LncRNA SNHG6 promotes breast cancer progression and epithelial-mesenchymal transition via miR-543/LAMC1 axis

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Received: 2 December 2020 / Accepted: 10 March 2021 / Published online: 29 March 2021
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Abstract

Purpose Breast cancer (BC) is the most prevalent cancer in women with an estimated incidence of 10% and the leading cause of mortality due to its heterogenous property and high metastasis rate. Development of novel therapy is very necessary and requires an understanding of molecular mechanisms. We investigated the function of SNHG6/miR-543/LAMC1 axis in BC.

Methods Human BC tissues were obtained from diagnosed patients. BC cell lines and normal breast cells were used. QRT-PCR and Western blotting were employed to measure expression levels of SNHG6, miR-543, LAMC1, EMT-related proteins, and PI3K/AKT pathway. Dual-luciferase assay was performed to validate interactions of SNHG6/miR-543 and miR-543/LAMC1. Colony formation assay, flow cytometry, scratch wound healing assay, and transwell assay were utilized to assess the proliferation, apoptosis, migration, and invasion of BC cells. Nude mouse xenograft model was used to evaluate the function of SNHG6/miR-543 in tumor growth *in vivo*.

Results SNHG6 and LAMC1 were elevated, but miR-543 was reduced in BC tissues and cells. SNHG6 interacted directly with miR-543, while miR-543 targeted LAMC1. Knockdown of SNHG6 suppressed BC cell proliferation, migration, invasion, EMT, and PI3K/AKT pathway, but promoted cell apoptosis, while miR-543 inhibitor or overexpression of LAMC1 reversed those effects. Overexpression of LAMC1 also blocked the effects of miR-543 on BC cell proliferation, migration, invasion, and EMT. Knockdown of SNHG6 restrained BC growth *in vivo*, while miR-543 inhibitor inhibited that suppression.

Conclusion SNHG6 promoted EMT and BC cell proliferation and migration by acting as a miR-543 sponge and disinhibiting LAMC1/PI3K/AKT pathway. SNHG6/miR-543/LAMC1 axis could serve as candidates for the development of therapeutic strategies for BC.

Keywords Breast cancer · SNHG6 · MiR-543 · LAMC1 · EMT

Abbreviations

BC Breast cancer
LAMC1 Laminin subunit gamma 1
lncRNAs Long non-coding RNAs
miRNAs MicroRNAs

EMT Epithelial-mesenchymal transition
FBS Fetal bovine serum
DMEM Dulbecco's modified Eagle's medium
PFA Paraformaldehyde
PI Propidium iodide
TWIST1 Twist homolog 1

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Introduction

Breast cancer (BC) is the most frequent carcinoma among women throughout the world, and also ranks as the top cause of tumor-related deaths [1]. Despite great advances made in research and clinical diagnosis or treatment, the incidence and the mortality rate of BC remain constantly high, causing a big global health concern [1]. Breast cancer is extremely heterogeneous in its pathogenesis with diverse subtypes exhibiting varied biological behaviors and progressive

processes [2, 3]. Etiological factors of BC include genetics, alcohol age, diet, and endocrine factors [3]. Currently, the treatment options for BC are surgical resection together with chemo or radial therapy [4]. However, due to its drug resistance and local or distal metastasis, the prognosis of BC still needs to be improved [5]. It is necessary to understand the mechanisms of the tumorigenesis so that novel effective therapy could be developed.

Laminin subunit gamma 1 (LAMC1) is an extracellular matrix protein that belongs to the laminin family [6]. It regulates the assembly of basement membranes and has important roles in processes like cell growth, migration, development [6]. In addition, it has been implicated in many pathological diseases including cancers [7–9]. For example, LAMC1 has been shown elevated in hepatocellular cancer tissues and cells [8]. It promotes hepatocellular carcinoma progression via PETN/AKT pathway [8]. In BC, its expression has been observed upregulated, suggesting that it might be involved. However, the exact role of LAMC1 in BC remains further investigations.

Long non-coding RNAs (lncRNAs) are a class of non-coding RNAs with a length longer than 200 nucleotides [10]. They can regulate gene expression by sponging endogenous microRNAs (miRNAs) and thus have critical roles in many normal physiological processes like development, cell growth, as well as pathological processes including cancers [10, 11]. SNHG6 is an important lncRNA that has been implicated in various carcinomas, such as colorectal cancer and osteosarcoma [12, 13]. In BC, increased expression of SNHG6 has been reported [14]. Knockdown of SNHG6 promotes BC cell apoptosis and inhibits cell proliferation [14]. These studies show that SNHG6 plays a role in BC. Regarding its downstream targets and the mechanisms underlying SNHG6's function, it is not well understood.

