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 in normal tissues. Compared with BCa cells 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 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 E-cadherin reversed the inhibitory effect of miRNA-221-5p knockdown in migratory and invasive potentials of BCa cells.

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

Keywords:
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% 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 Cancer Statistics Report released by the American Cancer Society in 2016, BCa ranked first in the incidence of female malignant tumors 29%, while its mortality rate was up to [illegible]%. 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 pot[illegible] roles of miRNA-221-5p and E-cadherin[illegible] metastatic development of BCa, which [illegible]gs new ideas for the diagnosis and treatment of [illegible]

Patients and M[illegible]

Patients and BCa Sam[illegible]s

Tumor tissues and pa[illegible]er[illegible] 52 BCa patients (ag[illegible]5-70[illegible] undergoing radical mastector[illegible] were col[illegible] None of the patients ha[illegible]perative rad[illegible]apy or chemotherap[illegible]th[illegible]al classific[illegible]tion and staging crit[illegible] for BC[illegible]e based on breast cancer st[illegible]g criteria re[illegible]d by the Union for Int[illegible]tional Cancer Co[illegible]ol (UICC). Enrolle[illegible]tients [illegible]their families signed the informons[illegible]rior to[illegible]ample collection. This study [illegible]oved b[illegible]e Ethics Committee of [illegible]annu[illegible] Hos[illegible].

Ce[illegible]nes

[illegible]man BCa cell lines (MCF-7, MDA-M[illegible] SKBR3) and normal mammary [illegible]thelial cell line (MCF-10A) were purchased [illegible] American Type Culture Collection (ATCC; M[illegible]as, 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 [illegible]were seeded into 6-well plates. Until cell [illegible]reached about 50-70%, cells were [illegible]nsfecte[illegible] microRNA NC, inhibitor, [illegible]A NC or [illegible] E-cadherin respectivel[illegible]ransfe[illegible]d cells [illegible] routinely cultured an[illegible]vested[illegible]8 h for functional experiments.

Cell Prolifer[illegible]n Assay

Transfect[illegible] for 48 h we[illegible]ed into the 96-well p[illegible]w[illegible]00 cells per well. At 24 h, 48 h, 72 [illegible] and 96[illegible] counting kit-8 (CCK-8) rea[illegible] (Dojindo [illegible]atories, Kumamoto, Ja[illegible] supplied in [illegible] well. After 2 h of [illegible]re, the absorbance of each well at 490 nm [illegible] recorded us[illegible]a microplate reader.

[illegible]Wound [illegible]ling Assay

[illegible]s for 48 h were digested and adjusted [illegible]×10^5/mL. They were seeded into [illegible]4-well plates with 5×10^4 cells per well. Until [illegible]confluence, an artificial wound was creat[illegible] 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 corresp... secondary antibody. Band exposure wa... oped by chemiluminescence.

Statistical Analysis

Statistical Product and Se... Solution... (SPSS) 22.0 software (IBM, A... Y, USA) was utilized for statistical ...ysis. ... quantitative data were represen... as mean ... deviation ($\bar{x} \pm s$). Measu... d...

lyzed 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 Ka... er method. $p<0.05$ was considered ... significant.

Result...

MiRNA-221-5p Was ...ghly E...pressed... BCa Tissues and C... Lines

The expression lev... -221-5... BCa tissues was rem...bly ... than th... adjacent non-tum...ssues, an... di...nce was statistically ...cant (Figure ... addition, miRNA-2...p ... highly exp...ssed in BCa cells as w...l, espe... in MCF-7 and SKBR3 cells (Fi...re 1B). We s... these two cell lines fo... owing *in vitro* ...eriments.

RNA-221-5... xpression Was ...related W... Lymph Node and ...nce Met...asis in BCa

E... xpression level of miRNA-221-5p in th... pairs of BCa tissues, these tissues ...ere divided into high level and low level group. ...ently, we analyzed the correlation be...ec... iRNA-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).

