Original Article
MicroRNA-335 inhibits proliferation, invasion, and migration of breast cancer cells possibly via ERBB3

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Abstract: Aim: Previous studies have shown that miR-335 plays important roles in human breast cancer. Many targets of miR-335 have been found. However, the roles of miR-335 in breast cancer remain largely unknown. Methods: Expression of miR-335 was detected using qRT-PCR. Mimics were transfected into cells. Effects of miR-335 on cell proliferation were measured using MTT assays and colony formation. Transwell invasion and migration assays were also used to determine the effects of miR-335 on cell invasion and migration. Bioinformatics was employed to predict downstream targets of miR-335. Rescue assays were conducted by introduction of ERBB3. Results: The current study found that expression of miR-335 was downregulated in breast cancer tissues and cells. Ectopic expression of miR-335 inhibited proliferation, invasion, and migration of MCF7 and MDA-MB-435 cells. According to bioinformatics analysis, it was found that ERBB3 was a direct target of miR-335. Rescue assays confirmed that ERBB3 abolished miR-335-induced breast cancer cell inhibition. Conclusion: Present results indicate that miR-335 and its target ERBB3 are involved in carcinogenesis of breast cancer. They show potential as therapeutic targets for breast cancer.

Keywords: miR-335, ERBB3, cell proliferation, cell invasion, cell migration, breast cancer

Introduction

Human breast cancer is the most common cancer in women, worldwide. Breast cancer is influenced by many environmental factors and is characterized by molecular heterogeneity [1]. In recent decades, development of screening programs and new therapeutics have significantly reduced the mortality rate of breast cancer. However, molecular mechanisms underlying breast cancer are only partially understood [2].

MicroRNAs (miRNAs) are a class of small noncoding RNAs that play important roles in gene regulation. MicroRNAs repress expression of target genes through interacting with the 3'UTR. It has been shown that miRNAs play a crucial role in carcinogenesis and tumor progression, either as oncogenes or tumor suppressor genes [3]. They can regulate cell proliferation, apoptosis, invasion, and migration [4, 5]. They have also been used as potential diagnostic and therapeutic markers in various can-

cers, including breast cancer [6]. For instance, miR-335 has been shown to be involved in different cancers, including colorectal carcinoma [7], gastric cancer [8], pancreatic cancer [9], clear cell renal cell carcinoma [10], hepatocellular carcinoma [11], and small cell lung cancer bone metastases [12]. A cohort of genes related to different cancer pathways have been identified as target genes of miR-335, including PAX6 [13], Sox4 [14] and c-Met [15]. However, downstream targets of miR-335 in breast cancer remain largely unknown.

ERBB3, as a unique member of the ErbB family [16], plays an important role in the development of breast cancer [17]. Expression levels are often upregulated in breast cancer and ERBB3 overexpression is related with metastasis and recurrence of breast cancer [18, 19]. It has been reported that miRNAs (such as miR-143 and miR-145) can regulate ERBB3 expression in breast cancer [20]. Although both miR-335 and ERBB3 are involved in breast cancer,

whether miR-335 exerts its function through directly regulating ERBB3 remains unclear.

Therefore, the present study investigated the roles of miR-335 and ERBB3 in breast cancer cells. It was found that overexpression of miR-335 repressed growth, invasion, and migration of breast cancer cells. Results also showed that the 3'UTR of REBB3 contained a putative binding site for miR-335. Furthermore, miR-335 directly targeted the 3'UTR of ERBB3, according to luciferase reporter assays. Therefore, miR-335 could inhibit the development of breast cancer, at least in part, via ERBB3.

Materials and methods

Collection of patient samples

A total of 36 patients from the Department of Breast, Head, and Neck Surgery, Affiliated Tumor Hospital of Xinjiang Medical University, were enrolled in this study. None of the patients had received radiotherapy, chemotherapy, or any other treatment before and after the operation. Breast cancer tissues and adjacent normal tissues, at least 2 cm distal to tumor margins, were collected during surgery and snap frozen in liquid nitrogen. Written informed consent was obtained from all participants involved in this study. All experiments were approved by the Ethical Committee of the Affiliated Tumor Hospital of Xinjiang Medical University.

