Original Paper

LOC101930370/MiR-1471 Axis Modulates the Hedgehog Signaling Pathway in Breast Cancer

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Key Words

MiR-1471 • LOC101930370 • SHH • Gli-1 • Hedgehog pathway • Breast cancer

Abstract

Background/Aims: Non-coding RNAs (ncRNAs) play vital regulatory roles in many tumors. However, the functional roles of these transcripts responsible for their dysregulation in breast cancer (BC) are not thoroughly understood. **Methods:** We examined the expression of microRNA miR-1471 in BC specimens. Online analysis tools predicted that lncRNA LOC101930370 might act as an endogenous 'sponge' by competing for miR-1471 binding targets. Luciferase assays were used to prove the interaction of LOC101930370, miR-1471 and SHH. Edu, wound-healing and transwell assays were used to verify the contribution of miR-1471 and LOC101930370 on MCF-7 cells proliferation and metastasis. Gain and loss of function studies were performed to evaluate the relevance of Hedgehog pathway with LOC101930370/miR-1471 regulating axis in MCF-7 cells. **Results:** The expression of miR-1471 was markedly downregulated in BC. Inhibition of miR-1471 by LOC101930370 was proved by luciferase assay. Knockdown of LOC101930370 suppressed BC cells progression. MiR-1471 inhibitor resulted in a more aggressive metastasis of MCF-7 cells. Moreover, SHH and Gli-1 expression were significantly suppressed by LOC101930370 knockdown, and upregulated by miR-1471 inhibitor transfection. **Conclusions:** Collectively, our study reveals the interaction between LOC101930370 and miR-1471 for the first time. LOC101930370 positively regulates the expression of SHH by sponging miR-1471, which sheds new light on lncRNA-directed diagnostics and therapeutics in BC.

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Introduction

Breast cancer (BC) is a malignant neoplasm originating from breast tissue and the most common cause of death of women throughout the world [1, 2]. A central deficiency in our knowledge of cancer concerns how genomic changes drive the proteome to execute phenotypic characteristics. Advances in functional genomics have revealed that the human genome is actively transcribed. However, vast majority of the transcripts are non-coding RNA (ncRNA) including microRNAs and long non-coding RNAs (lncRNAs).

MicroRNAs (miRNAs), which are ~ 22 nucleotides in length, play important roles in the regulation of target gene expression by base pairing to complementary sites in 3'-untranslated regions (3'UTR) of the target mRNA [3, 4]. The microRNAs may act as oncogenes, tumor suppressors and modulators of tumor proliferation, invasion, apoptosis and therapy resistance [5-7]. In the case of BC, cDNA microarrays using probes for miRNAs have revealed that sets of miRNAs are differentially expressed in primary invasive carcinoma. Among them, some data have indicated that miR-1471 is related to BC oncogenesis [8]. However, the molecular mechanisms utilized by miR-1471 in BC are totally unclear. To clarify the potential role of miR-1471 in BC, we used TargetScan and microRNA.org to predict the putative targets of miR-1471. One of the most conserved is Sonic hedgehog (SHH), a crucial protein involved in Hedgehog pathway. Previous studies have shown that activation of the Hedgehog pathway can promote myofibroblast differentiation, stromal hyperplasia, and extracellular matrix (ECM) production, which facilitate the EMT process, invasion, and metastasis of BC [9-11]. Aberrant expression of SHH is implicated in the development of several tumors, including brain, pancreas, and BC [12-14]. Glioma-Associated Oncogene Family Zinc Finger 1 (Gli-1) is a transcriptional factor of the Hedgehog pathway [15-17]. Recent study has shown that the expression of Gli-1 is higher in TNBC cell lines and promotes angiogenesis by transcriptional modulation of the VEGFR2 [18]. Gli-1 levels also associate with the poor prognosis of BC [19-21]. However, whether miR-1471 regulates Hedgehog pathway by targeting SHH has not been clarified.

