European Review for Medical and Pharmacological Sciences 2020; 24: 3105-3112

MiR-548b-3p inhibits proliferation and migration of breast cancer cells by targeting MDM2

M.-X. SHA[1], X.-W. HUANG[2], Q. YIN[3]

[1]Interventional Operating Room, Yantai Affiliated Hospital of Binzhou Medical University, Yantai, China
[2]Department of Image, Yantaishan Hospital, Yantai, China
[3]Department of Breast Surgery, The People's Hospital of Rizhao, Rizhao, China

Mingxuan Sha and *Xingwen Huang* contributed equally to this work

Abstract. – OBJECTIVE: The aim of this study was to explore the expression and biological functions of micro ribonucleic acid (miR)-548b-3p in breast cancer (BC), and to investigate its potential molecular mechanism.

PATIENTS AND METHODS: The expression level of miR-548b-3p in BC tissues and cells was detected by quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). Subsequently, the impacts of miR-548b-3p on the proliferation, apoptosis, and cycle, as well as migration and invasion of BC cells, were explored using colony formation assay and 5-ethynyl-2'-deoxyuridine (EdU) staining, flow cytometry, and transwell assay, respectively. The possible downstream target genes of miR-548b-3p were predicted via bioinformatics and verified through qRT-PCR and Western blotting. Furthermore, Dual-Luciferase reporter gene assay was employed to confirm whether miR-548b-3p could directly bind to murine double minute 2 (MDM2).

RESULTS: QRT-PCR results showed that miR-548b-3p expression was significantly downregulated in 37 out of 43 BC tissues. Subsequent in-vitro experiments indicated that the overexpression of miR-548b-3p significantly inhibited the proliferation and metastasis, whereas promoted the apoptosis of BC cells. Bioinformatics predicted that MDM2 was the downstream target gene of miR-548b-3p. After overexpression of miR-548b-3p, qRT-PCR, and Western blotting results revealed that the expression of MDM2 was remarkably downregulated. Dual-Luciferase reporter gene assay further confirmed that miR-548b-3p could directly bind to MDM2.

CONCLUSIONS: MiR-548b-3p expression was significantly downregulated in BC. In addition, lowly expressed miR-548b-3p repressed the proliferation and metastasis of BC cells through targeted regulation of MDM2.

Key Words:
Breast cancer (BC), MiR-548b-3p, Proliferation, Metastasis, MDM2.

Introduction

Breast cancer (BC) is the most common malignant tumor in women, which is also the second major cause of tumor-related deaths in females around the world[1,2]. The mortality rate of BC patients has been significantly reduced due to the improvement in its early diagnosis and treatment strategies. Although most BC patients have favorable therapeutic effect at first, some may progress into more aggressive forms of tumor after a period of time and produce resistance to radiotherapy and chemotherapy at the same time[3]. Therefore, it is of important significance to seek for molecular biomarkers for the prevention, diagnosis, and treatment of BC.

Micro ribonucleic acids (miRNAs) are a category of small non-coding RNAs with about 22 nucleotides in length. They can bind to the 3'-untranslated region (3'-UTR) of their messenger RNAs (mRNAs) to trigger the degradation or post-transcriptional translational repression of mRNAs, thus regulating the target gene expression. A bioinformatics study[4] has illustrated that miRNAs may control more than 30% protein-coding genes. MiRNAs participate in regulating multiple factors of tumor progression, including growth and metastasis[5,6]. Meanwhile, they play vital roles in the occurrence and development of malignancies as anti-oncogenes or oncogenes. Currently, the expression profiles of miRNAs are becoming potential diagnostic and prognostic markers, which can facilitate individualized treatment and disease management[7].

The miR-548 family is a fairly big primate-specific miRNA family, with greater nucleotide difference and lower conservation than other big miRNA families[8]. As a member of the miR-548 family, miR-548b-3p exhibits dysregulated ex-

Corresponding Author: Qiang Yin, MM; e-mail: wzimzb@163.com

pression in a variety of tumors. Based on literature[9], miR-548b-3p suppresses the proliferation of hepatocellular carcinoma (HCC) cells by targeting CIP2A. In NSCLC, miR-548b-3p serves as an anti-oncogene by controlling the PI3K/AKT signaling pathway[10]. In this research, *in vitro* experiments showed that the expression of miR-548b-3p was downregulated in BC tissues and cells. Moreover, overexpressed miR-548b-3p repressed the proliferation and metastasis of BC cells.