Cell culturing

Breast cancer cell lines (MCF-10A, MCF-7, and MDA-MB-435) were purchased from ATCC and cultured in RPMI 1640 medium, supplied with 10% fetal bovine serum and 1% antibiotics (Invitrogen, USA).

Cell transfection

MCF-7 and MDA-MB-435 cells were seeded in 24-well plates overnight, then transfected with miR-335 mimics (RiboBio, Guangzhou, China) or miR-controls (RiboBio, Guangzhou, China) at a final concentration of 200 nM. In the recuse experiment, cells were transfected with miR-control or miR-335 mimics and pcDNA3.0-ERBB3. Transfection was performed using Lipofectamine 2000 (Invitrogen, USA), according to manufacturer instructions. At 48 hours after transfection, the cells were collected for further analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA extraction of the cells or tissue samples was performed using the mirVana miRNA Isolation Kit (Ambion, USA), according to manufacturer instructions. RNA (2 μg) was reverse-transcribed to cDNA using M-MLVRT (Promega, USA). Moreover, qRT-PCR analysis was performed using the SYBR Green I real-time PCR kit (GenePharma, Shanghai, China) and ABI 7300 Real-Time PCR System. Expression of miR-335 was normalized to levels of U6 RNA. Relative expression levels were calculated as fold change values using the $2^{-\Delta\Delta Ct}$ method. Each experiment was carried out in triplicate.

MTT assays

At 24 hours after transfection, the cells were trypsinized, counted, and seeded in 96-well plates, at a density of 8,000 cells/well (MCF-7) or 10,000 cells/well (MDA-MB-435). They were then incubated at 37°C. At 24, 48, and 72 hours after cell seeding, respectively, 10 μl MTT (0.5 mg/mL; Sigma, USA) was added to each well (20 μl/well). The cells were maintained at 37°C for another 4 hours. After removing the culture medium, 100 μl DMSO (Sigma, USA) was added. After shaking for 15 minutes, absorbance values were measured using a microplate reader (Spectramax M2, Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 570 nm. MTT assays were performed in triplicate.

Colony formation assays

At 24 hours after transfection, MCF-7 and MDA-MB-435 cells were counted and seeded in 12-well plates at a density of 200 cells/well. The plates were incubated at 37°C and 5% CO_2 in a humidified incubator. The medium was replaced every 3 days. After 14 days of culturing, the cells were stained with crystal violet and counted. Colonies containing at least 50 cells were considered for quantification. Colony formation assays were performed in triplicate.

Transwell invasion and migration assays

Invasion assays were performed with the Transwell chamber with 8 μm pores (Corning, USA). Briefly, 50 μl of diluted Matrigel (2 mg/mL, BD Biosciences, Bedford, MA) were placed on the inner surface. MCF-7 and MDA-MB-435

cells were transfected for 24 hours, re-suspended to a final concentration of $2×10^5$/mL, and placed on the top chamber. RMPI 1640 medium, supplied with 20% fetal bovine serum, was then added to the bottom chamber. After 24 hours, non-invading cells were removed from the top of the Matrigel with a cotton-tipped swab. Invading cells at the bottom of the Matrigel were fixed using methanol and stained with crystal violet. Invasion efficiency was determined by counting the penetrated cells under a microscope at ×200 magnification of 5 random fields in each well. Each experiment was performed in triplicate. The protocol of Transwell migration assays is similar to that of invasion assays, except that no Matrigel was on the inner surface of the chamber.

Bioinformatics prediction

TargetScan (http://www.targetscan.org/vert_71/) and RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/) were used to analyze potential targets of miR-335.

Dual luciferase reporter assays

Human ERBB3 3'UTR (harboring miR-335 target sequence) and the ERBB3-3'UTR-mut (harboring mutant miR-335 target sequence) were synthesized by GenPharm (Shanghai, China). For luciferase assays, $1×10^5$ cells were transfected with ERBB3-3'UTR, ERBB3-3'UTR-mut, miR-335 mimics, or miR-controls in a 24-well plate. Lipofectamine 2000 (Invitrogen) was then added. After 24 hours, firefly and Renilla luciferase activities were measured, consecutively, using the Dual Luciferase Assay kit (Promega).

