European Review for Medical and Pharmacological Sciences 2019; 23: 6983-6990

MiRNA-221-5p promotes breast cancer progression by regulating E-cadherin expression

X.-Y. NIU, Z.-Q. ZHANG, P.-L. MA

Department of Thyroid and Breast Surgery, Bayannur City Hospital, Bayannur, China

Abstract. – OBJECTIVE: To elucidate the role of miRNA-221-5p in the development of breast cancer (BCa) and its underlying mechanism.

PATIENTS AND METHODS: The expression level of miRNA-221-5p in 52 pairs of BCa tissues and adjacent normal tissues was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The correlation between miRNA-221-5p expression and pathological indicators of BCa was analyzed. MiRNA-221-5p expression in BCa cells was also determined by qRT-PCR. After transfection of miRNA-221-5p inhibitor in MCF-7 and SKBR3 cells, we detected the regulatory effects of miRNA-221-5p on cellular behaviors through cell counting kit-8 (CCK-8), wound healing, and transwell assay. Finally, the relationship between miRNA-221-5p and E-cadherin in BCa was elucidated.

RESULTS: QRT-PCR results showed that the expression level of miRNA-221-5p in BCa tissues was markedly higher than that in normal tissues. Compared with BCa patients with low expression of miRNA-221-5p, those with high expression had a higher incidence of lymph node metastasis and distant metastasis. However, miRNA-221-5p expression did not correlate with age and sex of BCa patients. MiRNA-221-5p was also highly expressed in BCa cells. Transfection of miRNA-221-5p inhibitor suppressed proliferative, invasive, and migratory potentials of BCa cells. Subsequently, we verified that E-cadherin was lowly expressed in BCa cells, and negatively correlated with miRNA-221-5p. In addition, rescue experiments confirmed that transfection of si-E-cadherin reversed the inhibitory role of miRNA-221-5p knockdown in migratory and invasive potentials of BCa cells.

CONCLUSIONS: The expression of miRNA-221-5p remained high in BCa, which was correlated with lymph node metastasis, distant metastasis, and poor prognosis of BCa. MiRNA-221-5p may promote the invasive and migratory potentials of BCa by regulating E-cadherin expression.

Key Words:

MiRNA-221-5p, E-cadherin, Breast cancer, Metastasis.

Introduction

Malignant tumors are the leading threat to human life. The mortality of malignant tumor in China ranks first in the world[1,2]. The incidence and mortality of malignant tumors in China has increased by 69% and 29.4% within the past two decades, respectively. On average, deaths from malignant tumors account for more than 25% of all deaths[2,3]. Breast cancer (BCa) is the most common malignant tumor in women. According to the National Cancer Statistics Report released by the American Cancer Society in 2016, BCa ranked first in the incidence of female malignant tumors as 29%, while its mortality rate was up to 14%[4,5]. With the improvement of diagnostic and therapeutic approaches, the mortality rate of BCa presents a decreasing trend. However, metastatic BCa is still a challenge in clinical treatment[5-7].

The seed and soil hypothesis has been extensively studied in tumor researches. Here, tumor cells are seed and the tumor microenvironment is soil. The adaptation between seed and soil allows the growth of tumor cells[8,9]. Tumor cells evolve during the metastatic process. They experience various pathological progresses, such as epithelial-mesenchymal transition (EMT), anti-apoptosis, and immune escape, etc. An appropriate soil (tumor microenvironment) provides good conditions for tumor metastasis[10]. In the past, changes of protein expressions in the interactive process between seed and soil were widely explored. Actually, tumor metastasis is influenced by regulation at multiple levels. MicroRNAs could regulate metastatic pathways at the post-transcriptional level by mediating oncogenes or tumor suppressors originated from seed or soil[8-10]. Due to the extensive role of miRNAs, it may be more effective in regulating or even reversing metastatic phenotypes of tumor cells[11].

MicroRNAs are a class of endogenous, short, non-coding RNAs that regulate gene expressions

Corresponding Author: Xiaoye Niu, MM; e-mail: 117695468@qq.com

by complementary pairing with the 3'UTR of the target gene mRNA, thereafter degrading or inhibiting translation of target mRNA[12]. The relationship between microRNAs and metastatic BCa has been well concerned nowadays[13,14]. It is of significance to clarify the functional microRNAs and the new functions of already known microRNAs[15]. Researches on target therapy and individualized therapy of tumors have achieved great advance. Searching for novel diagnostic and therapeutic targets of BCa is crucial for improving clinical outcomes of affected patients[5-7]. In this study, we first analyzed microarrays relative to metastatic BCa through bioinformatics and found that miRNA-221-5p is highly expressed in BCa with strong metastasis[13,14]. Studies have shown that miRNA-221-5p is also highly expressed in other tumors, showing a tumor-promoting role in pathological processes[16]. Regulation of target genes is the core function of microRNAs. Hence, target genes and involved signaling pathways in microRNAs are the focuses in tumor researches[12-14].

