Long non-coding RNA LINC00312 regulates breast cancer progression through the miR-9/CDH1 axis

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Abstract. Long non-coding RNAs (IncRNAs) are important mediators of the initiation and progression of tumors, including breast cancer (BC). The exact role of long intergenic non-coding RNA 00312 (LINC00312) in BC and its mechanism of action have not been reported to date. In the present study, LINC00312 was found to be downregulated in human BC tissues and cell lines by RT-qPCR. The findings of a functional study indicated that overexpression of LINC00312 suppressed the proliferation, colony forming ability, migration and invasiveness of BC cell lines. Mechanistically, LINC00312 was found to induce suppression of cell migration and invasion by directly binding to miR-9. Overexpression of LINC00312 increased the expression of cadherin 1 (CDH1), a direct target of miR-9, and decreased the expression of vimentin (VIM), a major cytoskeletal component of mesenchymal cells as determined by western blot analysis. miR-9 partly abrogated the upregulation of CDH1 and downregulation of VIM induced by LINC00312. Taken together, the results of the present study indicate a role for the LINC00312/miR-9/CDH1 axis in the progression of BC, and suggest a novel IncRNA-based diagnostic biomarker or therapeutic target for BC.

Introduction

Breast cancer (BC) is the second leading cause of female cancer-related mortality worldwide, and remains among the five most commonly diagnosed cancers, with an incidence of 11.6% (1,2). Despite the numerous approaches to the treatment of BC, including surgery, chemotherapy, radiotherapy and molecular-targeted therapy, the 5-year survival rate of patients with advanced BC is only 22% (3). Additionally, the heterogeneity and variability in treatment and survival response of BC highlight the need to elucidate the biological mechanisms driving this malignancy. Therefore, identification of critical molecules involved in BC progression may provide a breakthrough for targets and biomarkers of antitumor drugs against this malignant disease.

Long non-coding RNAs (IncRNAs) are a set of RNAs with a length of >200 nucleotides that lack protein translation ability (4). Aberrant expression of IncRNAs, which has been identified in a wide spectrum of cancers, has been proven to play a key role in a number of cellular processes, including cancer initiation, development, invasion and metastasis (4-6). Due to their tissue specificity, IncRNAs may be applied as diagnostic biomarkers and therapeutic targets for certain types of cancer. A long intergenic non-coding RNA 00312 (LINC00312), also referred to as NAG7, which is located on chromosome 3p25.3, has been identified as a new putative tumor-suppressor gene in different types of cancer, including bladder (7), lung (8-12), colorectal (13) and hepatocellular (14) cancers. In addition, a previous study also confirmed that LINC00312 may serve as a potential biomarker for the progression, metastasis and prognosis of nasopharyngeal carcinoma (15). However, the exact role of LINC00312 in BC progression and its mechanism of action remain unclear.

The aim of the present study was to investigate the expression status of LINC00312 in human BC tissues and cell lines, determine its effect on the proliferation, colony formation, migration and invasion of BC cell lines and elucidate the underlying mechanisms, in order to confirm whether LINC00312 may be used as a possible novel target for the treatment of BC.

Materials and methods

Tissue samples from patients with BC. Twenty-six pairs of BC tissues and corresponding adjacent normal tissues were collected during surgery from BC patients aged 32-69 years during October 2017 and February 2018 at the Shanghai Tenth People's Hospital. All patients provided informed...
consent according to procedures approved by the Shanghai Tenth People's Hospital Institutional Review Board (certificate no. SHSY-IEC-KY-4.0/17-23/01). All tissue specimens were rapidly frozen in liquid nitrogen and then stored at -80°C until extraction of RNA.