Western blotting assays

Cells were washed twice with cold PBS. Total cellular protein was extracted using a modified RIPA buffer with 0.5% sodium dodecyl sulfate, in the presence of proteinase inhibitor cocktail (Complete mini, Roche). Protein concentrations in the supernatants were determined using Bradford assays. Equal amounts of protein lysates were separated on SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% non-fat milk, followed by incubation with antibodies against ERBB3 (1:1000, Proteintech, China) or actin (1:1000, Proteintech, China). Actin was used as an internal control. Horseradish peroxidase (HRP) conjugated antibody was used as a secondary antibody. Signals were visualized with enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia; Buckinghamshire, UK), according to manufacturer protocol.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Differences were evaluated using Student's t-tests or one-way ANOVA. P<0.05 indicates statistical significance.

Results

miR-335 downregulated in breast cancer tissues and cells

Examining expression levels of miR-335 in human breast cancers, qRT-PCR was performed with 36 pairs of breast cancer tissues and adjacent normal tissues. As shown in **Figure 1A**, expression of miR-335 was significantly downregulated in cancer tissues, compared to that in adjacent normal tissues. In addition, expression of miR-335 was detected in breast cancer cells. Expression of miR-335 was also markedly downregulated in MCF-7 and MDA-MB-435 cells, compared with that in the non-malignant breast epithelial cell MCF-10A (**Figure 1B**). Thus, present results strongly suggest that miR-335 may function as a tumor repressor in breast cancer.
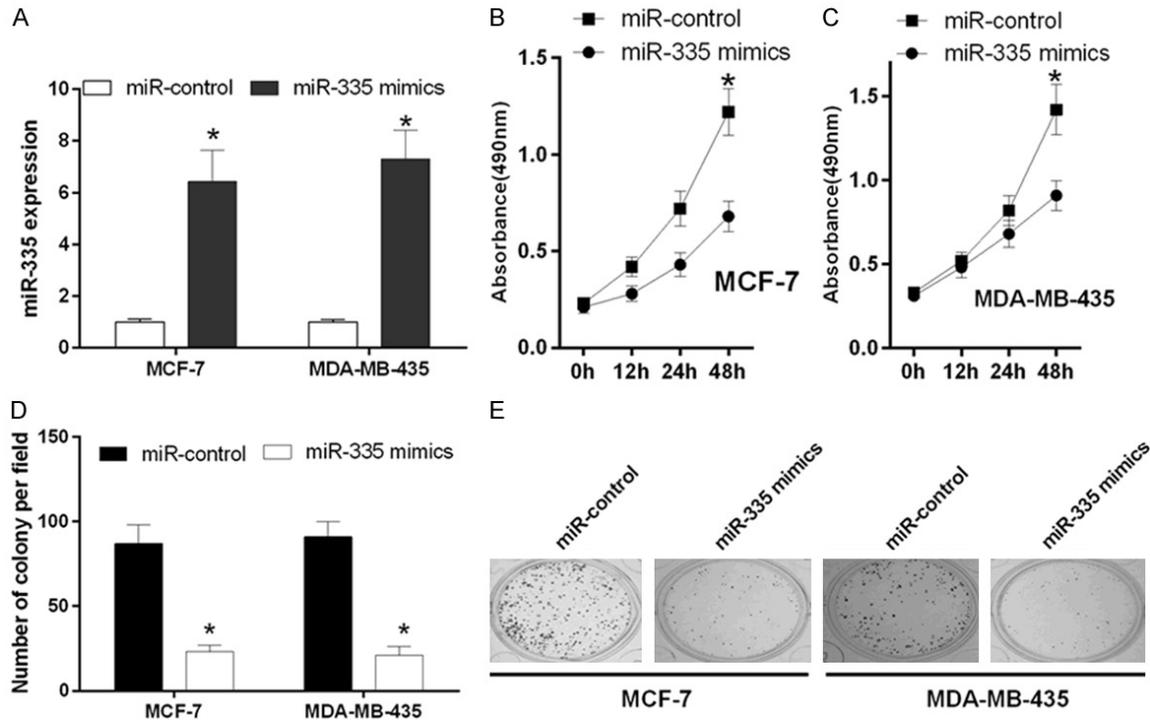
Ectopic expression of miR-335 suppresses proliferation of breast cancer cells

