

MiR-508-3p inhibits cell invasion and epithelial-mesenchymal transition by targeting ZEB1 in triple-negative breast cancer

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Abstract. – **OBJECTIVE:** Recently, studies have identified that microRNAs (miRNAs) are novel regulators for gene expression in tumor progression including breast cancer. The aim of the study is to investigate the clinical significance and underlying functions between miR-508-3p expression and triple-negative breast cancer (TNBC) development.

PATIENTS AND METHODS: Quantitative Real-time PCR (QRT-PCR) was performed to determine the expression of miR-508-3p in 54 pairs of TNBC specimens and adjacent non-tumor tissues. The association between miR-508-3p expression and clinicopathological factors was assessed using χ^2 -test. Transwell invasion assays were used to assess cell invasion ability. Luciferase reporter assay, Western blot analyses and qRT-PCR were performed to demonstrate ZEB1 was a direct target of miR-508-3p.

RESULTS: We demonstrated that miR-508-3p expression was remarkably decreased in TNBC tissues and cells. Lower miR-508-3p expression significantly associated with lymph node metastasis and distant metastasis in TNBC patients ($p < 0.05$). Ectopic expression of miR-508-3p significantly suppressed cell invasion ability of TNBC. MiR-508-3p overexpression suppressed cell epithelial-mesenchymal transition (EMT) phenomenon of TNBC by upregulating E-cadherin expression, but downregulating Vimentin expression. In addition, we revealed that ZEB1 was a direct target of miR-508-3p in TNBC cells. MiR-508-3p significantly suppressed cell EMT process by regulating ZEB1 expression.

CONCLUSIONS: We found that miR-508-3p may be a potential therapeutic target of TNBC.

Key Words:

Triple-negative breast cancer, MiR-508-3p, ZEB1, Cell invasion, Epithelial-mesenchymal transition.

ized as the mesenchymal phenotype that exhibits the worst prognosis among all breast cancer subtypes². Patients with triple-negative breast cancer are defined by a lack of expression of oestrogen, progesterone, and ERBB2 receptors³. TNBC patients did not benefit from therapy including anti-HER2 molecularly targeted therapy, endocrine therapy, and chemotherapy^{4,5}. Thus, to investigate underlying molecular regulatory mechanisms in TNBC could provide potential therapeutic value for TNBC.

MicroRNAs (MiRNAs) are small and endogenous single-stranded RNA, binding to the 3'-untranslated region (3'UTR) of their target mRNAs⁶. MiRNAs was reported to be crucial for cancer initiation and progression in triple-negative breast cancer. Some found miRNAs including microRNA-454⁷, miR-629-3p⁸, MiR-340⁹, miR-217¹⁰, and microRNA-223 affect cell proliferation, migration and invasion by functioning as oncogenes or tumor suppressors in TNBC¹¹. MiR-508-3p was observed to induce cell apoptosis and inhibit cell migration *in vitro* in renal cell carcinoma¹². However, the underlying role of miR-508-3p expression in TNBC remains unknown. In the study, we demonstrated that miR-508-3p expression was downregulated in TNBC tissues and cells. Upregulated miR-508-3p inhibited cell invasion and cell EMT process by regulating ZEB1 expression. Thus, these results indicated that miR-508-3p may be a potential target of breast cancer treatment.

Patients and Methods

Patients and Tissue Specimens

From March 2013 to July 2014, 54 pairs of human TNBC specimens (breast tumor and adjacent non-tumor tissues) were obtained from

Introduction

Breast cancer is one of the most common frequent tumors involved in female worldwide¹. Triple-negative breast cancer (TNBC) is character-

TNBC patients who underwent surgery at Institute of Cancer Research, Ganzhou Institute of Cancer Research. Tissue samples were collected after surgery, and immediately frozen in liquid nitrogen and stored at -80°C until for RNA analyses. The study was approved by the Ethics and Scientific Committees of Ganzhou Institute of Cancer Research. The written informed consents were obtained from all patients. The clinicopathological data was shown in Table I.

Cell Lines Culture and Cell Transfection

Two human TNBC cell lines including BT-549 and MDA-MB-231 cells and a non-tumorigenic MCF10A cell line were purchased from the Shanghai Institute of Biochemistry and Cell Biology of the Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were incubated at 37°C in a humidified atmosphere supplemented with 5% CO_2 . The miR-508-3p mimic or negative control (miR-NC) were designed and purchased from RiboBio Co., Ltd. (Guangzhou, Guangdong, China). Cells were transfected with miR-508-3p mimic or miR-NC at the

concentration of 100 nM using Lipofectamine[™] 3000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were harvested 48 h after cells transfection.