Many lncRNAs regulate gene expression via binding and inhibiting their targets of miRNAs [10]. It is well known that miRNAs interact with mRNAs and negatively regulate their expression [15]. In addition, various miRNAs are involved in cancer development and progression [15, 16]. miR-543 has been implicated in BC as a cancer suppressor by facilitating cell apoptosis and inhibiting cancer cell proliferation [17]. Our preliminary analysis with starbase suggested that miR-543 might bind both SNHG6 and LAMC1. We, therefore, hypothesized that SNHG6/miR-543/LAMC1 axis could play some roles in BC.

In the current study, we sought to investigate the function of SNHG6/miR-543/LAMC1 axis in BC. We confirmed the changes of SNHG6/miR-543/LAMC1 in BC tissues and cells, with SNHG6 and LAMC1 increasing but miR-543 decreasing. In addition, we validated the interactions of SNHG6/miR-543 and miR-543/LAMC1. Knockdown of SNHG6 promoted BC cell apoptosis but inhibited cell proliferation, migration, invasion, and epithelial-mesenchymal

transition (EMT) via miR-543/LAMC1/PI3K/AKT pathway. Moreover, knockdown of SNHG6 greatly restrained BC growth *in vivo*, while miR-543 inhibitor reversed that. Altogether, our results reveal an essential role of SNHG6/miR-543/LAMC1 in BC and suggest that suppressing SNHG6/LAMC1 or activation of miR-543 could be beneficial for BC therapy.

Materials and methods

Human breast tissues

Human breast cancer specimens were acquired from 28 diagnosed breast cancer patients during surgery at the Second Affiliated Hospital of University of South China. Nearby non-tumor tissues were obtained simultaneously from same patients. Patients did not receive preoperative treatments. The patients have consented to the study. The study was reviewed and received approval from the Ethics Committee of the Second Affiliated Hospital of University of South China. All specimens were put immediately in the liquid nitrogen after collection and stored at the freezer (−80 °C).

Cell culture

Four breast cancer cell lines (MCF-7, SK-BR-3, MDA-MB-231, BT-549) and one normal breast cell line (MCF-10A) were utilized. Cells were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were seeded and grown in 10% fetal bovine serum (FBS, Thermo Fisher Scientific, MA, USA) containing Dulbecco's modified Eagle's medium (DMEM) (Gibco, CA, USA). 1% penicillin–streptomycin was included into the medium. The cells were maintained in the cell culture CO₂ incubator at 37 °C.

Plasmids and transfection

The LAMC1 full length was cloned into the overexpression plasmid (pcDNA) (Genepharma, Shanghai, China). MiR-543 mimics and inhibitor were purchased from Genepharma. Sh-SNHG6 (5'-CTGCGAGGTGCAAGAAAGCCT-3') and sh-NC were synthesized and obtained from Genepharma. Lipofectamine 3000 (Invitrogen, Missouri, USA) was utilized as the reagent for cell transfection. In brief, cells were cultured to 70% confluence, and then 1 µg of construct was added together with 1 µL Lipofectamine 3000 at a ratio of 1:1. Cells were harvested for further analysis 48 h after

transfection and qRT-PCR was employed to validate the transfection efficiency.

Immunohistochemistry (IHC)

BC tissue was fixed in 4% formalin and embedded in paraffin. Paraffin Sects. (20- μ m thick) were washed in PBS and briefly treated with 80% methanol containing 0.3% H_2O_2 followed by blocking with 10% normal goat serum for 2 h at room temperature. Primary antibody anti-LAMC1 (1:500, Sigma-Aldrich, HPA001909) was incubated with the slices for overnight at 4 °C, and then incubated with HRP-labeled secondary antibody for 1 h at room temperature. Signals were analyzed with 3,3'-diaminobenzidine substrate and counterstained with hematoxylin.