Currently, the regulatory effect of miRNA-221-5p on E-cadherin has not been reported yet. Therefore, this study elucidated the potential roles of miRNA-221-5p and E-cadherin in the metastatic development of BCa, which brings new ideas for the diagnosis and treatment of BCa.

Patients and Methods

Patients and BCa Samples

Tumor tissues and paracancerous tissues of 52 BCa patients (aged 45-76 years) undergoing radical mastectomy were collected. None of the patients had preoperative radiotherapy or chemotherapy. Pathological classification and staging criteria for BCa were based on breast cancer staging criteria released by the Union for International Cancer Control (UICC). Enrolled patients and their families signed the informed consent prior to sample collection. This study was approved by the Ethics Committee of Bayannur City Hospital.

Cell Lines

The human BCa cell lines (MCF-7, MDA-MB-231, and SKBR3) and normal mammary epithelial cell line (MCF-10A) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). They were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), and maintained in a 37°C, 5% CO_2 incubator.

Transfection

MDA-MB-231 and SKBR3 cells were seeded into 6-well plates. Until cell density reached about 50-70%, cells were transfected with microRNA NC, inhibitor, siRNA NC or siRNA E-cadherin respectively. Transfected cells were routinely cultured and harvested at 48 h for functional experiments.

Cell Proliferation Assay

Transfected cells for 48 h were seeded into the 96-well plate with 2000 cells per well. At 24 h, 48 h, 72 h, and 96 h, cell counting kit-8 (CCK-8) reagent (Dojindo Laboratories, Kumamoto, Japan) was supplied in each well. After 2 h of culture, the absorbance of each well at 490 nm was recorded using a microplate reader.

Cell Wound Healing Assay

Transfected cells for 48 h were digested and adjusted at 5×10^5/mL. They were seeded into 24-well plates with 5×10^4 cells per well. Until 90% of confluence, an artificial wound was created in the confluent cell monolayer using a 200 μL pipette tip. The images were taken at 0 and 24 h using an inverted microscope, respectively.

Transwell Migration and Invasion Assays

Transfected cells for 48 h were re-suspended in serum-free medium at a density of 2×10^5/mL. Transwell chamber pre-coated with or without Matrigel was placed in a 24-well plate. 200 μL of the suspension was added to the apical chamber. 500 μL of medium with 10% FBS was added to the basolateral chamber. After incubating for 24 h, fixation with methanol, trypan blue staining and phosphate-buffered saline (PBS) wash for three times were performed. Penetrating cells were photographed under a microscope.

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

Relative expressions of E-cadherin and miRNA-221-5p in BCa tissues and cells were determined by qRT-PCR. Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reversely transcribed into the cDNA using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). Primers were designed using Primer 5.0 software. The qRT-PCR reaction was performed

using SYBR® Premix Ex TaqTM (TaKaRa, Otsu, Shiga, Japan) and StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). β-actin and U6 genes were used as internal references. The relative gene expression was calculated by $2^{-\Delta\Delta Ct}$ method. Primer sequences were listed as follows: miRNA-221-5p: forward: 5'-TCCGCGCCCTTGCCCAGACC-3'; reverse: 5'-GTGCCTGGTGCTCTCTTACC-3'; U6: forward: 5'-CTCGCTTCGGCAGCACA-3'; reverse: 5'-AACGCTTCACGAATTTGCGT-3'; Cadherin: forward: 5'-TAGGTATTGTCTACTACTCTG-3', reverse: 5'-TATATCACTCTTGCTTCA-3'; β-actin: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-GCTGATCCACATCTGCTGGAA-3'.

Western Blot

Total protein was extracted using the cell lysate for determining the protein expression. The protein sample was quantified by bicinchoninic acid (BCA; Pierce, Rockford, IL, USA), separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and blocked with 5% skim milk. Membranes were then incubated with the primary antibody and corresponding secondary antibody. Band exposure was developed by chemiluminescence.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was utilized for statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x} \pm s$). Measurement data were analyzed by the *t*-test, whereas categorical data were analyzed by the χ^2-test or Fisher's exact test. Survival analysis was conducted by the Kaplan-Meier method. $p<0.05$ was considered statistically significant.

Results

MiRNA-221-5p Was Highly Expressed in BCa Tissues and Cell Lines

The expression level of miRNA-221-5p in BCa tissues was remarkably higher than that in adjacent non-tumor tissues, and the difference was statistically significant (Figure 1A). In addition, miRNA-221-5p was highly expressed in BCa cells as well, especially in MCF-7 and SKBR3 cells (Figure 1B). We selected these two cell lines for the following *in vitro* experiments.

