Original Article

Knockdown of IncRNA SNHG12 suppresses cell proliferation, migration and invasion in breast cancer by sponging miR-451a

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Abstract: Background: Breast cancer (BC) is a common cancer with high incidence in women worldwide. Although there are some studies focusing on the pathogenesis of BC, the regulatory mechanism needs to be further investigated. The function of IncRNA and miRNA has been demonstrated to participate in cell progression of BC. However, the function of SNHG12 has not been clearly elucidated. Methods: We detected the expression of SNHG12 and miR-451a using quantitative real-time PCR (qRT-PCR). The protein expression of AKT, p-AKT, mTOR and p-mTOR were measured using western blot. The relationship between SNHG12 and miR-451a was confirmed by luciferase reporter assay. Cell proliferation was measured using MTT assay. Transwell assay was used to detect cell migration and invasion. Xenograft transplantation was used to detect the function of SNHG12 in vivo. Results: In this study, we found that SNHG12 was significantly increased in BC tissues and cells. Knockdown of SNHG12 inhibited BC cell proliferation, invasion, and migration in vitro as well as suppressed tumor growth in vivo. In addition, miR-451a expression was obviously down-regulated in BC tissues and had negative correlation with SNHG12. Luciferase reporter assay determined that miR-451a was a target miRNA of SNHG12. Notably, SNHG12 knockdown decreased cell proliferation, migration, invasion, and AKT/mTOR pathway activation which could be reversed by down-regulation of miR-451a. Conclusion: Knockdown of SNHG12 inhibited cell proliferation, invasion, and migration by regulating miR-451a through suppression of AKT/mTOR pathway in BC.

Keywords: BC, SNHG12, miR-451a, cell progression, AKT/mTOR

Introduction

Breast cancer (BC) is the most common invasive cancer in women worldwide, which seriously affect women’s health and the quality of life [1]. At present, some regulatory mechanisms of BC have been identified in various reported. However, the pathogenesis of breast cancer has not been fully elucidated.

Long non-coding RNAs (lncRNAs), more than 200 nucleotides in length, are a class of nc-RNAs [2]. Currently, accumulating evidence suggested that lncRNAs play important roles in cell growth, tumor development, prognosis and chemoresistance in multiple cancers. For example, PVT1 contributed to cell proliferation and migration in pancreatic cancer [3]. Knockdown of ANRIL inhibited cell proliferation, metastasis and invasion in hepatocellular carcinoma [4]. A study of Augoff et al showed that ATB promoted trastuzumab resistance and invasion-metastasis cascade in BC [5].

MicroRNAs (miRNAs) belong to non-coding RNAs, which are 18~22 nucleotides RNA molecules [6]. Moreover, miRNAs are involved in a series of cellular processes including tumorigenesis and inflammatory. Recent studies reported that miR-200c, miR-195, miR-10a and miR-26b were associated with cell proliferation of BC [7-11]. Bai et al reported that miR-20a-5p promoted cell growth in triple breast cancer through targeting RUNX3 [12].

LncRNA can act as a ceRNA of miRNA to play indispensable roles, and the regulatory networks of between lncRNA and miRNA have
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been investigated to affect BC regulatory processes, including cell proliferation, migration, invasion and immune response [13-15]. For instance, MALATI/miR-124 led to cell progression by CDK4/E2F1 signal activation [16]. Zhang et al suggested that MEG3 suppressed epithelial-mesenchymal transition by regulating miR-451 [17].

In this study, we found that Long non-coding RNA small nucleolar RNA host gene 12 (SNHG12) was up-regulated in BC tissues and cells compared with normal tissues and cells. In addition, bioinformatics analysis and luciferase reporter assay indicated that miR-451 was a target miRNA of SNHG12 and expressed less in BC tissues and cells. Thus we speculated SNHG12 affected cell progression and tumor formation by targeting miR-451 in BC.