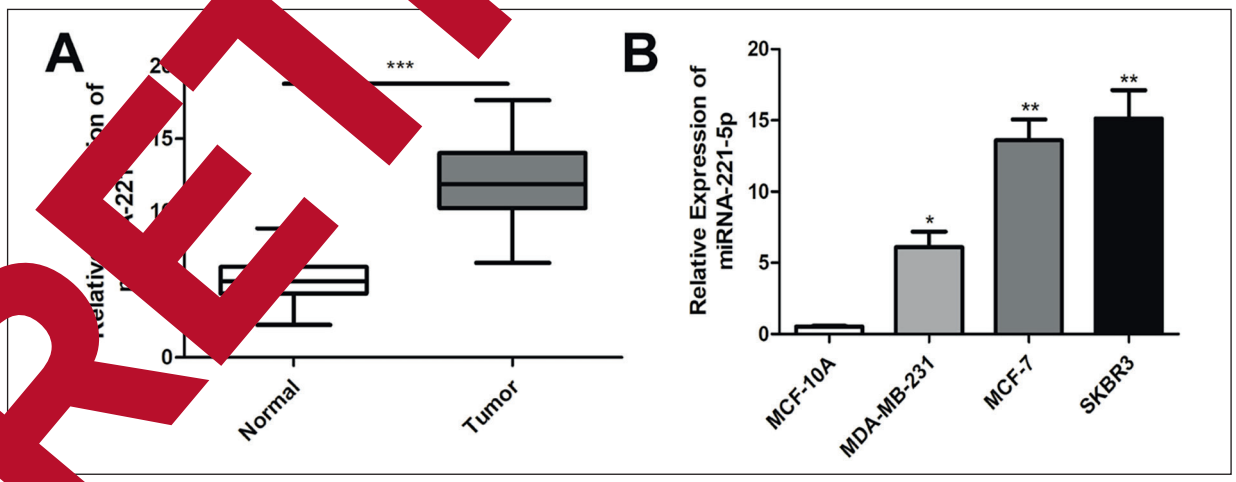

Fig... 1. MiRNA-221-5p was highly expressed in BCa tissues and cell lines. ***A,*** QRT-PCR showed higher expression level of miRNA-221-5p in BCa tissues than that in adjacent non-tumor tissues. ***B,*** QRT-PCR showed higher expression level of miRNA-221-5p in BCa cell lines, especially in MCF-7 and 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		
		Low (%)	High (%)	
Age (years)				0.857
< 60	20	12	8	
≥ 60	32	20	12	
Gender				
Male	25	14		
Female	27	18		
T stage				0.964
T1-T2	31	19	12	
T3-T4	21	13		
Lymph node metastasis				0.029
No	33	24		
Yes	19	8	11	
Distance metastasis				0.023
No	42	29	13	
Yes	10	3		

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 of cells with miRNA-221-5p knockdown. 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. Hence, 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 miR-NA-221-5p inhibitor presented an upregulated level of E-cadherin, further showing their negative correlation (Figure 3D).

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

Rescue experiments were conducted to elucidate if 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-