Transwell Invasion Assays

Cell invasion ability was assessed using 8 μm pore size diameter Transwell plates (Corning Life Sciences, Corning, NY, USA) and were pre-coated with Matrigel[®] basement membrane matrix (BD Biosciences, Franklin Lakes, NJ, USA). A total of 1×10^5 /well cells were transfected with miR-508-3p mimic or miR-NC. After cell transfection at 24 h, cells were seeded on the upper chamber. A total of 500 μl medium containing 10% FBS were added to the lower chamber. After incubation for 48 h, cells in the up chamber were removed and cells in the lower chamber were fixed using 100% methanol, and stained with crystal violet for 20 min at room temperature. Cells were counted in five randomly selected fields under a light microscope.

Western Blot Analyses

Transfected BT-549 and MDA-MB-231 cells were lysed in radioimmunoprecipitation assay (RAPA) lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Protein samples (50 μg) were separated using 10% SDS-PAGE and then transferred to polyvinylidene difluo-

Table I. The association between clinicopathological factors and miR-508-3p expression in TNBC patients.

Factors	No. of patients	miR-508-3p expression		<i>p</i> -value
		Lower (n = 26)	Higher (n = 28)	
Age (years)				0.967
< 45	31	15	16	
> 45	23	11	12	
Tumor size				0.106
≤ 2 cm	29	11	18	
> 2 cm	25	15	10	
TNM stage				0.441
I/II	36	16	20	
III	18	10	8	
Lymph node metastasis				0.028*
Negative	35	13	22	
Positive	19	13	6	
Distant metastasis				0.012*
Negative	40	15	25	
Positive	14	11	3	
Histological grade				0.439
G1/2	38	17	21	
G3	16	9	7	

**p* < 0.05.

ride (PVDF) membranes. The membranes were blocked with 5% non-fat milk for 1 h at room temperature. The membranes were incubated at 4°C overnight with the following primary antibodies: E-cadherin (dilution, 1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA), Vimentin (dilution, 1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA), ZEB1 (dilution, 1:1000; Abcam, Cambridge, MA, USA) and GAPDH (dilution, 1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA). After that, the membrane was treated with a horseradish peroxidase(HRP)-conjugated secondary antibody (dilution, 1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA) for 1 h at room temperature. The Western bands were detected using an enhanced chemiluminescence reagent (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China).

Luciferase Reporter Assays

In total, MDA-MB-231 cells were seeded in 96-well plates and grown to 80% confluency before cells transfection. Luciferase reporter vectors containing wild-type the ZEB1 3'-UTR or mutant ZEB1 3'-UTR were co-transfected with miR-508-3p mimic (50 nM) into the MDA-MB-231 cells using Lipofectamine® 3000 reagent according to the manufacturer's protocol. Following at 48 h after cell transfection, luciferase activity was detected using the Dual-Luciferase® Reporter assay kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. The values were normalized against Renilla luciferase gene activity.

Statistical Analysis

Statistical significance was determined using SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). Differences between two groups were analyzed by unpaired Student's *t*-test and more than two groups were assessed using one-way analysis of variance (ANOVA). The Student-Newman-Keuls method was used for post-hoc tests following performance of ANOVA. The experimental results were shown as mean $\pm$ SD. A $p < 0.05$ was considered to indicate a statistically significant difference. All experiments were performed at least three times.

Results

The Expression of MiR-508-3p is Significantly Downregulated in TNBC Tissues and Cells

The expression of miR-508-3p was detected in 54 cases of TNBC tissues and their adjacent normal tissues using qRT-PCR. As shown in Figure 1A, the miR-508-3p expression was significantly downregulated in tumor tissues compared to their adjacent normal tissues ($p < 0.05$). Furthermore, we also detected the expression of miR-508-3p in TNBC cell lines (BT-549 and MDA-MB-231 cells) were significantly downregulated compared to that in the normal breast epithelial cell line (Figure 1B, $p < 0.05$). According to the miR-508-3p expression measured by qRT-PCR analyses, the patients