Cell culture. Normal human mammary epithelial cells (HMECs) MCF-10A and human BC cell lines, including MCF-7, T47D, BT549, MDA-MB-231 and SKBR3 were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). The MCF-10A, MCF-7, MDA-MB-231 and T47D cell lines were incubated in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.). The BT549 and SKBR3 cell lines were incubated in Dulbecco's MEM (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and 1% penicillin-streptomycin. All cells were maintained in an incubator at 37°C with 5% CO2 in a humidified atmosphere.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was first isolated from tissues or cells using TRizol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse-transcribed to complementary DNA using a PrimeScript RT Reagent kit (TaKaRa). For the analysis of mRNA, qPCR was performed using the TaqMan MicroRNA Reverse Transcription kit and TaqMan Universal PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer’s instructions, and U6 small nuclear RNA (snRNA) was used as normalization control. The primer sequences were as follows: mir-9 forward, TCTTTGGTTATCTAGCTGTATGA and reverse, TGTTGTGCTTGAGTCG; U6 forward, CTCCGTTCTGGCA GCACA and reverse, AAGCCCTCACGAATTTCGCT. For analysis of mRNA, RT-qPCR was performed using SYBR® Premix ExTaq™ (TaKaRa) according to the manufacturer’s instructions, and GAPDH was used as the normalization control. PCR was conducted in a total volume of 20 µl under the following conditions: 95°C for 30 min and 60°C for 30 sec, for 30 cycles. The 2^ΔΔCt method (16) was used to calculate the relative levels of mRNA. The primer sequences were as follows: LINC00312 forward, TCTGCGCTGGTTGTGCTT GGA and reverse, GCTTAATTGCTTGGTCTCGT; and GAPDH forward, GCTGGCGCTGAGTACGCTGAGGT and reverse, CACAGCTTCTGGTGAGGAGT.

Transfection and lentivirus transduction. The mir-9 mimic and its negative control (NC) were purchased from Ribobio, and oligonucleotide transfection was performed using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The human LINC00312-cDNA was inserted into the pCDH-CMV-MCS-EF1-coGFP vector (System Biosciences) to construct a LINC00312-carrying lentiviral vector (Lv-LINC00312). The empty lentiviral vector was used as control (Lv-control). Lv-LINC00312 or Lv-control along with the packaging plasmid VSVG and psPAX2 were transfected into 293T cells to produce recombinant lentivirus, followed by infection of the MDA-MB-231 and SKBR3 cell lines using Polybrene (Sigma-Aldrich; Merck KGaA).

Cell proliferation analysis. Cell Counting Kit-8 (CCK-8) and colony formation assays were performed to detect the proliferation of MDA-MB-231 and SKBR3 cells transfected with Lv-LINC00312 or Lv-control. For CCK-8 assays, the cells were inoculated into 96-well plates at a density of 2,000 cells/well in 100 µl of complete medium. After 1, 2, 3, 4 and 5 days, 10 µl of the CCK-8 solution (Dojindo Molecular Technologies, Inc.) was added into each well, and the absorbance was then measured at 450 nm using a microplate reader after 2 h of additional incubation. For colony formation assays, cells were seeded into 12-well plates at a density of 1,000 cells/well and incubated in fresh medium for ~2 weeks to allow colony formation. Subsequently, the cells were fixed with 4% paraformaldehyde (PFA) and stained with 1% crystal violet solution for 20 min. The number of the cell colonies was automatically counted using ImageJ v1.8.0 software (National Institutes of Health, Bethesda, MD, USA) at a size of 80-infinity.

Transwell migration and invasion assays. Transwell migration and invasion assays were used to evaluate the effect of LINC00312 on the migration and invasion of MDA-MB-231 and SKBR3 cells transfected with Lv-LINC00312 or Lv-control. For the Transwell migration assays, 2x10⁵ cells in 100 µl of complete medium were seeded into the upper Transwell chamber (8-µm pore size, BD Biosciences) with medium containing 10% bovine serum albumin added to the lower compartments. After incubation for 36 h at 37°C, the cells adhering to the lower surface of the Transwell membrane were fixed in 20% methanol and stained with 0.1% crystal violet solution. The number of cells was calculated under an inverted light microscope (magnification, x100). For the Transwell invasion assays, the procedure was similar to that for the migration assay, except that the upper compartment was precoated with Matrigel (BD Biosciences).