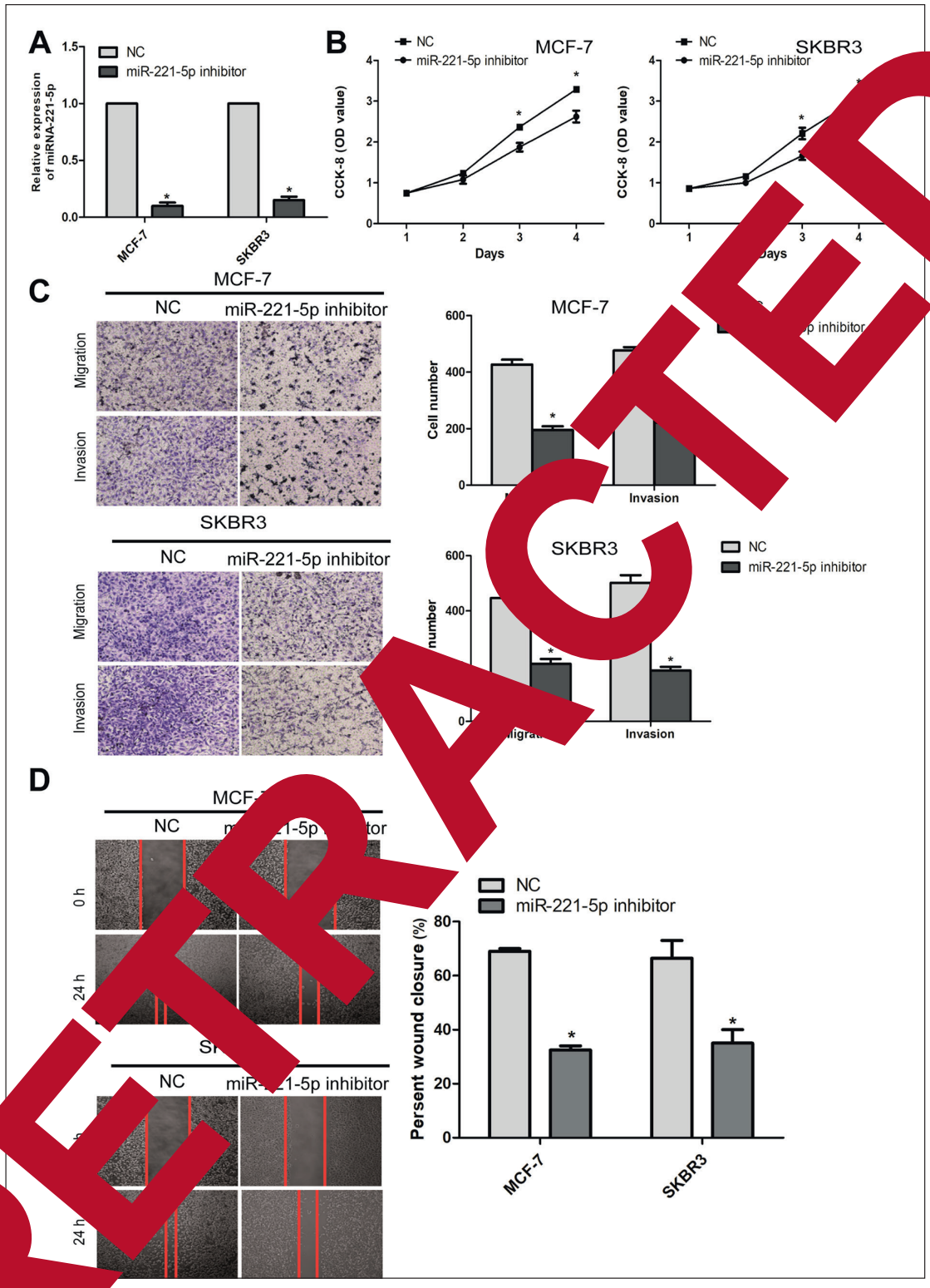

Figure 2. Knockdown of miRNA-221-5p inhibited proliferative, migratory and invasive potentials of BCa cells. ***A,*** Transfection efficacy of miRNA-221-5p inhibitor in MCF-7 and SKBR3 cells detected by qRT-PCR. ***B,*** CCK-8 assay showed that transfection of miRNA-221-5p inhibitor markedly suppressed 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×).