Materials and methods

Patients and specimens

Paired breast cancer and adjacent normal tissues were collected from 20 patients with breast cancer in the Beijing Obstetrics and Gynecology Hospital, Capital Medical University. This study was approved by the research Ethics committee of Beijing Obstetrics and Gynecology Hospital, Capital Medical University and written informed consent was obtained from all patients and their guardian. All patients were not underwent any pre-operative treatment, such as radiotherapy, chemotherapy, immunotherapy, and targeted therapy.

Cell culture and transfection

Normal breast cell lines MCF-10A and breast cancer cell lines MCF-7, BT-549, BT-549, SK-BR-3 and MDA-MB-231 were purchased from RiboBio Co. (Guangzhou, China) and cultured in DMEM medium with 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1% penicillin and streptomycin at 37°C with 5% CO₂.

sh-SNGH12, negative control (sh-NC), miR-451a, miR-451a inhibitor, and their negative control (miR-NC and NC inhibitor) were purchased from GenePharma (Shanghai, China). MCF-7 and MDA-MB-231 cell lines were transfected with sh-SNGH12, sh-NC, miR-451a inhibitor, NC inhibitor, miR-451a and miR-NC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Quantitative real-time PCR

Total RNAs were extracted from tissues and cells using TRIzol reagent Kit (Invitrogen) according to manufacturer's instructions. NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific) was applied to detect RNA concentration. TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used to reverse transcribe cDNA of miRNA. In addition, Reverse Transcription Reagents (Applied Biosystems) was used to reverse transcribed cDNA of mRNA. Real-time qPCR was performed using SYBR-Green Supermix (Bio-Rad, Hercules, CA, USA). The fluorescence was detected in an ABI 7300 System (Applied Biosystems). U6 and GAPDH acted as reference genes. LncRNA was normalized according to GAPDH. miRNA expression was normalized according to U6. The relative expression of miRNA and mRNA was calculated using 2-ΔΔCt method. The primer sequences: LncRNA SNHG12 forward, 5'-TCTGGTGATCGAGGACTTCC-3' and reverse, 5'-ACCTCCTCAGTATCACACACT-3'; miR-451a forward, 5'-ACACTCCA-GCTGGGAAACGGTACCATTACT-3' and reverse, 5'-CTGGTGTGACCGATGGCGA-3'; U6 forward, 5'-CTCGCTTCGGCAGCACAA-3' and reverse, 5'-AACGCTTCAGCAGACTACGAG-3'; GAPDH: forward 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse 5'-ATGGTGGTGACGAGCCAGT-3'.

Western blot

Total protein was extracted form cells lysed with RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China). 10 μg of protein samples were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to PVDF membrane (Millipore, Bedford, MA, USA). Then, the membrane was blocked in Tris-buffered saline (TBS) with 5% skim milk at room temperature for 1 h. The membrane was incubated with primary antibody against AKT, p-AKT, mTOR, p-mTOR and GAPDH (1:2000 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4°C overnight. After washed three times in TBST buffer, the membrane was incubated with secondary antibodies HRP-conjugated anti-mouse IgG (1:2000 dilution, Santa Cruz Bio-
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Cell proliferation

Cell proliferation was detected using MTT assay. Cells and transfected cells were seeded into 96-well plates at a density of 2×10^3 cells per well. After culture for 48 h, 20 μl MTT solution (5 mg/mL, Sigma, St. Louis, MO, USA) was added to stain cells. Subsequently, after the plates were incubated at 37°C for 4 h, 150 μL of dimethyl sulfoxide (DMSO; Sigma) were added into each well to dissolve the formazan crystal. The absorbance was detected at 490 nm using a micro-plate reader (Bio-Rad).