MiRNA-221-5p Expression Was Correlated With Lymph Node and Distance Metastasis in BCa

Based on the expression level of miRNA-221-5p in the 52 pairs of BCa tissues, these tissues were divided into high level and low level group. Subsequently, we analyzed the correlation between miRNA-221-5p expression with age, sex, clinical stage, lymph node metastasis, and distant metastasis. It is indicated that high expression of miRNA-221-5p was positively correlated to lymph node metastasis and distant metastasis, but was not correlated to age and sex of BCa patients (Table I).

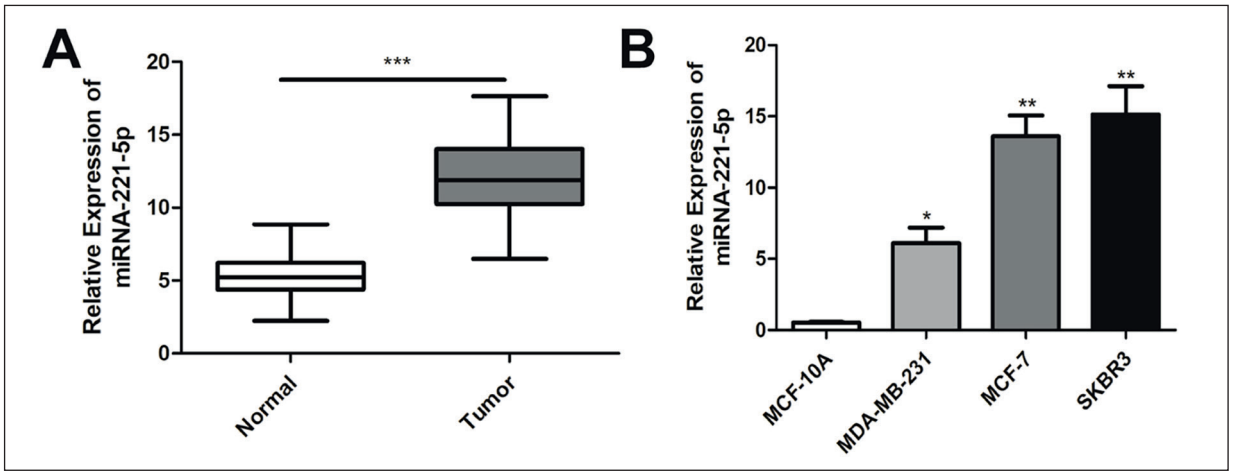

Figure 1. MiRNA-221-5p was highly expressed in BCa tissues and cell lines. ***A,*** QRT-PCR showed higher expression level of miRNA-221-5p in BCa tissues than that in adjacent non-tumor tissues. ***B,*** QRT-PCR showed higher expression level of miRNA-221-5p in BCa cell lines, especially in MCF-7 and SKBR3 cells. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Table I. Association of miRNA-221-5p expression with clinicopathologic characteristics of breast cancer.

Parameters	Number of cases	miRNA-221-5p expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.857
< 60	20	12	8	
≥ 60	32	20	12	
Gender				0.430
Male	25	14	11	
Female	27	18	9	
T stage				0.964
T1-T2	31	19	12	
T3-T4	21	13	8	
Lymph node metastasis				0.029
No	33	24	9	
Yes	19	8	11	
Distance metastasis				0.023
No	42	29	13	
Yes	10	3	7	

Knockdown of MiRNA-221-5p Inhibited Proliferative, Migratory and Invasive Potentials of BCa Cells

To explore the role of miRNA-221-5p in biological behaviors of BCa cells, we first constructed a miRNA-221-5p inhibitor and verified its transfection efficacy (Figure 2A). Subsequently, we evaluated proliferative, invasive, and migratory potentials of MCF-7 and SKBR3 cells. As CCK-8 data indicated, transfection of miRNA-221-5p inhibitor markedly suppressed proliferative rate (Figure 2B). Transwell assay confirmed decreased migratory and invasive rates in BCa cells with miRNA-221-5p knockdown (Figure 2C). Identically, the wound healing assay suggested a decreased wound closure in MCF-7 and SKBR3 cells transfected with miRNA-221-5p inhibitor (Figure 2D).