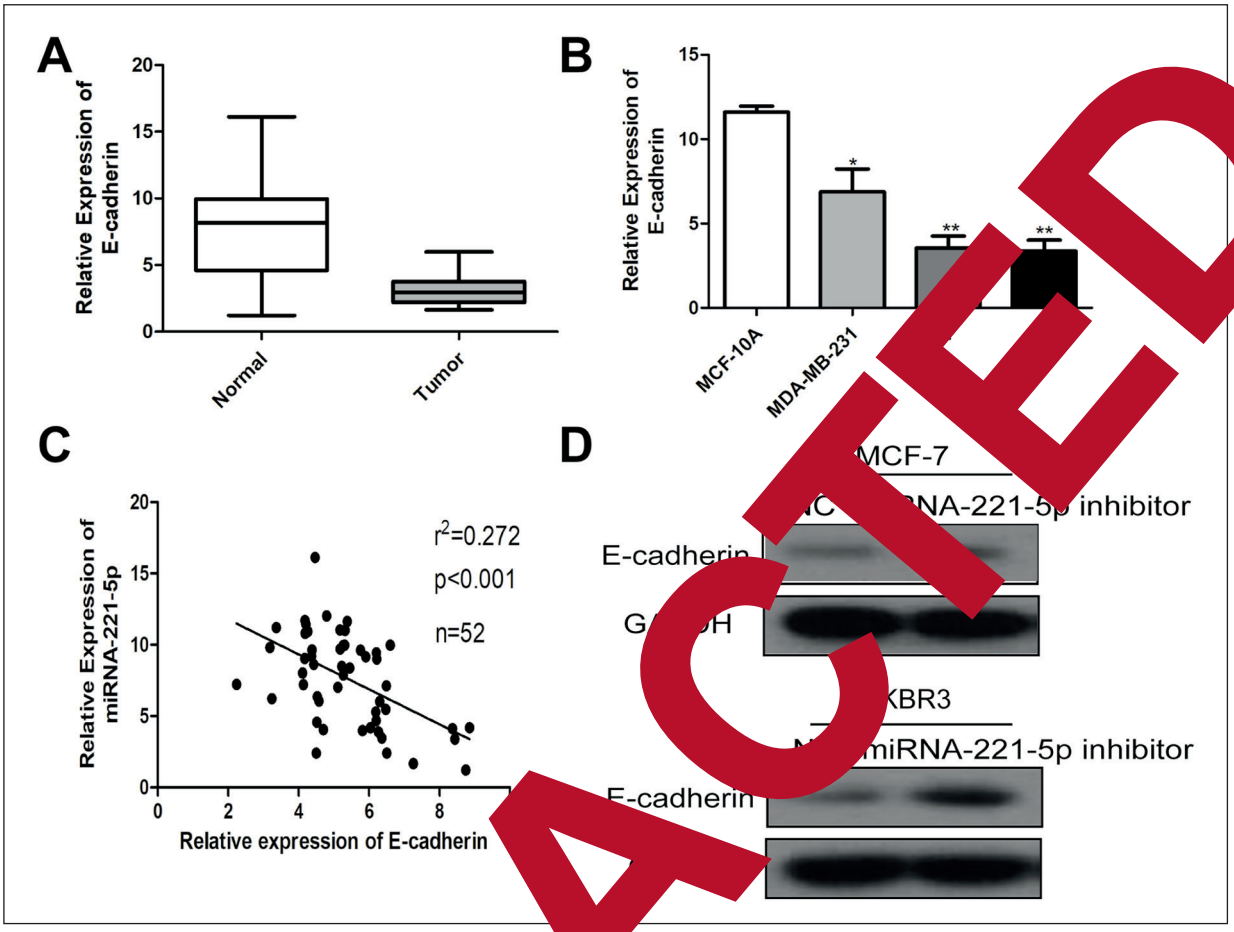

Figure 3. E-cadherin was lowly ex BCa tissu d cell lines. ***A,*** QRT-PCR showed lower expression level of E-cadherin in BCa tissues than tha jac n-tumor t ***B,*** QRT-PCR showed lower expression level of E-cadherin in BCa cell lines, especially in M and SK cells. ***C,*** A gative correlation between miRNA-221-5p and E-cadherin in BCa tissues. ***D,*** Western blot r showed t 7 and SKBR3 cells transfected with miRNA-221-5p inhibitor presented an upregulated level of E-cadh <0.0 <0.001.

mor metastasis promote or meta-static tumor c by acing several signaling pathways[8-10].

