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LINC00174 triggers the malignant development of breast cancer by negatively regulating miR-1827 level

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Abstract. – **OBJECTIVE:** Long non-coding RNAs (lncRNAs) are extensively involved in tumor development. In-depth researches on cancer-associated lncRNAs provide a theoretical basis for developing prognostic hallmarks and individualized therapeutic targets in breast cancer (BCa). This study aims to detect expression characteristics of LINC00174 in BCa and its biological role in regulating BCa cell phenotypes.

PATIENTS AND METHODS: LINC00174 levels in BCa and adjacent normal tissues were detected by quantitative real-time polymerase chain reaction (qRT-PCR). The influence of LINC00174 on pathological indicators of BCa was analyzed. In MCF-7 and MDA-MB-231 cells with LINC00174 knockdown, proliferative and migratory abilities were examined by cell counting kit-8 (CCK-8), colony formation and transwell assay, respectively. At last, molecular mechanisms of LINC00174 and its downstream gene miR-1827 in regulating BCa development were explored by Luciferase assay and rescue experiments.

RESULTS: LINC00174 was upregulated in BCa tissues than adjacent normal ones. High level of LINC00174 predicted advanced tumor staging, high metastasis rate and poor prognosis in BCa. Knockdown of LINC00174 attenuated proliferative and migratory abilities in BCa cells. MiR-1827 was the target gene binding LINC00174, showing a negative correlation between each other. Silence of miR-1827 abolished the regulatory effects of LINC00174 on proliferative and migratory abilities in BCa cells.

CONCLUSIONS: LINC00174 is upregulated in BCa samples. It is closely linked to tumor staging, metastasis and prognosis in BCa. By negatively regulating miR-1827 level, LINC00174 aggravates the malignant development of BCa.

Key Words:
LINC00174, MiR-1827, BCa.

Introduction

Breast cancer (BCa) is the most common malignancy in females worldwide[1,2]. According to the latest report, there were 252,710 newly onset cases of BCa in the United States in 2017 and 40,610 people will die of BCa[3,4]. The incidence and mortality of BCa in 2017, ranked the first and second of all malignancies, respectively. BCa is also highly prevalent in China, which seriously threatens the health and even lives[5]. In the past few decades, the development of surgery, chemotherapy, radiotherapy, endocrine therapy and molecular targeted therapy has greatly improved the therapeutic effectiveness of BCa[5,6]. Nevertheless, challenges occur in the prevention and treatment of BCa[6]. As a highly heterogeneous disease, and tumorigenesis of BCa can be driven by different genes. The pathogenesis, and potential genes and regulatory networks involved in BCa remain largely unclear[9,10]. In addition, individualized therapeutic response to anti-BCa treatment astonishingly occurs even in BCa cases with the same tumor staging and histological type. Effective indicators for risk stratification and prognosis evaluation in BCa patients are still lacking[7,8]. Drug resistance is a problem that cannot be avoided during chemotherapy, endocrine therapy and molecular targeted therapy in BCa patients[11]. Sensitive hallmarks of BCa in screening, diagnosing and assessing the progression of BCa are urgently required[12].

Non-coding RNAs (ncRNAs) cannot encode proteins. According to whole-genome and transcriptome sequencing studies, protein-encoding mRNAs account for only 2% of all genes, and the remaining are ncRNAs[13,14]. Non-coding RNAs are classified into short-chain ncRNAs

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(miRNAs, tRNAs and rRNAs) and long non-coding RNAs (lncRNAs)[14]. They display important biological functions in life activities and disease development[15]. LncRNAs, which were previously considered to be transcription noises, have become another research hotspot in the life sciences following miRNAs because of their important functions in gene regulation[16]. LncRNAs have diverse effects and mechanisms on BCa regulation. Individualized therapy developed based on BCa-associated lncRNAs contribute to improving the prognosis and life quality in affected people[17,18]. Previous studies have shown that LINC00174 is upregulated in glioma and colorectal cancer cases[19,20]. It is involved in tumor invasiveness and metastasis. Its functions in BCa, however, are unclear.