E-Cadherin Was Lowly Expressed in BCa Tissues and Cell Lines

Through bioinformatics analyses, we found that E-cadherin may be related to miRNA-221-5p. Here, E-cadherin was lowly expressed in BCa tissues than adjacent normal tissues (Figure 3A). Identically, E-cadherin also remained at a low level in BCa cell lines (Figure 3B). By detecting expression levels of miRNA-221-5p and E-cadherin in enrolled BCa tissues, their negative correlation was identified (Figure 3C). Moreover, MCF-7 and SKBR3 cells transfected with miRNA-221-5p inhibitor presented an upregulated level of E-cadherin, further showing their negative correlation (Figure 3D).

MiRNA-221-5p Regulated Cellular Behaviors of BCa Cells by Targeting E-Cadherin

Rescue experiments were conducted to elucidate how miRNA-221-5p inhibited the malignant progression of BCa. We first verified the transfection efficacy of si-E-cadherin in MCF-7 and SKBR3 cells at both mRNA and protein levels (Figure 4A and 4B). Of note, the transwell assay showed that E-cadherin reversed the regulatory effects of miRNA-221-5p on migratory and invasive potentials of BCa cells (Figure 4C and 4D).

Discussion

MicroRNA was first discovered in 1993. It contains 19-24 nucleotides in length. Functionally, microRNA inhibits translation and degrades target mRNA by an incompletely complementary pair, thus participating in the physiological or pathological process[12-15]. One microRNAs can regulate multiple mRNA expressions in a one-to-many manner. Bioinformatics software is usually utilized for predicting the target gene of microRNAs, whereas these predictive results are needed to be further verified by functional experiments[8-10]. Previous studies[12-14] have suggested that dysregulated microRNAs can serve as tumor biomarkers. MicroRNAs are related to many pathological behaviors of tumors, and of which, tumor metastasis is mostly concerned. Multiple microRNAs have been identified to regulate tu-