Patients and Methods

Tissue Specimens

Tissue specimens were collected from 43 patients receiving radical mastectomy in Shandong Provincial Third Hospital from January 2017 to June 2018. No patient received radiotherapy, chemotherapy, endocrine therapy, targeted therapy, or other treatments before operation. The types of tissue specimens were defined pathologically. All collected tissues were immediately stored in liquid nitrogen at -180°C for use. This research was approved by the Ethics Committee of Shandong Provincial Third Hospital.

Cell Lines

Human BC cell lines (MDA-MB-231, MDA-MB-435, MCF-7, and ZR-75-1) and normal mammary epithelial cell line (MCF-10A) were purchased from the Shanghai Institutes for Biological Sciences, CAS (Shanghai, China). All cells were incubated in Roswell Park Memorial Institute-1640 (RPMI)-1640 medium (HyClone; South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in an incubator with 5% CO_2 at 37°C.

Reagents

The reagents used were: TRIzol reagent, reverse transcription (RT) kit, and SYBR® Green Mix (Vazyme Biotech Co., Ltd., Nanjing, China); miR-548b-3p mimics (Shanghai GenePharma, Shanghai, China); Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA); 5-ethynyl-2'-deoxyuridine (EdU) staining kit (Shanghai Beyotime Biotechnology Co., Ltd. , Shanghai, China); transwell chambers, 6-well plate, and 96-well plate (Corning, Corning, NY, USA); rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and murine double minute 2 (MDM2) antibodies (Cell Signaling Technology, Danvers, MA, USA).

Quantitative RT-Polymerase Chain Reaction (qRT-PCR)

Total RNAs were extracted from BC tissues and cells using TRIzol reagent. RNA concentration and quality were determined *via* a micro-plate reader. Next, extracted RNAs were reversely transcribed into complementary deoxyribonucleic acid (cDNAs). The amplification was performed using a real-time fluorescence qPCR instrument, with U6 as an internal reference. PCR procedures were as follows: pre-denaturation at 95°C for 3 min, denaturation at 95°C for 15 s, and annealing at 62°C for 10 s, for a total of 40 cycles. The primer sequences used in this study were as follows: U6 F 5'-GTGCTCGCTTCGGCAGCATA-3', R 5'-GGAACGCTTCACAATTTGCGTGTC-3'; miR-548b-3p F 5'-TACTCAAAATGGGGGCGCTT-3', R 5'-GGGACACCCCAAAATCGAAG-3'; MDM2 F 5'-GCGGAGTGATCGTCAATAACA-3', R 5'-GACCCGGTAAGGGGTAAGGT-3'. GAPDH F: 5'-TGACTTCAACAGCGACACCCA-3', R: 5'-GGAGTGTTGGAGAAGTCATATTAC-3'

Colony Formation Assay

BC cells (8×10^2) in the experimental group and control group were first cultured in 6 cm culture dishes. 14 d later, the culture medium was discarded. Then, the cells were rinsed with phosphate-buffered saline (PBS), fixed in 20% methanol, and stained with crystal violet. Next, the cells on the surface of the upper membrane were discarded. Finally, formed colonies were observed under a microscope, and the number of colonies was counted.

EdU Staining

The cells in the logarithmic growth phase were collected and seeded into a 24-well plate (400 μL/well) at a density of 8×10^5 cells/mL. Then, the cells were cultured in an incubator for plating. After adherence, DS intervention was applied in the experimental group. Meanwhile, an equal volume of PBS was added into the control group. At 24 h after intervention, EdU staining was conducted according to the kit instructions.

Cell Apoptosis

The grouping and steps of transfection were as above. 48 h after transfection, the digested cells were harvested and centrifuged to remove the

supernatant. Then, the cells were washed with pre-cooled PBS at 4°C twice, followed by centrifugation and collection. Later, cell concentration was adjusted to 1×10^6 cells/mL using l × Annexin V binding buffer. After that, each sample was mixed with 5 μL of PE Annexin V and 5 μL of 7-ADD, separately. After incubation in the dark at room temperature for 10-15 min, 400 μL of 1 × binding buffer was added. Finally, cell apoptosis was detected using a flow cytometer (Partec AG, Arlesheim, Switzerland).

Cell Cycle

Digested cells at 48 h after transfection were collected, and the supernatant was discarded after centrifugation. Subsequently, the cells were washed with pre-cooled PBS at 4°C twice, centrifuged, harvested, and re-suspended in 500 μL of PBS. Next, the cells were quickly added with 5 mL of ice-cold 70% ethanol at -20°C. After fixation at 4°C overnight, the cells were centrifuged to discard the supernatant and washed with PBS for 3 times to remove the fixative. Next, 500 μL of propidium iodide/RNase staining solution was added for water bath in the dark at 4°C for 30 min. Finally, cell cycle distribution in each group was examined using the flow cytometer.