LncRNA-miRNA interaction displays cancer-promoting or anti-cancer effects through two mechanisms. The first is ceRNA hypothesis that lncRNA sponges miRNA to regulate downstream genes of the miRNAs. Secondly, truncation of lncRNAs leads to miRNA production[21,22]. In this paper, we first detected LINC00174 levels in 64 paired BCa and paracancerous tissues. Its biological functions in mediating BCa cell phenotypes were further analyzed.

Patients and Methods

BCa Patients and Samples

Paired tumor tissues and paracancerous tissues were surgically resected from 64 BCa patients. All tissues were pathologically confirmed and standardly graded. None of included BCa patients had preoperative chemotherapy/radiotherapy. These patients were then followed up, and the clinical data were recorded to further analysis the association between LINC00174 and clinicopathological parameters of the patients. All participants had signed the informed consents before collecting the tissues. In this study, tumor staging was assessed based on the guideline proposed by the Union for International Cancer Control (UICC). Besides, tumor tissues and paracancerous tissues were confirmed by two pathologists in our hospital respectively. In addition, this study was in line with the declaration of Helsinki clinical practice guidelines. This investigation has been approved by the Ethics Committee of Jiaxing University and it was conducted after informed consent of each subject.

Cell Lines and Reagents

BCa cell lines (MDA-MB-231, MDA-MB-453, MCF-7, SKBR3 and ZR-75-30) and a normal mammary epithelial cell line (MCF-10A) were purchased from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) in a 5% CO_2 incubator at 37°C. 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin were applied in culture medium. Cell passage was conducted when cells were grown to 80-90% confluence.

Transfection

Cells were inoculated in 6-well plates with 5×10^4 cells/well. After cell growth to 30-50% confluence, they were transfected with plasmids constructed by GenePharma (Shanghai, China) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Transfection efficacy was tested by quantitative real-time polymerase chain reaction (qRT-PCR).

Cell Counting Kit-8 (CCK-8) Assay

Cells were inoculated in a 96-well plate with 2×10^3 cells per well. At the appointed time points, absorbance value at 490 nm of each sample was recorded using the CCK-8 kit (RIBOBIO, Guangzhou, China) for plotting the viability curves.

Colony Formation Assay

Cells pre-inoculated in 6-weel plates were cultured for 2 weeks. Visible colonies were washed in phosphate-buffered saline (PBS). After incubation with methanol for 20 min and 0.1% crystal violet (Solarbio, Beijing, China) for another 20 min, colonies were washed and finally captured for calculating colony numbers.

Transwell Migration Assay

Transwell chambers (Millipore, Billerica, MA, USA) were inserted in each well of a 24-well plate. 200 μL of suspension (1×10^5 cells/mL) was applied in the upper layer of a chamber with 700 μL of medium containing 20% FBS at the bottom. After 48-h incubation, bottom cells were reacted with 15 min methanol, 20 min crystal violet and captured using a microscope. Migratory cells were counted in 10 random selected fields per sample.

QRT-PCR

Extracted RNAs by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I

treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using PrimeScript RT Reagent (TaKaRa, Komatsu, Japan). The obtained cDNAs underwent qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Komatsu, Japan). Each sample was performed in triplicate. Relative level was calculated by $2^{-\Delta\Delta Ct}$ and normalized to that of β-actin or U6. LINC00174: Forward: 5'-GGCCCAACACTTCCCTCAAA-3', Reverse: 5'-CAGGGAGAAACGACCTGGAG-3'; β-actin: Forward: 5'-TCCTCTGACTTCAACAGCGACAC-3', Reverse: 5'-CACCCTGTTGCTGTAGCCAAATTC-3'; MiR-1827: Forward: 5'-GGGGTGAGGCAGTAGATTG-3', Reverse: 5'-GTGCAGGGTCCGAGGT-3'; U6: Forward: 5'-CTCGCTTCGGCAGCACA-3', Reverse: 5'-AACGCTTCACGAATTTGCGT-3'.

