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LncRNA AK024094 aggravates the progression of breast cancer through regulating miRNA-181a

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Abstract. – OBJECTIVE: The aim of this study was to investigate the role of long noncoding ribonucleic acids (lncRNAs) AK024094 in regulating the progression of breast cancer (BCa) and the potential mechanism. Our findings might help to provide a theoretical basis for the targeted therapy of BCa.

PATIENTS AND METHODS: The relative expression level of lncRNA AK024094 in BCa and adjacent normal tissues was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The prognostic potential of AK024094 in BCa was assessed by the Kaplan-Meier method. Meanwhile, AK024094 level in BCa cell lines was detected by qRT-PCR as well. The regulatory effects of AK024094 on the proliferative, migratory, and invasive abilities of MDA-MB-468 and MCF-7 cells were evaluated by functional assays. The Dual-Luciferase Reporter Gene Assay was applied to verify the binding between AK024094 and miRNA-181a. In addition, the rescue experiments were conducted to uncover the role of AK024094/miRNA-181a in the progression of BCa.

RESULTS: AK024094 was significantly upregulated in BCa tissues and cell lines. Compared with BCa patients with low expression of AK024094, the tumor staging of those with a high level of AK024094 was remarkably worse. Meanwhile, the rate of distant metastasis was significantly higher, and the overall survival was shorter in BCa patients with high expression of AK024094. The knockdown of AK024094 significantly attenuated the proliferative, migratory, and invasive abilities of MDA-MB-468 and MCF-7 cells. Subsequently, miRNA-181a was predicted and verified as the target of AK024094. A negative correlation was identified between the expression levels of AK024094 and miRNA-181a in BCa. Furthermore, the knockdown of miRNA-181a partially reversed the effect of AK024094 on cellular behaviors of BCa cells.

CONCLUSIONS: AK024094 aggravates the malignant progression of BCa, and is closely related to tumor staging, distant metastasis, and poor prognosis of BCa. In addition, AK024094 accelerates the proliferation and metastasis of BCa cells by targeting miRNA-181a.

Key Words:
AK024094, MiRNA-181a, Breast cancer (BCa).

Introduction

Breast cancer (BCa) is one of the most common malignancies worldwide, which is also the leading cause of tumor death in women[1,2]. BCa accounts for about 15% of newly tumor cases each year in China. Meanwhile, it ranks first in cancer-related mortality among females under 45 years[3,4]. More seriously, the morbidity and mortality of BCa are on the rise. This poses a great threat to female health and even their lives[4]. The tumor cell migration has been well concerned in the research of tumor metastasis[5,6]. Compared with primary tumors, the metastatic tumors are resistant to chemotherapy and cannot be operated. About 90% of tumor deaths result from distant metastasis[7]. Unfortunately, the molecular mechanism underlying the proliferation and metastasis of BCa has not been fully elucidated. Currently, there is still a lack of sensitive and effective biomarkers that can predict the malignant progression of BCa[8,9]. Therefore, the search for key molecules and regulatory pathways in the progression of BCa contributes to guide early intervention and improve the prognosis of BCa patients[9,10].

High-throughput sequencing has revealed that only a small part of mammalian genomes can be transcribed into protein-coding genes. Most of them are transcribed into non-coding RNAs[11,12]. Long noncoding ribonucleic acids (lncRNAs) are a class of RNA molecules with no protein-encoding function[13,14]. In recent years, the role of lncRNAs in the metastasis of tumors has been widely reported. For example, HOTAIR stimulates the metastasis of BCa by inducing chromatin rearrangement[14,15]. MALAT1 is a key regulator in the metastasis of lung cancer cells[16]. NKILA inhib-

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its the metastasis of BCa by blocking IkB phosphorylation[17]. Previous studies have confirmed that lncRNA AK024094 is highly expressed in many tumors. Moreover, its expression level is closely related to the pathological grade, clinical stage, and prognosis. However, the exact role of AK024094 in BCa has not been reported.

The competitive endogenous RNA (ceRNA) hypothesis suggests that endogenous RNAs, such as lncRNAs, pseudogenes, and mRNAs, can abolish the inhibition of the target genes by competitively binding to common miRNAs. Therefore, the expression levels of the target genes are significantly up-regulated, thereby influencing many biological progressions[18-20]. In this paper, we first detected the expression pattern of AK024094 in BCa tissues and cells. The correlation between AK024094 level and pathological indexes of BCa patients was analyzed as well. Subsequently, the molecular mechanism of AK024094 in regulating the cellular behaviors of BCa cells was further explored. Our findings aimed to provide a theoretical basis for improving the therapeutic efficacy of BCa patients.