Transwell Assay

Matrigel (8-12 mg/mL) (Corning, Corning, NY, USA) was diluted 30-40 times to 200-300 μg/mL. 0.1 mL of diluted Matrigel was carefully injected into transwell chambers using a yellow pipette for 2 h of incubation at 37°C. Next, partial supernatant was absorbed carefully, and the cells were diluted by serum-free medium into cell suspension (2×10^5/mL). 100 μL of serum-free cell suspension was added into the upper chamber. Meanwhile, 800 μL of 10% FBS + RPMI-1640 medium was added into the lower chamber. Then, the cells were incubated in an incubator at 37°C for 24 h. Next, transwell plates were taken out, washed with PBS twice, fixed in methanol for 30 min, and stained with crystal violet for 30 min. The cells on the upper surface were wiped out using cotton balls, and the migrating cells were observed under a microscope.

Western Blotting

The cells in the experimental group and control group were harvested and washed in PBS twice to remove the supernatant. The total proteins were extracted and extracted protein concentration was measured *via* a bicinchoninic acid kit. Every 100 μL of protein sample was added with 25 μL of loading buffer for 5× SDS-polyacrylamide gel electrophoresis (PAGE), followed by denaturation at 100°C for 5-10 min. Later, the protein samples were subjected to PAGE and wet transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and soaked in methanol previously. The membranes were then sealed in 50 g/L skimmed milk powder for 1.5 h and washed with Tris-Buffered Saline-Tween 20 (TBST) for 3 times (10 min for each). Next, the membranes were incubated with primary antibodies (1:1000) on a shaking table at 4°C overnight. On the next day, the membranes were washed with TBST for 3 times (10 min/time), and incubated with the corresponding secondary antibodies (1:2000) at room temperature for 1.5 h. Immuno-reactive bands were finally exposed *via* enhanced chemiluminescence (ECL) method (Thermo Fisher Scientific, Waltham, MA, USA).

Dual-Luciferase Reporter Gene Assay

Target genes were predicted by means of online web TargetScan (http://www.targetscan.org/vert_72). Finally, MDM2 was screened as a possible target molecule. BC cells in the logarithmic growth phase were inoculated into 6-well plates and conventionally cultured for 24 h. Then, the cells were transfected with pmirGLO-MDM2-wild-type (WT) + miR-548b-3p mimics, pmirGLO-MDM2-WT + negative control (NC), pmirGLO-MDM2-mutant-type (Mut) + miR-548b-3p mimics and pmirGLO-MDM2-Mut + NC, respectively. At 24 h after transfection, the fluorescence intensity of MDM2 promoter was detected in accordance with Dual-Luciferase assay kit (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS Inc., Chicago, IL, USA) was utilized for all statistical analysis. The measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$). The *t*-test was adopted to compare the differences between the two groups. One-way analysis of variance was applied to compare the differences among multiple groups, followed by post-hoc test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

MiR-548b-3p Expression was Downregulated in BC Tissues and Cells

Total RNA was first extracted from 43 BC tissues and reversely transcribed into cDNA. QRT-PCR assay showed that compared with para-carcinoma tissues, miR-548b-3p expression was significantly downregulated in 37 of 43 BC tissues (Figure 1A). Total RNA in BC cells was extracted using the same method. Subsequent qRT-PCR results indicated that the expression of miR-548b-3p significantly decreased in BC cells (Figure 1B). To investigate the function of miR-548b-3p in BC cells, its overexpressed mimics were designed and transfected into BC cells. Transfection efficiency was verified by qRT-PCR 48 h later (Figure 1C and 1D).

MiR-548b-3p Inhibited Proliferation of BC Cells

After miR-548b-3p overexpression in BC cells, colony formation assay, and EdU staining results manifested that the proliferation of BC cells was remarkably repressed (Figure 2A-2D).

MiR-548b-3p Promoted Apoptosis and Cycle Arrest and Inhibited Metastasis of BC Cells

To further explore the biological function of miR-548b-3p *in vitro*, miR-548b-3p mimics were transfected into BC cells. The changes in the apoptosis and cycle distribution of BC cells were detected *via* flow cytometry. The results displayed that the apoptosis rate of BC cells was significantly upregulated after overexpression of miR-548b-3p (Figure 3A and 3B). Meanwhile, cell cycle was distributed at G1/G0 phase (Figure 3C and 3D). Subsequently, the influences of miR-548b-3p on the migratory and invasive abilities of BC cells were explored by transwell assay. The results exhibited significantly repressed migratory and invasive abilities of BC cells after miR-548b-3p overexpression (Figure 3E and 3F).