Luciferase Assay

Wild-type or mutant-type LINC00174 vector was constructed by inserting corresponding sequences into the pmirGLO vector. Cells were pre-seeded in a 24-well plate. They were co-transfected with NC/miR-1827 mimic and pmirGLO-LINC00174-WT/pmirGLO-LINC00174-MUT/pmirGLO, respectively. After 48 h cell culture, they were lysed for measuring luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between groups were analyzed by the *t*-test. Chi-square test was conducted for analyzing the relationship between LINC00174 level and pathological indicators of BCa patients. $p<0.05$ was considered as statistically significant.

Results

Upregulation of LINC00174 in BCa

We collected 64 paired BCa tissues and paracancerous ones. As qRT-PCR data revealed, LINC00174 was upregulated in BCa tissues than controls (Figure 1A). Besides, it was also highly expressed in BCa cell lines, suggesting a potential oncogenic role of LINC00174 in BCa (Figure 1B). MCF-7 and MDA-MB-231 cells showed the highest abundance of LINC00174 among the 5 tested BCa cell lines, which were used in the *in vitro* experiments.

LINC00174 Expression Was Correlated with Tumor Staging, Metastasis and Prognosis in BCa

Included 64 BCa patients were classified into two groups based on their corresponding LINC00174 levels in tumor tissues. Chi-square test results uncovered that LINC00174 level was positively linked to tumor staging, lymphatic metastasis and distant metastasis in BCa (Table I). Higher level of LINC00174 was observed in T3-T4 BCa patients, and those with lymphatic metastasis or distant metastasis (Figure 1C).

Knockdown of LINC00174 Inhibited Proliferative and Migratory Abilities in BCa

LINC00174 knockdown was conducted by transfection of sh-LINC00174 in MCF-7 and MDA-MB-231 cells (Figure 2A). Viability and relative colony number markedly decreased after knockdown of LINC00174 in BCa cells, suggesting the inhibited proliferative ability (Figure 2B, 2C). Moreover, transwell assay showed a decline in migratory cell number in BCa cells transfected with sh-LINC00174 than those of controls (Figure 2D). It is concluded that LINC00174 could promote proliferative and migratory abilities in BCa.

Interaction Between LINC00174 and MiR-1827

Binding sequences were found in the 3'UTR of LINC00174 and miR-1827 by bioinformatics analysis (miRDB). Overexpression of miR-1827 decreased Luciferase activity in pmirGLO-LINC00174-WT, and it failed to influence luciferase activity in pmirGLO-LINC00174-MUT or controls, demonstrating the binding between LINC00174 and miR-1827 (Figure 3A). In BCa tissues, miR-1827 was markedly downregulated, and it displayed a negative correlation with LINC00174 level (Figure 3B).

MiR-1827 Abolished the Regulatory Effects of LINC00174 on BCa

We next explored the potential functions of miR-1827 in BCa. Transfection efficacy of miR-1827 inhibitor was first tested in MCF-7 and MDA-MB-231 cells (Figure 4A). Of note, higher viability and migratory cell number were seen in BCa cells with co-silenced LINC00174 and miR-1827 than those with solely LINC00174 knockdown (Figure 4B, 4C). It is indicated that the inhibitory effects of silenced LINC00174 on proliferative and migratory abilities in BCa were abolished by miR-1827 knockdown.