Patients and Methods

Patients and BCa Samples

BCa tissues and matched adjacent normal tissues were surgically resected from 40 BCa patients. None of these patients received preoperative anti-tumor therapies. Meanwhile, the clinical indexes and the follow-up data were collected for subsequent analyses. The informed consent was obtained from each patient and their families. This study was approved by the Ethics Committee of Cancer Hospital of China Medical University.

Cell Culture

Normal mammary epithelial cell line (MCF-10A) and BCa cell lines (MDA-MB-231, SK-BR-3, MDA-MB-468, and MCF-7) were provided by American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Hyclone, South Logan, UT, USA) and maintained in a 37°C, 5% CO_2 incubator. Culture medium was replaced every 2-3 days. The cell passage was conducted at 80-90% of confluence.

Cell Transfection

The transfection plasmids were provided by GenePharma (Shanghai, China). The cells were pre-seeded into 6-well plates. Cell transfection was performed according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at 70% of confluence. 48 h after transfection, the cells were harvested for subsequent experiments.

Cell Counting Kit-8 (CCK-8) Assay

The cells were first seeded into 96-well plates, with 2×10^3 cells per well. At appointed time points, absorbance (A) at 450 nm of each well was recorded using CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan). Finally, the viability curve was plotted.

5-Ethynyl-2'-Deoxyuridine (EdU) Proliferation Assay

The cells were inoculated into 96-well plates at a density of 1×10^5 cells per well. Then, the cells were labeled with 100 μL of EdU reagent (50 μM) per well for 2 h. After washing with Phosphate-Buffered Saline (PBS), the cells were fixed with 50 μL of fixation buffer, decolored with 2 mg/mL glycine, and permeated with 100 μL of penetrant. Subsequently, the cells were washed again with PBS once, followed by staining with AdoLo and 4',6-diamidino-2-phenylindole (DAPI) in the dark for 30 min. Finally, the EdU-positive ratio was determined under a fluorescent microscope.

Transwell Migration and Invasion Assay

The transfected cells for 48 h were adjusted to a dose of 5.0×10^5/mL. 200 μL/well cell suspension was added to the upper side of the transwell chambers (Millipore, Billerica, MA, USA) pre-coated with Matrigel. Meanwhile, 700 μL of medium containing 10% FBS was added to the bottom side. After 48 h of incubation, the cells invaded to the bottom side were fixed with methanol for 15 min and dyed with crystal violet for 20 min. The number of penetrating cells was counted under a microscope. 5 fields were randomly selected per sample. The migration assay was conducted in the same way except for Matrigel pre-coating.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and purified by DNase I treatment. Subsequently, extracted RNA was reverse transcribed into complementary deoxyribonucleic acids (cDNAs) using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). Obtained cDNA was subjected to qRT-PCR using SYBR®Premix Ex Taq™ (TaKa-

Ra, Otsu, Shiga, Japan). GAPDH and U6 were used as internal references for mRNA and miRNA, respectively. Each sample was performed in triplicate. The relative level of genes was calculated by the $2^{-\Delta\Delta Ct}$ method. iQ5 2.0 (Bio-Rad, Hercules, CA, USA) was used for data analysis. The primer sequences used in this study were as follows: AK024094, F: 5'-CCAGAGAACCGGCTGTTACAC-3', R: 5'-CAATAGCATGTAACCGCAGGTA-3'; microRNA-181a, F: 5'-GAATGTAGACCAGTTCTCCCTG-3', R: 5'-AGTGTGCCGTCGTCAGTCCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Dual-Luciferase Reporter Gene Assay

The cells were co-transfected with pmirGLO-AK024094-WT/pmirGLO-AK024094-MUT/pmirGLO and miRNA-181a mimics/NC using Lipofectamine 2000. 24 h later, the co-transfected cells were harvested. The Luciferase activity was determined using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Statistical Analysis

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was used for all statistical analyses. The experimental data were expressed as mean ± standard deviation. The intergroup differences were analyzed by *t*-test. The Kaplan-Meier curve was introduced for assessing the prognostic value of AK024094 in BCa. The Chi-square test was performed to evaluate the relationship between AK024094 and miRNA-181a. $p<0.05$ was considered statistically significant.