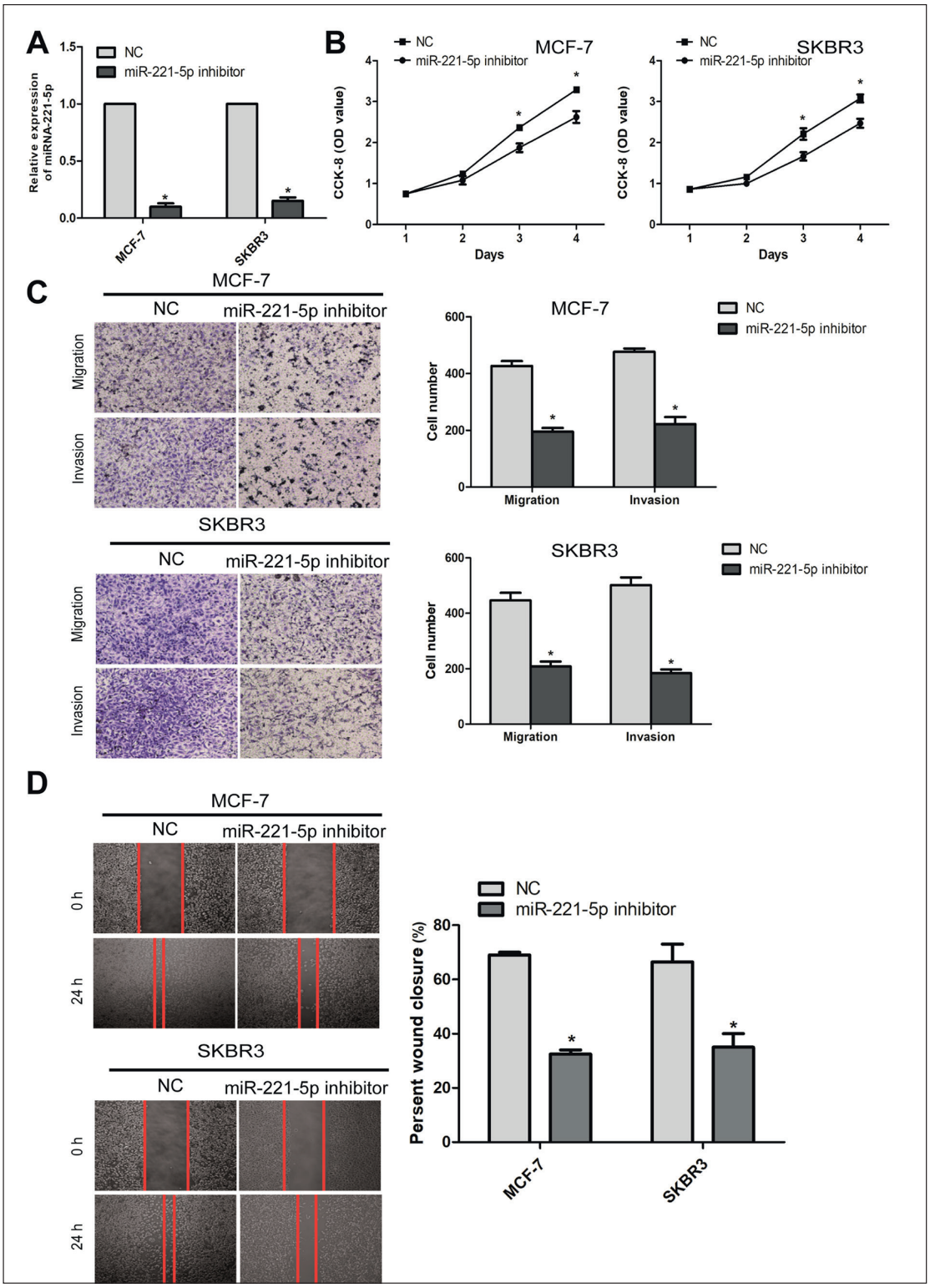

Figure 2. Knockdown of miRNA-221-5p inhibited proliferative, migratory and invasive potentials of BCa cells. ***A,*** Transfection efficacy of miRNA-221-5p inhibitor in MCF-7 and SKBR3 cells detected by qRT-PCR. ***B,*** CCK-8 assay showed that transfection of miRNA-221-5p inhibitor markedly suppressed the proliferative rate of MCF-7 and SKBR3 cells. ***C,*** Transwell assay showed that transfection of miRNA-221-5p inhibitor markedly suppressed migratory and invasive rates of MCF-7 and SKBR3 cells (Magnification: 40×). ***D,*** Wound healing assay showed that transfection of miRNA-221-5p inhibitor markedly reduced wound closure in MCF-7 and SKBR3 cells. *p<0.05 (Magnification: 10×).