RNA extraction and qRT-PCR

Trizol (Invitrogen, China) was employed to extract total RNAs from cultured cells and tissues as the manufacturer's instructions described. For miRNA analysis, total RNAs were isolated with the miRNeasy Advanced Mini Kit (QIAGEN, Hilden, Germany). DNaseI was included into the lysis buffer to avoid the contamination of DNA. Commercial kit (cDNA synthesis kits, Thermo Fisher, China) was utilized to generate cDNAs through reverse transcription. TaqMan MicroRNA Reverse Transcription kit (Thermo Fisher, China) was used for miRNA analysis. SYBR Green Master Mix (Invitrogen, China) was used for the quantitative PCR. Real-time qPCRs were performed in triplicate with this protocol: 2 min at 94 °C, followed by 40 cycles (30 s at 94 °C and 45 s at 55 °C). The $2^{-\Delta\Delta C_t}$ method was used to quantify the results. Relative RNA expression levels of SNHG6, LAMC1, or mi-543 were normalized to GAPDH or U6, respectively. The primers listed as follows were from Genepharma:

SNHG6 forward primer (F): 5'-ATACTTCTGCTTCGT TACCT-3';

SNHG6 reverse primer (R): 5'-CTCATTTTCATCATT TGCT-3';

miR-543 F: 5'-CCAGCTACACTGGGCAGCA-3';

miR-543 R: 5'-CTCAACTGGTGTCTGTGGA-3';

LAMC1 F: 5'-ACTCCTAATCTTGGACCATAC-3';

LAMC1 R: 5'-ACAACAGCACAACTTGAAC-3';

U6 F: 5'-GCTTCGGCAGCACATATACTAAAAT-3';

U6 R: 5'-CGCTTCACGAATTTGCGTGTTCAT-3'

GAPDH F: 5'-AGCCCAAGATGCCCTTCAGT-3';

GAPDH R: 5'-CCGTGTTCTACCCCAATG-3'.

Western blotting

Proteins from tissues or cells were extracted by utilizing the RIPA lysis buffer (Abcam, China) according to standard protocol. DC Protein Assay Kit (Bio-Rad, China) was utilized to quantify the protein concentrations. Equal protein from each sample was loaded into SDS-polyacrylamide gels and separated through electrophoresis. Later proteins in the gels were transferred to PVDF membranes (Sigma-Aldrich, China). 3% BSA was used to block the membranes for 30–60 min at room temperature and then specific primary antibodies were added to incubate at 4 °C overnight. The antibodies were discarded and TBST was utilized to wash the membranes 3 times before incubation with specific secondary antibodies for 1–2 h at room temperature. Protein band intensities were detected by using the standard ECL kit. Primary antibodies used in the study were: Anti-LAMC1 (1:2000; Sigma-Aldrich, HPA001909); Anti-Snail (1:1200; Abcam, ab53519); Anti-Vimentin (1:2000; Abcam, ab137321); Anti-E-cadherin (1:1200; Abcam, ab15148); Anti-p-PI3K (1:1200; Cell Signaling, USA, 4228S); Anti-PI3K (1:2000; Cell Signaling, 4292S); Anti-p-AKT (Ser473, 1:1000; Cell Signaling, 9271S); Anti-AKT (1: 2000; Cell Signaling, 9272S); Anti-GAPDH (1:5000; Abcam, ab9485).

Colony formation assay

Transfected BC cells or normal breast cells were plated in the 12-well culture plate at a density of 10^3 cells/well and grown for 2 weeks in the incubator. 4% paraformaldehyde (PFA, G-Clone, Beijing, China) was added to fix the observed colonies at room temperature for 13–15 min. PFA was washed out with PBS. Colonies were incubated with crystal violet (1%, Abcam) for 30 min for staining followed by imaging. The number of colonies was quantified by ImageJ software.

Scratch wound healing assay

Cells were seeded in 6-well plate and cultured to 80–90% confluence. The pipette tip (10 μ L) was utilized to make a scratch in the middle of the dish. Images were taken at the time of scratching and 24 h later. Migration distances were analyzed using the Image J software and the migration rates were counted.

Transwell assay

Transfected BC cells were placed in the culture medium without any serum on top of the filter membrane (8 μ m pore) in the transwell insert that was pre-coated with Matrigel

(Corning, NY, USA) overnight. Full culture medium that contains 10% FBS was put in the lower chamber. 24 h after the upper filter was discarded. Cells growing on the lower dish were cells invading there from the filter. They were fixed in 4% PFA first for 10–15 min, and then 0.2% crystal violet was added to stain the cells followed by imaging.

