D) The expression of miR-299-5p in MDA-MB-468 and MDA-MB-231 cells lines transfected with miR-NC or miR-299-5p mimic was determined by qRT-PCR. (E) Expression of SNHG19 in MDA-MB-468 and MDA-MB-231 cells after the overexpression miR-299-5p. (F) The expression of miR-299-5p in MDA-MB-468 and MDA-MB-231 cells lines transfected with miR-NC or miR-299-5p inhibitor was determined by qRT-PCR. (G) Expression of SNHG19 in MDA-MB-468 and MDA-MB-231 cells after the knockdown miR-299-5p. **P < 0.05.

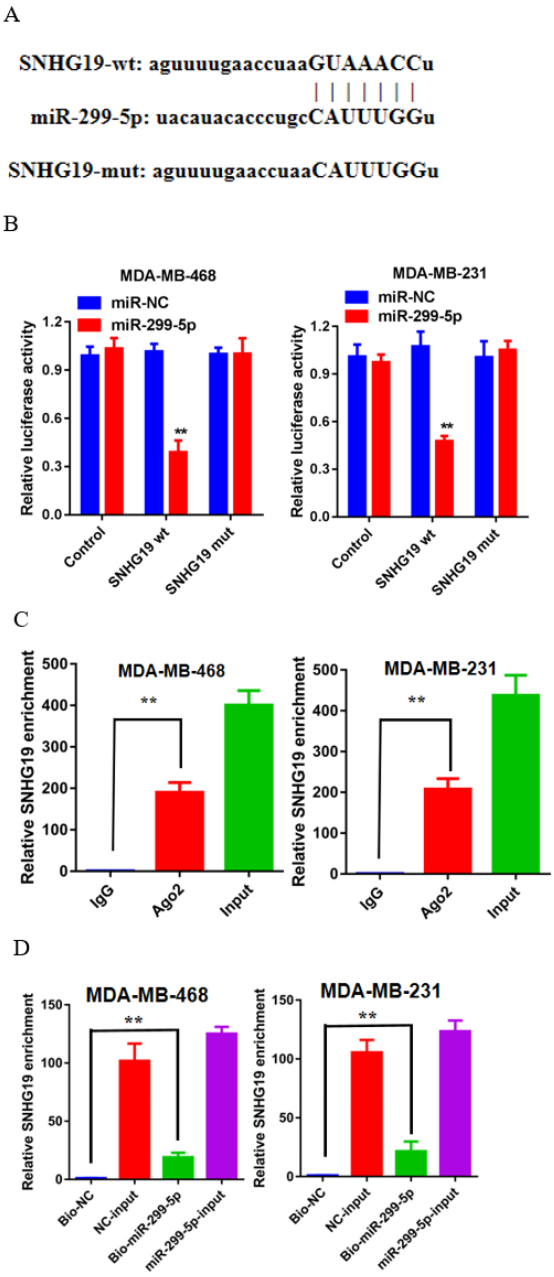

Figure 3

SNHG19 and miR-299-5p directly target each other. (A) Binding site of SNHG19 and miR-299-5p was predicted by online tools. (B) Relative luciferase activity in cells transfected with miR-299-5p mimic or mimic-NC and cotransfected with the control vector, SNHG19-wt or SNHG19-mut. (C) RIP assays were performed to determine the amount of SNHG19 bound to Ago2. (D) Mount of SNHG19 bound to biotinlabelled miR-299-5p mimic or biotin-labelled negative control. RNA pull-down assays were performed after 24 h of transfection. **P < 0.05.