Results

AK024094 Was Highly Expressed in BCa Tissues and Cells

40 paired BCa tissues and adjacent normal tissues were collected in this study. QRT-PCR data showed that AK024094 expression was significantly up-regulated in BCa tissues relative to normal controls (Figures 1A, 1B). AK024094 level in BCa cells was significantly higher than that of normal mammary epithelial cells as well (Figure 1C). MDA-MB-468 and MCF-7 cell lines were selected for subsequent experiments due to their high abundances of AK024094.

AK024094 Expression Was Correlated With Tumor Staging, Distant Metastasis, and Overall Survival of BCa Patients

Based on the median expression level of AK024094, BCa patients were divided into high-level group and low-level group, respectively. The Chi-square test was performed to uncover the correlation between AK024094 level and clinical indexes of BCa patients. The results indicated that AK024094 expression was positively correlated with tumor staging and distant metastasis of BCa patients, rather than age, gender, and lymphatic metastasis (Table I). The Kaplan-Mei-

Table I. Association of lncRNA AK027294 expression with clinicopathologic characteristics of breast cancer.

Parameters	Number of cases	AK027294 expression		*p*-value*
		Low (%)	High (%)	
Age (years)				0.864
<60	14	9	5	
≥60	26	16	10	
Gender				0.722
Male	12	7	5	
Female	28	18	10	
T stage				0.023
T1-T2	25	19	6	
T3-T4	15	6	9	
Lymph node metastasis				0.548
No	27	18	9	
Yes	14	8	6	
Distance metastasis				0.010
No	26	20	6	
Yes	14	5	9	

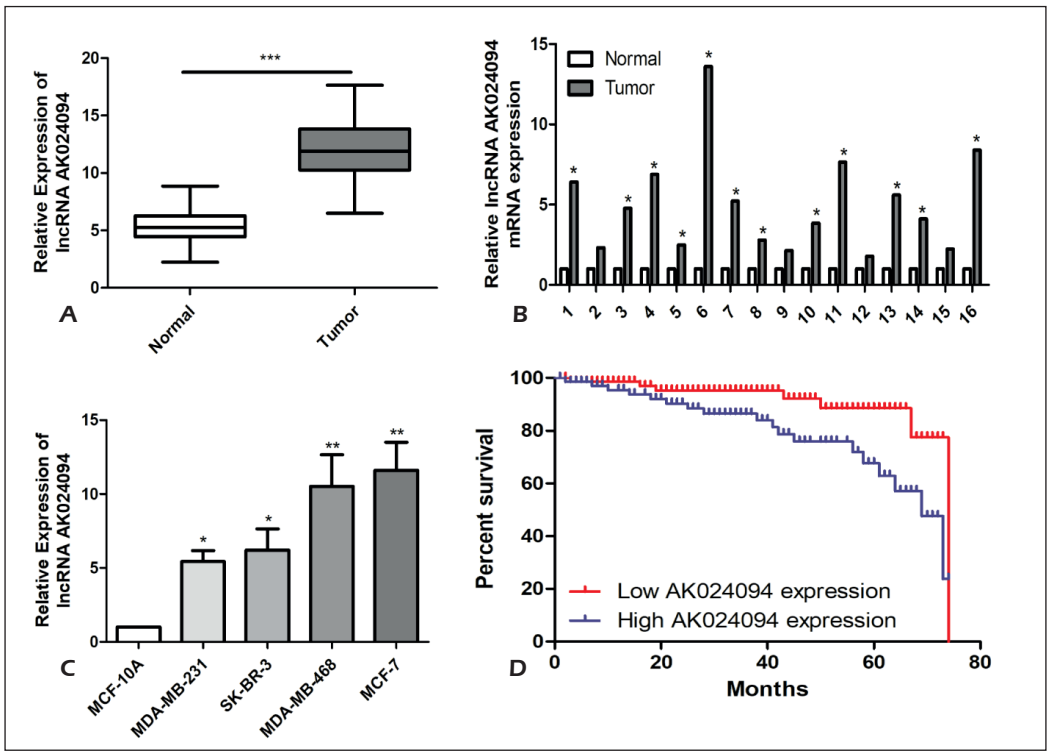

Figure 1. AK024094 was highly expressed in BCa tissues and cell lines. **A**, Relative level of AK024094 in BCa tissues and adjacent normal tissues. **B**, Relative level of AK024094 in 16 paired BCa and normal tissues. **C**, Relative level of AK024094 in normal mammary epithelial cells (MCF-10A) and BCa cells (MDA-MB-231, SK-BR-3, MDA-MB-468, and MCF-7). **D**, The Kaplan-Meier curve revealed the overall survival of BCa patients with high and low expression of AK024094.

er curve was plotted by analyzing the follow-up data of BCa patients. The results demonstrated that the prognosis of BCa patients with high-level AK024094 was significantly worse than that of those with low-level (Figure 1D).