Cell apoptosis

Cell apoptosis was measured by Annexin-V-FITC/Propidium iodide (PI) staining using Annexin-V-FITC/PI apoptosis Detection Kit (BD, USA) according to manufacturer's protocol. In brief, cell medium was discarded and ice cold PBS was used to wash cells. Binding buffer was added to resuspend the cells and then Annexin V-FITC and PI were added to stain cells for 15 min. The signaling were analyzed using a FACS Canto II flow cytometer (BD Biosciences, USA).

Dual luciferase reporter assay

cDNAs that included the wild type sequences or mutated binding sequences with miR-543 in SNHG6 or LAMC1 3' UTR were cloned into the pGLO luciferase reporter vector (Promega, WI, USA). Commercial kit (The Phusion Mutagenesis kit, Thermo Fisher Scientific) was utilized to mutate the predicted binding sites as the protocol described. BC cells were seeded in individual wells of the 24-well plates first overnight and then recombinant constructs were transfected into BC cells together with miR-543 mimics or mimics NC by using the lipofectamine 3000. 48 h following transfection, the cells were harvested to measure relative luciferase activities.

Nude mice xenograft experiments

Twenty-four adult nude mice (8–10 weeks old) were purchased from SJA Laboratory Animal Co., Ltd (Hunan, China;) and kept in the standard animal facility room. Experiments were approved by the Animal Care and Use Committee of the Second Affiliated Hospital of University of South China. Adult mice were implanted by unilateral subaxillary subcutaneous injection of 5×10^6 transfected BC cells (control sh-NC group, sh-SNHG6 group, sh-SNHG6 + inhibitor NC, sh-SNHG6 + miR-543 inhibitor) to induce tumors. Tumor growth was monitored every day and tumor volume was calculated according to the formula: tumor volume (mm^3) = $0.5 \times (W)^2 \times (L)$, where L represents the length and W represents the width. 30 days later, the mice were sacrificed and the tumor weight was measured and snap frozen in liquid nitrogen for further studies.

Statistical analysis

All experiments were performed with at least three biological replicates in triplicate. All statistical analyses were analyzed in GraphPad Prism 7. Statistical significance was determined by unpaired Student t test (for two groups) or one-way ANOVA (for groups more than two) as indicated in figure legends. The data were presented as Mean $\pm$ SD.

Results

SNHG6 and LAMC1 were elevated in breast cancer tissues and cells, while miR-543 was reduced

To investigate the role of SNHG6, miR-543, and LAMC1 in breast cancer (BC), we first measured their levels in BC tissues and cells. In BC tissues from patients, SNHG6 were elevated, while miR-543 was decreased compared to nearby non-cancer tissues (Fig. 1a, b). Consistently, LAMC1 mRNA and protein level were increased in BC tissues (Fig. 1c–e). In addition, we compared BC cell lines with normal breast cells and observed similar results. SNHG6 and LAMC1 mRNA levels were significantly higher in BC cells than in normal breast cells, while miR-543 was greatly diminished (Fig. 1f–h). These results show that SNHG6 and LAMC1 are upregulated, while miR-543 is downregulated in BC. The changes were bigger in BT-549 and SK-BR3 cells than in MDA-MB-321 and MCF-7 cells. Therefore, we used BT-549 and SK-BR3 cells for subsequent experiments.

SNHG6 directly bound miR-543, while miR-543 targeted LAMC1

It is well known that lncRNAs act by sponging miRNAs and that miRNAs exert functions through targeting downstream mRNAs. To study the regulatory relationships between SNHG6, miR-543 and LAMC1, we manipulated SNHG6 and miR-543 levels and examined their effects on expression of other molecules. Transfection of cells with sh-SNHG6 greatly decreased SNHG6 level (Fig. 2a). Interestingly, we saw a significant increase of miR-543 level but a decrease in LAMC1 mRNA and protein levels in cells transfected with sh-SNHG6 compared to cells transfected with sh-NC (Fig. 2a). Transfection of miR-543 mimics remarkably upregulated miR-543 level, while miR-543 inhibitor downregulated (Fig. 2b). Concomitantly, we observed a reduction of LAMC1 following overexpression of miR-543 and an elevation of LAMC1 level in miR-543 inhibitor-transfected cells (Fig. 2c), suggesting that miR-543 negatively regulated LAMC1 expression. Through bioinformatic analysis, we detected potential binding sites between SNHG6 and

Fig. 1 SNHG6 and LAMC1 were elevated in breast cancer cells, while miR-543 was reduced. (a) Relative SNHG6 levels in BC tissues and nearby non-cancer tissues. (b) Relative miR-543 levels in BC tissues and nearby non-cancer tissues. (c) Relative LAMC1 mRNA levels in BC tissues and nearby non-cancer tissues. (d) Relative LAMC1 protein levels in BC tissues and nearby non-cancer tissues ($N=28$).