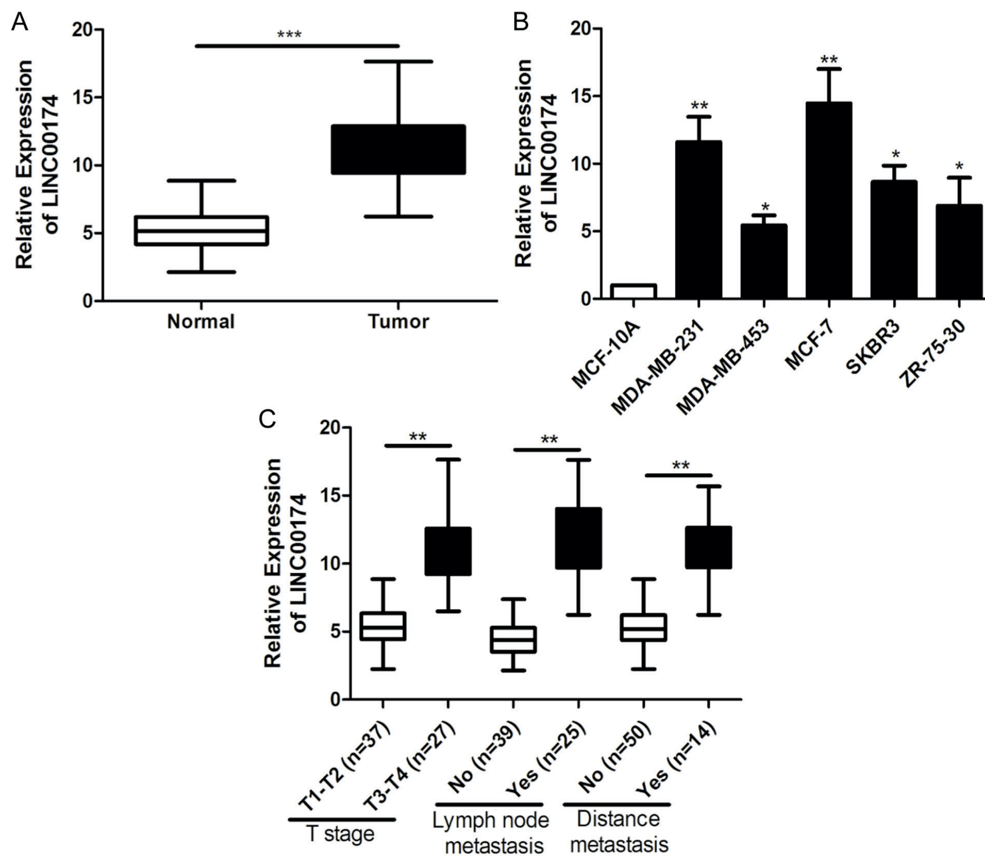

Figure 1. Upregulation of LINC00174 in BCa. **A,** Differential expressions of LINC00174 in BCa tissues and paracancerous ones. **B,** LINC00174 levels in BCa cell lines. **C,** Differential expressions of LINC00174 in BCa patients based on T stages or states of lymphatic metastasis and distant metastasis. Data were expressed as mean±SD. *p<0.05, **p <0.01, ***p <0.001.

Table I. Association of LINC00174 expression with clinicopathologic characteristics of breast cancer.

Parameters	No. of cases	LINC00174 expression		*p*-value
		Low (n = 37)	High (n = 27)	
Age (years)				0.310
<60	26	17	9	
≥60	38	20	18	
Tumor size (cm)				0.119
<4	31	21	10	
≥4	33	16	17	
T stage				0.004
T1-T2	37	27	10	
T3-T4	27	10	17	
Lymph node metastasis				0.021
No	39	27	12	
Yes	25	10	15	
Distance metastasis				0.012
No	50	33	17	
Yes	14	4	10	

EUA = emergency use authorization; FDA = the U.S. Food and Drug Administration; NAAT = nucleic acid simplification test; RT-PCR = reverse transcription polymerase chain reaction; SARS–CoV-2 = severe acute respiratory syndrome–related coronavirus-2; ID NOW = ID Now COVID-19; Xpert = Xpert Xpress SARS-CoV-2

Discussion

According to published data, about 15% of new female malignant tumor cases in China are BCa cases. BCa has become the most prevalent female malignant tumor in China, and it is also the leading cause of cancer death in women under 45 years of age[1,5]. More seriously, both the incidence and mortality of BCa have on the rise in China, which endangers the health and even life of women[5]. Tumor cell metastasis and subsequent destruction of normal functions are attributed to solid tumor death[6-8]. Compared with primary tumors, metastatic tumors cannot be surgically removed and are easily resistant to chemotherapy. 90% of cancer deaths are caused by distant metastases of the tumor[9,10]. However, the molecular mechanisms underlying metastasis of BCa are unclear, and molecular markers that can predict their progression and metastasis are still limited[11,12]. Therefore, the discovery of key molecules and regulatory pathways in the metastatic process of BCa should be well explored[12].

