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Long non-coding RNA LINC01287 promotes breast cancer cells proliferation and metastasis by activating Wnt/ß-catenin signaling

C. SONG, P. SUN, Q. HE, L.-L. LIU, J. CUI, L.-M. SUN

Department of Oncology, The 2nd Affiliated Hospital of Dalian Medical University, Dalian, Liaoning Province, China.

Abstract. – OBJECTIVE: Long noncoding RNA (lncRNAs) frequently exhibited abnormal levels in numerous tumors and other diseases in current biological researches. LINC01287, a newly discovered lncRNA, has been found to act as an oncogene in hepatocellular carcinoma. The aim of this research was to explore the expressions and functions of LINC01287 in breast cancer (BC).

PATIENTS AND METHODS: The relative expressions of LINC01287 in BC tissues and cells were determined using RT-PCR. The associations between the LINC01287 expression, the clinicopathological factors, and the overall survival of BC patients were statistically examined. The apoptosis and proliferation abilities of MCF-7 and MDA-MB-468 cells were analyzed by MTT and flow cytometry assay after LINC01287 knockdown. The effects of LINC01287 in migration and invasion were determined using wound-healing and transwell assays. The protein expressions of the Wnt/β-catenin pathway were determined using Western blot.

RESULTS: We showed that the levels of LINC01287 were significantly upregulated in BC tissues and BC cell lines, and the abnormal expressions of LINC01287 were correlated with TNM stage and lymph node metastasis. A distinct difference was observed and indicated that BC patients with higher LINC01287 expressions had significantly shorter overall survival than patients with lower LINC01287 expressions. The multivariate analysis demonstrated that LINC01287 expression was independently correlated with the overall survival. Si-LINC01287 transfection significantly inhibited the proliferation and metastasis of BC cells, and further promoted apoptosis. Besides, the knockdown of LINC01287 suppressed Wnt/β-catenin activation and affected the expressions of β-catenin, cyclin D1, and c-myc.

CONCLUSIONS: Our findings indicated that the new lncRNA LINC01287 was correlated with poor clinical outcome and may function as a novel prognostic biomarker and therapeutic target in the development of antineoplastic therapies for BC.

Key Words:
LINC01287, Breast cancer, Metastasis, Prognosis, Wnt/β-catenin pathway.

Introduction

Breast cancer (BC) is one of the most common malignancies among women throughout the world and is estimated to account for 26% of all new tumor cases in China in 2017[1,2]. BC is classified into numerous molecular subtypes, such as basal-like, HER2-positive, and luminal breast tumors[3,4]. Although the mortality of some BC patients decreased along with the development in surgery, radiotherapy, and chemotherapy, the overall survival of BC patients with advanced stages remains shorter[5,6]. Even though the great majority of tumors begin as primary central abrasions, the distant metastasis of these primary neoplasms is associated with advanced clinical stages, poorer clinical outcome, and eventual patients deaths[7,8]. For this cause, the key for the improvement of patients' survival is the exploration of biomarkers which can be used for the identification of tumors likely to metastasize.

Long noncoding RNA (lncRNA) is another class of noncoding RNA with more than two hundred nucleotides in length[9]. Multiple studies[10,11] have provided evidence that lncRNAs are involved in diverse cellular processes, including proliferation, stem cell pluripotency, invasion, cell cycle progress, and apoptosis, causing a paradigm adjustment in our concepts of genes modulation. Recently, some findings[12-14] indicate that the dysregulation of some functional lncRNAs expressions plays a tumor-suppressive or oncogenic role in the initial and progression of various tumors, and that the examination of these lncRNAs may be used as a novel approach for the development of tumor diagnosis and prognosis prediction. For

Corresponding Author: Li-Min Sun, MD; e-mail: sun2uunl1@aliyun.com

instance, lncRNA SOX21-AS1 was found to be highly expressed and predicted shorter overall survival in hepatocellular carcinoma patients. The overexpression of this lncRNA was also observed to suppress the metastasis of tumor cells via modulating the expressions of p21[15]. LncRNA SNHG1 expression was shown to be up-regulated in osteosarcoma and its knockdown displayed tumor-suppressive effects in tumor growth and metastasis by regulating miR-577[16]. In BC, it was found that the forced up-regulations of lncRNA SNHG14 promoted BC cells proliferation and invasion by modulating miR-193a-3p[17].