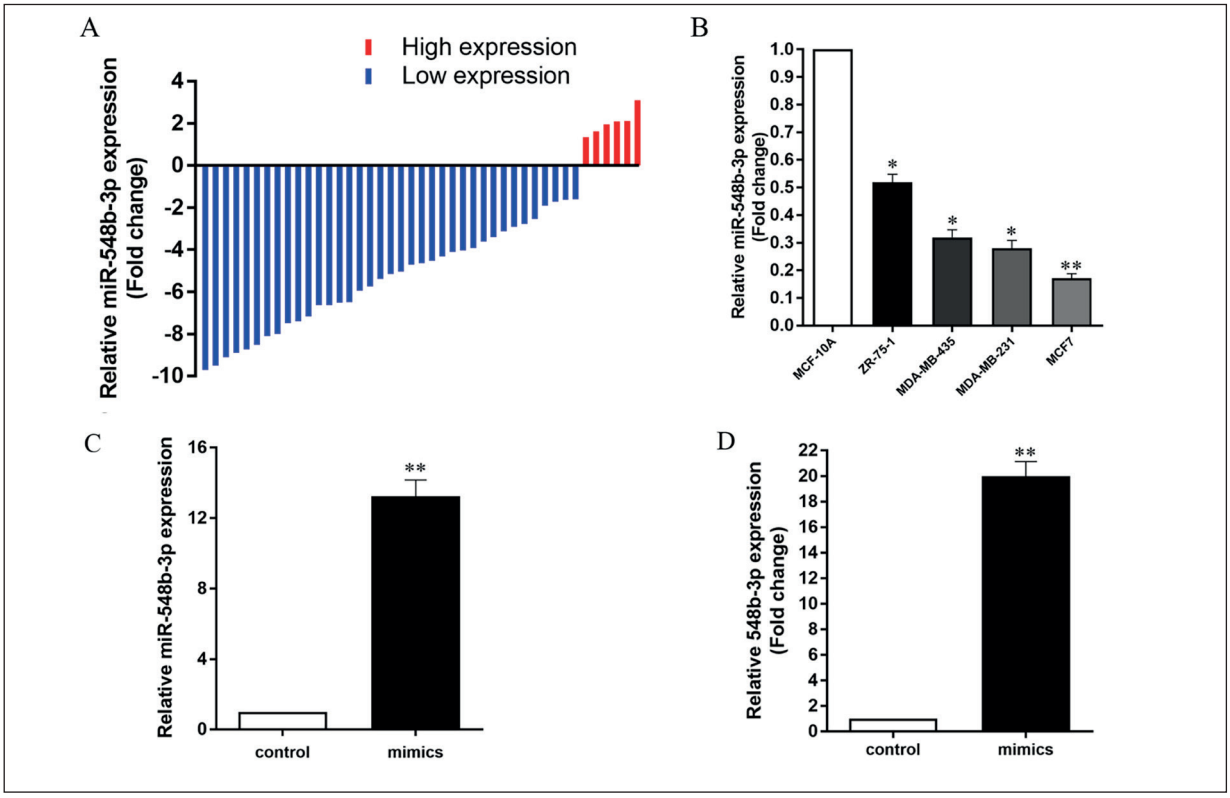

Figure 1. MiR-548b-3p expression is down-regulated in BC tissues and cells. **A,** Relative expression of miR-548b-3p in 43 cases of BC tissues detected *via* qRT-PCR. MiR-548b-3p expression is downregulated in 37 cases of BC tissues. **B,** Relative expression of miR-548b-3p in BC cells measured by qRT-PCR assay. **C,** and **D,** Overexpression efficiency determined through qRT-PCR after overexpression of miR-548b-3p.