Luciferase reporter assay. The potential binding sequences of miR-9 and LINC00312 wild-type (WT) and the mutant (MUT) miR-9-binding sequences in LINC00312 were co-transfected into MDA-MB-231 and SKBR3 cells with the miR-9 mimic or NC mimic using lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h of transfection, the luciferase activity was measured using a Dual Luciferase Reporter Assay kit (Promega Corp.).

Western blot analysis. Cells were lysed with lysis buffer [100 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate, 20% glycerol]. Total protein (30 µg per lane) was separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were then blocked with 5% skimmed milk for 60 min and incubated with primary antibodies against CDH1 (cat. no. 14472, Cell Signaling Technology, Inc.), vimentin (VIM; cat. no. 5741, Cell Signaling Technology, Inc.) and GAPDH (cat. no. 5174, Cell Signaling Technology, Inc.), followed by incubation with fluorescence-conjugated secondary antibodies (dilution 1:1,000). Bands were detected by a two-color infrared laser imaging system (Odyssey; LI-COR Biosciences).
Immunofluorescence assay. MDA-MB-231 and SKBr3 cells transfected with Lv-LINC00312 or Lv-control on coverslips were fixed with 4% paraformaldehyde for 15 min, then permeabilized and blocked with 5% bovine serum albumin in phosphate-buffered saline containing 0.2% Triton X-100 for 1 h, followed by incubation with primary antibodies against CDH1 (M168, Abcam) or VIM (RV202, Abcam) at 4˚C overnight. On the following day, the cells were incubated with the secondary antibodies DyLight TM 488-conjugated Affinipur donkey anti-mouse IgG (H + L) (M488, Jackson) at room temperature for 1 h, followed by staining and sealing with ProLong Gold Antifade Reagent plus DAPI (4,6-diamidino-2-phenylindole) (Invitrogen; Thermo Fisher Scientific, Inc.). Finally, images of the stained cells were captured using a laser scanning confocal microscope (magnification, x400; CarlZeiss AG).

Statistical analysis. Data are presented as the mean ± standard deviation. Two-way independent samples t-test for differences between two paired groups or one-way ANOVA followed by the Bonferroni post hoc test for multiple comparisons was performed using SPSS v.22.0 (IBM Corp.). "P<0.01 or "P<0.05 (as indicated by asterisk(s) in the graphical figures) were considered to indicate statistically significant differences. All experiments were repeated at least thrice independently.

Results

LINC00312 is downregulated in human BC tissues and cell lines. The expression levels of LINC00312 were first determined in 26 pairs of BC tissues and adjacent normal tissues by RT-qPCR analysis. As shown in Fig. 1A, the expression of LINC00312 was significantly downregulated in BC compared with that in the normal tissues (P<0.01). The expression of LINC00312 was next assessed in BC cell lines, namely MCF-7, T47D, BT549, MDA-MB-231 and SKBR3, and compared with that in normal human mammary epithelial cells (HMECs) MCF-10A. The results of the RT-qPCR analysis demonstrated that the expression of LINC00312 in all BC cell lines was significantly lower compared with that in the normal HMECs MCF-10A (Fig. 1B). These results confirmed that LINC00312 is downregulated in human BC.