LncRNA LINC01287 (LINC01287), located on 7q36.2, is a newly discovered lncRNA and was mainly expressed in the nucleus and highly conserved among mammalian tissues. The studies on LINC01287 in diseases are limited. Previously, the dysregulation of LINC01287 has been reported in gastric cancer and hepatocellular carcinoma[18,19]. However, the potential functions of LINC01287 in other tumors remain largely unclear. In this study, we firstly reported the frequent up-regulation of LINC01287 in BC. Then, we provided evidence that LINC01287 acted as a tumor suppressor in BC progression. Our findings highlighted the potential values of LINC01287 as a new prognostic biomarker and therapeutic target for BC patients.

Patients and Methods

Clinical Specimens

One hundred and twelve surgically resected BC specimens and adjacent normal tissue samples were obtained from the 2nd Affiliated Hospital of Dalian Medical University from April 2008 to March 2012. The patients enrolled in this study provided the written informed consents. Before surgery, none of the patients received local therapy. The specimens were immediately preserved at -80°C after resection. The study protocols were approved by the Ethical Committee of the 2nd Affiliated Hospital of Dalian Medical University Hospital. Table I showed the clinical features of BC patients.

Cell Transfection

The cells used in this study, including MCF-10A (as control cells), MDA-MB-468, MDA-MB-453, MDAMB-231, and MCF-7, were brought from RuiLu Biological company (Xiamen, Fujian, China). These cell lines were cultured using RPMI-1640 media with 10% FBS and maintained in an incubator with 5% CO_2 (at 37°C). For small interfering RNA (siRNA) transfection, the Lipofectamine 2000 reagent kits (MinYuan, Yantai, Shandong, China) were used. The siRNAs tar-

Table I. Correlation between LINC01287 expression and different clinicopathological features in BC patients.

Parameters	Number	LINC01287 expression		*p*-value
		Low	High	
Age (years)				NS
< 50	55	25	30	
≥ 50	57	32	25	
Tumor size				NS
<2.5 cm	70	39	31	
≥2.5 cm	42	18	24	
ER				NS
Positive	61	28	33	
Negative	52	29	23	
PR				NS
Positive	64	34	30	
Negative	48	23	25	
HER2 status				NS
Positive	63	35	28	
Negative	49	22	27	
Lymph node metastasis				0.015
Yes	31	10	21	
No	81	47	34	
TNM stage				0.019
I/II	37	13	24	
III	75	44	31	

geting LINC01287 (si-lncRNA#1, si-lncRNA#2) and the control siRNAs (si-NC) were brought form RiboBio Biological company (Guangzhou, Guangdong, China).

Real-Time PCR Analyses

TaKaRa RNAiso Plus kits (Shengxing, Nanjing, Jiangsu, China) were applied to extract the total RNAs. Then, the cDNA was reversely transcribed using the RT cDNA Mix kits (Jianda, Suzhou, Jiangsu, China). Thereafter, qPCR detection for LINC01287, β-catenin, cyclin D1, and c-myc was conducted using SYBR Green qPCR kits (YunGene, Ningbo, Zhejiang, China), according to the protocols provided by the manufacturer. The fold change was calculated using the $2^{-\Delta\Delta Ct}$ method. GAPDH was taken as an internal reference for LINC01287, β-catenin, cyclin D1, and c-myc detection. The primer sequences were as follow: GAPDH, 5'-GGAGCGAGATCCCTCCAAAAT-3' (forward) and 5'-GGCTGTTGTCATACTTCTCATGG-3' (reverse); LINC01287, 5'-CGAGTACTTCTAATACCCAGT-3' (forward) and 5'-AAAGGGTCCTCTCACTAAAAG-3' (reverse).

Western Blot Analyses

The Western blot analyses were conducted using the following antibodies: anti-caspase 3 antibody (1:800; PTG, Wuhan, Hubei, China), anti-caspase 9 antibody (1: 1000; PTG, Wuhan, Hubei, China), anti-β-catenin antibody (1:700; CST, Danvers, MA, USA), anti-cyclin D1 antibody (1:1100; BOSTER, Wuhan, Hubei, China), anti-c-myc antibody (1:800; Abcam, Cambridge, MA, USA), anti-GAPDH antibody (1:1500; BOSTER, Wuhan, Hubei, China). The cell lysates were collected using RIPA buffer (ShengTian, Guangzhou, Guangdong, China). Then, the protein extracted were separated using SDS-PAGE (8-12%), followed by transfection onto polyvinylidene difluoride (PVDF) membranes. After being blocked in 5% BSA-TBST buffer for 2.5 h, the membranes were incubated with the primary antibodies described above. After being probed using antibodies overnight at 4°C, the membranes were then washed using the Tris-Buffered Saline with Tween 20 (TBST), followed by incubation with matched secondary antibodies. Finally, the proteins were visualized by the enhanced chemiluminescence (ECL) kits (Zhongyuan, Xi'an, Shanxi, China).