Recent studies have found that in addition to about 21,000 protein-encoding mRNAs, there are 10,000 to 32,000 lncRNAs, 11,000 pseudogenes and about 9,000 miRNAs in human genomes[13,14]. Based on the length, ncRNAs shorter than 200 nt include miRNAs (post-transcriptional regulations on RNA degradation or translation inhibition) and tRNAs (regulations on mRNA translation). Those longer than 200 nt, that is, lncRNAs, are involved in various life activities[15,16]. LncRNAs are new potential regulators of normal cell development, and abnormally expressed lncRNAs may play a key role in tumorigenesis[16]. To seek for BCa-associated lncRNAs, bioinformatics analysis uncovered that LINC00174 is differentially expressed in BCa and it is further identified to be closely linked to the development of BCa[17,18]. The oncogenic role of LINC00174 has been previously reported. However, the relationship between linc00174 and breast cancer was not clear. Therefore, the objective of this study was firstly to elucidate the oncogenic role of LINC00174 in the progression of BCa, as well as the specific mechanism of LINC00174 regulating miR-1827. In this paper, LINC00174 was upregulated in BCa tissues, and it was related to advanced tumor staging, high metastasis rate and poor prognosis in BCa. Furthermore, *in vitro* evidence has shown that LINC00174 markedly stimulated proliferative and migratory abilities in BCa.