Cell invasion and migration

Cell invasion was performed using Transwell chamber with Matrigel (BD Biosciences, San Jose, CA, USA). Cell migration was displayed using Transwell chamber without Matrigel. 1×10^5 cells were cultured in non-serum medium in the transwell upper chamber. The transwell lower chamber was added in DMEM medium with 10% FBS. Incubated 24 h later at 37°C, cells on the upper chamber membrane were moved. Then the membrane was fixed with 90% ethyl alcohol and was stained with 0.1% crystal violet for 30 minutes. The number of cells with migration and invasion were counted using a phase contrast microscope.

Luciferase reporter assay

miRcode predicted that 3'-UTR of SNHG12 contained the binding sites of miR-451. SNHG12 wild-type sequence and mutate sequence was amplified and inserted into pMIR-REPORT™ (Thermo Fisher Scientific) to construct the reporters of SNHG12-WT and SNHG12-MUT. Then SNHG12-WT or SNHG12-MUT and miR-451a or miR-NC were transfected into MCF-7 and MDA-MB-231 cell lines. After we transfected 48 h, the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, E1910, Madison, WI, USA).

Animal experiment

Sh-SNHG12 and negative control (sh-NC) were stably transfected into MCF-7 cells 55. All female BALB/c nude mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd (China). All animal experiments were approved by the Institutional Animal Care and Use Committee of Beijing Obstetrics and Gynecology Hospital, Capital Medical University. Then sh-SNHG12 and sh-NC cell lines were injected subcutaneously into the right side of the posterior flank of female BALB/c nude mice at the age of 4-5-weeks. Tumor volumes were measured every 5 days. After injection for 30 days, all mice were sacrificed and tumor weight was measured.

Statistical analysis

All statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). Data were displayed as mean ± SD. Student’s t-test was used to analyze all comparison. Pearson’s correlation analysis was applied to analyze the correlation between SNHG12 expression and miR-451a expression in breast tissues. A value of P < 0.05 was considered significant.

Results

SNHG12 was upregulated in breast cancer tissues and cells

First, qRT-PCR was used to detect the SNHG12 in 20 pairs of specimens. The results showed that SNHG12 expression was upregulated in tumor tissues compared with adjacent tissues (Figure 1A). Furthermore, we obtained the nor-
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Then, MTT assay indicated that cell proliferation in sh-SNHG12 group was significantly inhibited in MCF-7 and MDA-MB-231 cells (Figure 2B). Meanwhile, analysis of transwell assay demonstrated that migrated cell numbers and invasive cell numbers were remarkably reduced in sh-SNHG12 group compared with control (Figure 2C and 2D). These findings indicated that down-regulated SNHG12 could suppress breast cancer cell proliferation, migration, and invasion.

Knockdown of SNHG12 suppressed tumor growth in vivo

To evaluate the effect of SNHG12 on tumor growth in vivo, MCF-7 cells stably transfected with sh-SNHG12 were subcutaneously injected into nude mice. We found that tumor volume in sh-SNHG12 groups was significantly decreased.
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Figure 3. Knockdown of SNHG12 suppresses tumor growth in vivo. (A and B) Tumor volume (A) and tumor weight (B) was measured in sh-NC and sh-SNHG12 groups in vivo on the indicated time point. (C) The expression of SNHG12 was detected in sh-NC and sh-SNHG12 groups using qRT-PCR in vivo. *P<0.05.

compared with controls (Figure 3A). After injection, the mice were sacrificed and tumor weight was measured in each group. Tumor weight in sh-SNHG12 group was notably less than control (Figure 3B). We also checked the sh-SNHG12 expression in tumors on day30 (Figure 3C). Therefore, the results showed that knockdown of SNHG12 could decrease tumor volume and tumor weight in vivo.

miR-451a was a target miRNA of SNHG12

To further explore the regulatory mechanism of SNHG12, we used the miRcode online database to predict the target miRNAs of SNHG12. Bioinformatics analysis showed that miR-451a was a potential target (Figure 4B). In our study, we found that sh-SNHG12 promoted miR-451a expression in MCF-7 and MDA-MB-231 cells (Figure 4A). Then, we constructed the luciferase vectors containing SNHG12 WT 3’UTR and SNHG12 MUT 3’UTR and co-transfected with miR-451 into MCF-7 and MDA-MB-231 cell lines, respectively. Compared with NC group, luciferase activity in SNHG12 WT + miR-451a group was significantly decreased, but not significant change in SNHG12 MUT + miR-451a group (Figure 4C). These results showed that miR-451a was a target miRNA of SNHG11 in MCF-7 and MDA-MB-231 cell lines.