Proliferation Evaluation

The cellular proliferation was detected by CCK-8 assays using CCK-8 detection kits (HuoDe, Hangzhou, Zhejiang, China). Briefly, the treated BC cells (MCF-7 and MDA-MB-468) were placed into plates (nighty-six well) with 2.5×10^3 cells/well. At the designated time points (48 h, 72 h, and 96 h), the cellular proliferation was detected by adding CCK-8 reagents (25 μl per well) into the plates. The plates were then placed in an incubator (37°C, 5% CO_2) for about 1.5 h, and the absorbance values at 450 nm were measured on a microplate reader system.

Clonogenic Formation Analysis

The treated BC cells (MCF-7 and MDA-MB-468) were placed into new plates (six-well) at a density of 800 cells/well. Then, 850 μl complete media (10% FBS) was added into the plates. The plates were then placed in an incubator (37 °C, 5% CO_2) for about 15 days. The media was changed every two days. Then, the visible colonies were fixed using formaldehyde (4%) for 15 min. After washing using PBS for two times, the crystal violet solution (0.1%) was applied to stain the colonies. Finally, the colonies were washed by PBS for three times and counted under a microscope.

Cell Apoptosis Detection

The percentages of apoptotic BC cells after treatment with LINC01287 siRNAs were detected by Apoptosis flow cytometry detection kits (YingBo; Chengdu, Sichuan, China). In short, the treated cells were trypsinized and collected in a centrifuge tube. Then, 350 μl binding buffer was added into the tube, and the cells were resuspended. Subsequently, 25 μl Annexin V-FITC and PI complexes were used to combine the cells, and they were kept at 4°C for 30 min. After being washed with PBS for two times, the stained cells were used for apoptosis detection on a flow cytometry machine.

Wound-Healing Assay

The treated BC cells were placed into plates (six-well) and allowed to grow to appropriate 100% cell confluent. The cells were then scraped by a pipette (200 μl) and washed with PBS. The media was added into the cells to allow the cells to migrate. The wound closures were observed and photographed by a microscope at 0 h and 48 h after the wounds were generated.

Transwell Assay

For transwell assays, the Corning Matrigel-coated transwell inserts (8 μm; RuiJian, Wuhan, Hubei, China) were put into twenty-four well plates. The

corresponding siRNAs-transfected cells (1.5×10^5 cells/well) were placed into the transwell inserts, and the serum-free media (200 μl/well) was also added into the cells. Then, the complete media (15 serum) were added into the lower wells. After incubation for 36 h, the invaded cells were fixed using formaldehyde (4%) for 15 min, followed by staining using crystal violet solution (0.1%). Then, a microscope was applied to take photographs.

Statistical Analysis

The statistical significance which was analyzed by SPSS 20.0 (IBM Corp., Armonk, NY, USA) was evaluated by the two-tailed Student's *t*-test or One-way ANOVA. The Kaplan-Meier method with the log-rank test was applied to calculate the overall survival curves. The multivariate survival assays were carried out for all parameters that were significant in univariate assays. The *p*-values less than 0.05 were considered significant.

Results

Identification of LINC01287 Which is Upregulated in BC Tissues and Cells Lines

Firstly, TCGA datasets were used for the downloading of microarray data involved in 1102 BC tissues and 113 normal breast tissues. Then, the differentially expressed lncRNAs in BC samples were shown using Heatmap (Figure 1A). Of note, LINC01287 expressions were found to be increased in BC tissues in the above datasets (Figure 1B). Similar findings were also observed in 112 paired BC samples, and adjacent non-tumor tissues examined using qRT-PCR. As showed in Figure 1C, the LINC01287 expressions were remarkably increased in tumor samples than in those of normal counterparts ($p<0.01$). Besides, by examining the expressions of LINC01287 in normal breast cells MCF-10A and in BC cell lines MDA-MB-468,