LINC00312 inhibits proliferation, colony formation, migration and invasion of BC cell lines. To determine the role of LINC00312 in BC, the LINC00312-carrying lentivirus (Lv-LINC00312) or control lentivirus (Lv-control) were transfected into the MDA-MB-231 and SKBR3 cell lines, which express LINC00312 at low levels. The RT-qPCR results demonstrated that the expression of LINC00312 was significantly upregulated in both types of cells using lentivirus-mediated human LINC00312-cDNA (Fig. 2A). The effect of LINC00312 on cell proliferation was first examined using CCK-8 and colony formation assays. As shown in Fig. 2B, overexpression of LINC00312 in both MDA-MB-231 and SKBR3 cell lines significantly inhibited cell viability compared with their corresponding controls. Consistently, LINC00312 overexpression significantly reduced the colony-forming ability of both MDA-MB-231 and SKBR3 cell lines (Fig. 2C). Subsequently, the effect of LINC00312 on cell migration and invasion was assessed using Transwell migration and invasion assays. The results demonstrated that MDA-MB-231 and SKBR3 cells overexpressing LINC00312 exhibited decreased migration and invasion ability compared with the negative control (Fig. 2D and E). Collectively, these findings confirmed that LINC00312 inhibited the proliferation, colony formation, migration and invasion of MDA-MB-231 and SKBR3 cells, suggesting a tumor-suppressive role of LINC00312 in BC.

LINC00312 directly binds to miR-9 in BC. To further explore the molecular mechanism of action of LINC00312 in BC, two different mRNA target-predicting algorithms, including miRcode (http://www.mircode.org/) and RNA22 (https://cm.jefferson.edu/rna22/interactive/), were used to predict the potential miRNAs that directly bind to LINC00312. miR-9, the level of which was found to be upregulated in BC cells (17), was identified as the most promising candidate. The potential binding sequences of miR-9 and LINC00312 are shown in Fig. 3A. RT-qPCR was first performed to examine the regulatory interconnection between LINC00312 and miR-9.
Figure 2. LINC00312 inhibits proliferation, colony formation, migration and invasion of breast cancer cell lines. (A) RT-qPCR analysis of LINC00312 expression in MDA-MB-231 and SKBR3 cell lines transfected with LINC00312-carrying lentivirus (Lv-LINC00312) or control lentivirus (Lv-control). (B) CCK-8 assays and (C) colony formation assays were performed to assess cell proliferation in MDA-MB-231 and SKBR3 cell lines transfected with Lv-LINC00312 or Lv-control. Columns (right panel in C) represent the colony numbers from three independent experiments, which were statistically analyzed using ANOVA method. (D) Transwell migration assays and (E) invasion assays were used to evaluate the effect of LINC00312 on cell migration and invasion in MDA-MB-231 and SKBR3 cell lines transfected with Lv-LINC00312 or Lv-control. Columns (right panels in D and E, respectively) represent the relative cell migration and relative cell invasion from three independent experiments, which were statistically analyzed using ANOVA method. Magnification, ×100. Data are presented as mean ± SD. **P<0.01 vs. Lv-control. LINC00312, long intergenic non-coding RNA 00312.
As shown in Fig. 3B, overexpression of LINC00312 in both MDA-MB-231 and SKBR3 cell lines significantly reduced the mir-9 levels compared with the corresponding control (P<0.01). Subsequently, the luciferase reporter vectors containing a WT or a Mut mir-9-binding sequence in LINC00312 were co-transfected into Mda-MB-231 and SKBr3 cells with the miR-9 mimic or nc mimic. A dual-luciferase reporter assay was performed to verify the direct interaction between LINC00312 and miR-9. As shown in Fig. 3C, miR-9 significantly decreased the luciferase activity in both Mda-MB-231 and SKBr3 cells that were fused to WT-linc00312, but not Mut-linc00312. Additionally, there was a notable inverse correlation between the levels of linc00312 and mir-9 in BC tissues (Fig. 3D). Taken together, these results confirmed that linc00312 directly binds to mir-9 in BC.