Knockdown of AK024094 Inhibited Proliferative, Migratory, and Invasive Abilities of BCa

To identify the biological function of AK024094 in BCa, we first constructed sh-AK024094-1 and sh-AK024094-2. The transfection of sh-AK024094-1 or sh-AK024094-2 in MDA-MB-468 and MCF-7 cells markedly down-regulated the expression level of AK024094 (Figure 2A). CCK-8 assay showed significantly reduced the viability of BCa cells transfected with sh-AK024094-1 or sh-AK024094-2 than that of controls (Figure 2B). Moreover, EdU assay revealed that the ratio of EdU-positive cells was remarkably reduced after the silence of AK024094 in MDA-MB-468 and MCF-7 cells. These findings suggested that AK024094 knockdown inhibited the proliferative ability of cells (Figure 2C).

The transwell assay was performed to assess the migratory and invasive abilities of BCa cells influenced by AK024094. After transfection of sh-AK024094-1 or sh-AK024094-2 in MDA-MB-468 and MCF-7 cells, the migratory and invasive abilities were markedly inhibited (Figures 3A, 3B). Collectively, the silence of AK024094 suppressed the proliferation, migration, and invasion of BCa cells.

AK024094 Could Bind to MiRNA-181a

Based on the binding sites between miRNA-181a and AK024094, pmirGLO-AK024094-WT, pmirGLO-AK024094-MUT, and pmirGLO were first constructed. Subsequently, the Dual-Luciferase Reporter Gene Assay was performed. The results indicated that the Luciferase activity significantly decreased after the co-transfection of pmirGLO-AK024094-WT and miRNA-181a mimics. This verified the binding relationship between miRNA-181a and AK024094 (Figure 4A). The transfection of sh-AK024094-1 or sh-AK024094-2 significantly up-regulated the expression level of miRNA-181a in MDA-MB-468 and MCF-7 cells (Figure 4B). In addition, miR-