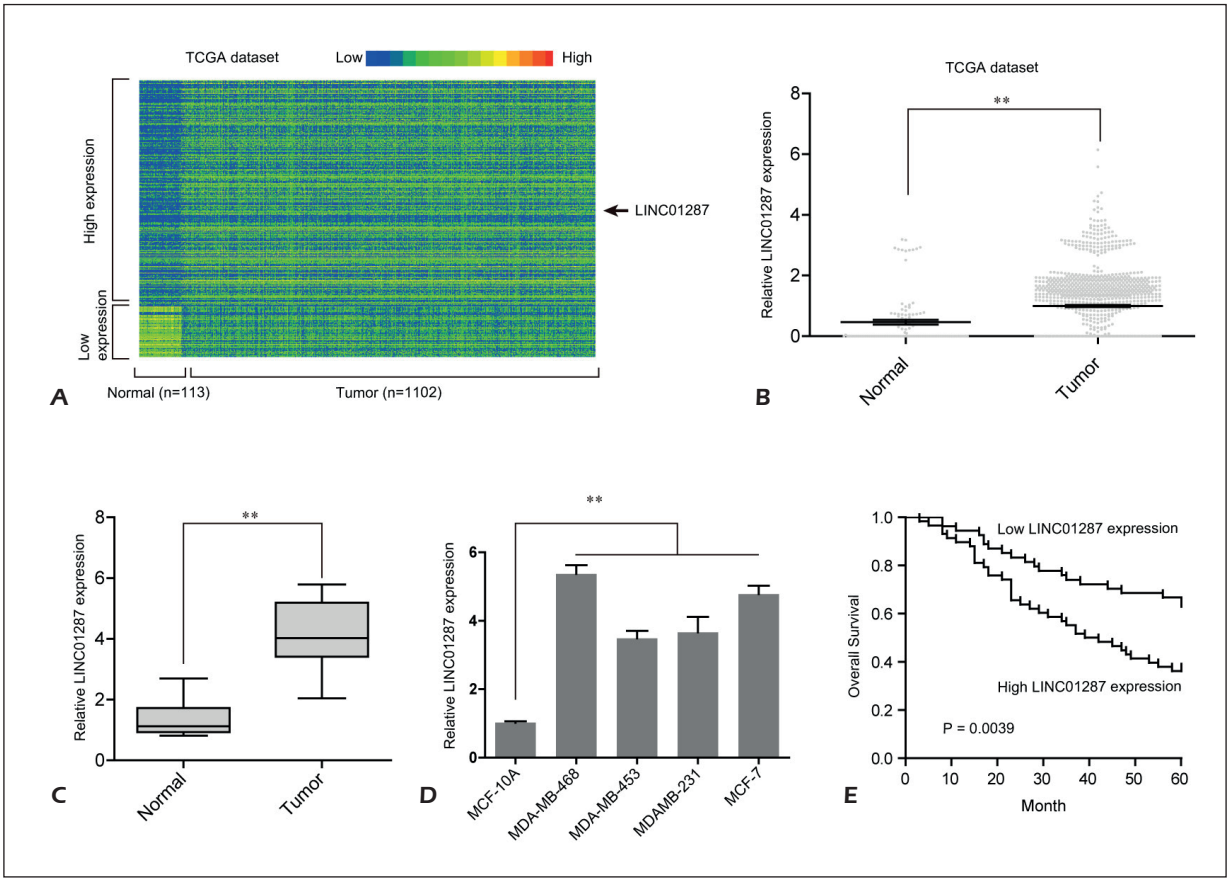

Figure 1. The levels of LINC01287 in BC and its clinical significance. **A**, Heat map of differential expressions between BC tissues and normal breast tissues by analyzing the microarray data from TCGA datasets. **B**, The above TCGA assays revealed that LINC01287 were significantly highly expressed in BC. **C**, The expressions of LINC01287 in BC tissues was significantly lower than those in adjacent non-tumor breast tissues. **D**, LINC01287 expressions in 4 BC cell lines and normal breast cells (MCF-10A) was detected using RT-PCR. E, Kaplan-Meier assays of the associations between LINC01287 high or low expression and overall survival in BC patients. *$p<0.05$, **$p<0.01$.

Table II. Univariate and multivariate Cox's hazards analysis on possible prognostic factors for patients with BC.