(e) IHC analysis of LAMC1 levels in BC tissues and nearby non-cancer tissues. (f) Relative SNHG6 levels in BC cells and normal breast cells. (g) Relative miR-543 levels in BC cells and normal breast cells. (h) Relative LAMC1 mRNA levels in BC cells and normal breast cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

miR-543, as well as between miR-543 and LAMC1 mRNA 3' UTR (Fig. 2d–e). To directly confirm these interactions, we performed dual luciferase activity assay. MiR-543 mimics substantially diminished the relative luciferase activities of WT-SNHG6 and WT-LAMC1, but had no effects on the activities of MUT-SNHG6 and MUT-LAMC1 where in the predicted binding sites with miR-543 were mutated (Fig. 2d–e). These data demonstrate that SNHG6 directly binds miR-543 to inhibit its expression, while miR-543 targets LAMC1 and negatively regulates its expression.

SNHG6 regulated BC cell proliferation and apoptosis via miR-543 and LAMC1

To determine the function of SNHG6 in BC, we tested how it regulated the proliferation and apoptosis of BC cells. As expected, transfection of cells with pcDNA-LAMC1 drastically increased mRNA and protein levels of LAMC1 (Fig. 3a–b). The colony formation assay results presented that knockdown of SNHG6 greatly decreased the number of colonies formed by BC cells. However, co-transfection of sh-SNHG6 with miR-543 inhibitor or pcDNA-LAMC1 could reverse the effects and recovered the numbers of colonies (Fig. 3c). With flow cytometry, we examined the cell apoptosis. We found that knockdown of SNHG6 alone significantly increased the percentage of cell apoptosis (Fig. 3d). Again,

Fig. 2 SNHG6 directly bound miR-543, while miR-543 targeted LAMC1. (a) Relative levels of SNHG6, miR-543, and LAMC1 in cells with transfection of sh-NC or sh-SNHG6. (b) Relative miR-543 levels in cells with transfection of miR-543 mimics, mimics NC, miR-543 inhibitor, or inhibitor NC. (c) Relative LAMC1 mRNA levels in cells with transfection of miR-543 mimics, mimics NC, miR-543 inhibitor, or inhibitor NC. (d) Complementary binding sites between

SNHG6 and miR-543 (left); Relative luciferase activities of WT-SNHG6 and MUT-SNHG6 in cells with transfection of mimics NC or miR-543 mimics (right). (e) Complementary binding sites between miR-543 and LAMC1 mRNA 3' UTR (left); Relative luciferase activities of WT-LAMC1 and MUT-LAMC1 in cells with transfection of mimics NC or miR-543 mimics (right). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

co-transfection of miR-543 inhibitor or pcDNC-LAMC1 blocked the effects and suppressed the increased induced by sh-SNHG6 (Fig. 3d). Taken all together, these results show that knockdown of SNHG6 inhibits BC cell proliferation but promotes cell apoptosis via miR-543 and LAMC1.

SNHG6 regulated BC cell migration and EMT via miR-543 and LAMC1

We next examined how SNHG6 regulated BC cell invasion, migration and EMT, a key process in tumorigenesis.

Fig. 3 SNHG6 regulated BC cell proliferation and apoptosis via miR-543/LAMC1. (a–b) Relative LAMC1 mRNA and protein levels in cells with transfection of pcDNA or pcDNA-LAMC1. (c) Representative images of colonies formed in cells with transfection of sh-NC, sh-SNHG6, sh-SNHG6+ inhibitor NC, sh-SNHG6+ miR-543 inhibitor, sh-SNHG6+pcDNA, and sh-SNHG6+pcDNA-LAMC1. (d) Flow cytometry analysis of cell apoptosis in cells transfected with sh-NC, sh-SNHG6, sh-SNHG6+ inhibitor NC, sh-SNHG6+ miR-543 inhibitor, sh-SNHG6+pcDNA, and sh-SNHG6+pcDNA-LAMC1. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Using transwell assay, we showed that SNHG6 knockdown significantly diminished the number of invasive cells (Fig. 4a). Again, the number was restored when miR-543 inhibitor or pcDNA-LAMC1 was co-transfected together

with sh-SNHG6 (Fig. 4a). With the scratch wound healing assay, we found that knockdown of SNHG6 remarkably decreased the migration distance of BC cells in 24 h (Fig. 4b). The migration rate was increased when the cells