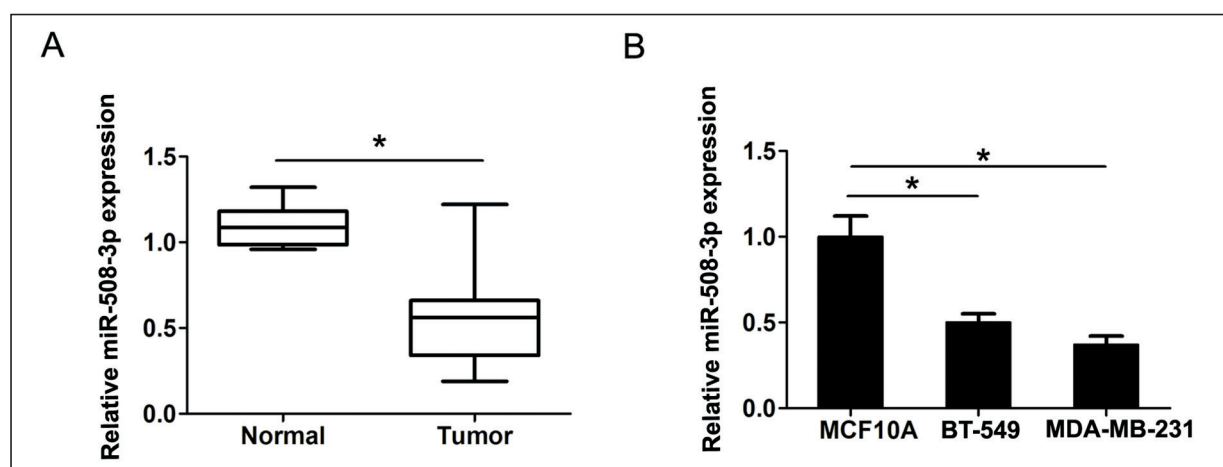


Figure 1. MiR-508-3p expression was downregulated in TNBC tissues and cells. **A**, QRT-PCR analysis of miR-508-3p expression in 54 cases of TNBC tissues and their adjacent normal tissues. GAPDH was used as the internal control. **B**, QRT-PCR analysis of miR-508-3p expression in two human TNBC cell lines including BT-549 and MDA-MB-231 cells and non-tumorigenic MCF10A cells. GAPDH was used as the internal control, * $p < 0.05$.

were divided into lower and higher miR-508-3p expression groups using the median expression as the cut-off point (0.55 fold). The χ^2 -test was used to assess the association between miR-508-3p and clinical parameters. The results showed that miR-508-3p expression was positively associated with lymph node metastasis (Table I, $p < 0.05$) and distant metastasis (Table I, $p < 0.05$), but no significant association was observed with other parameters, including age, tumor size, TNM stage and so on (Table I, all of $p > 0.05$).

Upregulation of MiR-508-3p Suppresses the Cell Invasion and EMT Process of TNBC in Vitro

To evaluate the functional effects of miR-508-3p on TNBC cells, we transfected miR-508-3p mimic or miR-NC into BT-549 or MDA-MB-231 cell lines to increased miR-508-3p expression (Figure 2A-2B). Transwell invasion assay was used to evaluate the cell invasive ability of TNBC cells. The results indicated that transfection of miR-508-3p mimic in MDA-MB-231 and BT-549