Factors	Univariate analysis		Multivariate analysis	
	p-value	HR (95% CI)	*p*-value	HR (95% CI)
Age	0.354	1.482(0.894-2.331)	–	–
Tumor size	0.158	1.582(0.558-2.572)	–	–
ER	0.137	1.429(0.582-2.881)	–	–
PR	0.382	0.989(0.692-1.885)	–	–
HER2 status	0.094	1.337(0.923-2.431)	–	–
Lymph node metastasis	0.011	2.895(1.324-4.729)	0.019	2.669(1.149-4.372)
TNM stage	0.015	2.562(1.219-4.428)	0.021	2.488(1.128-4.028)
LINC01287 expression	0.008	3.015(1.484-5.018)	0.016	2.886(1.268-4.774)

MDA-MB-453, MDAMB-231, and MCF-7, it was shown that LINC01287 was also significantly up-regulated in all four cell lines compared with MCF-10A cells. Taken together, these results indicated that the abnormal LINC01287 expressions may be related to BC progression.

Associations Between LINC01287 Expressions and the Clinical Outcome of BC Patients

The median value of the LINC01287 expressions was 5.262, according to the examination of RT-PCR in 112 cancer tissues of BC, which were classified into two groups using the median values of LINC01287 expressions as a cut-off. Subsequently, the clinical assays from our group between LINC01287 levels and clinical information in BC patients suggested that LINC01287 expressions in BC tumor were in distinct correlations with lymph node metastasis (p=0.015) and TNM stage (p=0.019) (Table I). To explore whether the levels of LINC01287 in BC tissues were correlated with the survivals of the BC patients, the Kaplan-Meier survival assays were conducted. As presented in Figure 1E, our data indicated that BC patients with high levels of LINC01287 revealed a short survival time in comparison with those with low levels of LINC01287 (p=0.0039). Multivariate assays confirmed that high LINC01287 expression was an unfavorable prognostic indicator for BC patients (HR=2.886, HR: 1.268-4.774, p<0.016, Table II).

LINC01287 Knockdown Suppressed BC Development In Vitro

To ascertain whether LINC01287 exhibited oncogenic effects in BC progression, the loss-of-function studies were performed using siRNAs targeting LINC01287 which was separately transfected into the BC cells. Then, the real time-PCR assays were carried out, and the results suggested that the knockdown efficiency of LINC01287 siRNAs was appropriate at 70% (Figure 2A). Afterward, we performed the CCK-8 analyses to determine whether LINC01287 influenced the cellular viability of the BC cells. The data validated that by suppressing the levels of LINC01287 it resulted in remarkably decreased cellular proliferation at 48 h, 72 h, and 96 h when compared with the controls (Figure 2B). The clonogenic assays also were performed to evaluate the impact of LINC01287 on the cell colony formation ability. It is shown in Figures 2C and D, that LINC01287 knockdown strongly suppressed the number of BC cell colonies, which indicated that the depletion of LINC01287 inhibited the clonogenic capacities of the BC cells. Thereafter, we assessed the effects of LINC01287 on the apoptotic capacity of the BC cells using flow cytometry analyses. The results confirmed that LINC01287 silencing markedly promoted apoptosis in BC (Figure 2E). Then, we determined the activities of caspase 3/9, two apoptosis-related molecules, in BC cells after treatment with LINC01287 siRNAs. It was found that the repression of the levels of LINC01287 notably accelerated the activities of caspase 3/9 in BC cells (Figure 2F). These findings indicated that the downregulation of LINC01287 was capable to depress BC development *in vitro*.

LINC01287 Inhibited the Metastatic Potentials of BC Cells

To investigate whether LINC01287 affected the mobility of the BC cells, we next transfected LINC01287 siRNAs into MDA-MB-468 and MCF-7 cells and subsequently employed the transwell assays and wound-healing assays to determine invasion and migration, respectively. By using the transwell assays, it was observed that the