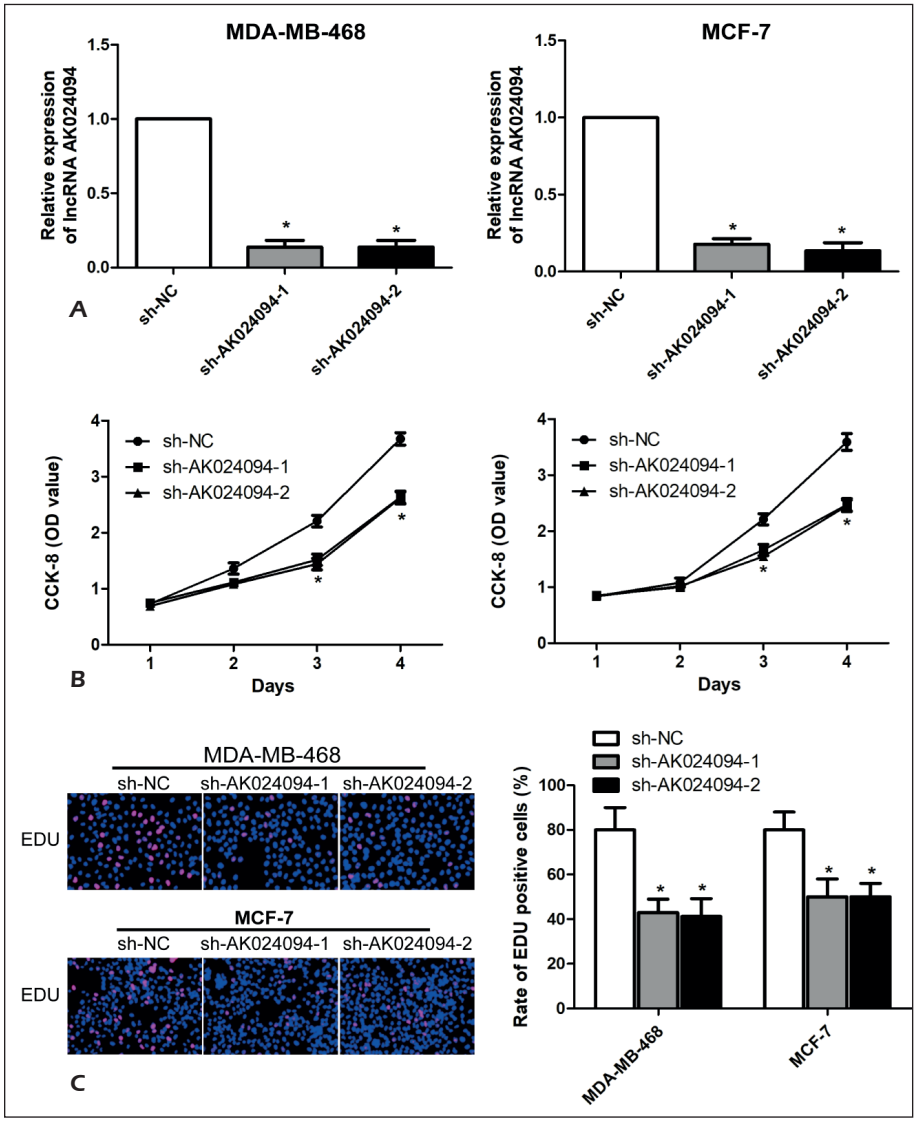

Figure 2. Knockdown of AK024094 inhibited proliferative ability of BCa. **A**, Transfection efficacy of sh-AK024094-1 and sh-AK024094-2 in MDA-MB-468 and MCF-7 cells. **B**, Viability in MDA-MB-468 and MCF-7 cells transfected with sh-NC, sh-AK024094-1, or sh-AK024094-2. **C**, EdU-positive ratio in MDA-MB-468 and MCF-7 cells transfected with sh-NC, sh-AK024094-1, or sh-AK024094-2 (magnification × 40).

NA-181a was found remarkably down-regulated in BCa tissues and cell lines (Figures 4C, 4D). Furthermore, a negative correlation was identified between the expression levels of miRNA-181a and AK024094 in BCa tissues (Figure 4E).

AK024094/MiRNA-181a Regulatory Network in BCa

According to the abovementioned results, we speculated that AK024094 might serve as a ceRNA to absorb miRNA-181a. In MDA-MB-468 and MCF-7 cells overexpressing AK024094, the transfection of miRNA-181a inhibitor markedly decreased the ratio of EdU-positive cells, as well as migratory and invasive abilities (Figure 5A). Interestingly, decreased the ratio of EdU-positive BCa cells owing to transfection of sh-AK024094-1 could be partially reversed after the co-transfection of miRNA-181a inhibitor (Figure 5B). Similarly, reduced migratory and invasive abilities by AK024094 knockdown were partially reversed after miRNA-181a knockdown (Figure 5C). All these findings demonstrated that AK024094 influenced the cellular behaviors of BCa by negatively regulating miRNA-181a.

Discussion

With the increase of tumor morbidity and mortality, it has become the leading cause of death in humans[1-3]. It is reported that there were about