Unlike microRNAs, lncRNAs are larger than 200 bps in length. They have emerged as key players in a wide variety of cellular processes, including carcinogenesis and tumour growth [22, 23]. One of well-characterized mechanisms is the lncRNA-mediated gene regulation through complementary interaction with miRNAs [24]. For instance, GAS5 inhibits the expression of miR-21 through the competing endogenous RNA mechanism [25, 26]. LncRNA MIR31HG exhibits oncogenic property in pancreatic ductal adenocarcinoma and is negatively regulated by miR-193b [25]. However, whether lncRNAs are involved in the down-regulation of miR-1471 in BC remains unclear.

Taken together, in the present study, we investigated the expression and roles of lncRNA, microRNA-1471, SHH and transcription factor Gli-1 in BC. Meanwhile, the interaction among lncRNA, microRNA-1471, SHH and Gli-1 in regulation of BC metastasis and the possible mechanisms were also revealed.

Materials and Methods

Patients and specimens

Human Breast cancer specimens (breast tumor and adjacent non-tumor tissue) were obtained from patients diagnosed with mammary cancer who underwent surgery at the Second Affiliated Hospital of Harbin Medical University (Harbin, China), from February 2017 to June 2017. The specimens from patients were frozen in liquid nitrogen immediately after the cancer ectomy, then stored at -80°C. A total of 15 patients were enrolled and the clinicopathological information of patients is shown in Table 1. The research methods in our study were approved by the Ethics and Scientific Committees of Harbin Medical University. Written informed consents were obtained from all the enrolled patients (HMUIRB20170017).

Cell culture

Four breast cancer cell lines (MCF-7, MDA-MB-231, BT-474, SKBR3) were used (Shanghai fuxiang biotechnology Co., Ltd). MCF-7, MDA-MB-231 and SKBR3 were grown in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at the temperature of 37 °C in 5% CO₂. BT-474 was cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS) at the same incubator. For transfection, The MCF-7 cells were starved in serum-free medium for 24 h, and then transiently transfected with 100nM miR-1471 mimics, miR-1471 inhibitors or negative controls (RiboBio Co., Ltd., Guangzhou, Guangdong, China), and si-lncRNA (LOC101930370 siRNA) or si-NC (non-sense control of si-lncRNA) (Invitrogen, USA). X-treme GENE siRNA transfection reagent (Shanghai GenePharma, Ltd, China) was used according to the manufacturer's instructions.

Quantitative real-time PCR

QRT-PCR were used to determine the expression levels of LOC101930370, miR-1471, miR-718, SHH and Gli-1 transcripts. Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, USA) according to manufacturer's instructions. The SYBR Green Real-time PCR Master Mix Kit (TOYOBO) was used in real-time PCR for relative quantification of RNAs. GAPDH and U6 were used as an internal control.

The following primers were used:

LOC101930370: Forward, 5'-ACCTCTCCCTTGTGGAGGAT-3'; Reverse, 5'-CAGGTCCCCTCTCTTGTGAG-3'.
miR-1471: RT, 5'-GTCGTATCCAGTGGGAGTGGGCAATTGC
ACTGGATACGACACCT-3'; Forward, 5' -TGGACCCTGGTCTACTCTCTG-3'; Reverse, 5'
-CAAGAGCCCCTGTACAGCAT-3'.
miR-718: RT, 5'- GTCGTATCCAGTGGGAGTGGGCAATTGCA
CTGGATACGACCGACGC-3'; Forward, 5'- GTTATTCTCCGCCCCGCCAG-3'; Reverse, 5'-
GGCTCGTGGAGTCGGCAATTG-3'.
SHH: Forward, 5' -CCAATTACAACCCGACATC-3'; Reverse, 5'-CTCTGAGTGGTGGCCATCTT-3'.
Gli-1: Forward, 5'-GTGCAAGTCAAGCCAGAACA-3'; Reverse, 5' -GGGGCCTGACTGGAGATATT-3'.
GAPDH: Forward, 5'-AAGAAGGTGGTGAAGCAGGC-3'; Reverse, 5' -TCCACCACCCAGTTGCTGTA-3'.
U6: Forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3'; Reverse, 5' -CGCTTCACGAATTTGCGTGTCTAT-3'.