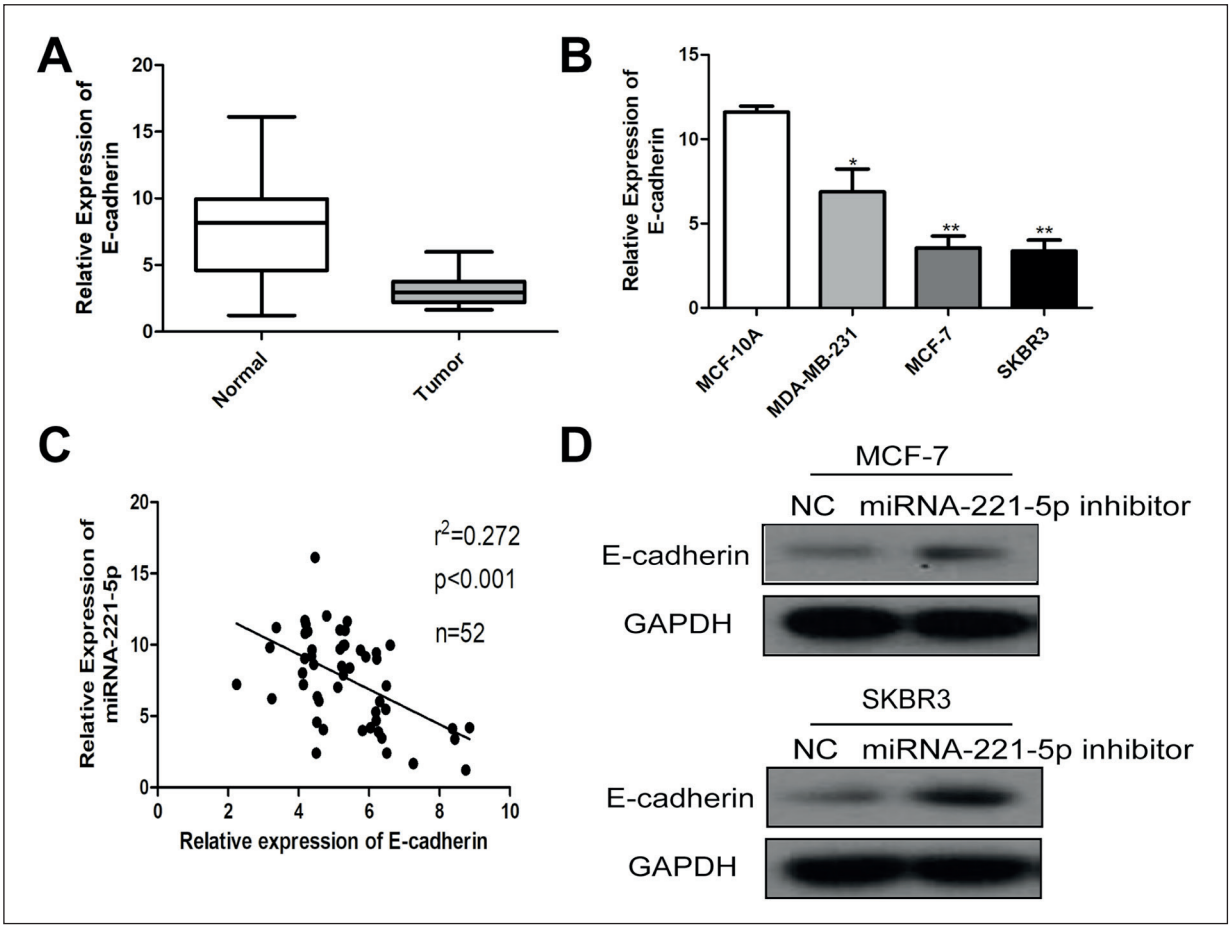

Figure 3. E-cadherin was lowly expressed in BCa tissues and cell lines. ***A,*** QRT-PCR showed lower expression level of E-cadherin in BCa tissues than that in adjacent non-tumor tissues. ***B,*** QRT-PCR showed lower expression level of E-cadherin in BCa cell lines, especially in MCF-7 and SKBR3 cells. ***C,*** A negative correlation between miRNA-221-5p and E-cadherin in BCa tissues. ***D,*** Western blot results showed that MCF-7 and SKBR3 cells transfected with miRNA-221-5p inhibitor presented an upregulated level of E-cadherin. *p<0.05, **p<0.01, ***p<0.001.

mor metastasis, which promote or inhibit metastatic tumor cells by influencing several signaling pathways[8-10].

BCa is a heterogeneous disease with complex origins. Its occurrence and development are closely related to various factors, including environmental factors and genetic factors[4,5]. With the development of surgical approaches, radiotherapy and chemotherapy, diagnostic and therapeutic approaches of BCa have made considerable progress[6-8]. However, distant metastasis remains one of the difficulties in the treatment of BCa. MicroRNAs could regulate the metastatic potential of tumor cells as oncogenes or tumor-suppressor genes[8-10]. Multiple microRNAs have been discovered to participate in the progression of metastatic BCa[12-14]. This study focused on the effects of miRNA-221-5p on biological behaviors of BCa cells. As our results revealed, miRNA-221-5p was highly expressed in BCa cells, suggesting a potential function in the development of BCa. QRT-PCR data showed higher expression of miRNA-221-5p in BCa tissues than adjacent normal tissues. Moreover, its expression was positively correlated with lymph node metastasis and distant metastasis of BCa. We, therefore, believed that miRNA-221-5p served as an oncogene in BCa.

In general, a miRNA can regulate hundreds of target genes. One gene can be regulated by hundreds of microRNAs, thus forming a complex regulatory pathway at the post-transcriptional level[5-7]. MicroRNAs act as regulators by inhibiting gene translation and participate in cell differentiation, metastasis, and other behaviors[17-18]. Our results constructed a miRNA-221-5p knockdown model by