Furthermore, to explore the interaction between miR-451a and SNHG12 expression, the expression of miR-451a was detected using qRT-PCR in NC inhibitor, miR-451a inhibitor, miR-451a inhibitor + sh-NC and miR-451a inhibitor + SNHG12 groups. The results showed that miR-451a transfection inhibited miR-451a expression. However, down-regulated SNHG12 could improve the expression of miR-451a suppressed by miR-451a inhibitor (Figure 4D). Moreover, the expression of miR-451a was down-regulated in breast cancer tissues (Figure 4E). As expected, SNHG12 expression was negatively correlated with miR-451a expression in 20 pairs of breast cancer tissues (Figure 4F). These findings revealed that miR-451a was a downstream target of IncRNA SNHG12.

SNHG12/miR-451a axis regulated cell proliferation, migration and invasion in breast cancer

To evaluate the effect of SNHG12/miR-451a axis on cell proliferation, migration, and invasion in breast cancer, miR-451a inhibitor and sh-SNHG12 were co-transfected into MCF-7 and MDA-MB-231 cell lines. In the present study, cell proliferation, migration, and invasion was increased in miR-451a inhibitor group compared with that in NC inhibitor group (Figure 5A-D). In addition, cell proliferation, migration, and invasion in miR-451a inhibitor + sh-SNHG12 group were obviously lower than that in miR-451a inhibitor + sh-NC group (Figure 5A-D). These findings revealed that miR-451a inhibitor significantly promoted breast cancer cell proliferation, migration, and invasion. However, the suppressive effects of miR-451a inhibitor on cell growth in breast were impaired by down-regulation of SNHG12. Therefore, SNHG12/miR-451a might play an important role in breast cancer cell proliferation, migration, and invasion.

SNHG12/miR-451a axis affected cell growth by regulating AKT/mTOR pathway in breast cancer

Recent studies have shown that miR-451a was associated with AKT/mTOR pathway in cancers [18-20]. Thus we speculated that SNHG12/miR-451a axis was able to regulate AKT/mTOR pathway. To confirm that, the protein level of...
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AKT, p-AKT, mTOR and p-mTOR, which are key molecules in AKT/mTOR pathway, was measured using western blot. The results showed that miR-451a inhibitor improved AKT, p-AKT and p-mTOR protein expression, while down-regulation of SNHG12 reversed the effect of miR-451a inhibitor on AKT, p-AKT and p-mTOR protein expression. However, the protein expression of mTOR had no significant change in each group (Figure 6). Thus, these findings demonstrated that SNHG12/miR-451a axis affected breast cancer cell growth by regulating AKT/mTOR pathway.

Discussion

Many IncRNAs have already been demonstrated to participate in BC progression and inflam-
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Figure 5. SNHG12/miR-451a axis regulated cell proliferation, migration, and invasion in breast cancer. (A and B) Cell proliferation was detected in NC inhibitor, miR-451a inhibitor, miR-451a inhibitor + sh-NC, and miR-451a inhibitor + sh-SNHG12 groups in MCF-7 (A) and MDA-MB-231 (B) cells. (C and D) Cell migration (C) and invasion (D) were measured in NC inhibitor, miR-451a inhibitor, miR-451a inhibitor + sh-NC, and miR-451a inhibitor + sh-SNHG12 groups in MCF-7 and MDA-MB-231 cells. *P<0.05.