Fig. 4 SNHG6 regulated BC cell migration and EMT via miR-543/LAMC1. **(a)** Representative images of invasive cells transfected with sh-NC, sh-SNHG6, sh-SNHG6+ inhibitor NC, sh-SNHG6+ miR-543 inhibitor, sh-SNHG6+ pcDNA, and sh-SNHG6+ pcDNA-LAMC1. **(b)** Representative images of scratch wounds in cells transfected with sh-NC, sh-SNHG6, sh-SNHG6+ inhibitor NC, sh-SNHG6+ miR-543 inhibitor, sh-SNHG6+ pcDNA, and sh-SNHG6+ pcDNA-LAMC1. **(c)** Relative protein levels of Snail, Vimentin, and E-cadherin in

cells transfected with sh-NC, sh-SNHG6, sh-SNHG6+ inhibitor NC, sh-SNHG6+ miR-543 inhibitor, sh-SNHG6+ pcDNA, and sh-SNHG6+ pcDNA-LAMC1. **(d)** Relative protein levels of LAMC1, p-PI3K, and p-AKT in cells transfected with sh-NC, sh-SNHG6, sh-SNHG6+ inhibitor NC, sh-SNHG6+ miR-543 inhibitor, sh-SNHG6+ pcDNA, and sh-SNHG6+ pcDNA-LAMC1. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

were co-transfected with miR-543 inhibitor or pcDNA-LAMC1 compared to cells transfected with sh-SNHG6 alone (Fig. 4b). Through Western blotting, we measured the levels of several markers of EMT. Knockdown of SNHG6 decreased protein levels of Snail and Vimentin but increased E-cadherin level (Fig. 4c), suggesting that the reduction of SNHG6 suppressed EMT process. MiR-543 inhibitor or pcDNA-LAMC1 rescued the levels of Snail and Vimentin in sh-SNHG6-transfected cells and decreased E-cadherin level to baseline (Fig. 4c). We also examined PI3K/Akt signaling as it is involved in EMT and also previous study implicates LAMC1 in Akt pathway. Knockdown of SNHG6 decreased the levels of LAMC1, p-PI3K and p-AKT, while co-overexpression of miR-543 inhibitor or pcDNA-LAMC1 recovered the levels (Fig. 4d). Altogether, these results show that inhibition of SNHG6 decreases the migration ability of

BC cells and suppresses EMT and PI3K/AKT signaling via miR-543 and LAMC1.

MiR-543 regulated BC cell proliferation and apoptosis via LAMC1

We then tested the role of miR-543/LAMC1 axis in BC cell proliferation and apoptosis. Results of colony formation assay showed that overexpression of miR-543 remarkably decreased the number of colonies formed, while co-overexpression of pcDNA-LAMC1 recovered the number (Fig. 5a). The flow cytometry results indicated that transfection of miR-543 mimics significantly increased the percentage of cell apoptosis, and that this increase was inhibited by pcDNA-LAMC1 transfected cells (Fig. 5b). Therefore, we

Fig. 5 miR-543 regulated BC cell proliferation and apoptosis via LAMC1. (a) Representative images of colonies formed in cells with overexpression of mimics NC, miR-543 mimics, miR-543 mimics + pcDNA, and miR-543 mimics + pcDNA-LAMC1. (b) Flow

cytometry analysis of cell apoptosis in cells with overexpression of mimics NC, miR-543 mimics, miR-543 mimics + pcDNA, and miR-543 mimics + pcDNA-LAMC1. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Fig. 6 miR-543 regulated BC cell migration and EMT via LAMC1. **(a)** Representative images of invasive cells transfected with mimics NC, miR-543 mimics, miR-543 mimics + pcDNA, and miR-543 mimics + pcDNA-LAMC1. **(b)** Representative images of scratch wounds in cells with overexpression of mimics NC, miR-543 mimics, miR-543 mimics + pcDNA, and miR-543 mimics + pcDNA-LAMC1. **(c)** Relative protein levels of Snail, Vimentin, and E-cadherin in cells

with overexpression of mimics NC, miR-543 mimics, miR-543 mimics + pcDNA, and miR-543 mimics + pcDNA-LAMC1. **(d)** Relative protein levels of LAMC1, p-PI3K, and p-AKT in cells with overexpression of mimics NC, miR-543 mimics, miR-543 mimics + pcDNA, and miR-543 mimics + pcDNA-LAMC1. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

conclude that miR-543 suppresses BC cell proliferation but facilitates cell apoptosis via targeting LAMC1.