Western blotting

MCF-7 cell protein samples were grinded in 200 ul of ripa containing a cocktail of protease inhibitor. Lysates were centrifuged at 13500 r/s for 20 min. Protein concentration in the supernatant was measured by BCA Protein Assay (Beyotime, Shanghai, China). Equal amounts of protein samples (80 ug) were fractionated on a 10% SDS-PAGE gel electrophoresis and transferred onto nitrocellulose membranes. Then the membranes were blocked in the defatted milk (5%) for 2 h and incubated on the shaker at 4°C overnight with the primary antibodies of SHH (Abcam, Cambridge, MA, USA), Gli-1 (Abcam, Cambridge, MA, USA), vimentin (Wanlei, Inc., Shenyang, China), E-cadherin (Wanlei, Inc., Shenyang, China), α-SMA (Wanlei, Inc., Shenyang, China) with GAPDH (Zhongshanjinqiao, Inc., Beijing, China) as an internal control. Appropriate secondary antibody was (Invitrogen) incubated for 1 h at room temperature. Protein bands were visualized and quantified by Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA).

Cell proliferation

Cell proliferation was measured using a Cell Counting Kit-8, CCK8 (Dojindo, Kumamoto, Japan). MCF-7 cells were plated in 24-well plates at 3x10⁵ cells/well. Then cells were incubated in 10% CCK8 which was

Table 1. Clinicopathological information of 15 breast cancer specimens

	Cases n=15
Age at diagnosis, years	43 (21, 85)
Tumor stage	
pT1a	0
pT1b	5 (33%)
pT1c	8 (54%)
pT2	2 (13%)
Lymph node involvement	
pN0	7 (47%)
pN1	5 (33%)
pN2	3 (20%)
Grading	
G1	5 (33%)
G2	7 (47%)
G3	3 (20%)
Estrogen receptor status	
Positive	12 (80%)
Negative	3 (20%)
Progesterone receptor status	
Positive	13 (87%)
Negative	2 (13%)

diluted in normal culture medium at 37°C for color conversion. Proliferation rates were determined at 24 h after transfection.

EdU assay kit

Cells plated on coverslips in 6-well culture plates were treated as experiment design. The proliferation of MCF-7 cells were detected by EdU kit (RiboBio, China) according to the manufacturer's instructions. A fluorescence microscope (Olympus, Japan) was used to acquire the images. Nuclei that double labeled with EdU and DAPI were considered to be positive cells.

Cell migration and Invasion

To analyze wound healing, the cells were seeded in six-well plates with complete DMEM medium. After 48 h, the cell monolayer was wounded using a plastic pipette tip. Then the cells were rinsed with PBS and cultured with serum-free DMEM for 24 h. The wound closure was observed and photographed under a microscope. For the Transwell assays, 8-µm pore size chambers (Corning, NY, USA) were used with an insert coated with Matrigel (BD Bioscience). Twenty-four hours after transfection, 1×10^5 cells in serum-free medium were added to the upper chamber. The lower chamber was filled with 10% FBS DMEM. After 24 h of incubation, the cells remaining on the upper surface of the membrane were removed, whereas the cells that had invaded through the membrane were fixed with 100% methanol for 15 minutes, stained with 0.1% crystal violet for 20 minutes. Images of MCF-7 cells were taken under a phase-contrast microscope (Olympus, Japan).