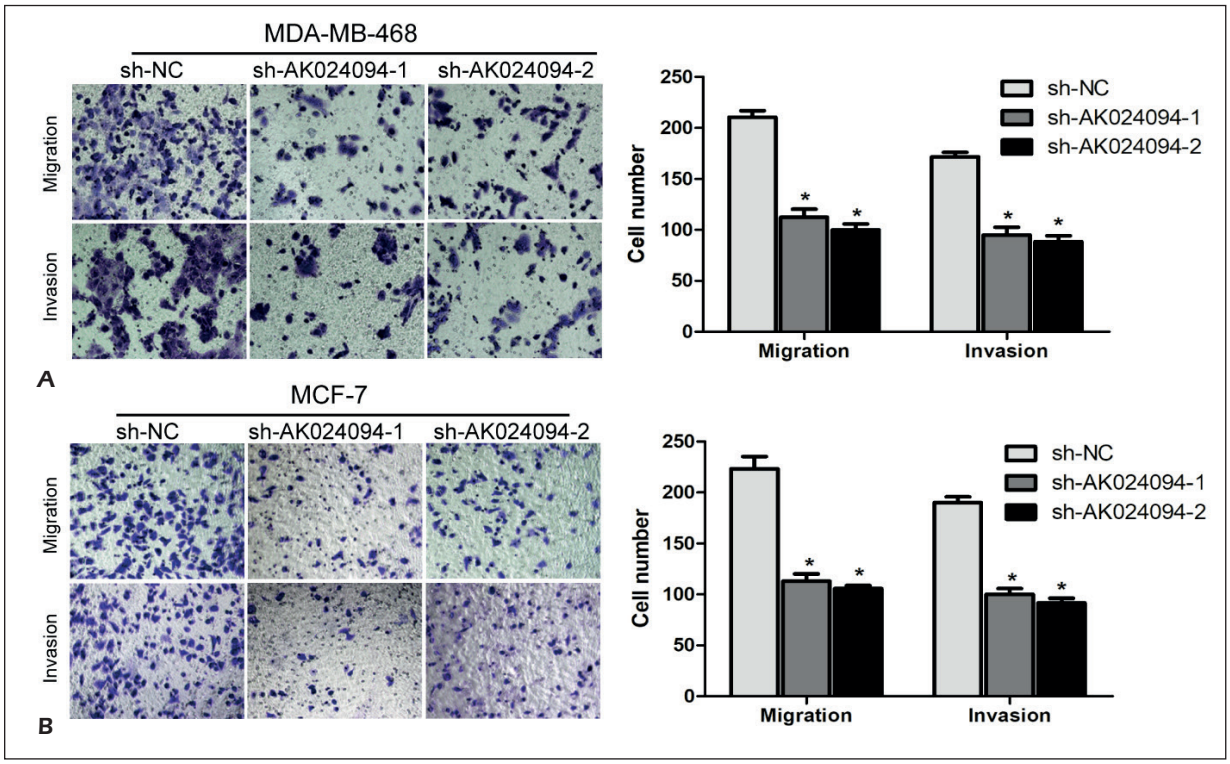

Figure 3. Knockdown of AK024094 inhibited the migratory and invasive abilities of BCa. **A**, Migratory and invasive abilities of MDA-MB-468 cells transfected with sh-NC, sh-AK024094-1, or sh-AK024094-2 (magnification × 40). **B**, Migratory and invasive abilities of MCF-7 cells transfected with sh-NC, sh-AK024094-1, or sh-AK024094-2 (magnification × 40).

4292,000 new cancer cases and 2814,000 cancer deaths in China in 2015[3,4]. Breast cancer, lung cancer, stomach cancer, colorectal cancer, and esophageal cancer are common types affecting females[4]. Currently, BCa has become the most prevalent cancer that seriously threatens female health. Therefore, it is necessary to investigate the mechanism of metastatic BCa, thus improving the therapeutic efficacy of BCa patients[5-7].

Some studies[11,12] have found approximately 21,000 protein-encoding mRNAs, 10,000-32,000 lncRNAs, 11,000 pseudogenes, and 9,000 miRNAs. According to the molecular length of non-coding RNA, it can be divided into two types, namely: short non-coding RNAs less than 200 nt (i.e., miRNA, tRNA) and lncRNA with over than 200 nt long[12]. LncRNAs are widely involved in chromatin modification, transcriptional regulation, etc. As a key regulator, the abnormally expressed lncRNA may serve as important regulators in tumorigenesis[13,14].

To uncover the potential role of lncRNAs in the malignant progression of BCa, we analyzed differentially expressed lncRNAs in BCa through bioinformatics. Finally, lncRNA AK024094 was screened out. In this study, AK024094 was found significantly upregulated in BCa tissues. Through analyzing the follow-up data of BCa patients, we found that AK024094 level was positively correlated with tumor staging, distant metastasis, and poor prognosis of BCa. Hence, it was speculated that AK024094 exerted a carcinogenic role in the progression of BCa. *In vitro* experiments demonstrated that the silence of AK024094 significantly attenuated the proliferative, migratory, and invasive abilities of MDA-MB-468 and MCF-7 cells.

The ceRNA hypothesis proposes a novel regulatory network involving mRNAs, pseudogenes, lncRNAs, and circRNAs. Such a network is of great significance in tumor progression[18-20]. Previous investigations[19,20] have found that lncRNAs can competitively bind to common miRNA alongside with mRNAs, thus serving as oncogenes or tumor suppressors. Our results proved the binding relationship between miRNA-181a and AK024094. Besides, a negative correlation was identified between the expressions of miRNA-181a and AK024094 in BCa.