MiR-543 regulated BC cell migration and EMT via LAMC1

We evaluated the function of miR-543/LAMC1 in BC cell migration and EMT. Transwell assay results indicated that miR-543 mimics significantly reduced the number of invasive BC cells, while co-expression of pcRNA-LAMC1 restored it (Fig. 6a). Then, we used scratch wound healing assay to determine the migration

ability of BC cells. Transfection of cells with miR-543 mimics greatly diminished the migration rate of BC cells. However, co-overexpression of LAMC1 enhanced the migration (Fig. 6b). Western blotting results showed that miR-543 mimics decreased Snail and Vimentin but increased E-cadherin, while co-transfection with pcDNA-LAMC1 reversed the effects of miR-543 mimics on the expressions of those proteins (Fig. 6c). Similarly, miR-543 mimics downregulated the levels of LAMC1, p-PI3K, and p-AKT, while co-transfection with pcDNA-LAMC1 recovered those levels (Fig. 6d). These results indicate that miR-543 restrains the migration of BC cells and

Fig. 7 Knockdown of SNHG6 suppressed BC growth in vivo via miR-543. (a) Representative images of tumors from each group of mice. (b) Average tumor volumes in each group of mice with time. (c) Average tumor weight after 28 days of xenograft in each group of mice. (d) Relative SNHG6 levels in the tumors from each group of mice. (e) Relative miR-543 levels in the tumors from each group

of mice. (f) Relative LAMC1 mRNA levels in the tumors from each group of mice. (g) Relative protein levels of Snail, Vimentin, and E-cadherin in the tumors from each group of mice. (h) Relative protein levels of LAMC1, PI3K, p-PI3K, AKT, and p-AKT in the tumors from each group of mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

suppresses EMT and PI3K/AKT signaling via targeting LAMC1.

Knockdown of SNHG6 suppressed BC growth *in vivo* via miR-543

Finally, we examined the role of SNHG6/miR-543 in BC *in vivo* through the nude mouse xenograft model. As shown in Fig. 7a–c, the tumor grew rapidly with time in the mice implanted with sh-NC-transfected BC cells. However, both tumor weight and volume were consistently and significantly smaller in the mice bearing sh-SNHG6 transfected BC cells compared to control group (Fig. 7a–c). Notably, miR-543 inhibitor reversed the effects of sh-SNHG6 on tumor growth (Fig. 7a–c). As expected, we confirmed that sh-SNHG6 diminished SNHG6 expression, as well as LAMC1 mRNA level, but increased miR-543 level (Fig. 7d–f). miR-543 inhibitor suppressed those changes induced by sh-SNHG6 (Fig. 7d–f). Consistent with *in vitro* results, we found that knockdown of SNHG6 via sh-SNHG6 *in vivo* reduced protein levels of Snail and Vimentin but upregulated E-cadherin level, suggesting a suppression on EMT process (Fig. 7g). Again miR-543 inhibitor normalized those levels (Fig. 7g). Similarly, sh-SNHG6 diminished LAMC1 protein level, as well as p-PI3K and p-AKT, while miR-543 inhibitor restored the levels (Fig. 7h). Taken together, these findings demonstrate that SNHG6 knockdown suppresses EMT and restrains BC growth *in vivo* via miR-543/LAMC1.

Discussion

As the most prevalent cancer and the top cause of deaths among women, BC affects nearly 10% of women worldwide [1, 5]. Metastasis is the primary cause of BC-related death and EMT is largely involved in the metastasis of cancer cells [1, 18]. Here, we studied the molecular mechanisms of EMT and BC development, with the focus on the SNHG6/miR-543/LAMC1 axis. We showed that SNHG6 and LAMC1 were upregulated, while miR-543 was downregulated in BC tissues and cells. SNHG6 functioned to sponge miR-543 which directly targeted LAMC1. Knockdown of SNHG6 greatly restrained BC cell proliferation, migration, invasion, and EMT process, but promoted cancer cell apoptosis via miR-543/LAMC1/PI3K/AKT. Additionally, miR-543 modulated BC cell proliferation and EMT via LAMC1/PI3K/AKT signaling. These results clearly indicate that SNHG6 promotes BC cell proliferation, migration, and EMT via sponging miR-543 to disinhibit LAMC1/PI3K/AKT signaling.