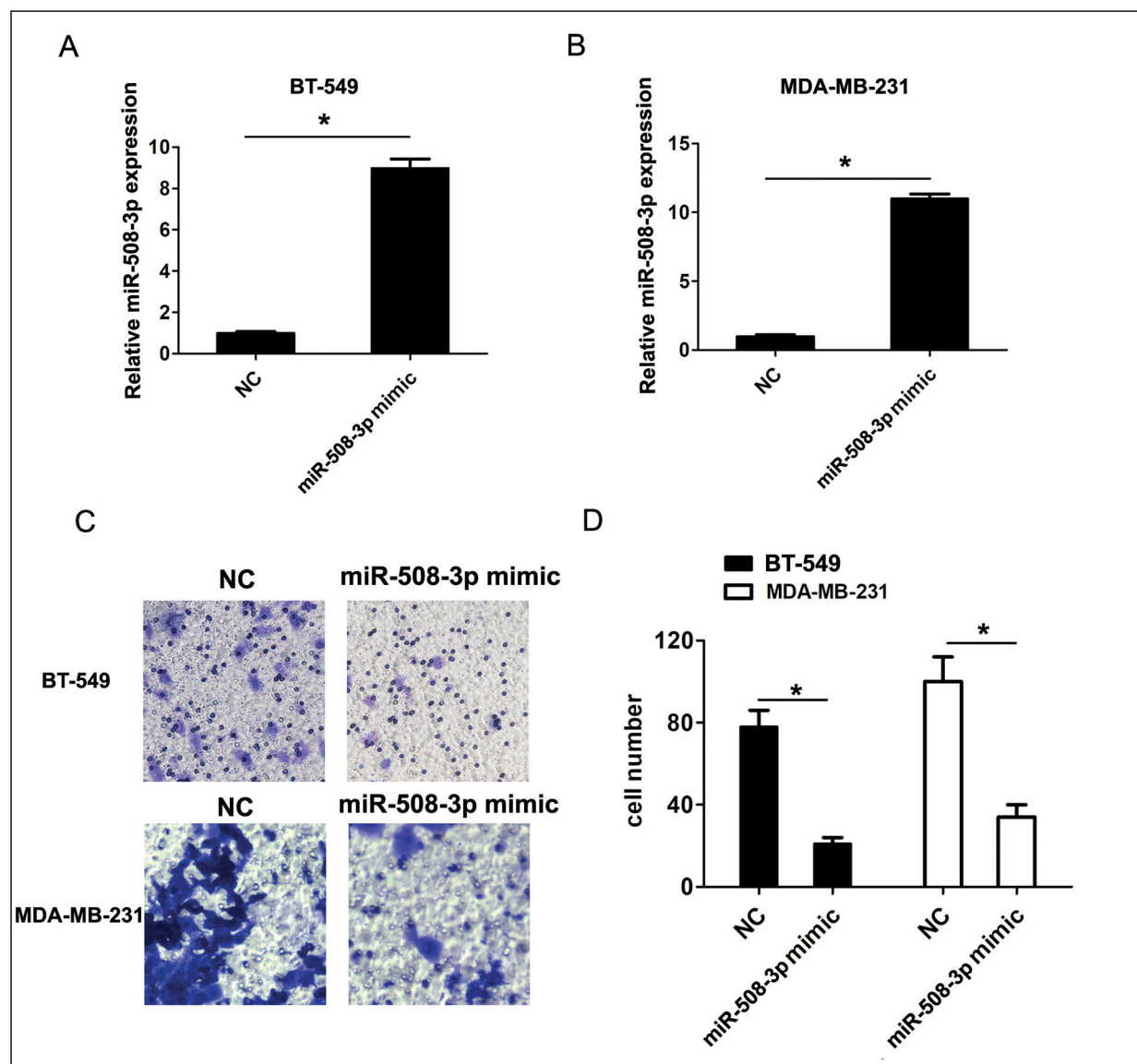


Figure 2. Upregulation of miR-508-3p expression inhibited cell invasion ability in TNBC cells. **A-B**, QRT-PCR analysis of miR-508-3p expression after BT-549 and MDA-MB-231 cells were transfected with miR-508-3p mimic or miR-NC at 48 h after cell transfection. **C-D**, Transwell cell invasion assays were used to assess cell invasion ability after BT-549 and MDA-MB-231 cells were transfected with miR-508-3p mimic or miR-NC at 48 h after cell transfection, $*p < 0.05$.

cells significantly inhibited cell invasion ability compared to miR-NC groups ($p < 0.05$, Figure 2C-2D). Moreover, we found that the cell EMT makers E-cadherin expression was increased, while Vimentin expression was decreased when MDA-MB-231 and BT-549 cells were transfected with miR-508-3p mimic compared to miR-NC groups (Figure 3A-3B). Thus, these results indicated that upregulation of miR-508-3p could suppress the cell invasion and EMT process of TNBC *in vitro*.

ZEB1 is Identified as a Direct Target of MiR-508-3p

To explore the potential molecular mechanism underlying the miR-508-3p mediated regulation of cell invasion and EMT process, we performed the bioinformatics analysis based on computer-aided algorithms (TargetScan and miRanda). We found that the candidate gene was ZEB1, which was confirmed to affects cell invasion and EMT process (Figure 4A). Furthermore, we found that ZEB1 mRNA and protein expression were significantly reduced compared to miR-NC groups after MDA-MB-231 and BT-549 cells were transfected with miR-508-3p mimic ($p < 0.05$, Figure 4B-4E). To identify whether miR-508-3p could directly regulate ZEB1, a dual-luciferase reporter assay was performed. Luciferase reporter vectors containing the wild-type 3'-UTR of ZEB1 (WT) or mutant 3'-UTR of ZEB1 (MUT) were constructed (Figure 4A). MDA-MB-231 cell

was transfected with miR-508-3p mimic showed a significant reduction in relative luciferase activity of wild-type 3'-UTR of ZEB1 (WT) reporter vector (Figure 4F, $p < 0.05$), but no significance was observed in the luciferase activity of mutant 3'-UTR of ZEB1 (MUT) reporter vector. Thus, these results indicated that ZEB1 was a direct target of miR-508-3p in TNBC cells. MiR-508-3p affected cell EMT process by regulating ZEB1 expression.