Investigating the function of miR-335 in breast cancer cells lines, MCF-7 and MDA-MB-435 were transfected with miR-335 mimics. It was found that miR-335 mimics significantly increased expression of miR-335 in MCF-7 and MDA-MB-435 cells, respectively (**Figure 2A**). MTT assays were then performed to examine the effects of miR-335 on cell growth *in vitro*. Data showed that cell growth was inhibited when transfected with miR-335 mimics (**Figure 2B**, **2C**). To further assess the effects of miR-335 on long-term proliferation capacity, colony formation assays were conducted. As shown in **Figure 2D**, **2E**, the number of colonies was markedly inhibited by miR-335 overexpression (P<0.05). Current results suggest that miR-335 could repress the proliferation of breast cancer cells.
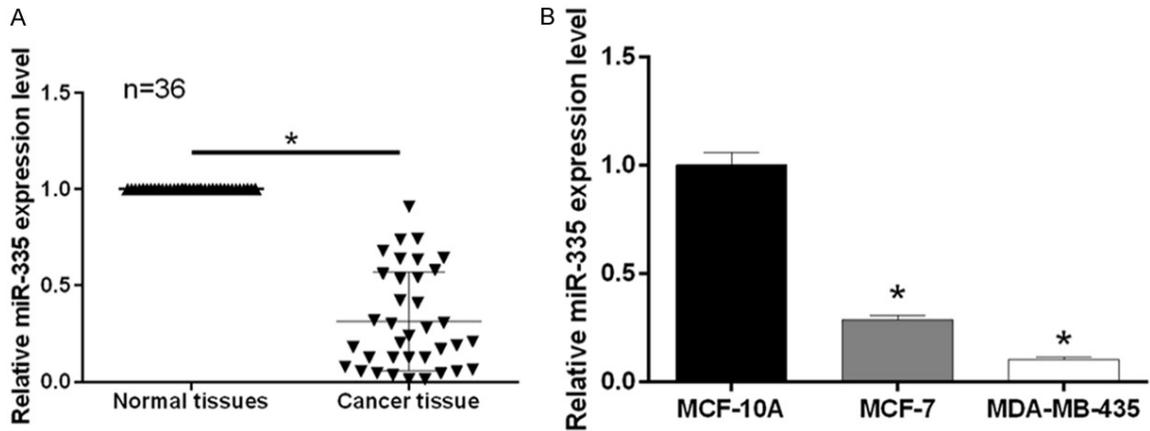
Figure 1. Expression of miR-335 in breast cancer tissues and cells. A. Expression of miR-335 in breast cancer tissues and adjacent normal tissues was examined using qRT-PCR assays (n=36). Student's t-test, *p<0.05; B. Relative expression of miR-335 in breast cancer cells (MCF-7 and MDA-MB-435) and non-malignant breast epithelial cell (MCF-10A) was measured using qRT-PCR assays. U6 was used as an internal control. One-way ANOVA, *p<0.05.


Figure 2. miR-335 inhibits the proliferation of breast cancer cells. A. Transfection efficiency of miR-335 mimics in MCF-7 and MDA-MB-435 cells was measured by qRT-PCR assays; B and C. Cell survival rates were determined by MTT assays; D and E. The long-term growth of cells was determined by colony formation assays. Student's t-test, *p<0.05.

Ectopic expression of miR-335 suppresses invasion and migration of breast cancer cells

To further investigate whether miR-335 affects cell metastasis, Transwell invasion assays and migration assays were performed. Results of