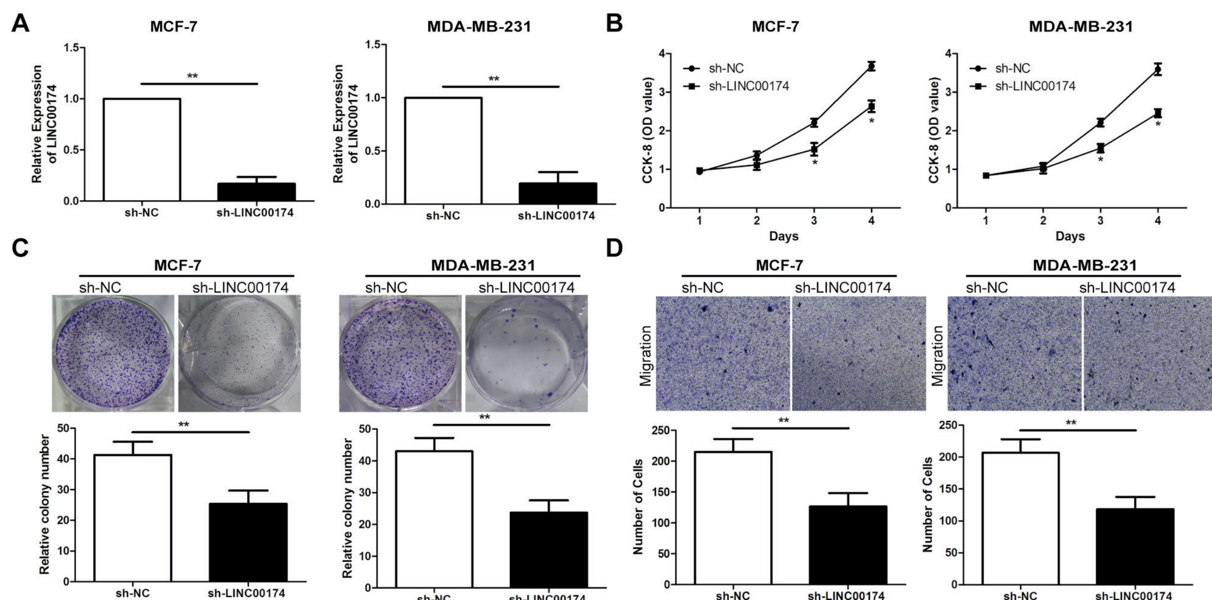

Figure 2. Knockdown of LINC00174 inhibited proliferative and migratory abilities in BCa. **A,** Transfection efficacy of sh-LINC00174. **B,** Viability in MCF-7 and MDA-MB-231 cells transfected with sh-NC or sh-LINC00174. **C,** Relative colony number in MCF-7 and MDA-MB-231 cells transfected with sh-NC or sh-LINC00174 (magnification: 10×). **D,** Migration in MCF-7 and MDA-MB-231 cells transfected with sh-NC or sh-LINC00174 (magnification: 40×). Data were expressed as mean±SD. *p<0.05, **p<0.01.

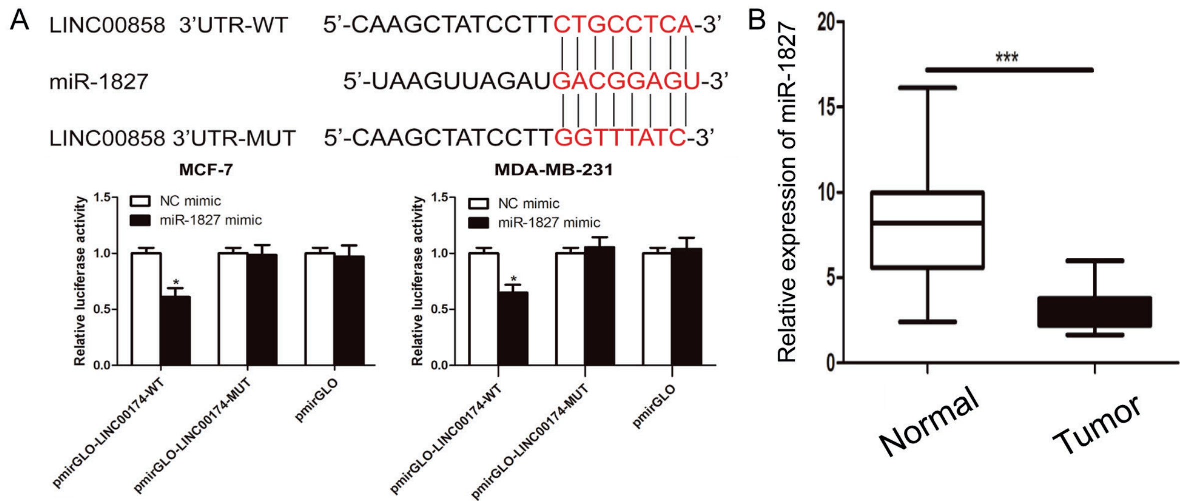

Figure 3. Interaction between LINC00174 and miR-1827. **A,** Luciferase activity in MCF-7 and MDA-MB-231 cells co-transfected with NC/miR-1827 mimic and pmirGLO-LINC00174-WT/pmirGLO-LINC00174-MUT/pmirGLO. **B,** Differential expressions of miR-1827 in BCa tissues and paracancerous ones. Data were expressed as mean±SD. *p<0.05, ***p<0.001.