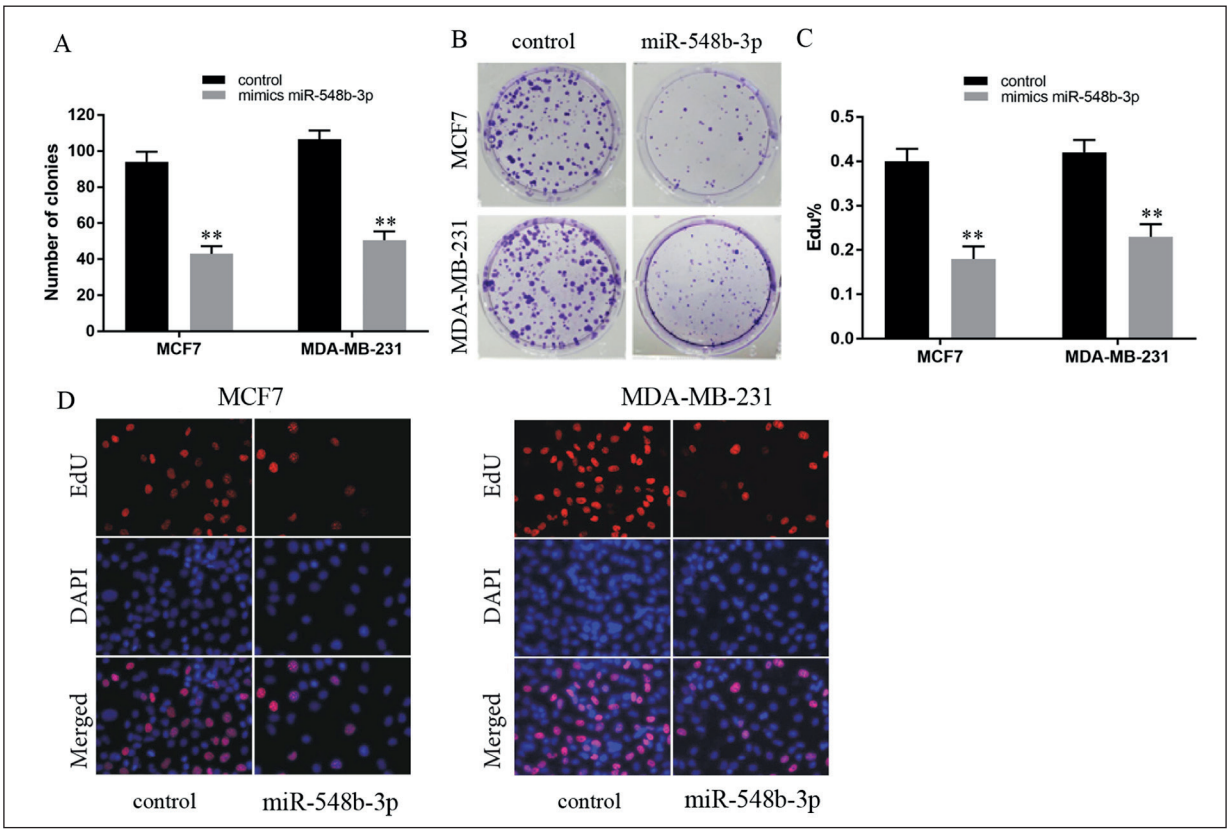

Figure 2. MiR-548b-3p inhibits proliferation of BC cells. **A,** and **B,** Changes in proliferative capacity of BC cells detected *via* colony formation assay after overexpressing miR-548b-3p (×20). **C,** and **D,** Changes in proliferative capacity of BC cells detected by EdU staining after overexpressing miR-548b-3p (×40).

MiR-548b-3p Targeted Regulation of MDM2 Expression

Target genes that could probably bind to miR-548b-3p were predicted *via* bioinformatics (Figure 4A). According to qRT-PCR results, the mRNA expression level of MDM2 was significantly downregulated after overexpression of miR-548b-3p (Figure 4B). Western blotting results indicated that the protein level of MDM2 decreased as well (Figure 4C). Dual-Luciferase reporter gene assay confirmed that miR-548b-3p was able to directly bind to MDM2 (Figure 4D). These findings suggested that miR-548b-3p was downregulated in BC and remarkably repressed the proliferation and metastasis of BC cells through targeted regulation of MDM2.

Discussion

BC, with an incidence rate of about 29% and death rate of 14%, ranks the first among malignant tumors in women. It seriously threatens the safety and quality of life in females worldwide[11]. According to relevant investigations, the malignant proliferation and metastasis of BC are the leading causes of death. Meanwhile, exploring the molecular biology, genetics, etiology, and cell origin of BC exhibits crucial effects in modifying treatment protocol and identifying prognosis[12,13].

MiRNAs are endogenous, non-coding, single-stranded RNAs capable of regulating gene expressions at the post-transcriptional level. Their aberrant expression involves such tumor processes as growth, invasion, and apoptosis[14,15]. Sun et al[16] and Mai et al[17] have found that miRNAs are abnormally expressed in various tumors, including lung cancer, colorectal cancer, and gastric cancer, serving as anti-oncogenes or oncogenes. Min et al[18] have indicated that miR-214 inhibits epithelial-mesenchymal transition by downregulating RNF8 expression in BC. Zhao et al[19] have reported that miR-665 accelerates BC metastasis through targeted regulation of NR4A3 expression.