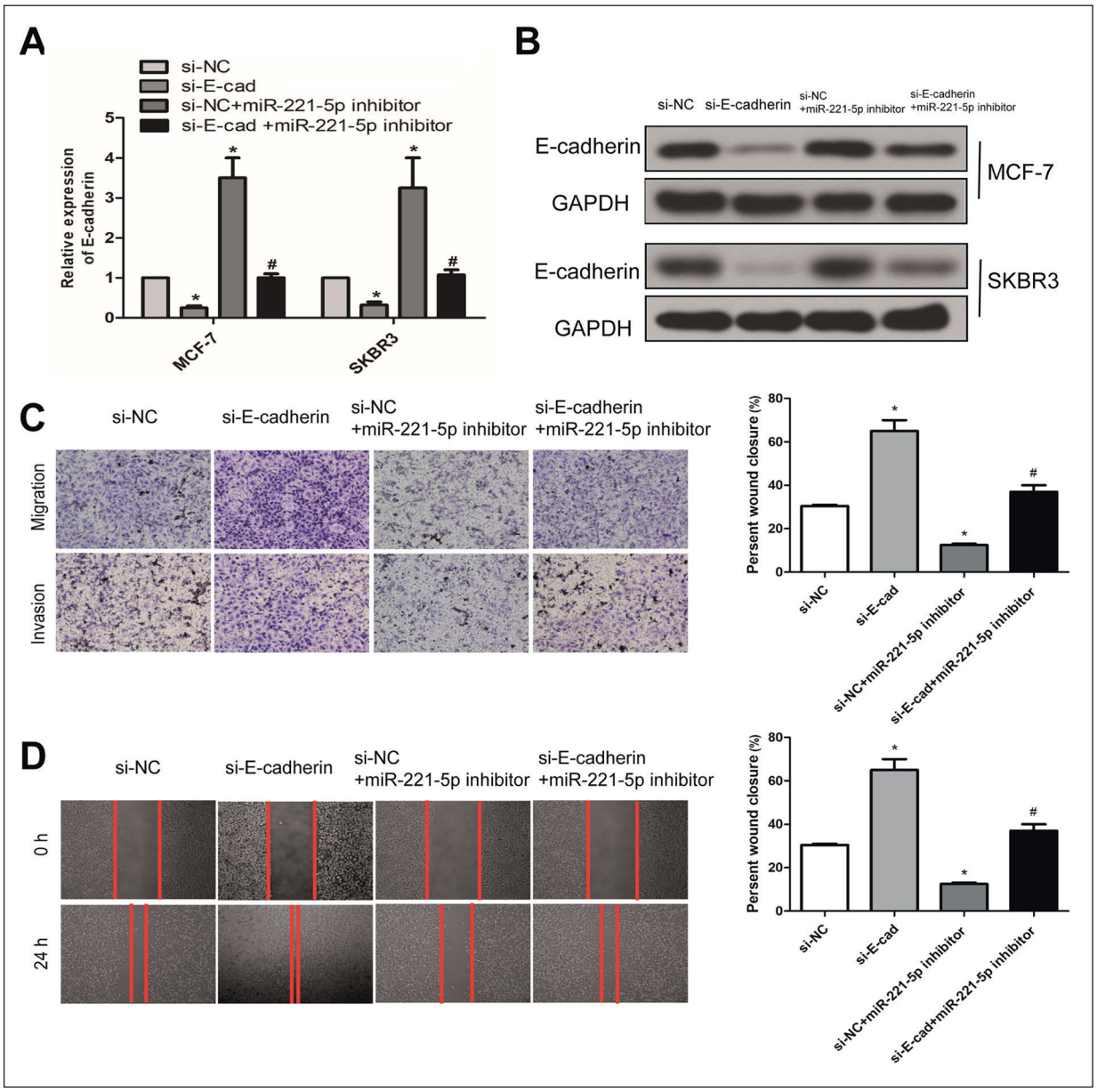

Figure 4. MiRNA-221-5p regulated cellular behaviors of BCa cells by targeting E-cadherin. MCF-7 and SKBR3 cells were transfected with si-NC, si-E-cadherin, si-NC+miRNA-221-5p inhibitor or si-E-cadherin+miRNA-221-5p inhibitor. ***A,*** The mRNA level of E-cadherin in each group. ***B,*** Western blot analysis of E-cadherin in each group. ***C,*** Transwell assay revealed migration and invasion in each group (Magnification: 40×). ***D,*** Wound healing assay revealed wound closure in each group. *p<0.05 (Magnification: 10×).

lentivirus transfection. Subsequent functional experiments proved that miRNA-221-5p knockdown promoted metastasis of BCa cells. However, the specific molecular mechanism remained unclear.

Tumor metastasis is the process in which tumor cells are *in situ* detached to spread to distant target organs and adapt to the new microenvironment. Tumor cell dissemination and survival are the two fundamental keys for metastasis[19-21]. The increased mobility of tumor cells is a manifestation of cell plasticity. Currently, EMT has been widely explored in tumor researches. EMT involves multiple gene changes, such as the down-regulation of E-cadherin (epidermal marker), up-regulation of vimentin (interstitial cell marker), and other molecules[21,22]. MicroRNAs participate in the regulation of life activities by regulating target genes[8-10]. Potential genes and pathways

involved in the disease regulation by microRNAs are the research focuses and difficulties[12-15]. In the present study, we found that E-cadherin was lowly expressed in BCa tissues and cell lines. Furthermore, E-cadherin was negatively regulated by miRNA-221-5p. To further clarify the biological function of miRNA-221-5p, rescue experiments were conducted. It is indicated that E-cadherin reversed the role of miRNA-221-5p in the malignant progression of BCa cells.