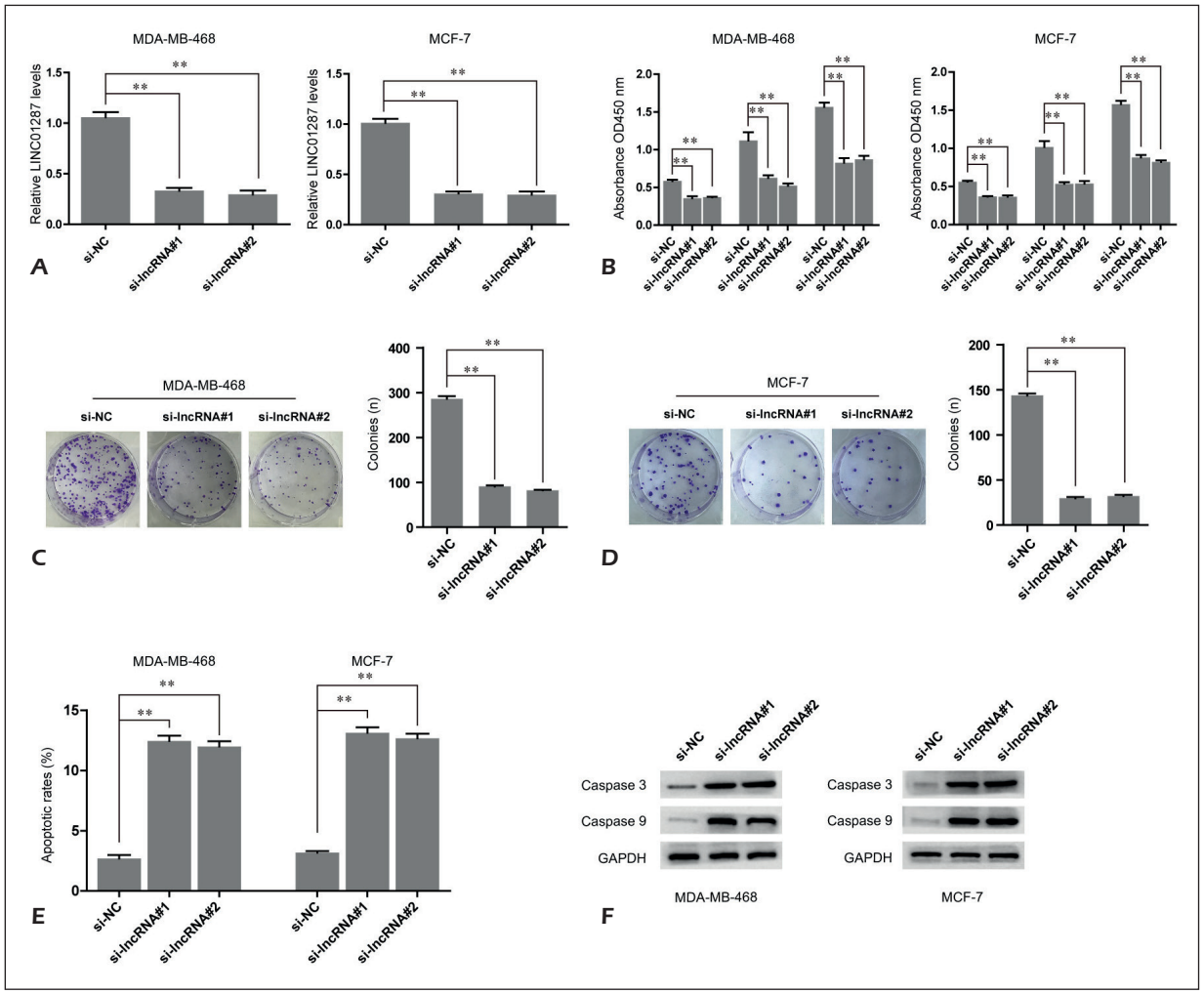

Figure 2. LINC01287 enhanced cell proliferation and inhibited cell apoptosis. **A**, The relative levels of LINC01287 in control and LINC01287-knockdown BC cells were measured by real time-PCR assays. **B**, The growth curves of the BC cells after transfection with LINC01287 siRNAs or the control siRNA were detected using CCK-8 assays. **C-D**, The colony formation analyses evaluated the clonogenic abilities of BC cells (Magnification: 10×). **E**, FACS analyses showed remarkably increased apoptosis of BC cells in the LINC01287 siRNAs-transfected groups. **F**, Western blot analyses examined the protein levels of caspase 3/9. *$p<0.05$, **$p<0.01$.

invaded cell number was reduced by more than 60% in LINC01287 silenced-BC cells than that of the controls (Figures 3A and B). Consistently, the wound-healing assays demonstrated that the depletion of LINC01287 led to evident decreased migratory capacities of BC cells (Figures 3C and D). Therefore, these results indicated that the suppression of LINC01287 inhibited metastatic potentials of BC cells.