Immunofluorescence staining and immunocytochemistry

For immunofluorescence staining, MCF-7 were fixed with 4% buffered paraformaldehyde in PBS. Blocking solution (1% BSA and 0.1% Triton-X in PBS) was used to penetrate and incubate fixed cells at room temperature for 2 h. Primary antibodies against SHH (1:200) and Gli-1(1:200) were placed in PBS overnight at 4 °C, followed by incubation with the appropriate secondary antibody (Invitrogen) for 1 h at room temperature. Then nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI; Beyotime, Shanghai, China) for 20 mins at room temperature. Immunofluorescence was examined and analyzed by laser scanning confocal microscopy (FV300, Olympus, Japan).

Statistical analysis

Group data was described as the mean $\pm$ S.E.M. Student's t-test or one-way ANOVA accompanied by Bonferroni's Multiple Comparison Test was used to analyze comparisons. $p < 0.05$ was considered to indicate a significant difference. Data were analyzed using the GraphPad Prism 5.0 software.

Results

MiR-1471 is down-regulated in breast cancer

RNA regulatory mechanisms have been connected to oncogenesis. Studies from whole genome and transcriptome analysis have indicated that miR-1471, miR-718 were down-regulated in breast cancer [8]. To further validate the possible roles of these two miRNAs in BC, we evaluated the miR-1471 and miR-718 levels in 15 paired breast cancer and adjacent breast tissue samples using qRT-PCR. The results showed that miR-1471 was significantly (0.43 fold changes) decreased in tumor tissues compared to adjacent breast tissues (Fig. 1a). However, downregulation of miR-718 in BC tissues was not such apparent (Fig. 1b). We then tested the expression level of miR-1471 in several breast cancer cell lines (MCF-7, BT-474, SKBR3, MDA-MB-231). Compared with non-tumor breast tissues, MCF-7 cells exhibited lowest expressed miR-1471 (Fig. 1c). Collectively, these results suggested that miR-1471 was down-regulated in BC cells and tissues. That might indicate a potential tumor suppressor role of miR-1471 in BC tumorigenesis.

LOC101930370 is a competing endogenous RNA (ceRNA) of miR-1471

Based on our recent studies [25], we selected lncRNAs connected to the control of miR-1471. Two databases LncTar and NCBI BLAST predicted that lncRNA LOC101930370

Fig. 1. MiR-1471 was dysregulated in breast cancer. a. Relative miR-1471 expression levels in 15 paired breast cancer and adjacent breast tissue specimens determined by qRT-PCR. b. Relative miR-718 expression levels in 15 paired breast cancer and adjacent breast tissue specimens determined by qRT-PCR. c. MiR-1471 expression levels in four breast cancer cell lines, MCF-7, MDA-MB-231, BT-474, SKBR3 and non-tumor breast tissue, determined by qRT-PCR. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ vs. Control. $n = 3$ batches of cells. The data are presented as mean $\pm$ SEM.

Fig. 2. LOC101930370 is a ceRNA of miR-1471. A. Sequence complementarity between LOC101930370 and miR-1471. b. Luciferase reporter constructs containing the binding sites of LOC101930370 and miR-1471, or the mutated binding sites. c, d. Luciferase reporter assay showed that transfected miR-1471 could reduce the luciferase activity in wild-type (wt) of Luciferase constructs but not in mutant (mut) one. ** $p < 0.01$, ## $p < 0.01$ vs. miR-1471. e. LOC101930370 expression levels in 15 paired breast cancer and adjacent breast tissue specimens determined by qRT-PCR. f. LOC101930370 expression levels determined by qRT-PCR. g. MiR-1471 expression levels determined by qRT-PCR. h. The influence of miR-1471 mimic and inhibitor on expression of LOC101930370. i. MiR-1471 expression levels after miR-1471 mimic and inhibitor transfection in MCF-7 cells. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ vs. Control. $n = 3$ batches of cells. The data are presented as mean $\pm$ SEM.