Transwell invasion assays showed that the number of invaded MCF-7 and MDA-MB-435 cells was significantly repressed by miR-335 mimics (**Figure 3A**, **3B**). Results of Transwell migration assays showed that the number of migrated MCF-7 and MDA-MB-435 cells was
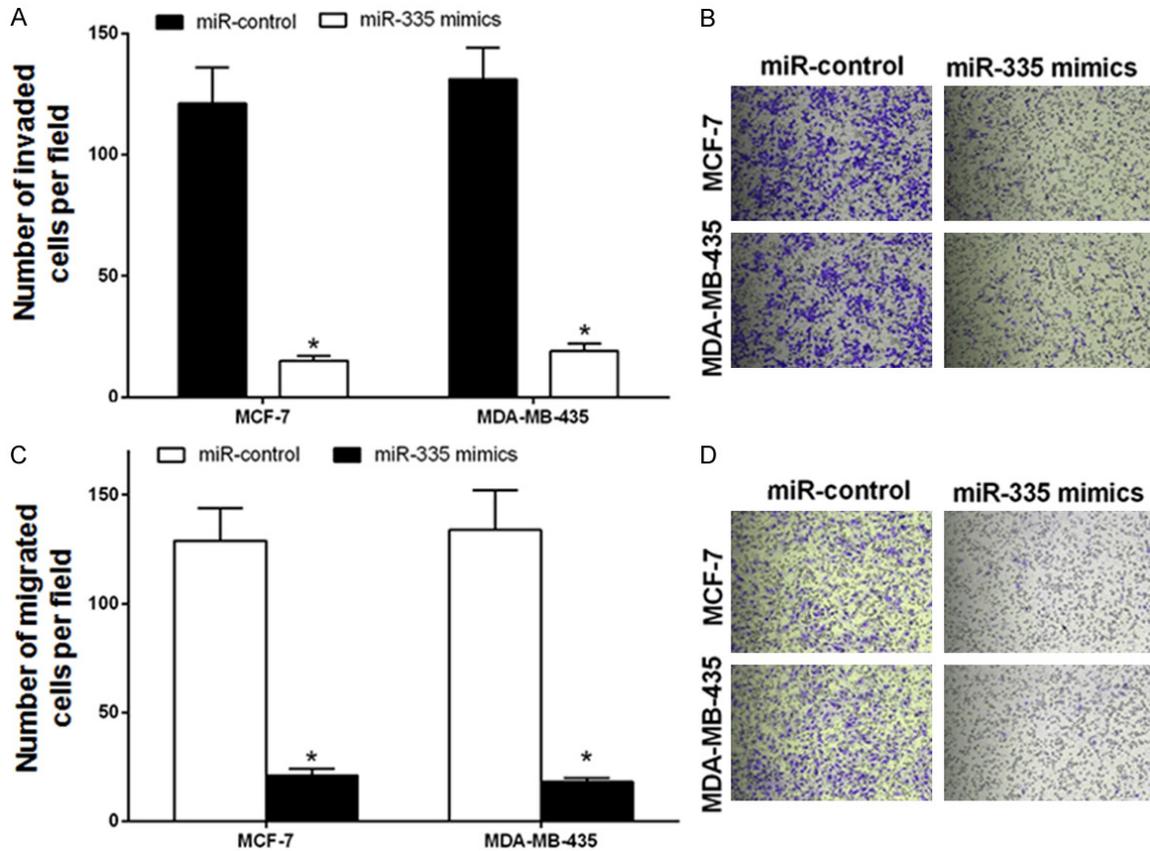
Figure 3. miR-335 represses the invasion and migration of breast cancer cells. A and B. Transwell invasion assays were performed in MCF-7 and MDA-MB-435 cells transfected with miR-335 or miR-control; C and D. Transwell migration assays were performed in MCF-7 and MDA-MB-435 cells transfected with miR-335 or miR-controls. Student's t-test, *p<0.05.

also significantly inhibited by miR-335 (**Figure 3C**, **3D**). Present results strongly indicate that miR-335 can repress invasion and migration abilities of breast cancer cells.
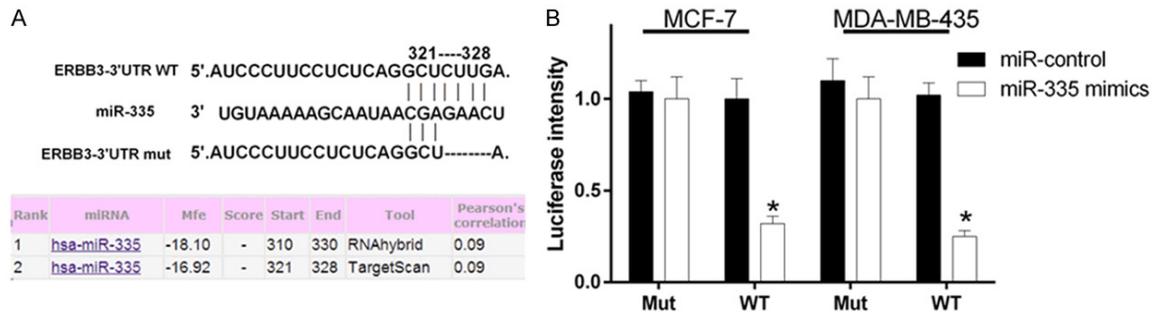
miR-335 directly targets the 3'UTR of ERBB3