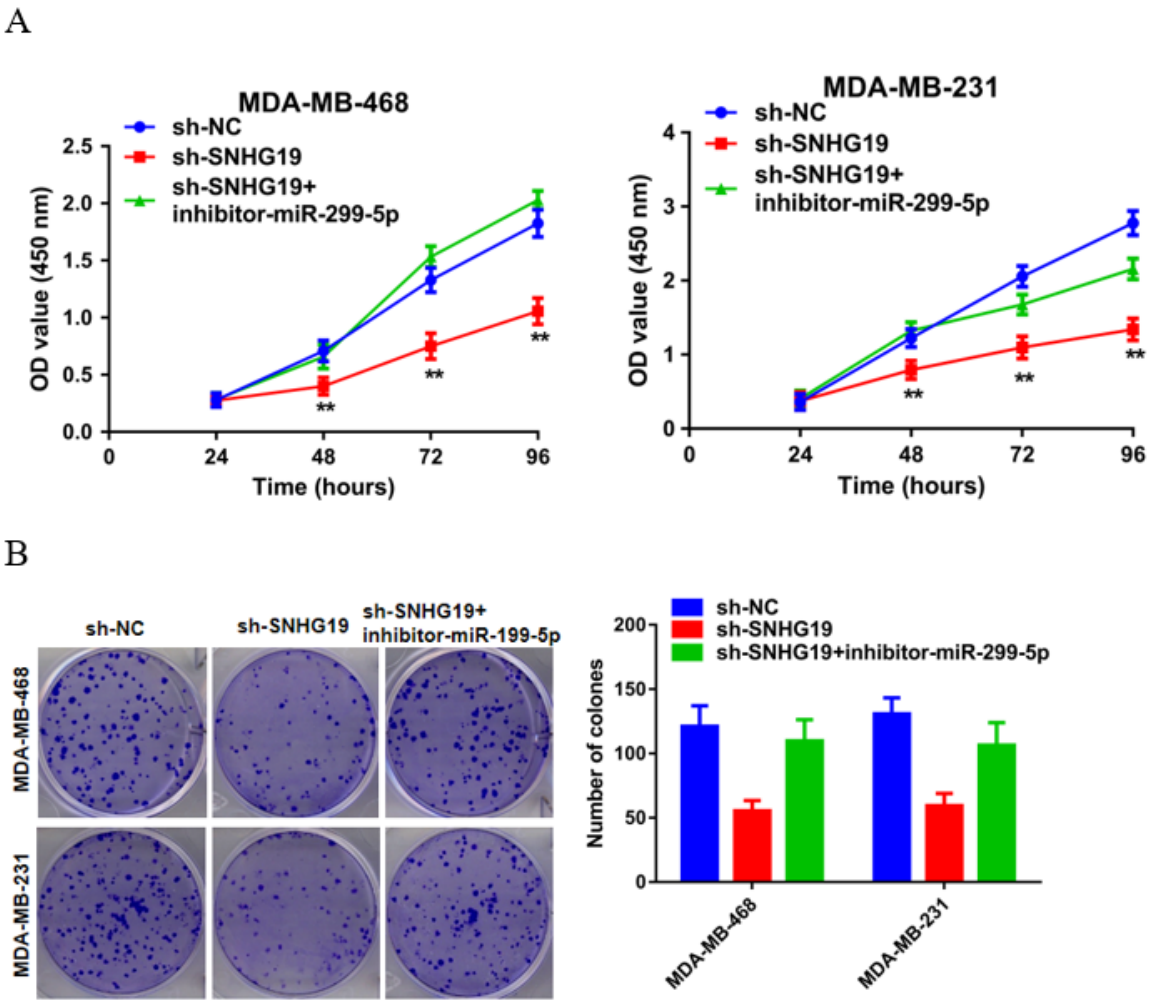

Figure 4

The role of SNHG19 and miR-299-5p in breast cancer cell proliferation. (A) CCK-8 cell viability assays were performed to evaluate the sh-NC, sh-SNHG19, and sh-SNHG19+miR-299-5p inhibitor groups. (B) Colony formation assay were used to determine cell proliferation of different groups. **P < 0.05.