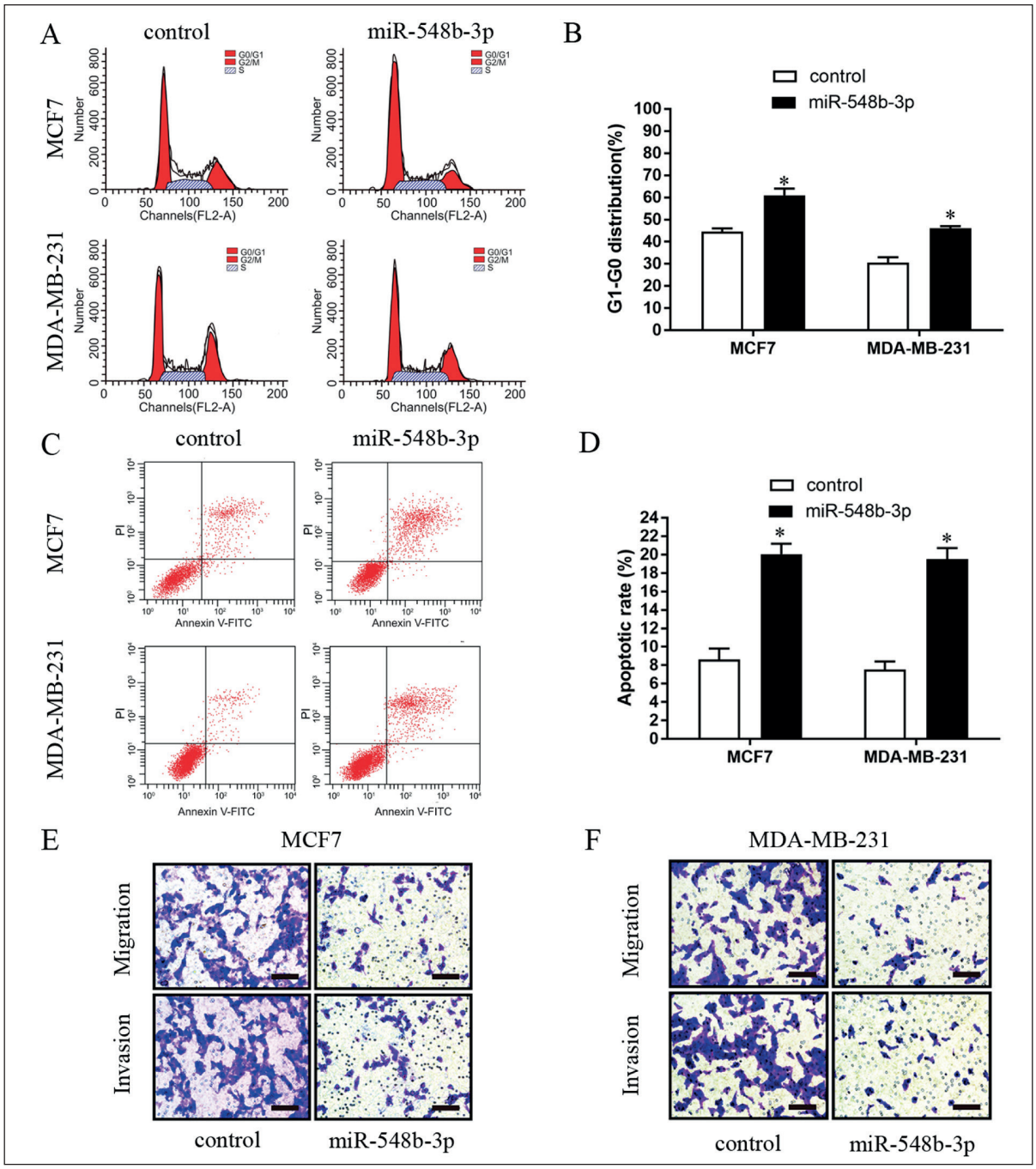

Figure 3. MiR-548b-3p promotes apoptosis and cycle arrest and inhibits metastasis of BC cells. **A,** and **B,** Apoptosis rate of BC cells determined *via* flow cytometry after overexpressing miR-548b-3p. **C,** and **D,** Cycle distribution of BC cells determined by flow cytometry after miR-548b-3p overexpression. **E,** and **F,** Changes in migratory and invasive abilities of BC cells detected through transwell assay after overexpression of miR-548b-3p (×40).