was complementary binding with miR-1471 (Fig. 2a). Luciferase assay was used to identify the inhibitory effect of LOC101930370 on miR-1471 (Fig. 2b). The results demonstrated that miR-1471 dramatically inhibited the luciferase activity of the constructed plasmid containing wild-type (WT) LOC101930370, but not that of the mutant (mut) plasmid (Fig. 2c and d). We then tested the expression of LOC101930370 in tumor specimens from BC patients. The results suggested a significant upregulation of LOC101930370 in BC tissues (Fig. 2e). In addition, LOC101930370 expression was remarkably upregulated in MCF-7 cells (for all online suppl. material, see www.karger.com/doi/10.1159/000491980, Suppl. Fig. 1). Therefore, we proceeded to explore a potential regulatory axis involving LOC101930370 and miR-1471. To verify the regulated interaction of the two ncRNAs, we transfected MCF-7 with small interfering RNA against LOC101930370 (si-lncRNA). LOC101930370 level was significantly suppressed (Fig. 2f). Cellular miR-1471 levels were significantly increased after the si-lncRNA transfection (Fig. 2g). To determine if miR-1471 has feedback regulatory effects on LOC101930370, miR-1471 mimics, miR-1471 inhibitor, and nonsense control were transfected to MCF-7 cells. Significant overexpression or knockdown of miR-1471 did not affect cellular LOC101930370 levels (Fig. 2h and i). Collectively, LOC101930370 is associated with the reduction of miR-1471 in BC.

Fig. 3. Knockdown of LOC101930370 inhibits MCF-7 cell proliferation. **a.** Representative images of Edu staining showing proliferation cells (stained in pink). Nuclei that double labeled with EdU (red) and DAPI (blue) were considered to be proliferative cells. Scale bar indicates 100 μ m. **b.** The Edu positive cells were counted. **c.** MCF-7 cells viability were detected by CCK8 assay. **d,f.** The effect of LOC101930370 downregulation on the invasion of MCF-7 cells was assessed using transwell assay. Scale bar indicates 100 μ m. **e,g.** Wound healing assay showed that LOC101930370 siRNA resulted in a slower closing of scratch wounds. ** p <0.01 *** p <0.001 vs. Control. n =3 batches of cells. Data are expressed as mean $\pm$ SEM.

Knockdown of LOC101930370 inhibits MCF-7 cell proliferation and metastasis

Next, we asked if decreasing LOC101930370 protected MCF-7 cells from malignancy. We transfected MCF-7 cells with siRNA against LOC101930370. 5-Ethynyl-2'-deoxyuridine (Edu) assay was performed for determining cell proliferation. The results showed that Edu positive cells were drastically reduced following LOC101930370 downregulation (Fig. 3a and b). Consistent with this, cell viability detected by CCK8 showed that LOC101930370 knockdown significantly decreased MCF-7 cell viability (Fig. 3c). To further determine whether LOC101930370 was associated with the progression of MCF-7, we analyzed the effect of LOC101930370 knockdown on the invasive behavior of MCF-7 cells. Using a transwell system, we found that the invasive ability of MCF-7 cells was significantly decreased following si-lncRNA mediated downregulation of LOC101930370 (Fig. 3d and f). In addition, the wound-healing assay showed that cells transfected with si-lncRNA underwent a slower closing of scratch wounds compared with the control groups (Fig. 3e and g). Taken together, these results suggested that LOC101930370 siRNA might act as a tumor suppressing tool through inhibiting cell proliferation, migration and invasion in BC.