BCa i heterogeneou ase with complex o s. Its occurrence d development are ly rel to various factors, including ronm l facto and genetic factors[4,5]. With lopmer surgical approaches, adiother nd ch otherapy, diagnostic and eutic es of BCa have made considerable progr . However, distant metastasis ins one of the difficulties in the treatm MicroRNAs could regulate the tastatic potential of tumor cells as oncogenes mor-suppressor genes[8-10]. Multiple microR-N ve been discovered to participate in the prog ession 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

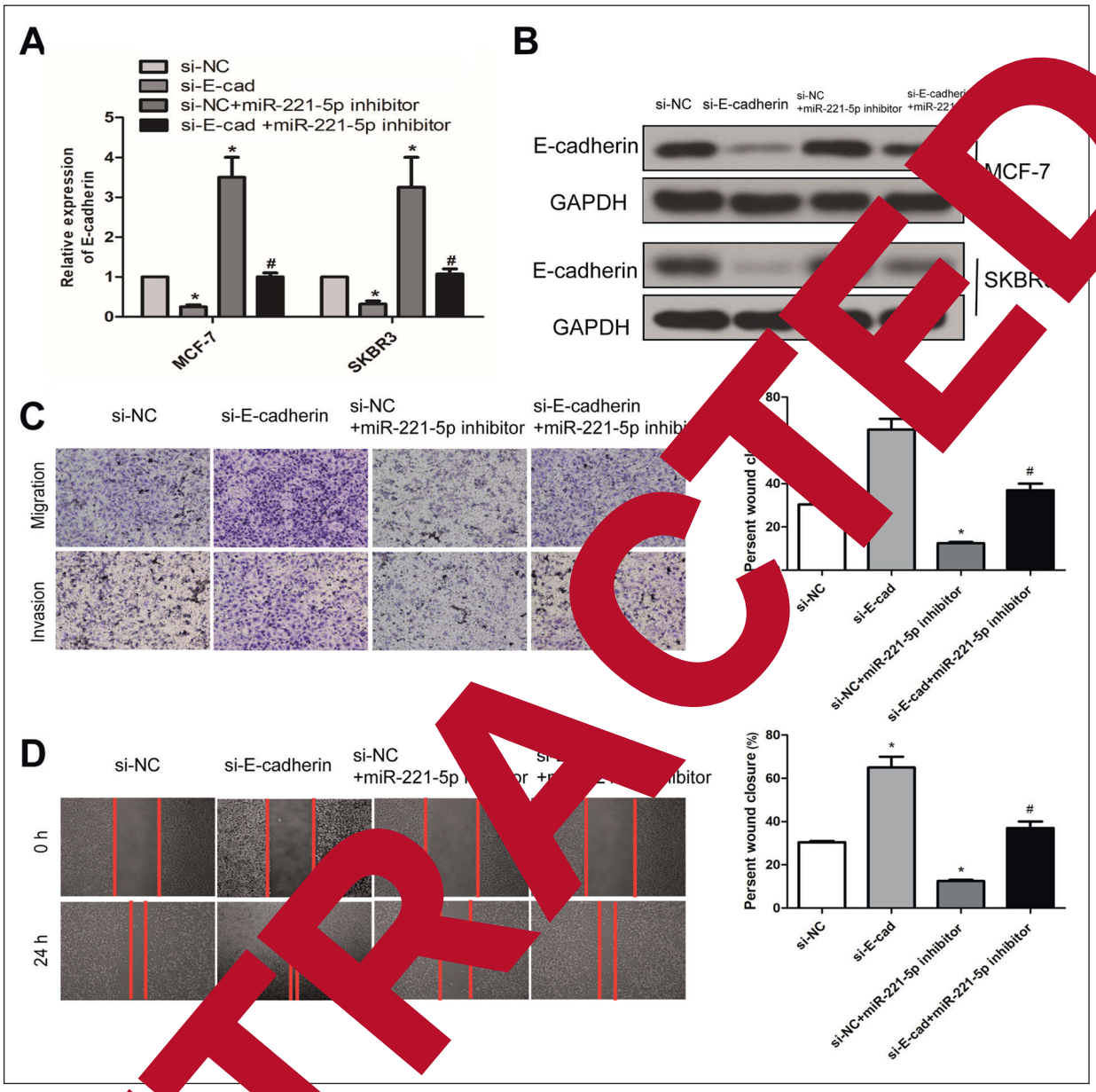

Figure 4. MiRNA-221-5p regulates cellular behaviors of BCa cells by targeting E-cadherin. MCF-7 and SKBR3 cells were transfected with si-NC, si-E-cadherin, si-NC+miRNA-221-5p inhibitor or si-E-cadherin+miRNA-221-5p inhibitor. ***A,*** The mRNA expression of E-cadherin in each group. ***B,*** Western 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< (Magnification: 10×)

...us tr... Subsequent functional experiments prove... that miRNA-221-5p knockdown pro... metastasis of BCa cells. However, the s... lar 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 modeli human cancers. Front Oncol 2018; 8: 429.
2) Lovegrove CE, Matanhelia M, P Eldred-E ans D, Tam H, Miah S, Winki HU, Shah TT. Prostate imaging feat hat inc e benign or malignant pathology biopsy. Androl Urol 2018; 7: S420-S4
3) Marcell SA, Malm ezel ara Y, Betancourt LH, Rivas D rffy B, M arga G. Challenging the he eneity of dise esentation in malignant a-impact on nt treatment. Cell To 018 Oct 24. doi: 10.1007/s10565-01 9446-9. [ahead of print].