Exploring potential targets of miR-335, two computational algorithms, TargetScan and RNAhybrid, were employed. Many different genes were predicted to be potential targets of miR-335, including Sox4, PAX6, and c-Met, which have been validated as miR-335 targets previously [13-15]. Of these targets, ERBB3 was identified as a novel target of miR-335. The wild type and mutant type binding sequences of miR-335 on ERBB3 are shown in **Figure 4A**. To understand whether the effects of miR-335 on ERBB3 are specific, dual luciferase reporter assays were conducted. Results showed that miR-335 significantly inhibited

luciferase activity when co-transfected with wild type ERBB3 in MCF-7 and MDA-MB-435 cells (P<0.05) (**Figure 4B**). However, this effect was abrogated when the binding site of 3'UTR was mutated. Current results imply that miR-335 can bind the 3'UTR of ERBB3 mRNA directly.

Ectopic expression of ERBB3 could abolish breast cancer cell inhibition by miR-335

To address whether the effects of miR-335 are due to repression of ERBB3, rescue experiments were performed. As shown in **Figure 5A**, expression of ERBB3 protein was rescued by ERBB3 overexpression vector without its 3'UTR. Colony formation assays also showed that repression of cell proliferation by miR-335 was rescued by transfection of ERBB3 (**Figure 5B**). Furthermore, transfection of ERBB3 in MCF-7 and MDA-MB-435 cells abolished re-

Figure 4. miR-335 can directly target the 3'UTR of ERBB3. A. A schematic diagram of pmiRGLO/3'UTR-luciferase plasmid, in which the ERBB3-3'UTR (wild-type or mutated version) were ligated upstream of the luciferase gene; B. MCF-7 and MDA-MB-435 cells were transfected with miR-335 or miR-controls. The luciferase intensity of pGL3/Luciferase-ERBB3-3'UTR-WT or ERBB3-3'UTR-mut reporter was then measured using the Dual Luciferase Assay kit. Student's t-test, *p<0.05.
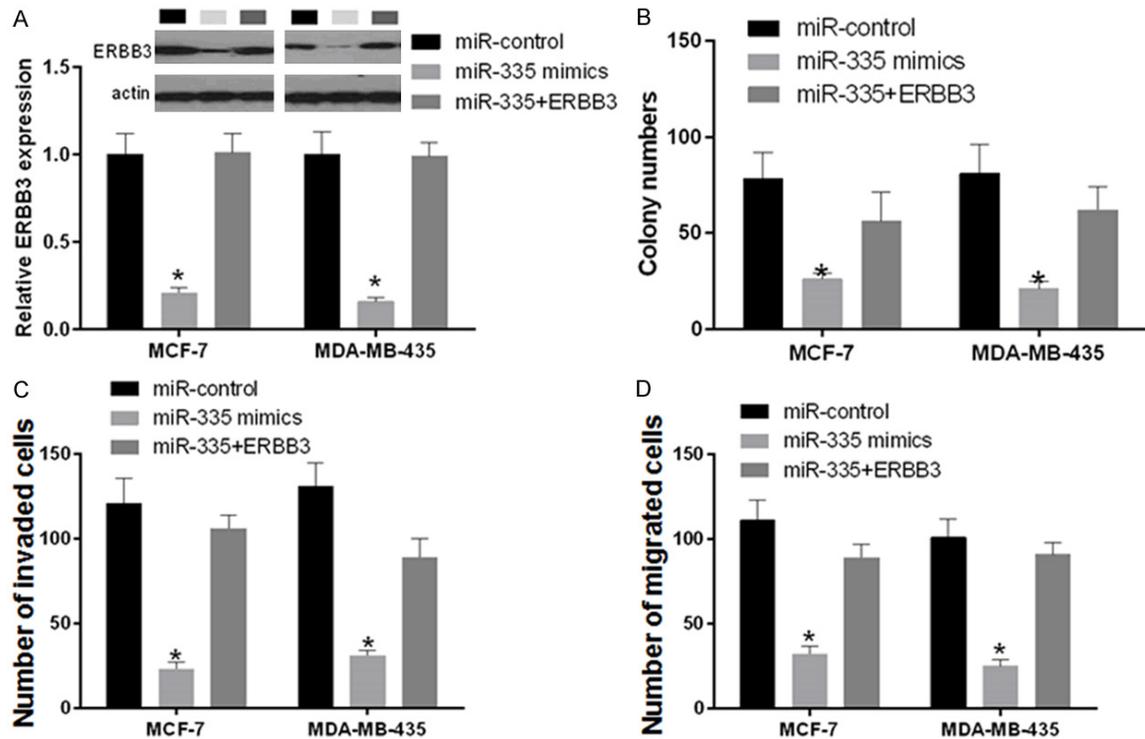
Figure 5. Inhibition of cell growth and invasion by miR-335 was reversed by overexpression of ERBB3 in breast cancer cells. (A) Relative expression levels of ERBB3 were detected in the breast cancer cells transfected with miR-335 or control mimics and co-transfected with miR-335 and ERBB3 using qRT-PCR and Western blot assays; (B) Cell viability of breast cancer cell transfected with miR-335, control mimics, or co-transfected with miR-335 and ERBB3 was measured by colony formation assays; (C) Migration and (D) invasion abilities of breast cancer cells transfected with miR-335, control mimics, or co-transfected with miR-335 and ERBB3 are measured using Transwell assays. The experiments were performed in triplicate. One-way ANOVA, *p<0.05.