The miR-9/CDH1 axis is involved in the anti-BC effect of LINC00312. After confirming the direct interaction between mir-9 and LINC00312, the role of miR-9 in LINC00312-induced suppression of BC was further investigated. The miR-9 mimic was transfected into MDA-MB-231 and SKBR3 cells over-expressing LINC00312, and the cell migration and invasion abilities were assessed using Transwell migration and invasion assays, respectively. The results demonstrated that miR-9 partly reversed the suppressive effect of LINC00312 on cell migration and invasion (Fig. 4A and B), suggesting that miR-9 is involved in the anti-BC effect of LINC00312.

miR-9 has been demonstrated to directly target CDH1, the E-cadherin-encoding mRNA, leading to downregulation of E-cadherin and increased motility and invasiveness of BC cells (17). The morphological changes in BC cells transfected with Lv-LINC00312 or Lv-control were first examined using inverted microscopy. The results demonstrated that both MDA-MB-231 and SKBR3 cells underwent a morphological change from a spindled to a rounded or cobblestone-like shape upon LINC00312 overexpression (Fig. 5). We then investigated whether LINC00312 affects the expression of CDH1 and E-cadherin. As shown in Figs. 4C and 6, overexpression of LINC00312 in both MDA-MB-231 and SKBR3 cell lines significantly increased the expression of CDH1 and decreased the expression of the mesenchymal marker VIM. Additionally, we also observed that miR-9 partly abrogated the upregulation of CDH1 and downregulation of VIM induced by LINC00312 in both MDA-MB-231 and SKBR3 cells (Fig. 4D). Taken together, these results indicate that the miR-9/CDH1 axis may be involved in the anti-BC effect of LINC00312.

Discussion

Only ~1.5% of the genome is actually responsible for protein coding (18,19). The non-coding RNAs (ncRNAs), which represent a large group of RNAs, may be divided into two categories, namely housekeeping and regulatory, according to their functions (20). The regulatory ncRNAs may be
broadly classified into two major classes: Short ncRNAs and long ncRNAs (lncRNAs) (21). The latter are one of the key members of the ncRNA family, and have been demonstrated to be involved in the regulation of tumorigenesis and progression as tumor-suppressor genes or oncogenes (22-24).

linc003312 is a long intergenic non-coding RNA, which has been reported to be associated with the onset, progression and prognosis of several types of cancer. The levels of linc003312 were previously reported to be significantly decreased in non-small cell lung cancer (NSCLC) tissues (11,25). linc003312 has been demonstrated to be regulated by HOXa5, leading to inhibition of proliferation and induction of apoptosis in NSCLC cells (9). In colon cancer, linc003312 has been demonstrated to suppress the proliferation and metastasis of cancer cells through regulation of the miR-21/PTEN axis (13,26). In hepatocellular carcinoma, linc003312 has been reported to downregulate the expression of cyclinB1 and inhibit the proliferation of cancer cells in vitro as well as in vivo (14). In thyroid cancer, LINC00312 has been demonstrated to inhibit the invasion and migration of cancer cells by downregulating the PI3K/Akt signaling pathway and microRNA-197-3p (27,28). Additionally, Zhang et al (15) and Huang et al (29) reported that the expression of LINC00312 was negatively correlated with the size of nasopharyngeal carcinoma, and that it may inhibit the invasion of cancer cells through upregulation of the JNK2/AP-1/MMP1 pathway. However, a recent study reported that linc00312 promotes the metastasis and invasion of lung adenocarcinoma cells by directly binding to the transcription factor YBX1 (10). To the best of our knowledge, the present study was the first to report that linc00312 is downregulated in human breast cancer tissues and cell lines, and that overexpression of linc00312 suppresses the proliferation, colony formation, migration and invasion of BC cell lines, suggesting that linc00312 may serve as a tumor-suppressor gene in BC.