LINC01287 Depressed the Activity of Wnt/ß-Catenin Signaling

We next aimed to clarify the detail mechanisms by which LINC01287 exerted its oncogenic roles in BC. Among these signaling pathways which modulated diverse aspects of malignant cancer behaviors, we focused on Wnt/β-catenin signaling because it had been widely reported that this signaling was a crucial regulator in the development of BC[20,21]. We thereby performed real time-PCR analyses to assess whether the change of LINC01287 levels was able to alter the expression of Wnt/β-catenin signaling. Interestingly, the qRT-PCR data certified that the depression of the levels of LINC01287 resulted in a notable decline of mRNA levels of β-catenin, cyclin D1, and c-myc in BC cells, which indicated that LINC01287 could effectively modulate Wnt/β-catenin signaling in BC cells (Figure 4A). Therefore, our group applied Western blot analyses to detect the protein level changes of Wnt/β-catenin signaling in BC cells after the changes in their LINC01287

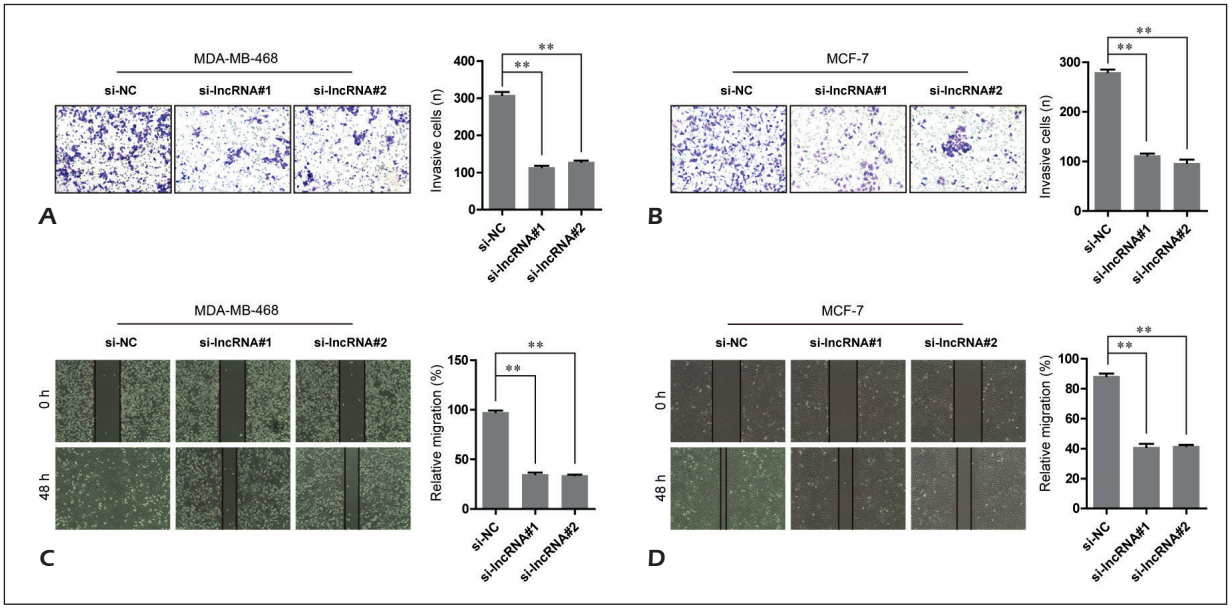

Figure 3. The invasion and migration of BC cells were inhibited by LINC01287 knockdown. **A-B**, The transwell assays assessed the influence of LINC01287 on the cellular invasion of BC cells (Magnification: 40×). **C-D**, Wound healing assays determined the changes of the migration capacities in BC cells after LINC01287 knockdown (Magnification: 10×). *p<0.05, **p<0.01.

levels. The results were similar to that of the real time-PCR analyses, which indicated that LINC01287 knockdown significantly reduced the protein expressions of β-catenin, cyclin D1, and c-myc (Figure 4B). Overall, our study revealed that LINC01287 was a critical player in the regulation of Wnt/β-catenin pathway in BC.

Discussion

BC is the most commonly diagnosed cancer among women in China. Up to date, the establishment of therapeutic schedules is primarily in terms of clinical stages and the presences of several discovered biomarkers, such human epidermal growth factor receptor-2, and progesterone receptors[22,23]. Unfortunately, with the development of personalized therapies and/or precise therapies, the above-mentioned staging systems and the cancer biomarkers do not meet the increasing clinical requirements[24,25]. The determination of the markers which can be used for the accurate prediction of clinical outcome and targeted therapies remains one of the critical challenges for BC.