However, there has been no report about the role of miR-548b-3p in BC yet. Therefore, our study investigated the role of miR-548b-3p in BC and found that miR-548b-3p was lowly expressed in BC cells and overexpression miR-548b-3p could inhibited proliferation and metastasis of BC cells *via* promoting apoptosis and cycle arrest.

The oncogene MDM2 was initially detected in spontaneously transformed 3T3DM cells containing DMs. It is located in chromosome 12q13-14,

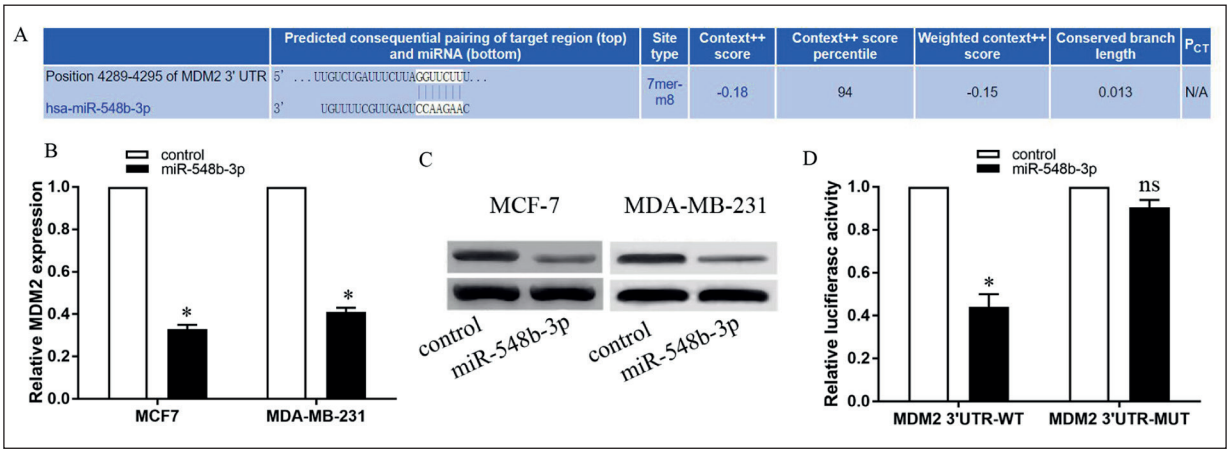

Figure 4. MiR-548b-3p regulates MDM2 expression in a targeted manner. **A,** MiR-548b-3p binding to MDM2 according to prediction *via* bioinformatics. **B,** Decreased MDM2 expression after overexpression of miR-548b-3p determined through qRT-PCR. **C,** Decreased MDM2 expression after overexpressing miR-548b-3p detected *via* Western blotting. **D,** Dual-Luciferase reporter gene assay verifies that miR-548b-3p can directly bind to MDM2.

with a full length of approximately 2.372 kb and a relative molecular weight of 90 kD. MDM2 protein products can form complexes with p53 protein, so as to reduce the expression and even inactivate p53, thereby inducing malignant tumors[20]. Based on a literature report[21], miR-339-5p promotes the occurrence and development of colorectal cancer by modulating the MDM2 pathway. In this research, functional assays confirmed that miR-548b-3p repressed the proliferation and metastasis of BC cells by directly binding to MDM2.

Conclusions

MiR-548b-3p expression was significantly downregulated in BC. Moreover, lowly expressed miR-548b-3p repressed the proliferation and metastasis of BC cells through targeted regulation of MDM2.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Gupta V, Haque I, Chakraborty J, Graff S, Banerjee S, Banerjee SK. Racial disparity in breast cancer: can it be mattered for prognosis and therapy. J Cell Commun Signal 2018; 12: 119-132.

2) Sunar V, T DH, Sarici F, Ates O, Akin S, Baspinar B, Aksoy S, Altundag K. Association between androgen receptor status and prognosis in triple negative breast cancer. J BUON 2018; 23: 1325-1330.

3) Hu W, Tan C, He Y, Zhang G, Xu Y, Tang J. Functional miRNAs in breast cancer drug resistance. Onco Targets Ther 2018; 11: 1529-1541.

4) Weng J, Zhang H, Wang C, Liang J, Chen G, Li W, Tang H, Hou J. MiR-373-3p targets DKK1 to promote EMT-induced metastasis via the Wnt/β-catenin pathway in tongue squamous cell carcinoma. Biomed Res Int 2017; 2017: 6010926.