MiR-1471 inhibitor enhances tumor growth and tumorigenesis

Next, we sought to determine the effect of miR-1471 on the proliferation and tumor-propagating potential of MCF-7 cells. We conducted gain-of-function experiment using miR-1471 mimics and loss-of-function experiments using miR-1471 inhibitor (Fig. 2i). After transfection, MCF-7 cells proliferation and viability were examined by Edu assay (Fig. 4a and b) and CCK8 (Fig. 4c). Transfected with miR-1471 mimic exhibited a significant decrease of cell proliferation and cell viability. But miR-1471 inhibitor caused a marked increase in the proliferation and viability of MCF-7 cells. Following the same treatment regime, we also examined cell invasion and migration. Invasion of MCF-7 cells was significantly reduced following miR-1471 mimic transfection as shown by transwell assay. While MCF-7 cell invasion increased after miR-1471 inhibitor transfection compared with control group (Fig. 4d and f). Also, the cell migration was suppressed by miR-1471 mimic, upregulated by miR-1471 inhibition (Fig. 4e and g). Taken together, these results indicated the tumor suppressing roles of miR-1471 in MCF-7 cells, which was opposite to its ceRNA LOC101930370.

SHH is the downstream target of miR-1471

The next question is how LOC101930370/miR-1471 modulates the MCF-7 cell phenotypes. In an effort to identify the putative target of miR-1471, we used TargetScan

Fig. 4. MiR-1471 inhibitor enhances tumor progression. a. Representative images of Edu staining showed proliferation cells (stained in pink). Nuclei that double labeled with Edu (red) and DAPI (blue) were considered to be proliferative cells. Scale bar indicates 100 μ m. b. The Edu positive cells were counted. c. MCF-7 cells viability were detected by CCK8 assay. d,f. The effect of miR-1471 mimic and miR-1471 inhibitor transfection on the invasion of MCF-7 cells was assessed using transwell assay. Scale bar indicates 100 μ m. e.g. Wound healing assay showed that miR-1471 overexpression resulted in a slower closing of scratch wounds, miR-1471 inhibition caused a faster closing of scratch wounds. **p<0.01 ***p<0.001 vs. Control. n=3 batches of cells. Data are expressed as mean $\pm$ SEM.

Fig. 5. SHH acts as a downstream target of miR-1471. a. Sequence complementarity between miR-1471 and SHH 3'UTR in human. b. Luciferase reporter constructs containing 3'UTR of SHH or mutated 3'UTR of SHH. c,d. Luciferase reporter assay showed that transfected miR-1471 could reduce the luciferase activity in wild-type (wt) of SHH but not in mutant (mut) 3'UTR of SHH. **p<0.01, ##p<0.01 vs. miR-1471. e. SHH expression levels in 15 paired breast cancer and adjacent breast tissue specimens determined by qRT-PCR. f. Gli-1 expression levels in 15 paired breast cancer and adjacent breast tissue specimens determined by qRT-PCR. **p<0.01 vs. Control. n=3 batches of cells. The data are presented as mean $\pm$ SEM.

and microRNA.org to predict its complementary binding targets. Our attention was focused on SHH, a major pleiotropic developmental gene involved in Hedgehog signaling pathway (Fig. 5a). Together with its downstream transcription factor Gli-1, they control epithelial-mesenchymal transition, proliferation, migration and invasion of different cancer cells [27]. Furthermore, we used luciferase assay to confirm the inhibitory effects of miR-1471 on SHH (Fig. 5b). As the results demonstrated, the luciferase activity of the constructed plasmid containing wild-type (WT) 3'UTR of SHH was inhibited by miR-1471, but not that of the mutant (Mut) 3' UTR (Fig. 5c and d). Our results showed that SHH and Gli-1 mRNA levels were significantly up-regulated in tumor specimens of BC patients (Fig. 5e and f).

LOC101930370/miR-1471-mediates BC progression by regulation of SHH/Gli-1

Accordingly, we hypothesized that SHH may serve as a biologically relevant target of miR-1471 and participate in LOC101930370/miR-1471-mediated regulation of breast cancer development. As the downstream regulatory targets, si-lncRNA suppressed SHH and

Gli-1 mRNA and protein expression in MCF-7 cellular nucleus, exhibited by qRT-PCR (Fig. 6a and b), WB (Fig. 6c and d) and immunofluorescence (Fig. 5e and f) (see online suppl. material, Suppl. Fig. 2) results. We also detected the levels of soluble SHH in the cell culture supernatants. The results showed that the secreted SHH was decreased by LOC101930370 siRNA transfection (see online suppl. material, Suppl. Fig. 3a). All these results suggested an important role of LOC101930370 in modulating SHH by competitively binding miR-1471.