In this study, our group identified a new BC-related lncRNA LINC01287 whose up-regulation was firstly demonstrated in our tumor samples and cells using RT-PCR. Then, by analyzing the clinical data, higher levels of LINC01287 were

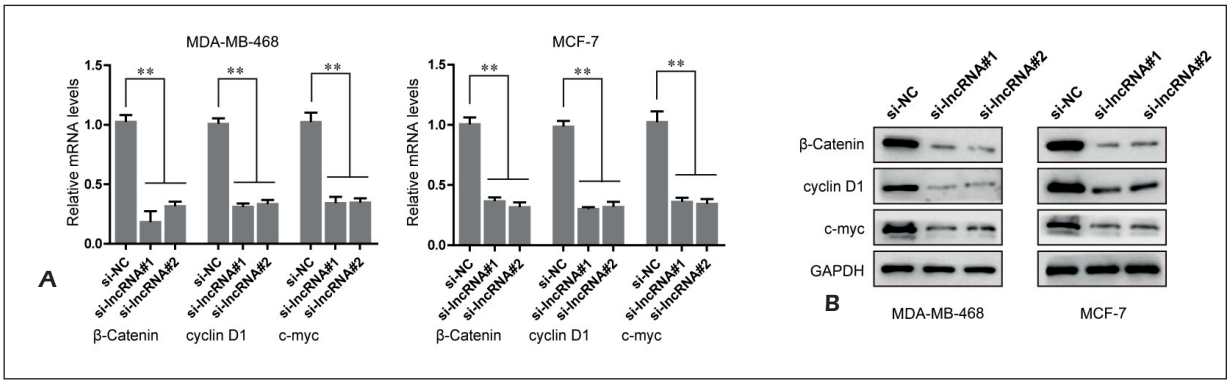

Figure 4. LINC01287 attenuated the activity of Wnt/β-catenin signaling. **A**, The relative levels of β-catenin, cyclin D1, and c-myc in control and LINC01287-knockdown BC cells. **B**, Western blot analyses examined the protein levels of β-catenin, cyclin D1, and c-myc. *p<0.05, **p<0.01.

observed to be associated with lymph node metastasis, TNM stage, and poorer clinical prognosis, which suggested that LINC01287 acted as a regulator in the clinical progress of BC via unclear biological mechanisms. In order to explore whether LINC01287 possessed the values as a novel prognostic biomarker, we conducted multivariate assays, finding that a higher LINC01287 expression was an unfavorable prognostic factor independent for BC patients. Notably, Mo et al[26] also confirmed the potential of LINC01287 as an independent biomarker for hepatocellular carcinoma. Taken together, our data developed our understanding of the clinical effects of LINC01287 in BC.

In recent years, growing *in vitro* and *in vivo* assays by performing gain-of-functions and lost-of-functions indicated that lncRNAs played an important role in the onset of tumorigenesis and the progress of metastasis[27,28]. LINC01287 was also reported to be elevated in hepatocellular carcinoma. Functionally, the overexpression of LINC01287 inhibited the cells growth and invasion by modulating miR-298/MYB axis[26]. Subsequently, our group studied the potential effects of LINC01287 on tumor cell behaviors in BC through loss-of-function assays. As we expected, the downregulation of LINC01287 suppressed cell proliferation, migration, and invasion and induced apoptosis. Our results demonstrated that LINC01287 acts as a positive regulator in BC progress.

Then, the potential mechanisms of LINC01287 in BC cells were further explored. It has been frequently reported that lncRNAs exert their functions in multiple ways among which serving as a modulator in the activity of various tumor-related signaling pathway is a critical path for lncRNAs[29,30]. Wnt/β-catenin pathway, a tumor-related signaling pathway which is also called the canonical Wnt pathway, is crucial to stem cellular differentiation and internal balance for organisms[31]. The studies of tumor biology have detected that the dysregulations of activations of this pathway could result in uncontrolled cells proliferation and cells malignant changes[21,32]. Recently, lncRNAs were emerging as a novel regulator in modulating metastasis of various tumor cells by influencing Wnt/β-catenin pathway[33,34]. In this study, by using Western blot and RT-PCR, we observed that LINC01287 down-regulations suppressed the levels of β-catenin, cyclin D1, and c-myc, indicating that LINC01287 may promote BC progression by increasing the activity of the Wnt/β-catenin pathway.

Conclusions

We demonstrated that LINC01287 was distinctly overexpressed in BC and correlated with poor clinical outcome. The silence of LINC01287 expressions suppressed BC cells growth, metastasis, and tumorigenesis through the modulation of Wnt/β-catenin pathway. Although numerous regulators contribute to the progression of BC, the present research provided a new approach to delay the progression of BC.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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