Discussion

Recent investigations¹³ have showed that the dysregulation of miRNA contributes to carcinogenesis by regulating target genes expression at the transcriptional and/or post-transcriptional. In TNBC, miRNAs could serve as effective biomarkers for the prognosis and treatment of patients. MiR-143-3p targeting LIM domain kinase 1 suppresses the progression of triple-negative breast cancer cells¹⁴. MiR-217 inhibits triple-negative breast cancer cell growth, migration, and invasion through targeting KLF5¹⁵. MiR-18a upregulation decreases Dicer expression and confers paclitaxel resistance in triple negative breast cancer¹⁶. MiR-199a-5p confers tumor-suppressive role in triple-negative breast cancer¹⁷. These studies indicated that miRNAs were involved in TNBC progression. In the present study, we showed

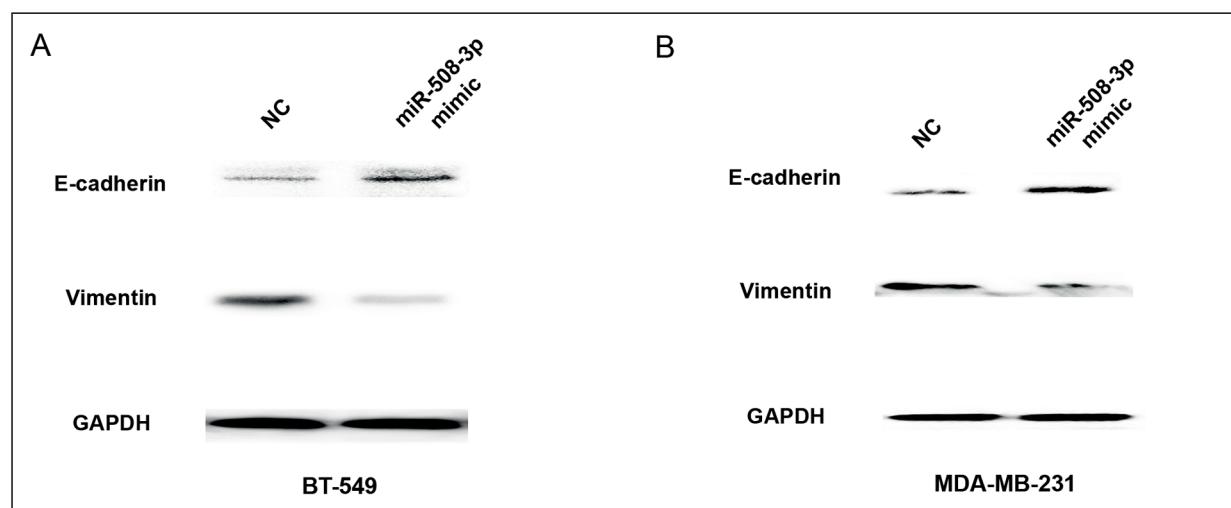


Figure 3. Upregulation of miR-508-3p expression inhibited cell EMT process in TNBC cells. **A**, Western blot analysis was used to detect the protein expression of E-cadherin and Vimentin after BT-549 cells were transfected with miR-508-3p mimic or miR-NC at 48 h after cell transfection. **B**, Western blot analysis was used to detect the protein expression of E-cadherin and Vimentin after MDA-MB-231 cells were transfected with miR-508-3p mimic or miR-NC at 48 h after cell transfection, * $p < 0.05$.