Figure 6. SNHG12/miR-451a axis affected cell growth via regulating AKT/mTOR pathway in breast cancer. The protein expression of AKT, p-AKT, mTOR and p-mTOR was measured using western blot in NC inhibitor, miR-451a inhibitor, miR-451a inhibitor + sh-NC, and miR-451a inhibitor + sh-SNHG12 groups in MCF-7 and MDA-MB-231 cells. *P<0.05.

Expression induced by c-myc could regulate cell progression in triple-negative breast cancer.
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[24]. However, the underlying regulatory mechanism of SNHG12 has not been fully elucidated.

IncRNA SNHG12 has been demonstrated to be involved in cell progression in various cancers, including gastric cancer, NSCLC, papillary thyroid carcinoma, and colorectal cancer [25-28]. SNHG12/miR-199a/b-5p regulated cell proliferation and invasion in gastric cancer [29]. In this study, we investigated potential function of IncRNA SNHG12 in BC in vivo and in vitro. The results showed that SNHG12 was upregulated in BC tissues and cells. Then, functional investigation indicated that knockdown of SNHG12 decreased the capacities of BC cell proliferation, migration, and invasion in vitro. Subsequently, we also found that SNHG12 knockdown could inhibit tumor growth in vivo. These findings suggested that SNHG12 was an important regulatory molecule in cell growth and tumorigenesis of BC.

Next, we found that miR-451a contained sequences partially complementary to the 3'UTR of SNHG12 using bioinformatic analysis. Accumulating evidence shows that miR-451a widely takes part in tumor tumorigenesis, diagnosis, prognosis and drug-resistances in multiple types of cancer, such as lung cancer, colorectal cancer, bladder cancer and breast cancer [30-32]. Zeng et al reported that miR-451 was obviously decreased in bladder cancer and overexpression of miR-451a inhibited cell growth and induced apoptosis in bladder cancer [31]. In addition, miR-451, acts as a prognostic biomarker and tumor suppressor, and was closely associated with diagnosis, therapy and prognosis of cancers [33]. Interestingly, in breast cancer, most studies on miR-451 have been linked to drug resistance and cell progression [32, 34, 35]. For example, miR-451 was downregulated in BC cells and in paclitaxel-resistant (PR) cells. Moreover, overexpression of miR-451 weakened the capacities of cell migration and invasion as well as induced apoptosis and cell-cycle arrest in paclitaxel-resistant of BC cells [35]. In the present study, the results showed that miR-451 was significantly down-regulated in BC tissues compared with that in adjacent tissues and was negatively correlated with SNHG12. Thus, we thought that SNHG12/miR-451 may play important roles in BC. To further verify the network of between SNHG12 and miR-451 in BC cell growth, the miR-451a inhibitor was co-transfected with sh-SNHG12 into BC cell lines. These findings displayed that miR-451a inhibitor promoted cell proliferation, migration, and invasion, which could be reversed by down-regulation of SNHG12. Therefore, SNHG12 affected cell proliferation, migration, and invasion in BC cells by regulating miR-451a.

Previous studies reported that miR-451a was associated with regulation of signaling pathway [18-20]. In papillary thyroid carcinoma, overexpression of miR-451a inhibited cell growth and attenuated AKT/mTOR pathway activation [18]. In addition, PI3K/AKT pathway was inhibited by miR-451a in human glioma [36]. In our study, we also found that AKT, p-Akt and p-mTOR, had changed in miR-451a inhibitor and miR-451a inhibitor + sh-SNHG12 groups. The results showed that down-regulated miR-451a increased the protein expression of AKT and mTOR, whereas SNHG12 knockdown reduced the protein expression of AKT and mTOR by up-regulating miR-451 through inhibiting AKT/mTOR pathway activation.

In conclusion, these findings revealed that SNHG12 was associated with progression in BC. Furthermore, knockdown of SNHG12 inhibited cell proliferation, migration, and invasion by up-regulating miR-451 through inhibiting AKT/mTOR.

Disclosure of conflict of interest

None.

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