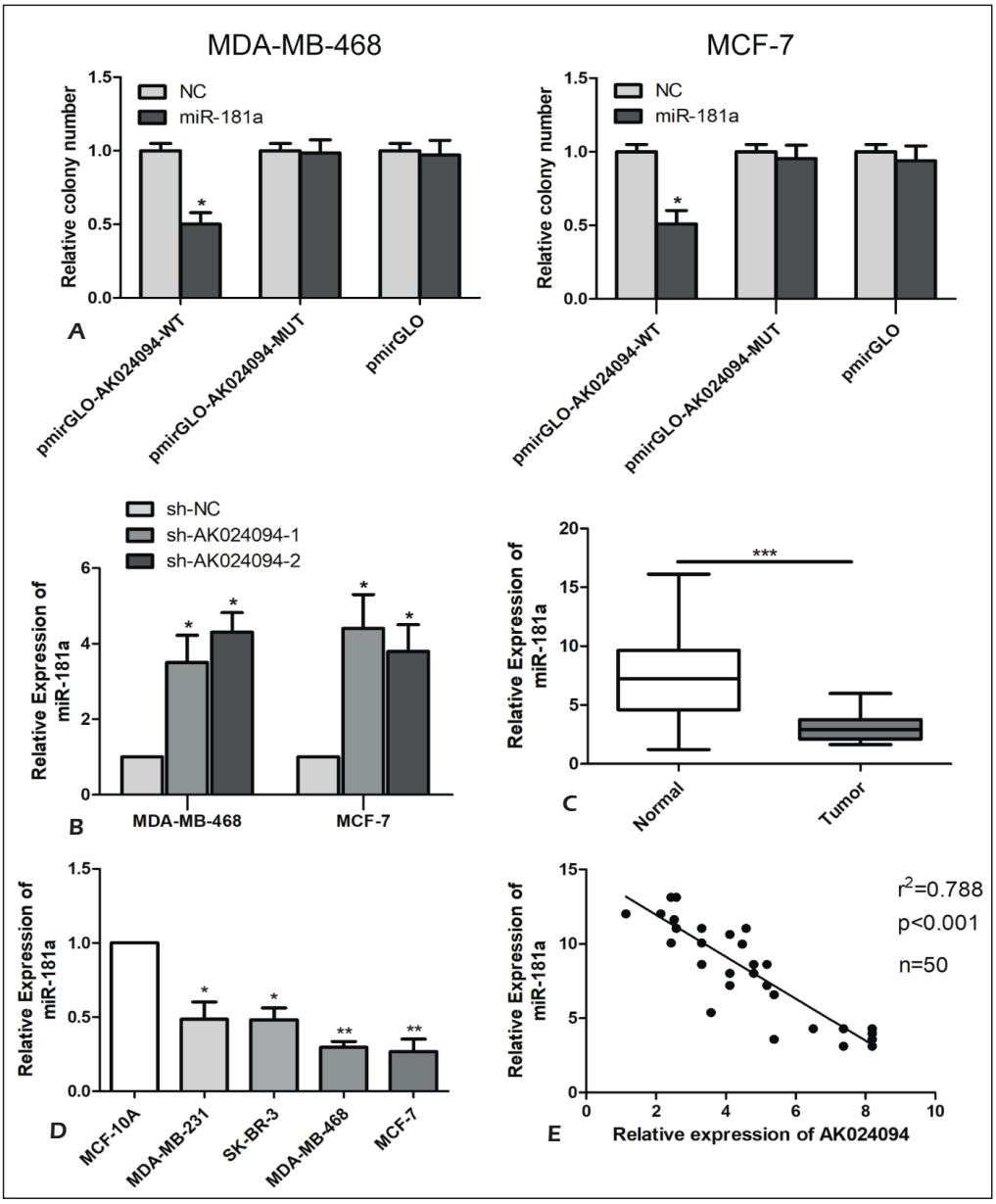

Figure 4. AK024094 could bind to miR-181a. **A**, Luciferase activity in MDA-MB-468 and MCF-7 cells co-transfected with pmirGLO-AK024094-WT/pmirGLO-AK024094-MUT/pmirGLO and miR-181a mimics/NC. **B**, Relative level of miR-181a in MDA-MB-468 and MCF-7 cells transfected with sh-NC, sh-AK024094-1, or sh-AK024094-2. **C**, Relative level of miR-181a in BCa tissues and adjacent normal tissues. **D**, Relative level of miR-181a in normal mammary epithelial cells (MCF-10A) and BCa cells (MDA-MB-231, SK-BR-3, MDA-MB-468, and MCF-7). **E**, A negative correlation was observed between the expression levels of miR-181a and AK024094.

Subsequently, we speculated that AK024094 might serve as a ceRNA to absorb miRNA-181a. To confirm our speculation, a series of rescue experiments were conducted *in vitro*. Notably, the knockdown of miRNA-181a partially reversed the effect of AK024094 on cellular behaviors of BCa cells. All our findings suggested that the regulatory network AK024094/miRNA-181a aggravated the progression of BCa.