Figure 4. The miR-9/CDH1 axis is involved in the anti-breast cancer effect of LINC00312. (A) Transwell migration and (B) invasion assays were performed to assess the cell migration and invasion in MDA-MB-231 and SKBR3 breast cancer cell lines transfected with Lv-control, Lv-LINC00312 or Lv-LINC00312 and miR-9 mimic. Columns (right panels in A and B, respectively) represent the relative cell migration and relative cell invasion from three independent experiments, which were statistically analyzed using ANOVA method. (C) Western blot analysis of the expression of CDH1 and VIM in MDA-MB-231 and SKBR3 cell lines transfected with Lv-LINC00312 or Lv-control. (D) Western blot analysis of the expression of CDH1 and VIM in MDA-MB-23 and SKBR3 cell lines transfected with Lv-control, Lv-LINC00312 or Lv-LINC00312 and miR-9 mimic. Magnification, x100. Data are presented as mean ± SD. **P<0.01. LINC00312, long intergenic non-coding RNA 00312; CDH1, cadherin 1; VIM, vimentin.

Figure 5. Phase contrast images of MDA-MB-231 and SKBR3 breast cancer cell lines transfected with Lv-LINC00312 or Lv-control (NC). Magnification, x200. LINC00312, long intergenic non-coding RNA 00312.
role for miR-9 in multiple types of cancer, including BC (17). A previous study also reported that miR-9 was upregulated in BC tissues and cells (32). We herein identified miR-9 as the most likely target of LINC00312. Therefore, it was hypothesized that LINC00312 may regulate BC progression by binding to miR-9. The overexpression of LINC00312 in BC cells was found to significantly reduce the levels of miR-9. These findings also confirm the direct interaction between LINC00312 and miR-9 in BC. Of note, a recent study reported a cross-talk between LINC00312 and miR-21 in colon cancer (13). As an important oncogenic miRNA, miR-21 is overexpressed in BC and has been reported to be involved in transforming growth factor β1-induced chemoresistance and invasion by targeting PTen in BC (33). These findings suggest that other miRNAs may also be involved in the regulation of BC by interacting with LINC00312.

miRNAs often regulate the expression of downstream genes by binding to the 3'-untranslated region of target mRNAs (30). miR-9 has been demonstrated to directly target CDH1, leading to downregulation of E-cadherin and increased motility and invasiveness of BC cells (17). We further demonstrated that overexpression of LINC00312 significantly increased the levels of CDH1 and decreased the levels of VIM, a major cytoskeletal component of mesenchymal cells. Additionally, miR-9 partly abrogated the upregulation of CDH1 and downregulation of VIM induced by LINC00312 in BC cells, suggesting that the miR-9/CDH1 axis may be involved in the anti-BC effect of LINC00312. Epithelial-to-mesenchymal transition (EMT) is crucial for tumor cells to invade and metastasize (34), and CDH1 has been proven to be an important molecule involved in EMT. Whether LINC00312 is involved in the EMT of BC cells through miR-9 requires further research, and ongoing investigations on related animal models in vivo are currently being conducted in our laboratory.

In conclusion, the data of the present study are the first to demonstrate that LINC00312 may regulate BC progression as a tumor-suppressor gene. Additionally, LINC00312 was shown to increase the expression of CDH1 by directly binding to miR-9 during the progression of BC, suggesting that LINC00312 may prove to be of value as a novel diagnostic and therapeutic target for BC.

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Figure 6. Overexpression of LINC00312 in MDA-MB-231 and SKBR3 breast cancer cell lines increases the expression of CDH1 and decreases the expression of the mesenchymal marker vimentin (VIM). Immunofluorescence analysis of the expression of CDH1 (A) and VIM (B) in MDA-MB-231 and SKBR3 cell lines transfected with Lv-LINC00312 or Lv-control (NC). Magnification, x400. LINC00312, long intergenic non-coding RNA 00312; CDH1, cadherin 1; VIM, vimentin.
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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

XG and YC designed the experiments, analyzed data and prepared the manuscript. YC, FQ, LH, WL, LL, CJ, XZ, LQ and ML performed the experiments. All authors discussed the results and approved the final version of the manuscript.

Ethics approval and consent to participate

All patients provided informed consent according to procedures approved by the Shanghai Tenth People’s Hospital Institutional Review Board (certificate no. SHSY-IEC-KY-4.0/17-23/01).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References