MiR-1471 inhibits Hedgehog signaling pathway by targeting SHH

We then evaluated the influence of miR-1471 on SHH and Gli-1 expression using the similar transfection procedure. For this purpose, we transduced the MCF-7 cells with miR-1471 mimic for overexpression and miR-1471 inhibitor for inhibition. Consistent with our prediction, SHH and Gli-1 transcripts were reduced after transfection of miR-1471 mimic, and increased by miR-1471 inhibitor (Fig. 7a and b). In agreement, western blot (Fig. 7c and d) and immunofluorescence (Fig. 7e and f) (see online suppl. material, Suppl. Fig. 4) analysis showed a decrease in SHH and Gli-1 protein levels with transfection of miR-1471 mimic over control group. A remarkable increase of these two protein expression was also observed after miR-1471 inhibitor transfection. Meanwhile, soluble SHH in the cell culture supernatants had a marked decreasing after miR-1471 mimic transfection, while increased by miR-1471 inhibitor (see online suppl. material, Suppl. Fig. 3b). Epithelial-mesenchymal

Fig. 6. Downregulation of LOC101930370 inhibits SHH and Gli-1 expression. QRT-PCR analysis of SHH (a) and Gli-1 (b) mRNA levels after LOC101930370 siRNA transfection. Western blot analysis of SHH (c) and Gli-1 (d) protein levels. Representative images of immunofluorescence staining for SHH (green) (e), Gli-1 (green) (f) in MCF-7 cells. The nuclei were stained in blue with DAPI. Scale bar indicates 100 μ m. *** p <0.001 vs. Control. n =3-5 batches of cells. Data are expressed as mean $\pm$ SEM.

Fig. 7. SHH and Gli-1 are the downstream targets of LOC101930370/ miR-1471 axis. QRT-PCR analysis of SHH (a) and Gli-1 (b) mRNA levels after miR-1471 mimic and inhibitor transfection. Western blot analysis of SHH (c) and Gli-1 (d) protein levels. Representative images of immunofluorescence staining for SHH (green) (e), Gli-1 (green) (f) in MCF-7 cells. The nuclei were stained in blue with DAPI. Scale bar indicates 100 μ m. * p <0.05 *** p <0.001 vs. Control. n =3-5 batches of cells. Data are expressed as mean $\pm$ SEM.

transition (EMT) program is the major result of Gli-1 activation in cancer. We then found the upregulation of epithelial cell marker E-cadherin, and downregulation of mesenchymal cell marker vimentin and α -SMA after miR-1471 mimic transfection. While miR-1471 inhibitor transfection caused the opposite results (see online suppl. material, Suppl. Fig. 5). That demonstrated the suppressive effect of miR-1471 on EMT through targeting SHH. Taken together, SHH/Gli-1 signaling pathway is the downstream effector of LOC101930370/miR-1471 mediated breast cancer metastasis.

Discussion

BC is a clinically challenging disease involving complicated changes in the genome and multiple related signaling pathways [28]. To date, changes of epigenetic modification in cancer related genes have been the major focus [29-31]. Among them, the role of Hedgehog signaling pathway in breast cancer has been recognized long ago. Most of the interest in the Hedgehog signaling pathway in cancer is in its role as a tumor suppressor [18, 32, 33]. SHH ligand is necessary for Hedgehog signaling activation. In BC, SHH has been proved to be overexpressed in tumor tissues and serum of BC patients [34]. Inhibition of SHH contributed to the anti-tumor activity of thioestrepton in BC [35]. Moreover, its downstream regulator Gli-1, was crucial for invasion of BC and associated with unfavourable overall survival of BC [19, 36]. Consistent with these studies, our research also suggested a remarkable upregulation of SHH and Gli-1 in BC tissues. Downregulation of SHH and Gli-1 by miR-1471 caused the suppression of EMT process in MCF-7 cells. While upregulation of SHH and Gli-1 exerted the opposite effects.