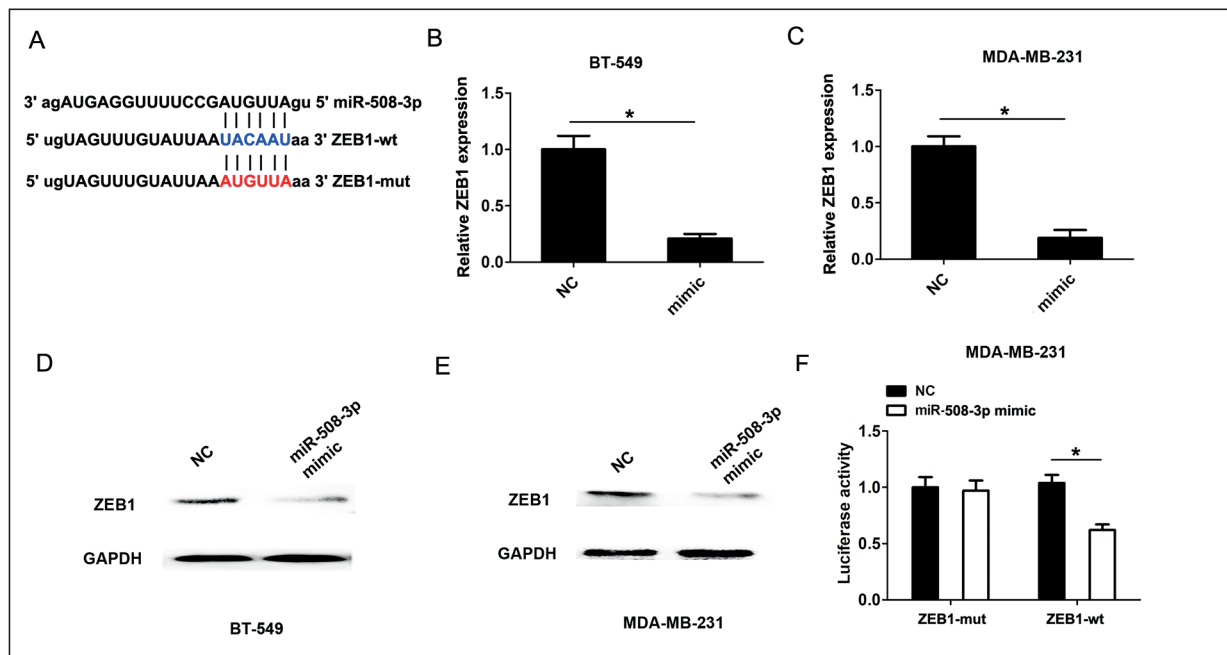


Figure 4. ZEB1 was regulated by miR-508-3p in TNBC cells. **A**, The seed sequence of miR-508-3p is complementary to the 3'UTR of ZEB1. **B-C**, QRT-PCR analysis of ZEB1 expression after BT-549 and MDA-MB-231 cells were transfected with miR-508-3p mimic or miR-NC at 48 h after cell transfection. **D-E**, Western blot analyses of ZEB1 expression after BT-549 and MDA-MB-231 cells were transfected with miR-508-3p mimic or miR-NC at 48 h after cell transfection. **F**, Luciferase activity was analyzed in MDA-MB-231 cells co-transfected with miR-508-3p mimic or negative control with 3'UTR-ZEB1-mut or 3'UTR-ZEB1-wt luciferase reporter vectors, * $p < 0.05$.

that miR-508-3p was lower expression in TNBC tissues and cells. Lower miR-508-3p expression was positively associated with lymph node metastasis and distant metastasis, but no significant association was observed with other parameters. These results indicated that miR-508-3p may associate with tumor progression. Thus, we further investigate the effects of miR-508-3p on cell invasion of TNBC. We found that upregulated miR-508-3p inhibited TNBC cells invasion ability. MiR-508-3p overexpression also dramatically suppressed cell epithelial-mesenchymal transition progression of TNBC by upregulating EMT maker E-cadherin expression and downregulating Vimentin expression. Thus, our findings indicated that miR-508-3p overexpression suppressed cell invasion and EMT process of TNBC.

ZEB1 is a zinc finger-containing transcription factor and aberrant activation of ZEB1 had been shown to regulate cancer cell invasion and EMT process by suppressing E-cadherin expression¹⁸. Furthermore, we found that ZEB1 mRNA and protein expression were significantly reduced after TNBC cells were transfected with miR-508-3p

mimic compared to miR-NC. These results indicated that ZEB1 was regulated by miR-508-3p in TNBC cells. To identify whether miR-508-3p could directly regulate ZEB1, we performed a dual-luciferase reporter assay. After that, we revealed that ZEB1 was a direct target of miR-508-3p in TNBC cells. Thus, we demonstrated that miR-508-3p suppressed cell invasion and epithelial-mesenchymal transition by targeting ZEB1 expression.

Conclusions

We showed that miR-508-3p was downregulated in TNBC and cells. Furthermore, we demonstrated that miR-508-3p inhibited cell invasion and EMT process by targeting ZEB1 expression. Thus, these data indicated that miR-508-3p may be a potential target of TNBC treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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