Conclusions

We detected that the expression of miRNA-221-5p remained high in BCa, which was correlated with lymph node metastasis, distant metastasis and poor prognosis of BCa. MiRNA-221-5p may promote the invasive and migratory potentials of BCa by regulating E-cadherin expression.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Gargiulo G. Next-generation in vivo modeling of human cancers. Front Oncol 2018; 8: 429.
2) Lovegrove CE, Matanhelia M, Randeva J, Eldred-Evans D, Tam H, Miah S, Winkler M, Ahmed HU, Shah TT. Prostate imaging features that indicate benign or malignant pathology on biopsy. Transl Androl Urol 2018; 7: S420-S435.
3) Marcell SA, Malm J, Rezeli M, Sugihara Y, Betancourt LH, Rivas D, Gyorffy B, Marko-Varga G. Challenging the heterogeneity of disease presentation in malignant melanoma-impact on patient treatment. Cell Biol Toxicol 2018 Oct 24. doi: 10.1007/s10565-018-9446-9. [Epub ahead of print].
4) Agostini D, Natalucci V, Baldelli G, De Santi M, Donati ZS, Vallorani L, Annibalini G, Lucertini F, Federici A, Izzo R, Stocchi V, Barbieri E. New insights into the role of exercise in inhibiting mTOR signaling in triple-negative breast cancer. Oxid Med Cell Longev 2018; 2018: 5896786.
5) Li J, Shen L, Xiao XG, Fang L. MicroRNAs in breast cancer and breast cancer stem cells and their potential for breast cancer therapy. Chin Med J (Engl) 2013; 126: 2556-2563.
6) Altundag K. Reducing dietary argininine restriction may decrease the metastatic potential of primary breast cancer. J BUON 2018; 23: 1202-1203.
7) Sharma D, Kumar S, Narasimhan B. Estrogen alpha receptor antagonists for the treatment of breast cancer: a review. Chem Cent J 2018; 12: 107.
8) Langley RR, Fidler IJ. The seed and soil hypothesis revisited--the role of tumor-stroma interactions in metastasis to different organs. Int J Cancer 2011; 128: 2527-2535.
9) Gil J, Ramsey D, Pawlowski P, Szmida E, Leszczynski P, Bebenek M, Sasiadek MM. The influence of tumor microenvironment on ATG4D gene expression in colorectal cancer patients. Med Oncol 2018; 35: 159.
10) Wang JJ, Lei KF, Han F. Tumor microenvironment: recent advances in various cancer treatments. Eur Rev Med Pharmacol Sci 2018; 22: 3855-3864.
11) Sun Z, Shi K, Yang S, Liu J, Zhou Q, Wang G, Song J, Li Z, Zhang Z, Yuan W. Effect of exosomal miRNA on cancer biology and clinical applications. Mol Cancer 2018; 17: 147.
12) Wang Y, Chen F, Zhao M, Yang Z, Zhang S, Ye L, Gao H, Zhang X. MiR-107 suppresses proliferation of hepatoma cells through targeting HMGA2 mRNA 3'UTR. Biochem Biophys Res Commun 2016; 480: 455-460.
13) Aakula A, Leivonen SK, Hintsanen P, Aittokallio T, Ceder Y, Borresen-Dale AL, Perala M, Ostling P, Kallioniemi O. MicroRNA-135b regulates ERalpha, AR and HIF1AN and affects breast and prostate cancer cell growth. Mol Oncol 2015; 9: 1287-1300.
14) Plantamura I, Cosentino G, Cataldo A. MicroRNAs and DNA-damaging drugs in breast cancer: strength in numbers. Front Oncol 2018; 8: 352.
15) Howard EW, Yang X. microRNA regulation in estrogen receptor-positive breast cancer and endocrine therapy. Biol Proced Online 2018; 20: 17.
16) Shao N, Ma G, Zhang J, Zhu W. miR-221-5p enhances cell proliferation and metastasis through post-transcriptional regulation of SOCS1 in human prostate cancer. BMC Urol 2018; 18: 14.
17) Ji DG, Jiang CW, Luo X, Liu Z, Huang WJ, Zhang XH, Yu TH, Zhang LR. MicroRNA-23a induces apoptosis of hepatocarcinoma cell line MHCC97H via down-regulating KIAP: a mechanism study. Eur Rev Med Pharmacol Sci 2018; 22: 5899-5905.
18) Yu T, Ma P, Wu D, Shu Y, Gao W. Functions and mechanisms of microRNA-31 in human cancers. Biomed Pharmacother 2018; 108: 1162-1169.
19) Chen J, Wu Y, Zhang L, Fang X, Hu X. Evidence for calpains in cancer metastasis. J Cell Physiol 2018 Oct 28. doi: 10.1002/jcp.27649. [Epub ahead of print].
20) Blomberg OS, Spagnuolo L, de Visser KE. Immune regulation of metastasis: mechanistic insights and therapeutic opportunities. Dis Model Mech 2018; 11: dmm036236.
21) Ghosh D, Dawson MR. Microenvironment influences cancer cell mechanics from tumor growth to metastasis. Adv Exp Med Biol 2018; 1092: 69-90.
22) Natividad RJ, Lalli ML, Muthuswamy SK, Asthagiri AR. Golgi stabilization, not its front-rear bias, is associated with EMT-enhanced fibrillar migration. Biophys J 2018; 115: 2067-2077.