Conclusions

Taken together these results proved that AK024094 aggravates the malignant progression of BCa, and is closely related to tumor staging, distant metastasis, and poor prognosis of BCa. Furthermore, it accelerates the proliferation and metastasis of BCa cells by targeting miRNA-181a.

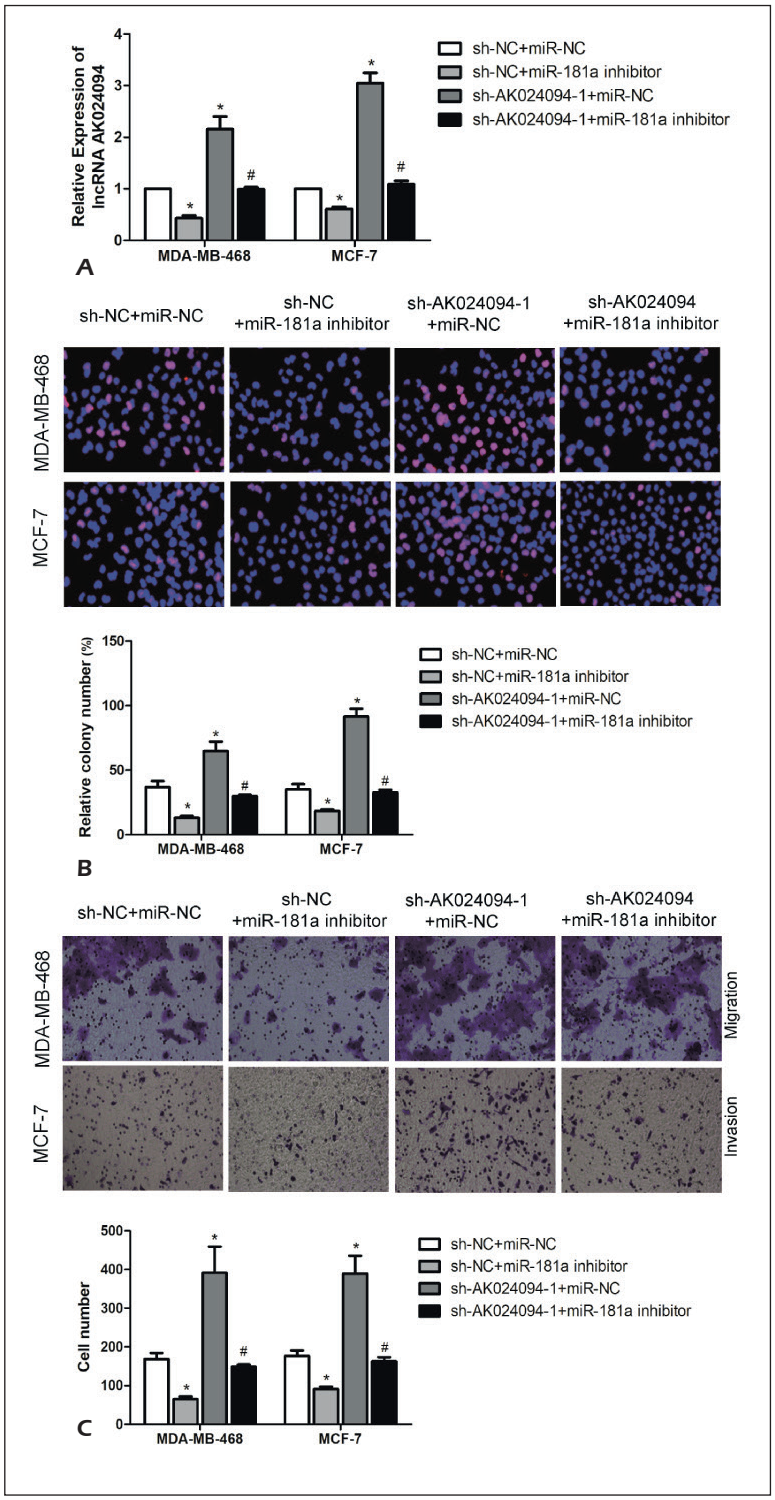

Figure 5. AK024094/miR-181a regulatory network in BCa. MDA-MB-468 and MCF-7 cells were transfected with sh-NC+miR-NC, sh-NC+miR-181a inhibitor, sh-AK024094-1+miR-NC, or sh-AK024094-1+miR-181a inhibitor. ***A***, Relative level of AK024094. ***B***, EdU-positive ratio (magnification × 40). ***C***, Migratory and invasive cell numbers (magnification × 40).

Conflict of Interests

The authors declared that they have no conflict of interests.

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