pressed cell invasion and migration by miR-335 mimics (**Figure 5C**, **5D**). Results suggest that repressed proliferation, invasion, and migration of breast cancer cells by miR-335 are mediated by ERBB3.

Discussion

MicroRNAs have been found to be involved in various physiological and pathological processes, especially in cancer development. Unusual

expression of miRNAs is a common feature of many tumors, including breast cancer. At present, a series of miRNAs have been experimentally confirmed to be associated with cellular proliferation, invasion, and migration, including miR-204, miR-210, miR-214, and miR-335 [21-24]. The current study found that miR-335 could act as an inhibitor of breast cancer cells. It was found that overexpression of miR-335 negatively affected proliferation, invasion, and migration of MCF-7 and MDA-MB-435 cells. Present results were consistent with previous reports [13, 15], further confirming the suppressive roles of miR-335 in breast cancer.

MicroRNAs exert diverse roles on expression of target genes [25]. Identified target genes of miR-335 include PAX6 [13], Sox4 [14], and c-Met [15]. To identify target genes of miR-335, bioinformatics analysis was conducted. Results showed that ERBB3 was identified as a novel candidate gene for miR-355. Dual luciferase reporter assays showed that expression of the wild type ERBB3 3'UTR was repressed by miR-335 and the introduction of mutated sites abolished this effect. Thus, present data suggests that miR-335 could downregulate ERBB3 expression by binding to its 3'UTR directly.

ERBB3 genes encode one of the epidermal growth factor receptors of tyrosine kinases [26]. This membrane-bound protein has a neuregulin binding domain but lacks an active kinase domain. Therefore, ERBB3 can bind the ligand, but cannot convey the signals into cells through protein phosphorylation [27]. However, it still can form heterodimers with other epidermal growth factor receptor family members without kinase activity. Heterodimerization leads to the activation of pathways and cell proliferation or differentiation [28]. Overexpression of ERBB3 has been reported in numerous cancers, including prostate, bladder, and breast tumors [29-31]. Elevated expression of ERBB3 receptors also induces resistance of breast cancer to therapeutic agents, such as trastuzumab [32]. It has been shown that ERBB3 induced EMT, cell migration, and invasion through PI3k/Akt-phospho-Smad2-Snail signaling pathways in SK-BR-3 and MCF7 cells [33]. However, no significant correlation between ERBB3 expression and breast cancer survival has been shown. The current study demonstrated that inhibition of breast cancer cell pro-

liferation, invasion, and migration by miR-335 was mediated by ERBB3. Current data provides more information for the regulation network of miR-335 in breast cancer carcinogenesis.

Taken together with previous results, present results demonstrate that miR-335 plays a negative role on proliferation, invasion, and migration of breast cancer cells. ERBB3 is a direct target of miR-335. Therefore, miR-335 may inhibit the development of breast cancer, at least in part, via ERBB3. Current findings provide further experimental evidence, revealing the mechanisms of breast cancer development.

Disclosure of conflict of interest

None.

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