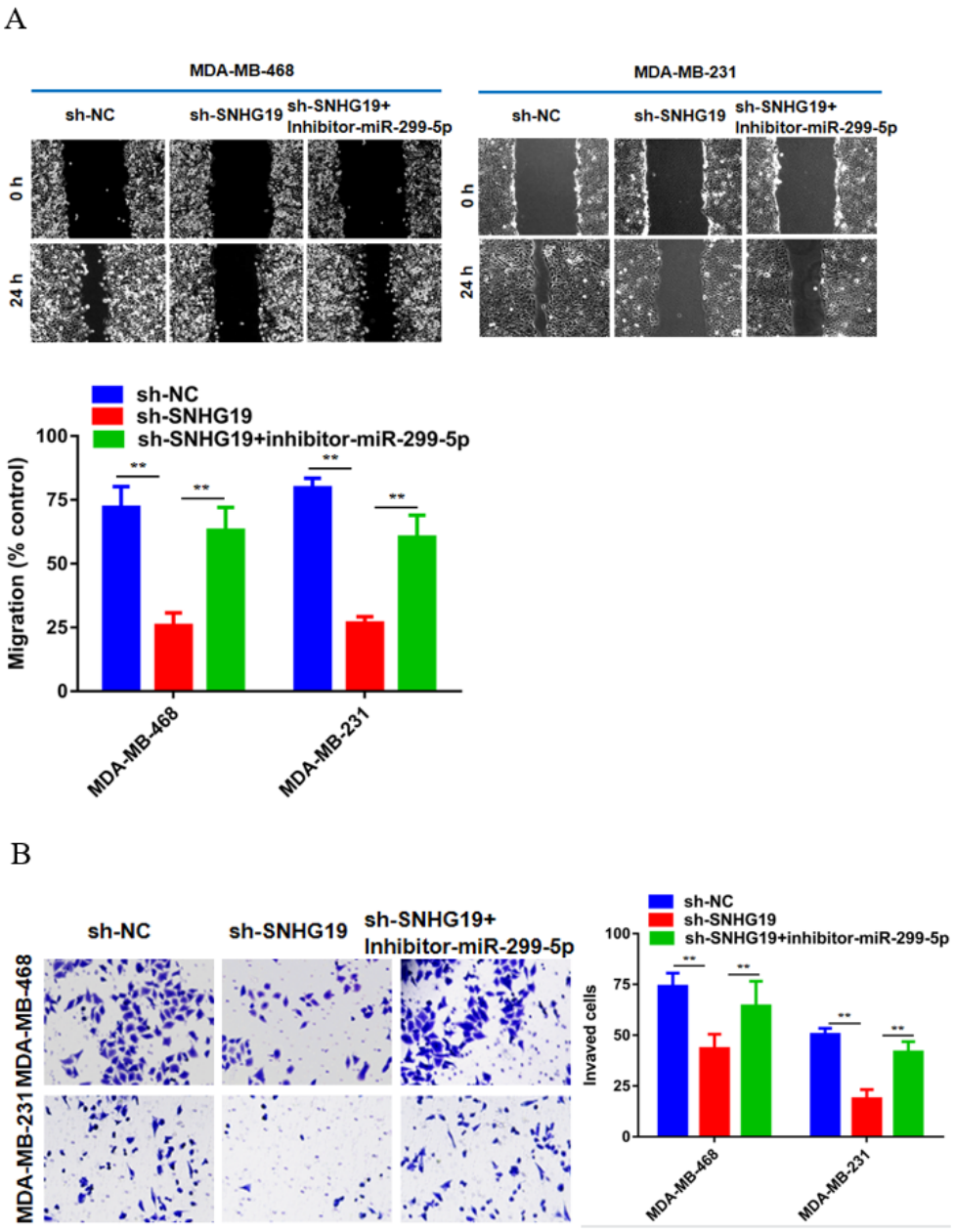

Figure 5

The role of SNHG19 and miR-299-5p in breast cancer cell migration and invasion. (A) Wound-healing assays were performed to detect the cell migration ability of each group. (B) Transwell invasion assays were conducted to examine the cell invasion ability of each group. **P < 0.05.

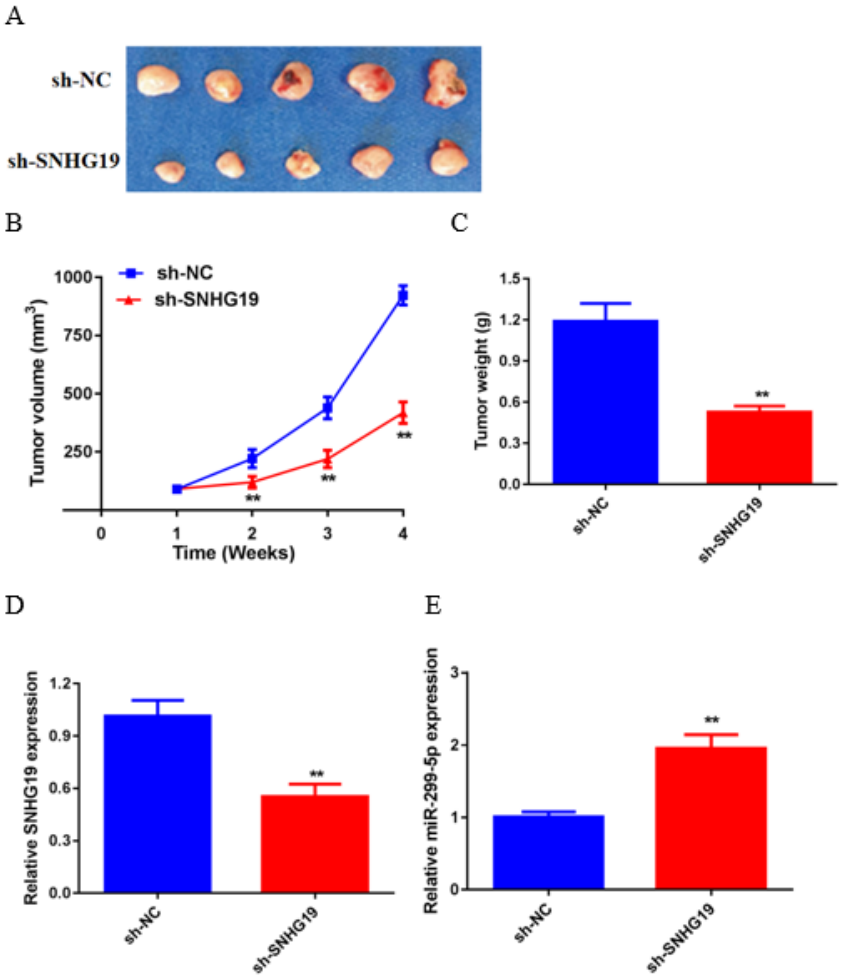

Figure 6

Knockdown of SNHG19 inhibits tumor growth in vivo. (A) The tumor volumes in the sh-SNHG19 and sh-NC groups were evaluated at 4 weeks after injection. (B) The tumor volumes were evaluated every week after the cell injection. (C) Four weeks after cell injection, the mice were killed and the tumor weight was detected. (D) Expression levels of SNHG19 in tumors. (E) Expression levels of miR-299-5p in tumors. **P < 0.05.