Moreover, it became apparent that ncRNAs were potent regulators of the pathway in this context. The importance of ncRNAs is demonstrated by the fact that a substantial part of genome is devoted to their transcription [24, 37]. As the power of miRNA in regulation of mRNA has been explored elaborately, we are beginning to recognize the complex interactions of miRNAs and lncRNAs and ensuing RNA regulatory networks. As the potent upstream regulators of Hedgehog signaling pathway, interaction of miRNAs with lncRNAs revealed in this study discloses the missing links in epigenetic regulation of SHH and provides novel therapeutic strategies for BC.

In our study, we firstly identified the anti-metastatic functions of miR-1471 on MCF-7 cells. Schrauder et al suggested that miR-1471 was downregulated in BC for the first time [8]. However, some studies reported that miR-1471 was overexpressed in lung adenocarcinoma [38] and hepatocellular carcinoma [39]. But the molecular mechanisms of miR-1471 in cancer development were still unknown. Here we also observed a downregulation of miR-1471 in BC tissues and proved miR-1471 as a tumor suppressor by targeting SHH dependent Hedgehog signaling pathway. Moreover, gain-of-function as well as loss-of-function experiments also revealed that the proven oncogenesis effects of SHH rely on the miR-1471. Our results showed that miR-1471 significantly downregulated SHH expression in transcript and protein levels. After transfection with miR-1471 mimic, we recorded a trend in SHH downregulation. On the contrary, SHH expression was significantly increased after miR-1471 inhibitor transfection, whereas the expression levels remained unchanged in miR-1471 NC-transfected cells.

Emerging evidences have demonstrated that lncRNAs can function as a natural miRNA sponge, interfere with miRNA pathways and modulate the repression of miRNA targets [40, 41]. LncRNA-H19/MiR-675-5p Pathway mediated the inhibitory effects of Huaier Extract on BC Progression [42]. MALAT1 induced migration and invasion of human BC cells by competitively binding miR-1 with cdc42 [43]. Here we identified LOC101930370 might responsible for the downregulation of miR-1471 in BC. Luciferase assay proved the inhibition effect of LOC101930370 on miR-1471. Real-time PCR showed that LOC101930370 levels were significantly elevated in BC tissues and MCF-7 cells. As expected, LOC101930370 could positively regulate SHH expression through sponging miR-1471 in MCF-7 cells. And

knockdown of LOC101930370 by its siRNA inhibited MCF-7 cell metastasis via upregulation of miR-1471. In the meanwhile, the downstream transcriptional factor of SHH, Gli-1, was significantly down-regulated by LOC101930370 siRNA transfection. Nevertheless, further investigation of LOC101930370 may also contribute to understanding of SHH biology and SHH-driven oncogenesis.

In conclusion, miR-1471 exerts anti-metastatic effects by suppression of SHH-dependent Hedgehog signaling pathway in MCF-7 cells. Besides, we identify LOC101930370 as an oncogene, which exerts a crucial role in the proliferation and metastasis in BC. Moreover, for the first time, our study sheds light on the interaction between LOC101930370 and miR-1471, and reveals that LOC101930370 positively regulates the expression of SHH by sponging miR-1471 in BC. Our findings will help to gain knowledge about the molecular mechanisms of Hedgehog signaling pathway in BC and show that RNAi-based approaches may contribute to the development of more effective cancer therapies.

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Disclosure Statement

The manuscript is original work that has not been submitted to and is not under consideration for publication by another journal. We confirm that all the listed authors have participated actively in the study and have seen and approved the submitted manuscript. The authors declare to have no competing interests.

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