Accumulating evidence suggest that lncRNAs are master regulators of tumorigenesis [19]. Dysregulated lncRNAs have been observed in varieties of cancers, such as lung cancer, colorectal cancer, and hepatocellular cancer [20–22].

In BC, aberrant expressions of many lncRNAs have been shown, including LINC01787, LINC00628, and LINC00511 [23–25]. Previous studies have reported that SNHG6 goes up in primary breast cancers and this elevation promotes cancer cell proliferation [14]. Here, consistently we observed increased level of SNHG6 in human BC tissues and cells. In addition, we found that knockdown of SNHG6 suppressed BC cell proliferation and promoted cell apoptosis. Knockdown of SNHG6 also inhibited BC cell migration, invasion and the EMT process. Besides BC, SNHG6 has been implicated as an important player in various tumors, such as hepatocellular carcinoma, gastric cancer, and bladder cancer [26–28]. Elevated SNHG6 level has been reported in those cancer cells and this elevation accelerates cancer cell proliferation and migration, indicating that SNHG6 has a conserved role as an oncogenic factor. SNHG6 mostly functions by sponging tumor suppressor miRNAs including miR-26a-5p, miR-101-3p, and miR-139-5p [12, 28, 29]. By acting as ceRNAs, SNHG6 regulates EMT process, TGF- β /Samd signaling, and PI3K/AKT pathway [30–32]. In BC, we demonstrated that SNHG6 exerted its function via sponging miR-543 to activate LAMC/PI3K/AKT signaling. It will be interesting to examine whether other mechanisms or miRNAs are involved in BC.

MiR-543 is involved in various cancers and its function varies depending on the type of cancers. In cancers like ovarian cancer and colorectal cancer, it has been indicated that miR-543 suppresses the tumor growth [33, 34]. On contrary, in cancers including oral squamous cell carcinoma and hepatocellular carcinoma, miR-543 acts as an oncogene [35, 36]. Here, in BC, we found that miR-543 functions as a tumor suppressor. Its expression level was decreased in BC tissues and cells. Further, overexpression of miR-543 suppressed BC cell proliferation, migration, invasion, and EMT. MiR-543 has multiple targets and that is probably the reason why it has distinct functions in different cancers. For instance, by targeting Twist homolog 1 (TWIST1), miR-543 restrains ovarian cancer cell proliferation and invasion [34]. In contrast, miR-543 promotes the development and progression of non-small cell lung cancer through targeting metastasis-associated protein 1 [37]. In BC cells, we identified that LAMC1 was the downstream target of miR-543 and we provided evidence showing that miR-543 regulated BC cell proliferation and migration via LAMC1. Further studies are necessary to examine whether other targets participate in BC.

LAMC1 is the gamma 1 chain of laminin, the most common subunit of laminins, and has crucial roles in cell migration and invasion [6]. It is also largely involved in the EMT during tumorigenesis [38]. Dysregulated LAMC1 is implicated in numerous cancers, such as hepatocellular cancer and colorectal cancer [9, 39, 40]. For example, silencing LAMC1 restrained prostate cancer cell proliferation and

migration [40]. LAMC1 is also involved in BC through unknown mechanisms. In the study, we showed that SNHG6/miR-543 is the upstream signaling of LAMC1 in BC. Interestingly, LAMC1 has been shown to promote the progression of hepatocellular cancer via PI3K/AKT signaling [8]. Here, we observed similar regulations in BC. Reduced level of LAMC1 induced by sh-SNHG6 or miR-543 mimics was accompanied by lower activity of PI3K/AKT signaling. These suggest that LAMC1/PI3K/AKT pathway might be a conserved signal among diverse cancers.

In summary, we demonstrate that SNHG6 promotes BC cell proliferation and migration and EMT via acting as a miR-543 sponge to activate LAMC1/PI3K/AKT pathway. Targeting SNHG6/miR-543/LAMC1 axis could be a promising avenue to treat BC.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10549-021-06190-y>.

Acknowledgements We would like to give our sincere gratitude to the reviewers for their constructive comments.

Funding This work was supported by Clinical Research Center for Breast & Thyroid Disease Prevention in Hunan Province (2018SK4001) and The Guiding Project of Clinical Medical Technology Innovation in Hunan Province (2018SK51513).

Declarations

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval The study was reviewed and received approval from the Ethics Committee of the Second Affiliated Hospital of University of South China.

Informed consent The informed consent is obtained from study participants.

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