5) Hu W, Liu Q, Pan J, Sui Z. MiR-373-3p enhances the chemosensitivity of gemcitabine through cell cycle pathway by targeting CCND2 in pancreatic carcinoma cells. Biomed Pharmacother 2018; 105: 887-898.

6) Zhao J, Jiang GQ. MiR-4282 inhibits proliferation, invasion and metastasis of human breast cancer by targeting Myc. Eur Rev Med Pharmacol Sci 2018; 22: 8763-8771.

7) Zeng Y, Gao T, Huang W, Yang Y, Qiu R, Hou Y, Yu W, Leng S, Feng D, Liu W, Teng X, Yu H, Wang Y. MicroRNA-455-3p mediates GATA3 tumor suppression in mammary epithelial cells by inhibiting TGF-β signaling. J Biol Chem 2019: 294: 15808-15825. doi: 10.1074/jbc.RA119.010800.

8) Liang T, Guo L, Liu C. Genome-wide analysis of mir-548 gene family reveals evolutionary and functional implications. J Biomed Biotechnol 2012; 2012: 679563.

9) Lin L, Wang Y. MiR-548b-3p regulates proliferation, apoptosis, and mitochondrial function by targeting CIP2A in hepatocellular carcinoma. Biomed Res Int 2018; 2018: 7385426.

10) Wang Z, Wu X, Hou X, Zhao W, Yang C, Wan W, Chen L. MiR-548b-3p functions as a tumor suppressor in lung cancer. Lasers Med Sci 2019. doi: 10.1007/s10103-019-02865-7. [Epub ahead of print].

11) Cai Z, Liu Q. Understanding the Global Cancer Statistics 2018: implications for cancer control. Sci China Life Sci 2019. doi: 10.1007/s11427-019-9816-1. [Epub ahead of print].

12) Giuliano S, Dufies M, Ndiaye PD, Viotti J, Borchiellini D, Parola J, Vial V, Cormerais Y, Ohanna M, Imbert V, Chamorey E, Rioux-Leclercq N, Savina A, Ferrero JM, Mograbi B, Pages G. Resistance to lysosomotropic drugs used to treat kidney and breast cancers involves autophagy and inflammation and converges in inducing CXCL5. Theranostics 2019; 9: 1181-1199.

13) Lian M, Zhang C, Zhang D, Chen P, Yang H, Yang Y, Chen S, Hong G. The association of five preoperative serum tumor markers and pathological features in patients with breast cancer. J Clin Lab Anal 2019; 33: e22875.

14) Mullany LE, Slattery ML. The functional role of miRNAs in colorectal cancer: insights from a large population-based study. Cancer Biol Med 2019; 16: 211-219.

15) Cui A, Zhang J, Bai W, Sun H, Bao L, Ma F, Li Y. Signal-on electrogenerated chemiluminescence biosensor for ultrasensitive detection of microRNA-21 based on isothermal strand-displacement polymerase reaction and bridge DNA-gold nanoparticles. Biosens Bioelectron 2019; 144: 111664.

16) Sun H, Zhou X, Bao Y, Xiong G, Cui Y, Zhou H. Involvement of miR-4262 in paclitaxel resistance through the regulation of PTEN in non-small cell lung cancer. Open Biol 2019; 9: 180227.

17) Mei JW, Yang ZY, Xiang HG, Bao R, Ye YY, Ren T, Wang XF, Shu YJ. MicroRNA-1275 inhibits cell migration and invasion in gastric cancer by regulating vimentin and E-cadherin via JAZF1. BMC Cancer 2019; 19: 740.

18) Min L, Liu C, Kuang J, Wu X, Zhu L. MiR-214 inhibits epithelial-mesenchymal transition of breast cancer cells via downregulation of RNF8. Acta Biochim Biophys Sin (Shanghai) 2019; 51: 791-798.

19) Zhao XG, Hu JY, Tang J, Yi W, Zhang MY, Deng R, Mai SJ, Weng NQ, Wang RQ, Liu J, Zhang HZ, He JH, Wang HY. MiR-665 expression predicts poor survival and promotes tumor metastasis by targeting NR4A3 in breast cancer. Cell Death Dis 2019; 10: 479.

20) Sirous H, Chemi G, Campiani G, Brogi S. An integrated in silico screening strategy for identifying promising disruptors of p53-MDM2 interaction. Comput Biol Chem 2019; 83: 107105.

21) Jansson MD, Damas ND, Lees M, Jacobsen A, Lund AH. MiR-339-5p regulates the p53 tumor-suppressor pathway by targeting MDM2. Oncogene 2015; 34: 1908-1918.