4) Agost Natalucci V, Ba G, De Santi M, Donat Vallorani L, Annibalin Lucertini F, Federic zo R, S hi V, Barbieri E. New insights into ole of cise in inhibiting mTOR signaling le- tive bre cancer. Oxid Med Cell Lo ; 2018 6786.
5) Li J, Si Xiao ang L. MicroRNAs in breast ncer a cancer stem cells and their po- tial for bi cancer therapy. Chin Med J (En- 2013; 126: 2556-2563.
6) Reducing dietary argininine restriction may decrease the metastatic potential of primary breast cancer. J BUON 2018; 23: 1202-1203.
7) rma 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 szczynski Bebenek M, Sasiadek MM. The in ce of tumor microenvironment on ATG4D pression in colorectal cancer patients. Med 2018; 35: 159.
10) Wang JJ, Lei KF, Han F or microenv recent advances in us ca er treat Eur Rev Med Ph col Sci 8; 22: 3 3864.
11) Sun Z, Shi K, Y S, Q, W , Song J, Li Z, Zhan uan W of ex nal miRNA on ca biology an plications. Mol Ca 8; 17: 147.
12) Wang hen o M, Yang Z, Zhang S, Ye L, Gao H, hang X. 7 suppresses proliferation of h atoma cells t targeting HMGA2 mRTR. Biochem B s Res Commun 2016; 0: 455-460.
13) Aakula A, Le n SK, Hintsanen P, Aittokallio T, Ceder Y, Borre Dale AL, Perala M, Ostling P, Kallioniemi O. Mic NA-135b regulates ERalpha, AR HIF1AN affects breast and prostate cancer. Mol Oncol 2015; 9: 1287-1300.
14) Plant I, Cosentino G, Cataldo A. MicroRNAs and DNA-damaging drugs in breast cancer: ength in numbers. Front Oncol 2018; 8: 352.
15) rd 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.