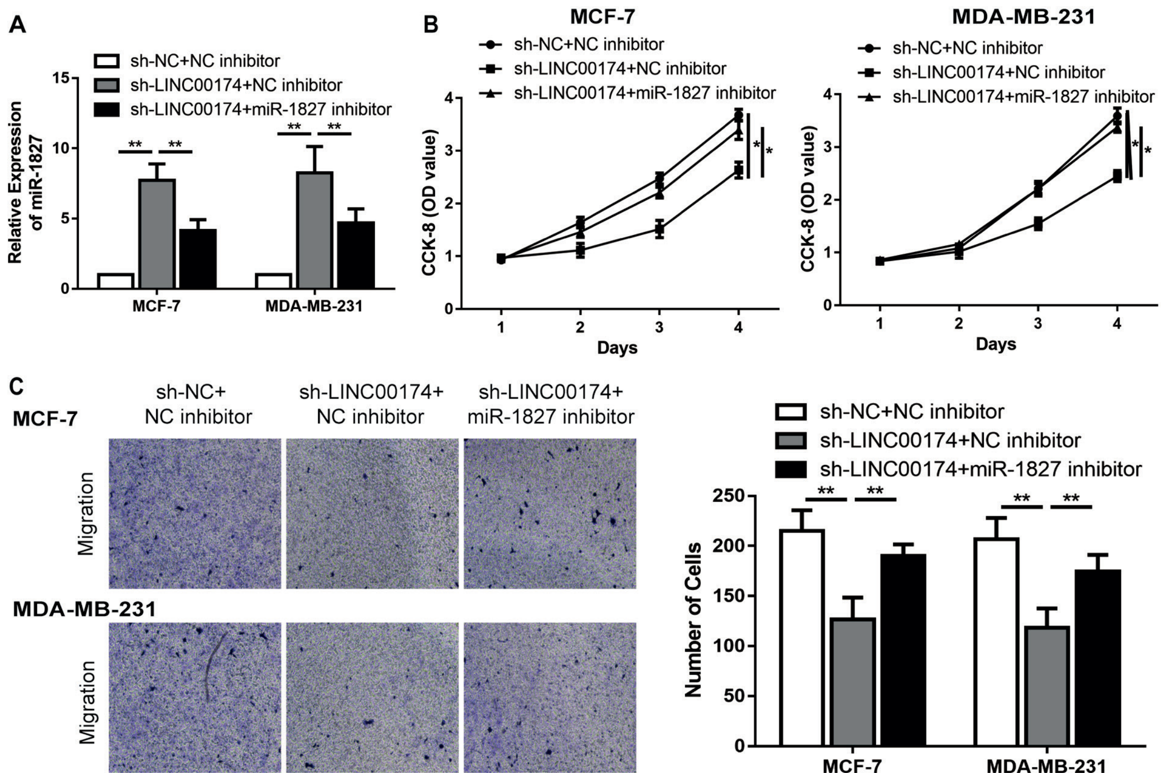

Figure 4. MiR-1827 abolished the regulatory effects of LINC00174 on BCa. MCF-7 and MDA-MB-231 cells were co-transfected with sh-NC+NC inhibitor, sh-LINC00174+NC inhibitor or sh-LINC00174+miR-1827 inhibitor. **A,** Relative level of miR-1827. **B,** Viability curves at day 1 to day 4. **C,** Migratory cell number (magnification: 40×). Data were expressed as mean±SD. *p<0.05, **p<0.01.

LncRNAs exert anti-cancer or cancer-promoting effects by competitively combining miRNAs with mRNAs[21,22]. In previous studies, bioinformatics analysis predicted that miR-1827 might interact with LINC00174. MiR-1827 binding site was contained in the 3'UTR of LINC00174. In the next Luciferase assay, we verified the direct binding of LINC00174 to the downstream target miR-1827 since wild-type LINC00174 vector successfully enriched miR-1827. MiR-1827 was found to be downregulated in BCa samples, and negatively regulated by LINC00174. It is indicated that the transcription activity of miR-1827 may be mediated by LINC00174. To investigate the involvement of miR-1827 in BCa development, rescue experiments were conducted. Co-silence of LINC00174 and miR-1827 obtained higher viability and migratory cell number in BCa cells than those with solely LINC00174 knockdown. Collectively, we have demonstrated a negative feedback loop that LINC00174 stimulated proliferative and migratory abilities in BCa *via* negatively regulating miR-1827 level. These findings provided new insights and ideas for clinical prediction and treatment of BCa and might become a key to making breakthrough progress in the future.

Conclusions

LINC00174 is upregulated in BCa samples. It is closely linked to tumor staging, metastasis and prognosis in BCa. By negatively regulating miR-1827 level, LINC00174 aggravates the malignant development of BCa.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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